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Evolution of Visual and Non-visual Pigments

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Chapter 1
The Evolution and Diversity of Pineal and Parapineal Photopigments

Emi Kawano-Yamashita, Mitsumasa Koyanagi, and Akihisa Terakita

Abstract Pineal and related organs are major extraocular photoreceptors in non-mammalian vertebrates. The pineal organ contains several types of photoreceptor cells, which contribute to regulating light-dependent melatonin secretion and the neural light response, including irradiance detection and wavelength discrimination. Visual opsins and pineal-specific opsins have been identified from the pineal and related organs in a wide variety of non-mammalian vertebrates. Pinopsin and parapinopsin are key opsins for understanding melatonin secretion in the chicken pineal organ and wavelength discrimination in the lamprey pineal organ, respectively. Interestingly, parapinopsin has the molecular characteristics of both vertebrate and invertebrate opsin-based pigments, making it an important photopigment for understanding the molecular evolution of vertebrate visual opsins. In this chapter, we discuss the opsin-based pigments functioning in the pineal and related organs with a focus on parapinopsin.

Keywords Rhodopsin • Parapinopsin • Pineal organ • Wavelength discrimination • Photoreceptor cell

1.1 Pineal and Related Organs

The pineal organ is common among vertebrates and is known to be involved in synthesizing and secreting the pineal hormone melatonin, a regulator of circadian rhythms (Underwood 1985; Gern and Greenhouse 1988; Falcon et al. 1989; Samejima et al. 1997, 2000). In mammals, melatonin synthesis is controlled by light information from the retina via the suprachiasmatic nuclei (SCN); however, most non-mammalian vertebrates possess an intrinsically photosensitive pineal organ that regulates melatonin synthesis (Oksche 1971; Collin et al. 1986; Falcon 1999; Okano and Fukada 2001; Bell-Pedersen et al. 2005). In addition to melatonin secretion, pineal photoreceptor cells of lower vertebrates transduce a light signal to an electrical response,

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which is transmitted to the brain through pineal ganglion cells (Dodt and Heerd 1962; Morita 1969; Dodt 1973). Interestingly, pineal and related organs of lower vertebrates have the ability of not only irradiance detection but also wavelength discrimination, namely "color discrimination" (Dodt and Heerd 1962; Morita 1966; Dodt 1973; Morita and Dodt 1973). Recent studies have revealed that pineal photoreception involves various types of opsin-based pigments including those that are pineal-specific. The photoreceptive mechanism in the pineal and related organs will be discussed in more detail, with a focus on opsin-based pigments in lower vertebrates.

1.1.1 Anatomical and structural Observation of the Pineal and Related Organs

The pineal organ is an outgrowth of the dorsal diencephalon and mostly located just below the skull (Fig. 1.1) (Oksche 1965; Collin et al. 1989; Falcon 1999). Most pineal and related organs in non-mammalian vertebrates are directly photosensitive.

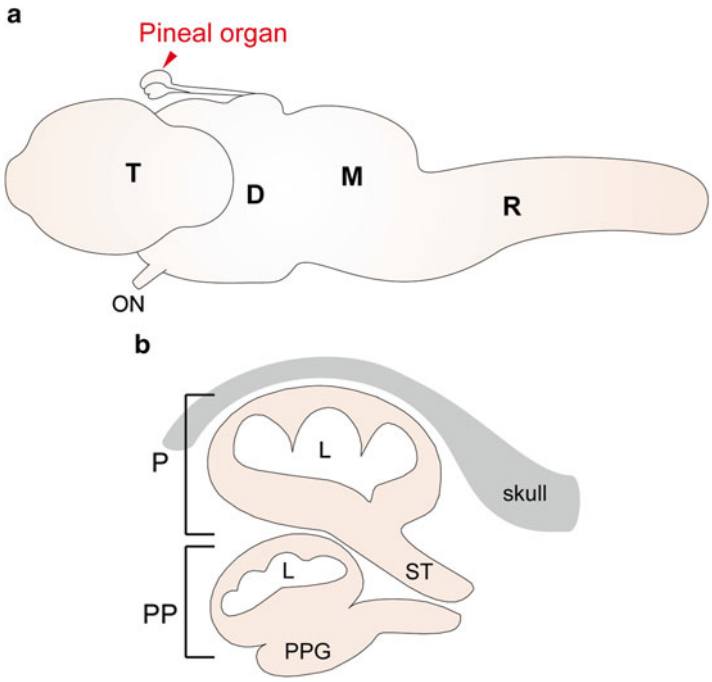

Fig. 1.1 Schematic drawings of the lamprey brain. (**a**) Lateral view of the lamprey brain. The pineal organ is situated at the dorsal diencephalon. *D* diencephalon, *M* mesencephalon, *ON* optic nerve, *R* rhombencephalon, *T* telencephalon. (**b**) The lamprey pineal and parapineal organ. The pineal and parapineal organ are located just below the skull, and are connected to the brain by the pineal stalk. The parapineal organ has a neuronal area called the parapineal ganglion. *L* lumen, *P* pineal organ, *PP* parapineal organ, *PPG* parapineal ganglion, *ST* pineal stalk

It is generally accepted that the pineal organ receives sufficient light to activate pineal photoreceptor cells through the overlaying tissues, which absorb, scatter, and reflect light (Dodt and Meissl 1982). The pineal organ is mainly composed of photoreceptor cells (often called pineal cells) as well as neural cells and glial cells. The pineal photoreceptor cells are broadly classified into two morphological types, the typical and the modified photoreceptor cells (Meiniel 1980). The typical photoreceptor cells in the lamprey, fish, and amphibian pineal organs possess relatively developed morphological features similar to those of the cone photoreceptor cell of the retina, such as outer segments and synaptic ribbons. Furthermore, the typical photoreceptor cells connect synaptically with the second-order neurons, which relay the neural light response (Vigh et al. 2002). The axons of the second-order neurons converge on the pineal stalk to form the pineal nerve that projects to the brain. In contrast, the modified photoreceptor cells have regressed outer segments and are involved in melatonin synthesis and secretion (Meiniel 1979, 1980; Collin et al. 1989). The modified photoreceptor cells are present in pineal organs of a wide variety of non-mammalian vertebrates and are predominant in the reptile and bird pineal organs. It is speculated that during the course of vertebrate evolution, the typical photoreceptor cells were gradually replaced by the modified photoreceptor cells and in mammals by the pinealocytes that do not have the membrane stacks or the capacity for photoreception. This hypothesis is supported by the presence of phototransduction-related molecules, such as opsin and arrestin, in mammalian pinealocytes (Korf et al. 1985a, b).

Lower vertebrates have pineal-related organs, such as the parapineal organ (lampreys and fishes), the frontal organ (anuran amphibians), and the parietal eye (lizards), in addition to the pineal organ. The parapineal organ is located in the intracranial region below the pineal organ. In the lamprey, the parapineal organ has a well-developed structure similar to the pineal organ, and a characteristic neuronal area called the parapineal ganglion, containing many ganglion cells (Fig. 1.1b). In teleosts, the parapineal is very small and has a vestigial morphology. The frontal organ of anuran amphibians is found in the extracranial region and is connected to the pineal organ by the nerve fiber. The lizard parietal eye, situated on the top of the head, has a lens and a retina, similar to the lateral eyes. It is speculated that the frontal organs and parietal eyes developed from a frontal part of the pineal organ and parapineal organ at an ancestral stage, respectively (Kappers 1965; Oksche 1965). The frontal organ and parietal eye exhibit a neural light response, which will be discussed later.

1.1.2 Functional Properties of Pineal and Pineal-Related Organs and Functional Opsins

As described above, pineal and related organs are involved in both light-dependent melatonin secretion and neural light-sensing. Opsins involved in melatonin secretion will be discussed first followed by neural light-sensing opsins, with a particular focus on wavelength discrimination.

1.1.2.1 Opsins Important for Melatonin Secretion in the Pineal Organ

The pineal photoreceptor cells that secrete melatonin, which correspond to the modified photoreceptor cells through morphological classification, have been identified immunohistochemically (Meiniel 1979, 1980). In the lamprey pineal organ, the melatonin-secretory photoreceptor cells were identified by both antibodies against serotonin, a precursor of melatonin, and against opsins (Tamotsu et al. 1990, 1994). These studies revealed that a red-sensitive cone opsin was present in most of the melatonin-secretory photoreceptor cells in the lamprey. A red-sensitive cone opsin was also detected in the lizard and frog pineal organs by immunohistochemistry (Masuda et al. 1994), suggesting that melatonin secretion may be controlled by long wavelength light in lower vertebrates.

In the chicken pineal organ, the red-sensitive cone opsin (iodopsin) is also present and considered to be involved in melatonin secretion (Okano et al. 1994, 1997). However, the involvement of other opsins in controlling melatonin secretion was investigated because the spectral sensitivity of red-sensitive opsin-based pigments could not completely account for the action spectrum of the inhibitory effect of light on serotonin N-acetyltransferase (NAT) activity, a key enzyme in the synthetic pathway of melatonin in the chicken pineal organ (Deguchi 1981). "Pinopsin" was identified as the first extraocular photopigment in the chicken pineal organ (Okano et al. 1994; Max et al. 1995). Pinopsin was successfully expressed in HEK293 cells and a recombinant pinopsin pigment reconstituted with 11-*cis* retinal exhibited an absorption maximum at 468 nm, identifying it as a blue sensitive pigment (Okano et al. 1994). The photoproduct of the recombinant pinopsin activates a visual G protein transducin in vitro in a light-dependent manner (Nakamura et al. 1999). The absorption spectrum of pinopsin together with that of the red-sensitive opsin-based pigment accounts for the action spectrum of inhibition of pineal NAT activity in the chicken pineal organ (Okano et al. 1994; Okano and Fukada 1997).

It has been suggested that pinopsin may play a role in synchronizing the phase of the endogenous circadian oscillator with an environmental dark–light cycle, such as the photic entrainment of the melatonin production rhythms (Okano and Fukada 1997, 2001). In chicken pineal cells, the rhythmic production of melatonin is regulated by two pathways, the acute suppression pathway and a photic-input pathway to the oscillator to reset the circadian clock (the phase-shifting effect of the circadian clock). Two types of G protein, Gt1 and Gq/11, colocalize with pinopsin in the chicken pineal photoreceptor cells (Matsushita et al. 2000). It has been hypothesized that pinopsin triggers two types of phototransduction cascades involving Gt1 and Gq/G11. On the other hand, recent studies have shown that melanopsin, which functions as a circadian photoreceptor in mammals (Provencio et al. 1998; Hattar et al. 2002; Panda et al. 2002), is also expressed in the chicken pineal organ (Bailey and Cassone 2005). It is of interest to see how melanopsin, which is suggested to drive a Gq/G11-type G protein signaling (Panda et al. 2002; Koyanagi and Terakita 2008; Bailes and Lucas 2013), contributes to the regulatory mechanisms cooperatively with pinopsin.

Reptiles (Kawamura and Yokoyama 1997) and amphibians (Yoshikawa et al. 1998) also possess the pinopsin gene, but fish and mammals do not. In the toad (*Bufo japonicus*), pinopsin is localized to the brain, specifically the anterior preoptic nucleus of the hypothalamus (Yoshikawa et al. 1998). Interestingly, pinopsin is localized in both the retina and pineal of a diurnal gecko (*Phelsuma madagascariensis longinsulae*) (Taniguchi et al. 2001), whereas in lizards, pinopsin is expressed only in the pineal [ruin lizard (*Podarcis sicula*), iguana (*Iguana iguana*)] and parietal eye [ruin lizard and side-blotched lizard (*Uta stansburiana*)] (Frigato et al. 2006; Su et al. 2006; Wada et al. 2012).

1.1.2.2 Opsins Involved in the Neural Light Response in the Pineal and Related Organs

In addition to the photoreceptor cells secreting melatonin, pineal organs in most lower vertebrates, such as lamprey, fishes, amphibians, and reptiles, contain typical photoreceptor cells (based on morphology) that transduce a captured light signal to an electrical response, which is then transmitted to the brain via the pineal ganglion cells (Dodt and Heerd 1962; Morita 1969; Dodt 1973). The frequency of neural discharge in the pineal ganglion cells is modulated by light, demonstrating that the pineal organ provides light information to the brain (Dodt 1973). In addition, several electrophysiological studies have established that the pineal organs of lampreys, fishes, and frogs have two types of ganglion cells, which show chromatic and achromatic responses. In the lamprey, the neural activity of achromatic-type ganglion cells is inhibited by visible light that can be detected by green-sensitive photoreceptor cells (Fig. 1.2a). On the other hand, chromatic-type ganglion cells receive and integrate light signals from two types of the photoreceptor cells, UV- and green-sensitive, and the neural activity of chromatic-type ganglion cells is inhibited and excited by UV and visible light, respectively (Fig. 1.2b).

The functional relation of the pineal photoreceptor cells to the ganglion cells has been well investigated for the pineal organ of the river lamprey, *Lethenteron japonicum* (Morita et al. 1989). The achromatic-type ganglion cells of the lamprey pineal show a maximum sensitivity at 525 nm, which correlates with the wavelength of maximal sensitivity of the green-sensitive pineal photoreceptor cells (Uchida et al. 1992). In addition, recombinant lamprey rhodopsin bound to the native chromophore 3,4-dehydroretinal (retinal A2) exhibits an absorption maximum at ~525 nm (Fig. 1.3a). The absorption spectrum of lamprey rhodopsin corresponds to the spectral sensitivity of the green-sensitive photoreceptor cells (Fig. 1.3a). Immunohistochemical analysis clearly showed that lamprey rhodopsin was distributed to the outer segments of the pineal green-sensitive cells, which were identified by electrical recordings (Fig. 1.3c). These results suggest that the achromatic-type ganglion cells receive and integrate light signals from the green-sensitive photoreceptor cells containing rhodopsin in the lamprey pineal organ (Fig. 1.2a). On the other hand, in the pineal organ of the river lamprey, the maximum sensitivities of the

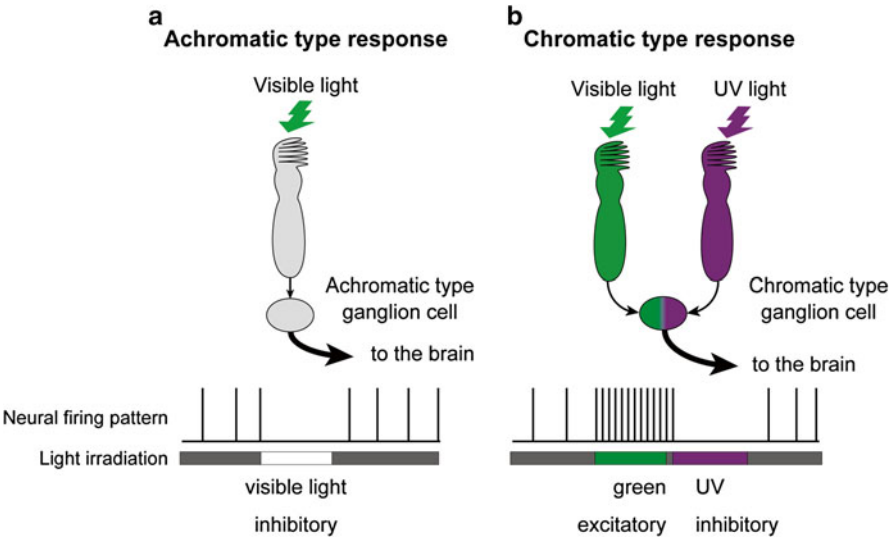

Fig. 1.2 Two types of the neural light responses in the lamprey pineal ganglion cells. (**a**) The neural activity of achromatic-type ganglion cells is inhibited by visible light that is received by green-sensitive photoreceptor cells. (**b**) The neural activity of chromatic-type ganglion cells is inhibited by UV light and excited by visible light. The chromatic-type ganglion cells receive and integrate light signals from two types of the photoreceptor cells, UV- and green-sensitive photoreceptor cells

inhibitory and excitatory responses of the chromatic-type ganglion cell were recorded at approximately 380 nm and 540 nm, respectively (Uchida and Morita 1994). Comparison of the spectral sensitivity of the achromatic and the antagonistic chromatic responses indicates that the pineal UV sensitivity is involved in the antagonistic chromatic response, which underlies the pineal wavelength discrimination (Uchida and Morita 1994), but not the achromatic response (Uchida et al. 1992), even though the green-sensitivity relates to both the chromatic and achromatic responses in the river lamprey.

1.1.2.3 UV Photopigment Underlying Wavelength Discrimination in the Pineal Organ

Tamotsu and Morita (1990) showed that a UV-sensitive opsin-based pigment with molecular properties different from those of vertebrate visual pigments may be present in the pineal organ of the lamprey (Tamotsu and Morita 1990), namely reversible photoisomerization of the chromophore retinal with UV and visible light, thereby suggesting the existence of a novel UV-sensitive opsin-based pigment. In fact, an opsin underlying UV sensitivity, including a member of the UV/violet group, has not been isolated from the pineal organ or eye of the river lamprey, although rod opsin (rhodopsin in rod photoreceptors) and red-sensitive cone opsins were

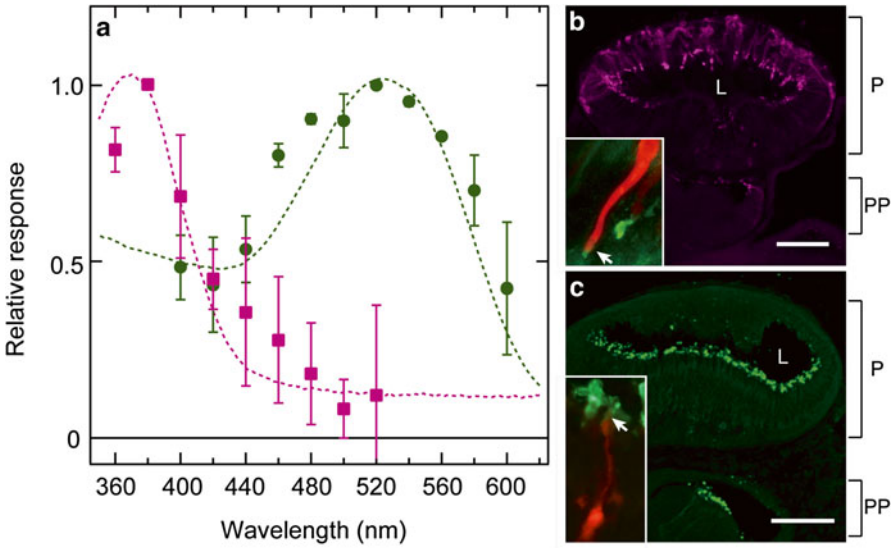

Fig. 1.3 Comparison of spectral sensitivities of UV- and green-sensitive photoreceptor cells and absorption spectra of opsin-based pigments in the lamprey pineal organ. (**a**) Relative spectral sensitivities of UV-sensitive cells (*magenta squares*) and green-sensitive cells (*green circles*). The response amplitude to each wavelength light stimulus was normalized by the maximum amplitude. The individual relative response curves were averaged ($n = 3$). Vertical bars indicate standard deviations. The absorption spectra of the recombinant parapinopsin (*magenta broken curve*) and green-sensitive porphyropsin (retinal A2-based rod opsin pigment) (*green broken curve*), which bind to the native chromophore, 3,4-dehydroretinal (retinal A2) and exhibit absorption maxima at ~370 nm and ~525 nm, respectively, are superimposed with the relative spectral sensitivities. (**b**) Immunohistochemical localization of parapinopsin (*magenta*) at the dorsal region in the lamprey pineal organ. Scale bar, 100 μm. *L* lumen, *P* pineal organ, *PP* parapineal organ. (*Inset* in **b**) UV-sensitive cell is labeled with intracellularly injected neurobiotin (*red*) after recording spectral sensitivities as shown in **a**. The parapinopsin immunoreactivity (*green*) is observed in the outer segment of neurobiotin-labeled cell (*arrow*). (**c**) Immunohistochemical localization of rod opsin (porphyropsin, *green*) at the ventral region in the lamprey pineal organ. (*Inset* in **c**) Green-sensitive cell is labeled with intracellularly injected neurobiotin (*red*) after recording spectral sensitivities as shown in **a**. The rod opsin immunoreactivity (*green*) is observed in the outer segment of neurobiotin-labeled cell (*arrow*)

identified immunohistochemically (Tamotsu et al. 1990, 1994). This stimulated the search for a novel UV-sensitive pigment in the pineal organ.

Parapinopsin, which was first identified from the catfish pineal and parapineal organs (Blackshaw and Snyder 1997), is such a UV-sensitive pigment (Koyanagi et al. 2004); spectroscopic analysis of recombinant lamprey parapinopsin containing native chromophore 3,4-dehydroretinal (retinal A2) showed that parapinopsin exhibits an absorption maximum at 370 nm in the UV region, clearly indicating that parapinopsin is a UV-sensitive opsin (Fig. 1.3a). Interestingly, the phylogenetic tree of opsins, including parapinopsin, indicates that two lines of UV pigments, UV cone opsin (UV/violet group) and parapinopsin (PP group), have evolved independently

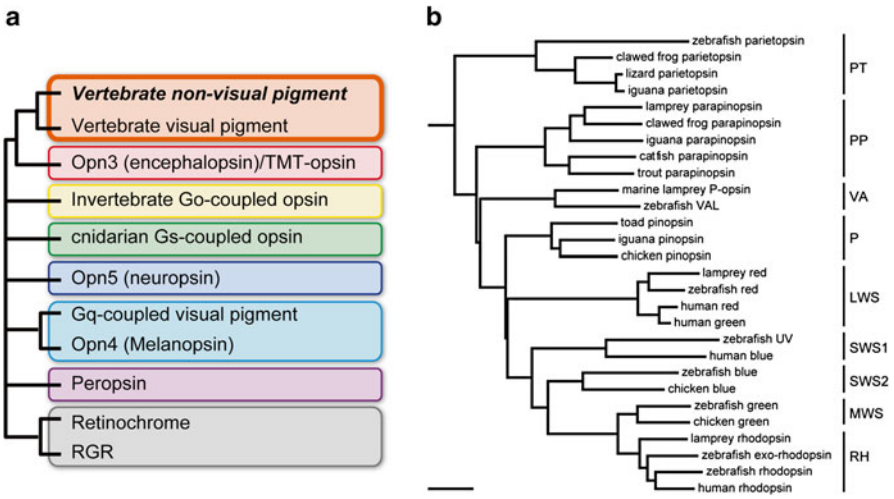

Fig. 1.4 Phylogenetic positions of opsins specific to the pineal and related organs. (**a**) Opsins have been classified into eight groups based on amino acid sequence similarity. (**b**) Phylogenetic relationships of vertebrate visual and non-visual pigments. Opsins specific to pineal and related organs, such as pinopsin, parapinopsin, parietopsin, and exo-rhodopsin, belong to the vertebrate visual pigment/non-visual pigment group. *PT* parietopsin, *PP* parapinopsin, *VA* VA-opsin, *P* pinopsin, *LWS* long wavelength-sensitive pigment, *SWS1* short wavelength-sensitive pigment 1, *SWS2* short wavelength-sensitive pigment 2, *MWS* middle wavelength-sensitive pigment, *RH* rhodopsin (rod opsin). The *scale bar* indicates 0.1 substitutions per site

in the vertebrate lineage, providing a striking example of convergent evolution (Fig. 1.4b). In addition, we also revealed that parapinopsin has molecular properties different from that of vertebrate visual pigments, yet is similar to those of invertebrate visual pigments, in agreement with the finding of reversible photoisomerization of chromophore retinal in the lamprey pineal organ by Tamotsu et al. (1990).

The possible expression of parapinopsin in the UV-sensitive photoreceptor cells of the pineal organ was investigated using electrophysiological and immunohistochemical techniques (Koyanagi et al. 2004). Intracellular hyperpolarizing responses demonstrate the highest sensitivity at ~380 nm from the lamprey pineal cells. The spectral sensitivity closely resembles the absorption spectrum of recombinant parapinopsin (Fig. 1.3a). The immunohistochemical analysis clearly shows that parapinopsin is distributed in the outer segments of the UV-sensitive cells that were identified by electrophysiological recordings (Fig. 1.3b). These results demonstrate that parapinopsin is responsible for UV reception in the lamprey pineal organ. In addition, parapinopsin expression is found only in the pineal (Kawano-Yamashita et al. 2007), suggesting that parapinopsin involvement may be limited to pineal UV reception.

Parapinopsin homologs have also been isolated from rainbow trout and the clawed frog pineal and related organs, where the UV sensitivity has been electrophysiologically detected (Morita 1966; Korf et al. 1981; Koyanagi et al. 2004). Recently, parapinopsin was found in the parietal eye of the green iguana, *Iguana*

iguana, which responds to UV light (Wada et al. 2012). Therefore, parapinopsin may be a common molecular basis of pineal UV reception for wavelength discrimination.

1.1.2.4 Characterization of the Parapinopsin-Containing UV Photoreceptor Cells in the Lamprey Pineal Organ

In order to understand how UV light signals captured by parapinopsin are transmitted to ganglion cells, which generate the antagonistic chromatic response, it is important to investigate the distribution of parapinopsin-containing cells and their neural projections histologically. Parapinopsin is localized predominantly in the dorsal layer of the lamprey pineal organ (Fig. 1.3b) (Koyanagi et al. 2004). In contrast, rhodopsin is distributed predominantly in the ventral layer (Fig. 1.3c) (Koyanagi et al. 2004). Remarkably, both parapinopsin and rhodopsin are expressed in the peripheral region, which is the dorsoventral border region of the lamprey pineal organ; however, the two pigments are never colocalized in the same photoreceptor cell (Kawano-Yamashita et al. 2007).

Parapinopsin-containing cells possess important histological characteristics that allow the assignment of the properties of pineal UV reception and neural projection to chromatic-type ganglion cells. Dye coupling, which indicates cell connection through gap junctions, was characterized in the basal processes of UV photoreceptor cells by intracellular injections (Koyanagi et al. 2004; Kawano-Yamashita et al. 2007). The UV photoreceptor cells are connected to each other, making a large photoreceptive field of at least 250 μm × 100 μm in area. Coupling in a large photoreceptive field may enable an averaging of the UV light information reaching the pineal organ and to help cancel the effect of shade.

In the lamprey pineal organ, most of the ganglion cells are localized in the ventral and peripheral regions, whereas only a few are localized in the dorsal region; the localization pattern of the ganglion cells is therefore different from that of the parapinopsin-containing cells, implying that they do not interact directly. However, parapinopsin-containing cells form a wide neural network, and almost all the basal processes from these cells are in direct contact with the ganglion cells in the peripheral region (Kawano-Yamashita et al. 2007), where many responses of chromatic-type ganglion cells have been recorded (Uchida and Morita 1994). These results suggest that in the lamprey pineal organ, the ganglion cells of the peripheral portion receive UV light information from the UV photoreceptor cells and that this drives the antagonistic chromatic response.

1.1.2.5 Visible Light Absorption in Wavelength Discrimination in the Lamprey Pineal

How visible light is captured and transduced in wavelength discrimination of lamprey pineal organ is of high interest. In chromatic-type ganglion cells, neural firing is inhibited and excited by UV and visible light, respectively (Uchida and Morita

1994). UV photoreceptor cells show hyperpolarizing responses to all wavelengths of measuring light (Uchida and Morita 1990; Koyanagi et al. 2004). Taken together with the finding that chromatic-type ganglion cells directly receive UV light information from the parapinopsin-containing cells (Kawano-Yamashita et al. 2007), it has been suggested that hyperpolarization of UV photoreceptor cells may cause suppression of the release of excitatory transmitters, such as glutamate, and produce the subsequent inhibitory responses of chromatic-type ganglion cells, analogous to light response and phototransduction of retinal photoreceptor cells. However, it remains unclear whether the pineal photoreceptor cells involved in the excitatory response to visible light are depolarized or hyperpolarized by light stimulation and whether they connect indirectly with chromatic-type ganglion cells through an interneuron. The maximum sensitivities of the excitatory responses are reported to be 540 nm (Uchida and Morita 1994), which is not in accordance with the absorption maximum of lamprey rhodopsin (Hisatomi et al. 1997) bearing the native chromophore 11-*cis* 3,4-dehydroretinal (retinal A2) ($\lambda_{max} = 525$ nm) (Fig. 1.3a). Therefore, it is possible that photoreceptor cells containing an opsin other than lamprey rhodopsin could be involved in the excitatory response.

1.1.2.6 Opsin-Based Pigment Involved in the Electrophysiological Response in the Other Pineal-Related Organs

Parapineal organs, frontal organs, and parietal eyes are known as pineal-related organs. The lamprey has a well-developed parapineal organ, where parapinopsin and rhodopsin are present (Koyanagi et al. 2004); however, an electrophysiological response has not yet been reported. On the other hand, the lizard parietal eye, which evolved from the parapineal, can detect the ratio of UV/blue to longer wavelength light. Interestingly, in the parietal eye, chromatic antagonism resides in the photoreceptor cells themselves; that is, the photoreceptor cells hyperpolarize and depolarize to light in a wavelength-dependent manner (Solessio and Engbretson 1993; Su et al. 2006). This chromatic antagonism mechanism in a single photoreceptor cell is unique to the parietal eye photoreceptor cells. The photoreceptor cells in the side-blotched lizard (*Uta stansburiana*) have two antagonistic light signaling pathways, which lead to hyperpolarizing and depolarizing responses with maximal sensitivity to blue and green lights, respectively. Recently, the molecular basis of these two pathways was revealed: the blue-sensitive pinopsin and the green-sensitive parietopsin are colocalized in a single photoreceptor cell and underlie antagonistic light responses (Su et al. 2006). The hyperpolarizing response to light is mediated by pinopsin, which activates a cGMP-phosphodiesterase through gustducin to lower cGMP concentrations, consequently closing cyclic nucleotide-gated (CNG) channels. On the other hand, the depolarizing light response is mediated by parietopsin (named after a parietal eye opsin). Light absorption by parietopsin causes elevation of cGMP levels through activation of Go, which is suggested to inhibit the cGMP phosphodiesterase, consequently opening CNG channels. Interestingly, the recent study by Wada et al. (2012) indicated that parietopsin colocalizes with the UV-sensitive pigment parapinopsin instead of

pinopsin in the parietal eye of iguana (*Iguana iguana*), which can discriminate UV and visible light. This finding strongly suggests that parapinopsin may serve as a UV-sensitive pigment in the wavelength discrimination pathway in a single photoreceptor cell. It is very interesting that the photopigment used in combination with parietopsin varies between two lizard species.

The electrophysiological responses have also been recorded from chromatic- and achromatic-type ganglion cells in the frog frontal organ, which differentiated from a frontal portion of the pineal organ (Dodt and Heerd 1962). It was reported that the wavelengths of maximum sensitivity for inhibitory and excitatory responses were 355 nm and 515 nm, respectively. Because parapinopsin was isolated from the clawed frog (*Xenopus tropicalis*) tissue containing the frontal organ (Koyanagi et al. 2004), it is suggested that parapinopsin may control UV reception for the inhibitory response.

Overall, parapinopsin may be a common UV-sensitive pigment for wavelength discrimination of UV and visible light in various pineal-related organs.

1.2 The Evolution of Pineal and Parapineal Photopigments

More than 2,000 types of opsins have been identified thus far and are classified into at least eight groups on the basis of amino acid sequence similarity: vertebrate visual and non-visual opsin, Opn3 (encephalopsin)/TMT-opsin, invertebrate Go-coupled opsin, cnidarian Gs-coupled opsin, Opn5 (neuropsin), Gq-coupled visual opsin and Opn4 (melanopsin), peropsin and retinal photoisomerase (Fig. 1.4a) (Terakita 2005; Terakita et al. 2012). Since the discovery of pinopsin (Okano et al. 1994), multiple types of non-visual opsins have been identified. Several lines of evidence suggest that these different types of opsins are expressed in pineal and related organs in a wide variety of non-mammalian vertebrates. Immunohistochemical and molecular biological investigations revealed that non-visual opsins, pinopsin (Okano et al. 1994), parapinopsin (Blackshaw and Snyder 1997), exo-rhodopsin (Mano et al. 1999), VA opsin (Philp et al. 2000), melanopsin (Bailey and Cassone 2005), and parietopsin (Su et al. 2006) are present in the non-mammalian pineal and related organs in addition to the rod and cone visual pigments (Vigh et al. 2002). As seen in the phylogenetic tree, opsins specific to pineal and related organs, exo-rhodopsin, pinopsin, parapinopsin, and parietopsin, are closely related to vertebrate visual opsins, suggesting an important evolutionary connection to vertebrate visual opsins (Fig. 1.4b).

The molecular phylogenetic tree including the members of Opn3 (encephalopsin)/TMT opsin as an out-group suggests that visual opsins arose from non-visual opsins. Previous studies revealed that upon light absorption, non-visual opsin-based pigments, such as Opn3 (encephalopsin)/TMT-opsin, Opn4 (melanopsin), and Opn5 (neuropsin)-based pigments, convert to a stable photoproduct, which activates G proteins and then reverts back to the original dark state by subsequent light absorption, as found for invertebrate visual pigments (Gq-coupled visual opsin) (Koyanagi et al. 2005, 2013; Koyanagi and Terakita 2008; Yamashita et al. 2010;

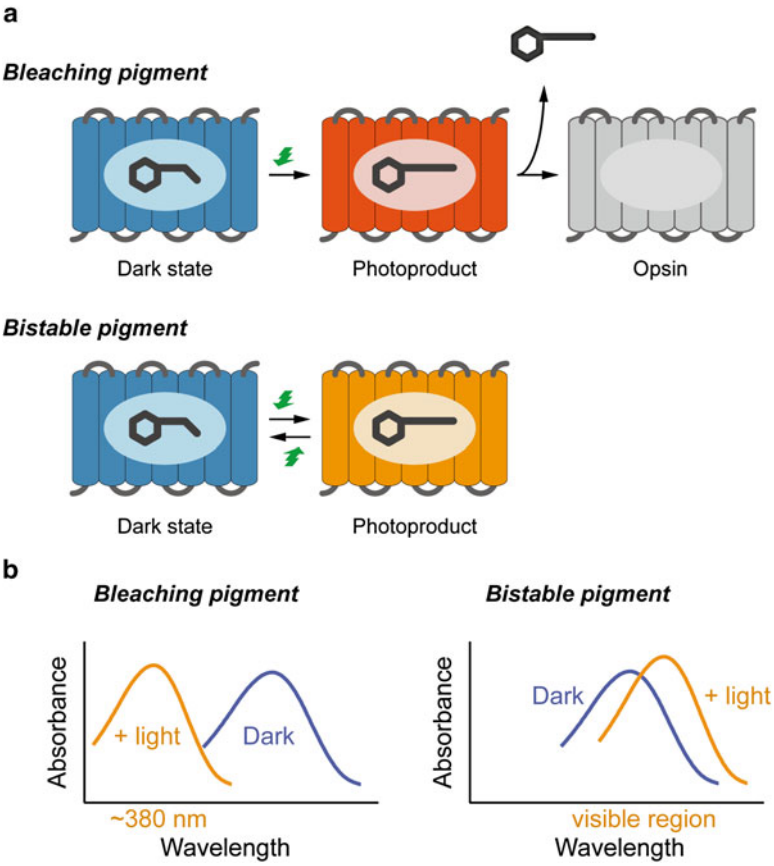

Fig. 1.5 Schematic drawings of molecular and spectroscopic properties of opsin-based pigments. (**a**) Opsin-based pigments are divided into two groups based on the molecular properties of their photoproducts, bleaching pigments and bistable pigments. (**b**) In the physiological conditions, the photoproduct of bleaching pigments become "colorless" with its absorption maximum at ~380 nm (UV region), as a result of chromophore release (**a**, *upper diagram*), whereas the photoproducts of bistable pigments have absorption maxima in the visible region, as a result of retaining the chromophore (**a**, *lower diagram*)

Kojima et al. 2011; Matsuyama et al. 2012). The photoreversible or photointerconvertible property of the two stable states (dark state and photoproduct) is called a bistable nature, which is quite different from the molecular properties of the vertebrate rod and cone visual pigments (Fig. 1.5) (Terakita 2005; Terakita et al. 2012). The photoproduct of such visual pigments releases the chromophore retinal and bleaches (becomes colorless) (Fig. 1.5). Detailed spectroscopic investigations indicated that pinopsin and exo-rhodopsin also have the bleaching property but their photoproduct (Meta II) decay rates are between those of rod and cone visual pigments (Nakamura et al. 2001; Tarttelin et al. 2011). In addition, parietopsin and

VA-opsin also have the bleaching property (Sato et al. 2011; Sakai et al. 2012). However, parapinopsin is bistable, similar to Opn3 (encephalopsin)/TMT-opsin homologs and Gq-coupled visual opsin, as suggested by reversible photoisomerization of chromophore retinal in the lamprey pineal organ (Tamotsu and Morita 1990).

1.2.1 The Molecular and Biochemical Properties of Parapinopsin as an Evolutionary Intermediate

Parapinopsin is a member of the Gt-coupled opsin group composed of vertebrate visual and non-visual pigments but has a bistable nature, unlike other members of this group (Koyanagi et al. 2004). Our previous studies suggested that the amino acid residue that serves as the counterion is related to the molecular properties of the photoproduct. For opsin-based pigments, the chromophore retinal binds to the highly conserved lysine residue at position 296 (Lys 296, bovine rod opsin (rhodopsin) numbering system) through a Schiff base linkage (Pitt et al. 1955; Hargrave et al. 1983; Findlay and Pappin 1986). Various types of opsin-based pigments with absorption maxima in the visible light region possess a "protonated" Schiff base linkage. In the protein moiety, the positive charge on the protonated Schiff base is unstable, and therefore a counterion, a negatively charged amino acid residue is needed to stabilize the positive charge. In vertebrate visual pigment, glutamic acid at position 113 serves as the counterion (Sakmar et al. 1989; Zhukovsky and Oprian 1989; Nathans 1990). In contrast, Glu181 acts as the counterion in invertebrate pigments as well as in retinochrome, a retinal photoisomerase in squid photoreceptor cells, suggesting that Glu181 was the counterion in the ancestral vertebrate visual pigments (Terakita et al. 2000, 2004). The counterion position is therefore a key site in defining the diversity of opsins and their pigments.

Although parapinopsin has an amino acid sequence similar to those of vertebrate visual pigments, it has the molecular properties of a bistable pigment, similar to invertebrate visual pigments (Gq-coupled visual opsin) and Opn3 (encephalopsin)/TMT-opsin-based pigments. These observations indicate that parapinopsin is one of the key pigments for understanding the molecular evolution of vertebrate visual pigments. Parapinopsin has glutamic acid residues at both positions 113 and 181, similar therefore to vertebrate visual pigments. However, mutational analyses have revealed that Glu181 is the functional counterion residue, as found for invertebrate rhodopsins (Terakita et al. 2004). Therefore, this suggests that the molecular properties of photoproducts, namely photoregeneration (bistability) and bleaching, may relate to counterion position and that vertebrate visual pigments having bleaching property might have evolved from an ancestral vertebrate bistable pigment similar to parapinopsin.

Interestingly, the G protein activation efficiency of both parapinopsin and invertebrate-type pigment, which have Glu181 as the counterion, is much lower (1/20–1/50) than that of vertebrate visual pigments (Terakita et al. 2004). However, previous studies revealed that the rhodopsin–G protein interaction sites are located

far from amino acid residues at positions 113 and 181, indicating that the different positions of counterion residues do not account by themselves for the difference in G protein activation efficiency. Thus, it is of interest to establish how the different G protein activation efficiencies are generated. Upon photoreception, some conformational changes take place in opsin-based pigments to activate the G protein. Site-directed spin labeling studies using bovine rod opsin-based pigment (rhodopsin) have revealed that movement of the cytoplasmic end of the sixth transmembrane helix is essential for pigment activation (Farrens et al. 1996; Hubbell et al. 2003; Altenbach et al. 2008). Using a site-directed fluorescence labeling technique, the difference in G protein activation efficiency between parapinopsin and bovine rod opsin-based pigment was investigated in relation to differences in the movement of helix VI (Tsukamoto et al. 2009). The movement of helix VI was similar in the two pigments, but the movement was greater in bovine rod opsin-based pigment than in parapinopsin. Amplitude differences of conformational changes likely led to the different G protein activation efficiencies between these pigments (see Chap. 7).

1.2.2 The Evolutionary Interaction of the Phototransduction Molecules with Opsins

The light response of vertebrate visual cells is achieved by light-absorbing visual pigments coupled to signal transduction proteins such as visual G protein transducin and visual arrestin, the latter of which binds to the light-stimulated visual pigment to shut off G protein-mediated signaling (Yau and Hardie 2009). As described above, the molecular properties of the photoproduct, which activates the G protein, are different between parapinopsin and vertebrate visual pigments. Therefore, we speculated that the signal transduction mechanism driven by parapinopsin was different from that of vertebrate retinal photoreceptors. Our immunohistochemical study suggested that transducin is the G protein coupled to the pigment in the parapinopsin-containing photoreceptor cells (Kawano-Yamashita et al. 2011). Arrestin binding to the parapinopsin photoproduct in the lamprey pineal organ however was found to involve β-arrestin, which is generally not bound to opsin-based G protein-coupled receptors (GPCRs); it is present in the parapinopsin-containing photoreceptor cells and translocates to the outer segments in a light-dependent manner (Kawano-Yamashita et al. 2011). These findings suggest that β-arrestin binds to light-stimulated parapinopsin to shut off signaling of the G proteins in the pineal photoreceptor cells, which is similar to the function of visual arrestin binding to light-activated rod opsin-based pigment (Philp et al. 1987).

In various mammalian GPCR systems, β-arrestin generally has two major functions that are carried out through binding to stimulated GPCRs (Lohse et al. 1990; Ferguson et al. 1996a, b; Goodman et al. 1996; Krupnick and Benovic 1998): termination of GPCR signaling to G proteins, and an involvement in the clathrin-mediated internalization process that removes receptors from the membrane to desensitize the cell. With respect to the latter function, β-arrestin has a clathrin binding domain,

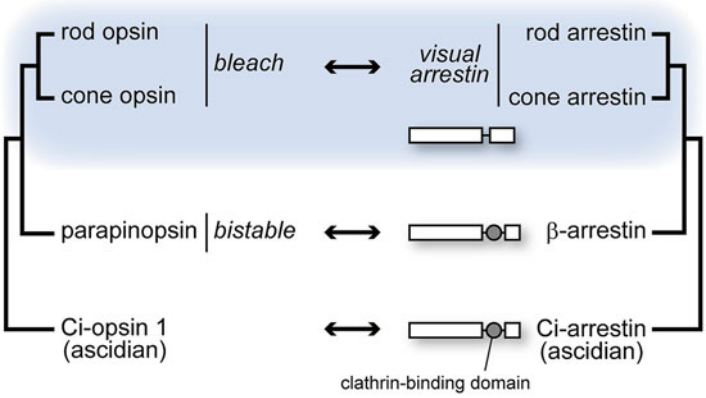

Fig. 1.6 A schematic presentation of the correlation between the molecular evolution of photopigments and arrestins. The *right* and *left trees* show the phylogenetic relationships of photopigments and arrestins, respectively. Three types of arrestins are shown but only two have a clathrin-binding domain (the *filled circle*). The *arrows* that connect the trees indicate the biochemical interactions between photopigments and arrestins

which visual arrestin lacks (Fig. 1.6). Interestingly, β-arrestin colocalizes with parapinopsin and not with the G protein in the granules observed in the parapinopsin-expressing cells under light illumination (Kawano-Yamashita et al. 2011). Our analysis of the interaction of parapinopsin with β-arrestin in HEK 293S cells revealed that lamprey β-arrestin modulated the internalization of parapinopsin in a clathrin- and light-dependent manner, similar to mammalian β-arrestin. It was therefore suggested that the granules in the cell body were generated in a light-dependent manner by β-arrestin-mediated internalization of parapinopsins from the outer segment, photoreceptive portions.

Internalization mediated by β-arrestin, namely, the removal of the light-activated parapinopsin, may be responsible for cell recovery after activation. The photoproduct of the visual pigment rhodopsin is unstable, and therefore, visual cells recover to the original dark state in the time taken to release of the chromophore, all-*trans* retinal, from the photoproduct. However, parapinopsin converts to a photoproduct that is stable and does not bleach. Therefore, the parapinopsin photoproduct does not release the chromophore retinal or is not degraded, even under strong light (Koyanagi et al. 2004). In this context, parapinopsin internalization mediated by β-arrestin may play an important role in photoproduct removal in the course of recovery to the original dark state. In *Drosophila* visual cells, where the visual pigment is converted to a stable photoproduct, the visual pigment interacts with invertebrate-type arrestins to terminate signal transduction (Dolph et al. 1993) and trigger light-induced internalization of visual pigments (Alloway et al. 2000; Kiselev et al. 2000; Satoh and Ready 2005). Interestingly, although invertebrate-type arrestins do not contain a clathrin-binding domain, they are implicated in light-induced clathrin-mediated internalization of visual pigments through interaction with another adaptor protein, AP-2 (Orem et al. 2006). As a result, this internalization leads to photoreceptor cell degeneration.

Therefore, it can be speculated that the arrestin-mediated internalization of bistable pigments is a general strategy for completely eliminating the light-activated pigment from the signaling cascades to restore photoreceptor cell conditions to the original dark state. In addition, the removal of photoproduct from the outer segments results in the down-regulation of parapinopsin function. This down-regulation may partially contribute to light adaptation and desensitization of photoreceptor cells to light, similar to the down-regulation of ligand-binding GPCRs through internalization (Lohse et al. 1990; Ferguson et al. 1996a, b; Goodman et al. 1996; Krupnick and Benovic 1998; Lefkowitz 1998).

Vertebrate visual arrestins are found in a wide variety of vertebrates, including the lamprey (Kawano-Yamashita et al. 2011). In most of these animals, visual arrestin is localized not only to the visual cells of the retinal but also to the pineal photoreceptor cells, which contain a pigment that bleaches (Collin et al. 1986). In other words, most visual arrestins function with bleaching pigments, regardless of their localization. This observation strongly supports the functional relationship between visual arrestin and bleaching visual pigments. Interestingly, in ascidians, which are the invertebrates that are most closely related to vertebrates, opsin-based pigments bind to arrestin, which has a function similar to the vertebrate β-arrestin (Nakagawa et al. 2002). Therefore, vertebrate visual arrestins appear to have diversified from their ancestral vertebrate "β-like" arrestin, for function in visual cells. It is possible therefore that vertebrate visual arrestins lack a clathrin-binding domain and are hence unable to function as a mediator of internalization because of the newly acquired bleaching property of the associated visual pigments that no longer require internalization for the inactivation of photoproducts. This is a strong argument in support of the notion that the evolution of visual pigments promoted the diversification of other signal transduction proteins and the acquisition of a phototransduction cascade that is unique to the vertebrate visual cell. That is, we can speculate that opsin evolution is correlated with the evolution of visual arrestin based on the findings of a pineal opsin, parapinopsin (Fig. 1.6).

1.3 Conclusion

Various types of opsin-based pigments are expressed in the pineals and related organs. Parapinopsin, which functions as the UV-sensitive photopigment in wavelength discrimination, is an important opsin-based pigment for understanding the molecular evolution of vertebrate visual opsins. Although parapinopsin has an amino acid sequence similar to those of vertebrate visual opsins, it shows photoreversibility and is therefore bistable in nature, similar to invertebrate visual pigments and unlike vertebrate visual pigments, which have a bleaching property. Based on the spectroscopic and biochemical properties of parapinopsin, vertebrate visual pigments that undergo bleaching may have evolved from the ancestral vertebrate opsin-based pigment with a bistable nature, similar to parapinopsin. Moreover, acquisition of the bleaching property during molecular evolution of vertebrate visual pigment may have promoted

the emergence of visual arrestin. Thus, we can predict that the bleaching property of opsin-based pigment may have facilitated the molecular evolution of other signaling proteins that specifically couple to bleaching visual pigments.

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Chapter 2
The Evolution and Function of Melanopsin in Craniates

Wayne I.L. Davies, Russell G. Foster, and Mark W. Hankins

Abstract In addition to well-characterised visual systems, many organisms, including the craniates, possess a complex sensory system of non-visual photoreceptors that detect light for a diverse array of non-image-forming tasks. Like the photoreceptors of image-forming systems, the pigments contained within non-visual photoreceptive cells comprise a protein component (opsin) linked to a light-sensitive retinal chromophore derived from vitamin A. In mammals, one of the most important of these non-visual pigments is melanopsin (encoded by the *OPN4* gene, specifically that of the "mammal-like" or "m-class"), which is restricted in expression to a subset of retinal ganglion cells and has been shown to be the conduit through which light regulates many physiological activities, including the photoentrainment of circadian systems (e.g. the sleep cycle) and the pupillary reflex response. In non-mammals, melanopsin exists as two distinct gene lineages, namely the m-class and x-class ("*Xenopus*-like"), and both are expressed in many different tissues, including the eyes, skin, fins, gills, brain and pineal gland; however, the functional roles mediated by melanopsin in these "lower" vertebrates remain to be fully elucidated. In this review, we discuss the evolutionary history of the melanopsin gene, its diverse patterns of expression and transcriptional output, the functional roles so far determined, and the clinical significance of this critical and phylogenetically most ancient opsin-based system of irradiance detection.

Keywords Evolution • Craniate • Circadian • Photopigment • Opsin • Melanopsin • opn4

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2.1 Introduction

Light detection or photoreception is paramount for the survival of most species, with photosensitive molecules being detection in both prokaryotes and eukaryotes (Briggs and Spudich 2005). Although these various light detection systems share a basic common function in converting quanta of electromagnetic waves (photons in this case) into chemical signals, the evolutionary origins and molecular signatures of the receptors involved are exquisitely diverse and include those that utilise light-sensitive retinal-based chromophores (e.g. vertebrate pigments), bilin-based chromophores (e.g. phytochromes), flavin-based chromophores (e.g. cryptochromes), amongst many others (Briggs and Spudich 2005).

Some organisms may utilise a distinct array of photosensitive molecules that are evolutionary unrelated, such as both red-light sensing photochromes and blue-light sensing cryptochromes in plants (Chaves et al. 2011; Devlin and Kay 2000; Somers et al. 1998), or Type I cryptochromes (Chaves et al. 2011; Emery et al. 1998; Stanewsky et al. 1998; Tomioka and Matsumoto 2010) and retinal-based rhodopsin pigments in insects (Lee et al. 1996; Montell 2012). Although mammals express a family of cryptochromes (Type II) (Chaves et al. 2011; Todo 1999), these molecules are not intrinsically sensitive to light and form a key component of the circadian clock (Chaves et al. 2011; Mohawk et al. 2012). In the craniates, the predominant light-sensing proteins are biophysiologically varied, although those studied thus far all utilise retinal as a photosensitive chromophore, an observation that is consistent with their related evolutionary origins (Davies et al. 2012a; Davies 2011; Shichida and Matsuyama 2009; Terakita 2005; Yokoyama 2000).

The principal type of craniate photopigment comprises a protein moiety (opsin) covalently linked to a retinoid chromophore that is based on vitamin A, via a Schiff base linkage formed at a lysine (Lys) residue at site 296 (based on bovine rod opsin numbering) (Fig. 2.1). Indeed, it is the presence of Lys296 that defines this class within a much larger superfamily of G protein-coupled receptors (GPCRs) (Davies et al. 2012a; Davies 2011; Shichida and Matsuyama 2009; Terakita 2005; Yokoyama 2000). Opsin proteins are classed as Type II polytopic transmembrane proteins as they consist of extracellular amino- and intracellular carboxyl-termini (N- and C-termini), respectively, which frame seven transmembrane (TM) domains, three extracellular loops (ECI-III) and three intracellular or cytoplasmic loops (CLI-III) (Fig. 2.1). Much of the work determining the structure–function relationships of opsin-based pigments (Sakmar et al. 2002) was performed by mutating the pigment expressed in rod photoreceptors: this rod opsin protein is encoded by the *RH1* gene and is also confusingly known as rhodopsin (e.g. often in the clinical context)—a term that defines the opsin-based pigments of invertebrate rhabdomeres, the craniate pigments that utilise a vitamin A_1-derived chromophore, as well as the rod light-sensing protein (Davies et al. 2012a). The manipulation of the rod pigment in this particular way is essentially historical as bovine rod opsin was the first opsin gene to be sequenced over 30 years ago (Hargrave et al. 1983; Nathans and Hogness 1983; Ovchinnikov Yu 1982). Subsequent studies have generated high-resolution crystal structures for the rod opsin pigment

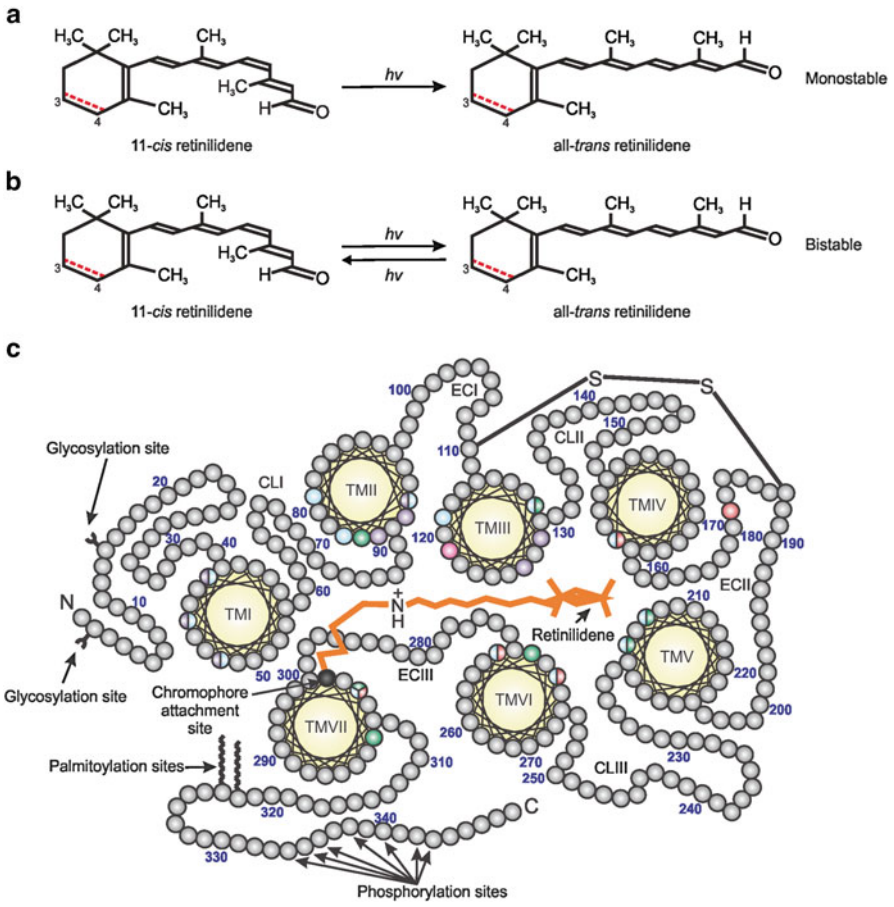

Fig. 2.1 A diagram of the structure of a typical photopigment (e.g. rod opsin). (**a**, **b**) The initial step in phototransduction consists of photon (*hv*) absorption by 11-*cis* retinal, which photoconverts to all-*trans* retinal. Vertebrate photopigments are broadly divided into rhodopsins that utilise a vitamin A_1-derived chromophore (*black line*) or porphyropsins that contain a vitamin A_2-derived chromophore (3,4-didehydroretinal). In the latter case, the presence of a double bond (i.e. C=C) between C_3 and C_4 is shown as a *dotted red line*. For many pigments (e.g. visual opsins), the conversion of 11-*cis* retinal to all-*trans* retinal is a unidirectional reaction (monostable), resulting in the hydrolysis and release of free all-*trans* retinal (**a**); however, in bistable pigments (e.g. melanopsin), the chromophore is not released from the retinal binding pocket and the pigment, upon absorbing a further photon, isomerases all-*trans* retinal to 11-*cis* retinal (**b**). (**c**) Mid-membrane section of a typical (opsin) photopigment, showing the presence of seven transmembrane domains (*yellow*) archetypal of the GPCR superfamily and their arrangement around the bound retinal chromophore (*orange*) (modified from Davies et al. (2012a)). The retinal attachment site (Lys296) (*black*) and counterion (Glu113) (*pink*) to the Schiff base ($-NH^+$) are shown. Opsin residues that cluster either around the Schiff base or ionone ring of the retinal chromophore are coloured to highlight the amino acids involved in the spectral tuning of long-wavelength-sensitive (LWS) (*red*), short-wavelength-sensitive-1 (SWS1) (*violet*), short-wavelength-sensitive-2 (SWS2) (*blue*) and rhodopsin-like-2 (RH2)/rhodopsin-like-1 (RH1 or rod opsin) (*green*) photopigments. Residues important for stabilising the tertiary structure (e.g. disulphide bridge (S-S), amino-terminal (N) glycosylation sites) and the activation/deactivation of photopigments (e.g. carboxyl-terminal (C) phosphorylation sites), as well as membrane anchorage (e.g. palmitoylation sites), are also shown. *TM* transmembrane, *CL* cytoplasmic loop, *EC* extracellular loop. The numbering is based on the bovine rod opsin (RH1) sequence

that continue to provide insights into the molecular mechanisms of photon capture and pigment activation (Palczewski et al. 2000; Ruprecht et al. 2004; Schertler 2005). Although the crystal structure of a cone pigment has yet to be determined, similar mutagenic approaches using opsin genes expressed in both cone and rod photoreceptors have shown that a number of residues surrounding the chromophore binding pocket are responsible for determining the spectral characteristics of a particular photopigment, defined by the wavelength at which maximum absorbance of light occurs (i.e. the spectral peak or λ_{max}) (Davies et al. 2012a; Davies 2011; Shichida and Matsuyama 2009; Terakita 2005; Yokoyama 2000) (Fig. 2.1). Despite consisting of a polypeptide of ~350 residues, only a relatively small number of amino acid substitutions appear to be tolerated in the generation of a functional, correctly folded protein, with just over 20 known tuning sites being employed by different groups of (visual) photopigments across many distinct classes or organisms. More, however, are likely to be discovered, especially given the disparity in pigment biochemistry (e.g. bistability vs. monostability) (Davies et al. 2007, 2012a; Yokoyama 2000). Thus it appears that convergent evolution has played a significant role in shaping the biochemical properties of the various craniate photopigment classes.

2.2 Visual Versus Non-visual Photoreception

In general, photosensory tasks in craniates are broadly divided into vision (image-forming) and non-vision (non-image-forming). Vision originates in the eye, where cone and rod photoreceptors of the duplex retina evolved to be sensitive under bright-light (i.e. photopic vision where rods are bleached leaving functional cones) and dim-light (i.e. scotopic vision where rods are active but cones cannot be stimulated due to sub-threshold light levels) conditions, respectively, or under mesopic circumstances where medium intensity light permits both cones and rods to be photoactive. In these cases, visual photopigments are housed within the outer segments of specialised cells and are able to detect external light, with their peak absorbances often being spectrally tuned to match the spectral composition of a specific habitat (Bowmaker 2008; Davies et al. 2012a; Davies 2011; Yokoyama 2000), with accompanying opsin gene loss or gain as an important substrate or consequence of adaptive evolution (Davies et al. 2009a, b, c, 2012a; Davies 2011; Yokoyama 2000).

Craniate visual pigments are classed into five main groups based on their molecular evolution and spectral sensitivities (Fig. 2.2). Encoded by four cone opsin genes, namely long-wavelength-sensitive (*LWS*), short-wavelength-sensitive-1 (*SWS1*), short-wavelength-sensitive-2 (*SWS2*), and rhodopsin-like-2 (*RH2*), as well as a single rhodopsin-like-1 (*RH1*) gene, these pigments are maximally sensitive to wavelengths that range from the ultraviolet (UV) to the yellow regions of the visible spectrum (~360–560 nm when utilising vitamin A_1-based chromophores) (Bowmaker 2008; Davies et al. 2012a; Davies 2011; Yokoyama 2000). During the past 10 years, it has been demonstrated that all five visual photopigments arose early in craniate evolution, firstly with the occurrence of five cone pigment genes (with the potential

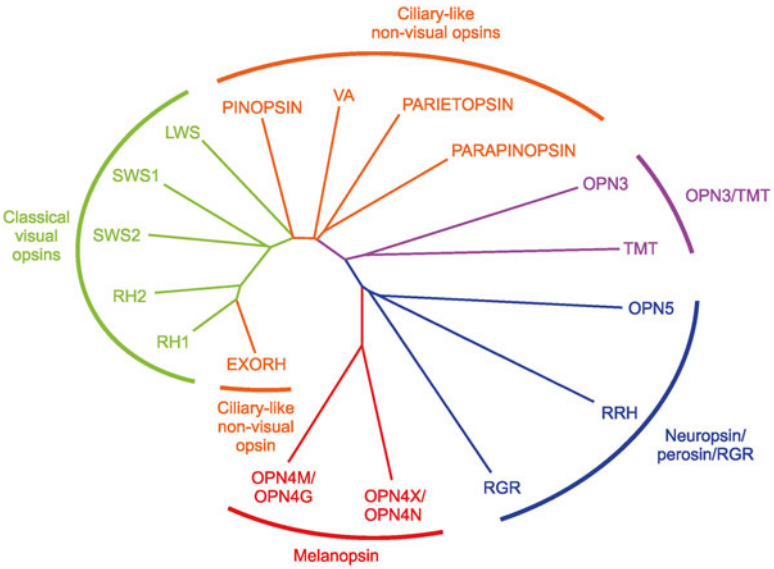

Fig. 2.2 A phylogenetic tree showing the evolution of known opsin-based pigments in the craniates and their classification into five main classes. Mammal-like/gnathostome melanopsin, OPN4M/OPN4G; *Xenopus*-like/non-therapsid melanopsin, OPN4X/OPN4N; retinal G protein-coupled receptor, RGR; retinal pigment epithelial (RPE)-specific rhodopsin homologue (RRH), peropsin; neuropsin, OPN5; panopsin/encephalopsin, OPN3; teleost multiple tissue opsin, TMT; parapineal gland-expressing opsin, parapinopsin; parietopsin-expressing opsin, parietopsin; vertebrate ancient opsin, VA; pineal gland-specific opsin, pinopsin; long-wavelength-sensitive opsin, LWS; short-wavelength-sensitive-1 opsin, SWS1; short-wavelength-sensitive-2 opsin, SWS2; middle-wavelength-sensitive rhodopsin-like-2 (*cone*), RH2; middle-wavelength-sensitive rhodopsin-like-1 (rod opsin), RH1; and extraretinal rod-like opsin, EXORH

for pentachromacy) in the common ancestor to both the jawless agnathans and the jawed gnathostomes (Collin et al. 2003, 2009; Davies et al. 2012a), followed by the conversion of the cone expressing the second *RH2*-like gene into a "true" rod (Collin et al. 2003; Davies et al. 2007; Okano et al. 1992; Yokoyama 2000) with a rod-specific phototransduction cascade (Ebrey and Koutalos 2001; Hisatomi and Tokunaga 2002).

During the previous 70 years, the eye and the visual system have been systematically well characterised at all levels of scientific research from the evolution of the molecules involved to the behavioural consequences (Arendt 2003; Davies et al. 2012a; Lamb et al. 2007, 2009; Nilsson 2013; Walls 1942). By contrast, the non-visual or non-imaging-forming systems of light detection have been less studied, at least with regard to the molecular basis of non-visual photopigments and their physiological roles, although non-visual responses have been the subject of investigation for many years. The first non-visual opsin was identified in 1994 and was named "pinopsin" after its discovery as an expressed sequence in the pineal glands of chickens (Okano et al. 1994) and later in the eyes of lizards (Taniguchi et al. 2001). This discovery

was augmented in rapid succession with the identification of other non-visual pigments, namely vertebrate ancient (VA) opsin in retinal horizontal cells and amacrine cells of the Atlantic salmon (Philp et al. 2000b; Soni and Foster 1997; Soni et al. 1998) and pineal gland of the lamprey (Yokoyama and Zhang 1997), parapinopsin in the catfish parapineal organ (Blackshaw and Snyder 1997) and lamprey pineal gland (Koyanagi et al. 2004), both initially found in 1997, and melanopsin in the skin of the African clawed frog (*Xenopus laevis*) in 1998 (Provencio et al. 1998b). To date, over 3,000 complete opsin sequences are listed in the NCBI sequence database (http://www.ncbi.nlm.nih.gov/nuccore/?term=opsin+complete+cds), all of which clade into about five distinct families of craniate visual and non-visual pigments based on their phylogenetic positions, expression profiles and putative functional roles (Peirson et al. 2009; Terakita 2005) (Fig. 2.2). These include (1) the melanopsins (Bellingham et al. 2006; Davies et al. 2010, 2012d; Provencio et al. 1998b); (2) a group consisting of neuropsin (OPN5), peropsin (also known as retinal pigment epithelial (RPE)-specific rhodopsin homologue (RRH)) and retinal G protein-coupled receptor (RGR) (Bellingham et al. 2003b; Shen et al. 1994; Sun et al. 1997; Tarttelin et al. 2003); (3) a class comprising panopsin/encephalopsin (OPN3) and teleost multiple tissue (TMT) opsin (Blackshaw and Snyder 1999; Halford et al. 2001; Moutsaki et al. 2003); the non-visual (ciliary) group consisting of parapinopsin (Blackshaw and Snyder 1997; Koyanagi et al. 2004), parietopsin (Su et al. 2006), VA opsin (Davies et al. 2010; Kojima et al. 2008; Soni and Foster 1997), pinopsin (Okano et al. 1994) and extraretinal rod-like opsin (EXO-RHO) (Philp et al. 2000a); and (4) the classical visual pigments comprising four cone opsins (LWS, SWS1, SWS2 and RH2) and a single rod opsin (RH1) (Davies et al. 2012a; Yokoyama 2000) (Fig. 2.2). Despite this diversity, very little is known about the physiological roles these pigments play except for the visual pigments and a small number of non-visual opsins, such as melanopsin (Bellingham et al. 2006; Davies et al. 2010, 2012d; Provencio et al. 1998b), VA (Davies et al. 2012c; Halford et al. 2009; Kojima et al. 2008; Soni and Foster 1997) and those expressed in the pineal gland (e.g. pinopsin (Okano and Fukada 1997; Okano et al. 1994)).

2.3 The Evolution and Expression of the Melanopsin Gene Family

Although not the first non-visual opsin to be discovered, melanopsin (encoded by the *opn4* gene) has received the most attention by far from physiologists, photobiologists and circadian scientists alike, with around 500 papers published on melanopsin sensory systems since the first description of the *opn4* gene sequence in 1998 (Provencio et al. 1998b). This is only superseded by the ~9,000 publications on the visual pigments that have appeared since the 1950s.

Based on the observation that the presence or absence of light caused dispersal and aggregation of melanin granules in the skin of frogs (Bagnara and Obika 1967), Provencio and colleagues (1998b,) analysed a melanophore cDNA library generated

from mRNA extracted from the dermis of *Xenopus laevis* (Provencio et al. 1998b). Using radiolabelled partial cone and rod opsin probes, the authors identified a novel pigment sequence that was named "melanopsin" in honour of the tissue in which it was discovered (Provencio et al. 1998b). Further analysis demonstrated that melanopsin transcripts were not restricted to melanophores, with the expression profile extending to multiple tissues of many other non-mammalian vertebrates, such as the eye and brain of teleosts (e.g. zebrafish, *Danio rerio*; cod, *Gadus morhua*; salmon, *Salmo salar*; roach, *Rutilus rutilus*; cichlid, *Astatotilapia burtoni*; catfish, *Ictalurus punctatus*) (Bellingham et al. 2002; Cheng et al. 2009; Davies et al. 2011; Drivenes et al. 2003; Grone et al. 2007; Jenkins et al. 2003; Sandbakken et al. 2012); amphibians (e.g. African clawed frog, *Xenopus laevis*) (Bellingham et al. 2006; Provencio et al. 1998b; Rollag et al. 2000); reptiles (e.g. ruin lizard, *Podarcis sicula*) (Frigato et al. 2006); turtles (e.g. red-eared slider, *Trachemys scripta elegans*) (Dearworth et al. 2010); and birds (e.g. chicken, *Gallus gallus*) (Bellingham et al. 2006; Chaurasia et al. 2005; Tomonari et al. 2007). In amphibians, melanopsin was detected in the hypothalamus (specifically the ventral part of the magnocellular preoptic nucleus and the suprachimatic nucleus (SCN)) and the iris, both structures known to be directly photosensitive, and more importantly the RPE and horizontal cells (Provencio et al. 1998b) (see Chap. 3 in this volume for further discussion of non-ocular melanopsin expression in the central nervous system). Given this insight, Provencio and co-workers searched for melanopsin in mammals and indeed found an *OPN4* orthologue in humans (*Homo sapiens*), which localised to a 1–2% subset of retinal ganglion cells (RGCs) (Provencio et al. 2000, 2002) (Fig. 2.3). Further analysis of the mammalian lineage has shown that melanopsin is present in the genomes of all three classes of mammals, namely the monotremes (e.g. platypus, *Ornithorhynchus anatinus*) (Bellingham et al. 2006; Davies et al. 2010), the marsupials (e.g. fat-tailed dunnart, *Sminthopsis crassicaudata*; grey short-tailed opossum, *Monodelphis domestica*) (Bellingham et al. 2006; Davies et al. 2010; Pires et al. 2009), and many crown group eutherians (Davies et al. 2010) studied thus far (e.g. mouse, *Mus musculus* (Provencio et al. 2000); rat, *Rattus norvegicus* (Hattar et al. 2002); blind mole rat, *Spalax ehrenbergi* (Hannibal et al. 2002); hamster, *Phodopus sungorus* (Hermann et al. 2005); cat, *Felis catus* (Semo et al. 2005); and human, *Homo sapiens* (Provencio et al. 2000)).

Initially it was assumed that all vertebrate melanopsin sequences were orthologous with the first sequence identified in amphibians (Provencio et al. 1998b). However, the percentage identity (~55%) between these genes, when analysing a core region from TM domain 1 to TM domain 7, was far less than expected, especially when compared to the ~85% similarity between vertebrate rod opsin sequences (Bellingham et al. 2006). Adopting a genomics approach, with subsequent molecular cloning, Bellingham et al. (2006) confirmed the presence of two melanopsin genes in non-mammalian species (e.g. chicken, *Gallus gallus*), where the percentage identity significantly increased from 55% to over 80% when similar melanopsin family members were compared (Bellingham et al. 2006). Based on historical grounds, one class was named "*Xenopus*-like" (*opn4x*) due to sequence similarity with the first melanopsin gene discovered. The other class resembled the melanopsins of mammals to a greater degree than the *opn4x* gene orthologues, thus, this

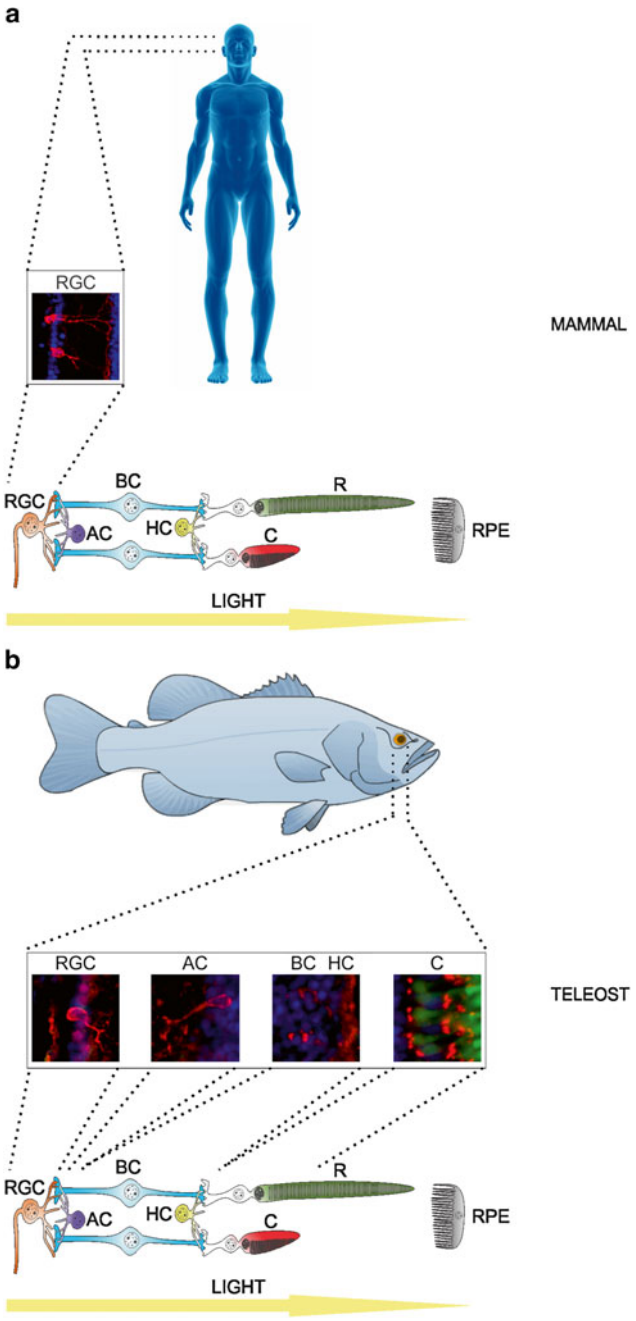

Fig. 2.3 A schematic showing immunocytochemically detected melanopsin expression (coloured *fluorescent red*) in the retina of (**a**) a typical mammal (e.g. human, *Homo sapiens*) compared to (**b**) a representative teleost (e.g. zebrafish, *Danio rerio*). In humans, the OPN4M protein is

gene family was renamed "mammal-like" (*OPN4M*) (Bellingham et al. 2006) (Figs. 2.2 and 2.4, and Table 2.1). Although both *opn4x* (x-class) and *opn4m* (m-class) genes are found in the majority of non-mammalian vertebrates, it would appear that *OPN4X* was lost in mammals prior to the marsupial/eutherian split (Bellingham et al. 2006; Pires et al. 2007). Similarly, an investigation of a representative monotreme (e.g. platypus, *Ornithorhynchus anatinus*) failed to identify an *OPN4X* gene in addition to the *OPN4M* orthologue, thereby suggesting that the *OPN4X* gene vanished from the "true" ancestral mammalian genome about 225 million years ago (MYA) (Davies et al. 2010). Before proceeding any further, a comment should be made with regard to nomenclature of the melanopsin gene families. Even though the terms "opn4m" and "opn4x" are useful in demonstrating the presence of two gene lineages, they are misnomers and confusing in their meaning: for example, "*Xenopus*-like" literally refers to a single genus, *Xenopus*, but orthologues in teleosts (e.g. zebrafish, *Danio rerio*) are just as related to those in the reptiles (e.g. ruin lizard, *Podarcis sicula*), so classing these genes as "teleost-like", or "podarcis-like" is equally incongruous. Similarly, "mammal-like" can be misinterpreted as "mammal-restricted" but melanopsin orthologues of this class are present in all mammalian and non-mammalian gnathostome ("jawed") vertebrates so far studied (Davies et al. 2010, 2012b), so they may also be paradoxically labelled as "non-mammal-like", accompanied by the obviously and inaccurate omission of all mammalian orthologues. A simpler nomenclature would be to label the two classes as *opn4a* and *opn4b* or more accurately as "gnathostome melanopsin (*opn4g*)" and "non-therapsid melanopsin (*opn4n*)" for *opn4m* and *opn4x* genes, respectively, if an evolutionary designation is required. Thus, the *opn4g* gene refers to melanopsin orthologues found in all jawed vertebrate and the *opn4n* gene indicates melanopsin orthologues in non-mammalian vertebrates. Nonetheless, in order to minimise confusion with unfamiliar terminology, "opn4m/opn4x" is used herein with both nomenclatures included in the table and figures where appropriate.

With the onset on whole genome sequencing projects, in addition to more traditional cloning approaches, additional vertebrate orthologues of melanopsin from both *opn4* classes have been recently identified from vertebrates that span from the teleosts to the eutherian mammals (Borges et al. 2012; Davies et al. 2011, 2012b; Dearworth et al. 2011; Dong et al. 2012; Sandbakken et al. 2012) (Fig. 2.4). Of particular interest is

Fig. 2.3 (continued) restricted to a subset of photosensitive retinal ganglion cells (pRGCs). By contrast, three of the five melanopsin orthologues, specifically *opn4m1-3* genes collectively, are expressed in all the major retinal layers of the zebrafish, with the presence of the opn4m2 photopigment also in the two classes of short-wavelength-sensitive cones that express the short-wavelength-sensitive-1 (*sws1*) and short-wavelength-sensitive-2 (*sws2*) opsin genes. A *yellow arrow* indicates the direction of light as it passes through the retina, firstly reaching the RGC layer before being primarily absorbed by the visual photoreceptor cells at the back of the eye. Retinal ganglion cell, RGC (*orange*); amacrine cell, AC (*purple*); bipolar cell, BC (*blue*); horizontal cell, HC (*yellow*); cone, C (*red*); rod, R (*green*); and retinal pigment epithelial cell, RPE (*grey*). Retinal panels are modified from (Davies et al. 2011; Ecker et al. 2010; Hughes et al. 2012a)

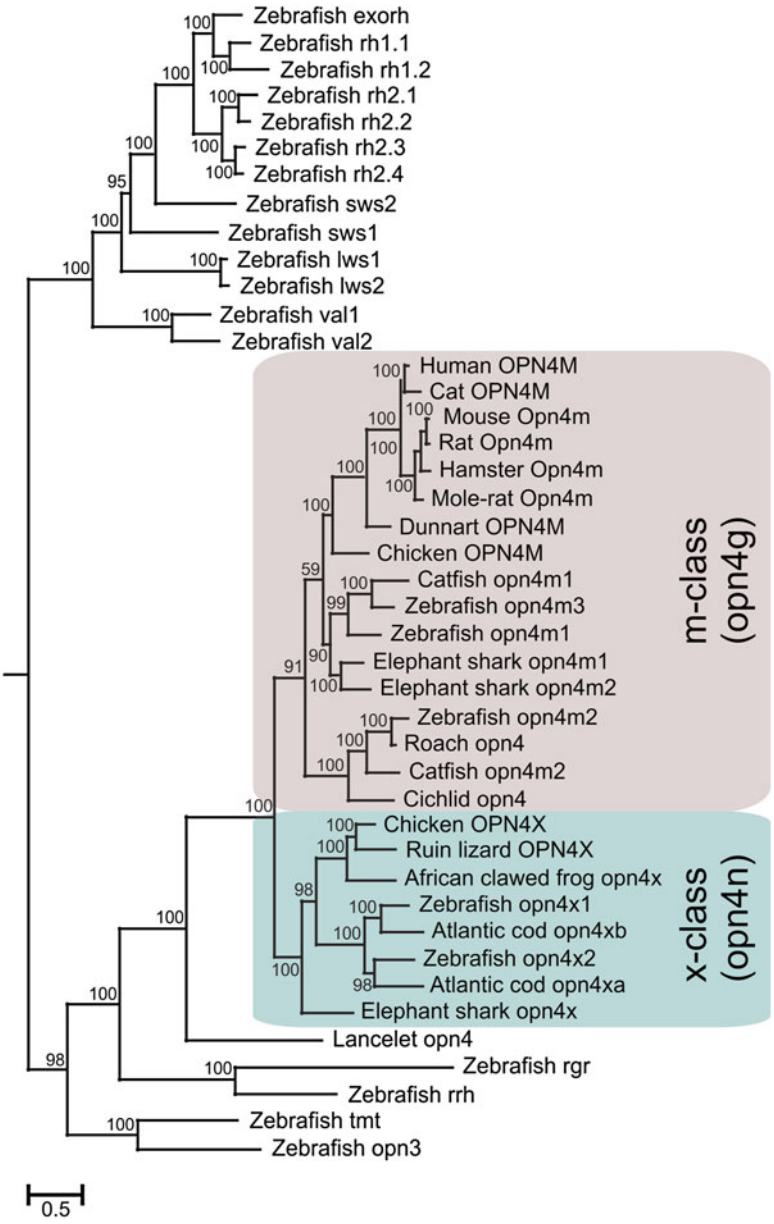

Fig. 2.4 The evolution of craniate melanopsin photopigments. Phylogenetic analyses based on a codon-matched nucleotide alignment of various chordate melanopsin cDNA sequences compared to published visual and non-visual photosensory pigments of the zebrafish (*Danio rerio*), showing the two main *opn4* clades, namely the m-class (opn4g) (purple) and the x-class (opn4n) (*blue*). A maximum composite likelihood (MCL) methodology (Tamura and Nei 1993) was applied to generate a bootstrapped (1,000 replications), neighbour-joining (NJ) phylogenetic tree (Saitou and Nei 1987) with the degree of internal branching expressed as a percentage. Evolutionary distances were calculated by using the MEGA Version 4 software (Tamura et al. 2007). The scale bar indicates

Table 2.1 Percentage identities between a number of published chicken (*Gallus gallus*) opsin protein sequences. Comparisons are made between full-length sequences (*upper right black shading*) and the third cytoplasmic domain (*lower left white shading*). Opsins are colour coded by class: (1) classical visual opsins (middle-wavelength-sensitive rhodopsin-like-1 (*rod*), RH1; middle-wavelength-sensitive rhodopsin-like-2 (cone), RH2; short-wavelength-sensitive-2 opsin (*cone*), SWS2; short-wavelength-sensitive-1 opsin (*cone*), SWS1; and long-wavelength-sensitive opsin (cone), LWS) (*green*); (2) ciliary-like non-visual pigments (pineal gland-specific opsin, PINOPSIN; and vertebrate ancient opsin, VA) (*orange*); (3) neuropsin/peropsin/RGR group (neuropsin, OPN5; retinal pigment epithelial (RPE)-specific rhodopsin homologue (RRH), peropsin; and retinal G protein-coupled receptor, RGR) (*blue*); and (4) two melanopsin subclasses (mammal-like/gnathostome melanopsin, OPN4M/OPN4G; and *Xenopus*-like/non-therapsid melanopsin, OPN4X/OPN4N; *red*). Modified from Davies et al. (2010)

	RH1	RH2	SWS2	SWS1	LWS	P-OPSIN	VAL	OPN5	RRH	RGR	OPN4M OPN4G	OPN4X OPN4N
RH1		72.5	49.2	45.6	39.7	42.7	35.1	22.1	24.0	21.3	17.7	16.8
RH2	80.8		51.8	48.0	41.5	43.5	39.9	22.1	21.5	18.0	17.7	16.8
SWS2	57.7	65.4		48.3	38.9	45.0	37.1	21.8	23.6	18.5	15.8	16.2
SWS1	61.5	69.2	73.1		41.0	43.5	38.4	19.8	20.0	18.4	19.7	16.4
LWS	46.2	46.2	46.2	61.5		42.2	36.8	20.9	23.3	18.5	18.0	18.5
P-OPSIN	57.7	57.7	61.5	65.4	50.0		41.4	22.9	25.1	20.5	17.4	17.7
VAL	23.1	34.6	42.3	38.5	19.2	30.8		23.2	26.2	19.2	17.9	17.4
OPN5	16.7	23.3	13.3	13.3	6.7	20.0	16.7		26.3	20.4	19.7	17.5
RRH	17.9	3.6	7.1	3.6	10.7	10.7	3.6	10.0		20.6	17.0	16.6
RGR	7.7	7.7	3.8	3.8	3.8	3.8	15.4	13.3	7.1		14.6	14.7
OPN4M/ OPN4G	13.2	13.2	7.9	13.2	10.5	7.9	10.5	21.1	10.5	10.5		40.4
OPN4X OPN4N	11.4	11.4	11.4	14.3	25.7	8.6	11.4	11.1	5.7	2.9	34.2	

Fig. 2.4 (continued) the number of nucleotide substitutions per site. The human GPR21 and GPR52 nucleotide sequences were used as outgroups (not shown). See Davies et al. (2012b) and Davies et al. (2011) for GenBank accession numbers. Pigment classes include are as follows: (1) extraretinal rod-like opsin (exorh); (2) middle-wavelength-sensitive rhodopsin-like-1 (rod) opsin (rh1); (3) middle-wavelength-sensitive rhodopsin-like-2 (cone) (rh2); (4) short-wavelength-sensitive-2 (sws2); (5) short-wavelength-sensitive-1 (sws1); (6) long-wavelength-sensitive (lws); (7) vertebrate ancient opsin (va); (8) mammal-like/gnathostome melanopsin (opn4m/opn4g or m/g-class); (9) *Xenopus-like*/non-therapsid melanopsin (opn4x/opn4n or x/n-class); (10) lancelet (*Branchiostoma belcheri*) melanopsin; (11) retinal G protein-coupled receptor (rgr); (12) retinal pigment epithelium (RPE)-specific rhodopsin homologue (rrh) (peropsin); (13) teleost multiple tissue opsin (tmt); and (14) panopsin/encephalopsin (opn3). The gene nomenclature used follows the guidelines adopted by the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene). In brief, the genes of all terrestrial species are in *uppercase*, except for rodents, where only the *first letter* is *capitalised*. The genes of all aquatic species, including amphibians, are in *lowercase*

the recent discovery of five melanopsin genes in the zebrafish (*Danio rerio*) (Davies et al. 2011; Matos-Cruz et al. 2011) and six in the Atlantic salmon (*Salmo salar*) (Sandbakken et al. 2012). In both teleost species, orthologues of *opn4m* and *opn4x* gene lineages exist that are accompanied by melanopsin class-specific duplications that give rise to *opn4m1-3* and *opn4x1-2* in the zebrafish (Davies et al. 2011; Matos-Cruz et al. 2011) and three orthologues of each class (named *opn4m1a1*, *opn4m1a2*, *opn4m2*, *opn4x1a*, *opn4x1b1* and *opn4x1b2*) in the salmon (Sandbakken et al. 2012). The inconsistent numbering of melanopsin genes in teleosts can be confusing, but in general duplications of each main *opn4* lineage have been identified in many modern bony fishes (Bellingham et al. 2006; Cheng et al. 2009; Davies et al. 2011; Drivenes et al. 2003; Matos-Cruz et al. 2011; Sandbakken et al. 2012) suggesting that (at least) two duplications occurred early in the evolutionary development of the ancestral teleost genome, most likely as a result of a whole genome duplication (WGD) event, a phenomenon that has been observed for many other genes, especially those of the GPCR superfamily (Amores et al. 1998; Jaillon et al. 2004; Meyer and Van de Peer 2005; Taylor et al. 2003). With regard to zebrafish melanopsin, this WGD has resulted directly in the retention of two genes in each *opn4* lineage (i.e. the *opn4m* class giving rise to *opn4m1* and *opn4m3*, and the *opn4x* class resulting in *opn4x1* and *opn4x2*) (Davies et al. 2011). In comparison, the salmon genome has undergone other melanopsin gene duplications (and losses): in particular the orthologue to zebrafish *opn4m3* has been lost in salmon and replaced by an independent species-specific duplication of the *opn4m1* gene to yield *opn4m1a1* and *opn4m1a2* (Sandbakken et al. 2012). Furthermore, the *opn4x1* orthologue identified in zebrafish has also duplicated further in salmon to result in *opn4x1b1* and *opn4x1b2* genes, in addition to a conserved *opn4x2* orthologue (Sandbakken et al. 2012). Interestingly, teleosts generally possess a third melanopsin class named *opn4m2* in zebrafish and other bony fishes (Davies et al. 2011). Unlike the other *opn4* genes, *opn4m2* is intronless and is likely to have arisen from the genomic reinsertion of a mature melanopsin mRNA via retrotransposition (Davies et al. 2011); such an event has precedent as it has been observed previously for the teleost rod opsin (*rh1*) gene (Bellingham et al. 2003a; Fitzgibbon et al. 1995). Not only are there differences at the genomic level with regard to the melanopsin gene complement in the zebrafish compared to the salmon, the expression profiles are markedly different. In the Atlantic salmon, the expression of both *opn4x* and *opn4m* melanopsin gene classes is limited to a small subset of RGCs, amacrine cells and horizontal cells (Sandbakken et al. 2012). However, in the zebrafish, melanopsin gene expression shows a wider tissue distribution with different isoforms detected in an overlapping, yet distinct pattern that encompasses all the major retinal layers (Davies et al. 2011). Of particular note is the identification of *opn4m2* expression at both transcript and protein levels in the photoreceptors of the zebrafish retina, specifically in the short-wavelength-sensitive cones that express the *sws1* and *sws2* opsin genes (Davies et al. 2011). The opn4m2 protein appears to form a ring-like structure close to the photoreceptor inner segment with a functional role that is, as of yet, unknown; however, it has been suggested that melanopsin expressed in this unexpected location may extend the spectral range of the photoreceptors involved, play a role in the circardian regulation of retinomotor

movements, modulate light adaptation under bright photic conditions or protect cone photoreceptors from calcium (Ca^{2+}) depletion under bright-light intensities (Davies et al. 2011). The molecular mechanism for the presence of opn4m2 transcripts in zebrafish cones requires confirmation since, being an intronless retrogene, it may have reinserted into the zebrafish genome downstream of regulatory elements that confer photoreceptor expression. Indeed, analysis of the upstream promoter region of the zebrafish *opn4m2* gene has identified two relevant transcription factor binding motifs, namely a cone photoreceptor regulatory element-1 (cpre-1) enhancer (in the proximal promoter) and a nuclear receptor subfamily 2 group E member 3-like site (in the distal promoter), that are not present in the promoter of the other four zebrafish melanopsin genes, which are not expressed in cones, and thus may account for the extraordinary expression pattern of the *opn4m2* gene (Davies et al. 2011). Therefore, given that retrogene genome reinsertion is a relatively random process, it is conceivable that other teleosts may lack these regulatory binding sites, and the ensuing photoreceptor expression profile, despite still possessing the intronless melanopsin gene. Nonetheless, the survival of the intronless *opn4* gene in the teleost genome must confer some functional advantage that is not solely dependent upon a role in visual photoreceptors.

Phylogenetically (and to a certain degree functionally as discussed below), the deuterostome melanopsin gene family is related to the rhodopsin class of invertebrates (e.g. *Rh1-6* of the fruit fly, *Drosophila melanogaster*; G_q-coupled rhodopsin of the scallop, *Mizuhopecten yessoensis*) (Borges et al. 2012; Peirson et al. 2009; Provencio and Warthen 2012; Terakita 2005) and, as such, is often described as "invertebrate-like" and classed as a rhabdomeric opsin (R-opsin) (Arendt 2003; Arendt et al. 2009; Lamb 2009, 2013). Although there are some signalling similarities (discussed further below), this designation once again is historical and results in a nomenclature that is confusing: vertebrates do not possess rhabdomeric photoreceptors and invertebrate rhodopsins could, given the same ruling, be renamed "vertebrate-like" which would be inaccurate and equally as perplexing. Evidence has been presented that suggests that all photoreceptors may be divided into two main structurally distinct classes, namely ciliary and rhabdomeric as these cells either contain a cilium that connects the inner and outer segments of the photoreceptor or comprise more villi-like projections, respectively (Arendt 2003; Arendt et al. 2009; Arendt and Wittbrodt 2001). Opsin pigments have equally been named either ciliary (C-opsin) or rhabdomeric (R-opsin) based on the type of photoreceptors that a particular organism possesses (Arendt 2003; Arendt et al. 2004; Lamb 2009, 2013; Lamb et al. 2009). However, this superimposed and derived argument is too simplistic as the majority of non-visual photoreceptors found in extant animals do not possess outer segments for clear morphological assignment (Davies et al. 2010). Furthermore, rapid successions of gene duplication and loss often affect the interpretation of gene origins and their linear progressions, with evolutionary relationships naturally falling into two groups as a result of the intrinsic binomial nature of phylogenetic analyses. A small number of studies have suggested that melanopsin-expressing cells (e.g. RGCs) exhibit developmental gene expression profiles that resemble those of rhabdomeres, with non-visual retinal cells (e.g. bipolar cells)

following a pattern that is similar to the classical ciliary visual photoreceptors, thus illustrating their distinct evolutionary origins (Arendt 2003; Arendt et al. 2009; Arendt and Wittbrodt 2001; Lamb 2009, 2013; Lamb et al. 2009). Once again the actual situation is far more complex with ocular non-visual photoreceptors often expressing both C- and R-opsin types (Davies et al. 2010), such as the presence of VA opsin (C-opsin) and melanopsin (R-opsin) in teleost horizontal cells (sometimes referred to an R-type cell) (Cheng et al. 2009; Jenkins et al. 2003) and the co-expression of *opn4m2* (R-opsin) and visual pigment (*sws1* and *sws2*; both C-opsins) genes in ciliary photoreceptors of the cyprinid zebrafish (*Danio rerio*) (Davies et al. 2011). Collectively, these data suggest that the use of such labels is misleading and inaccurate and should be used with caution (Davies et al. 2011).

Focussing on the chordate lineage, a single "melanopsin-like" gene was identified in the cephalochordate lancelet with a phylogeny that predates the duplication into the two *opn4* gene classes of the gnathostomes (Koyanagi et al. 2005) (Fig. 2.4). Recently, the complement of melanopsin genes was described in a modern representative of an early branch in the evolution of the cartilaginous fishes (chimaeras, sharks, rays and skates), namely the elephant shark, *Callorhinchus milii* (Davies et al. 2012b). The authors identified three *opn4* genes, with two belonging to the *opn4m* group that evolved from a species-specific gene duplication and the third being an *opn4x* orthologue, with expression patterns that included the eye, pineal gland, brain and skin (Davies et al. 2012b) (Fig. 2.4). Thus, it is now possible to date the chordate melanopsin gene duplication event to approximately 450–630 million years ago (MYA) (Sansom et al. 1996; Swalla and Smith 2008; Swalla and Xavier-Neto 2008).

2.4 General Considerations into the Structure of the Melanopsin Photopigment

Currently, a high-resolution crystal structure for melanopsin does not exist so much of the knowledge that exists regarding protein structure is inferred bioinformatically with homology modelling (e.g. melanopsin orthologues of the Djungarian hamster, *Phodopus sungorus* (Hermann et al. 2005) and the mouse (Sekharan et al. 2012)) or through comparisons with other known pigments such as the bovine rod opsin for which the overall three-dimensional structure is known (Palczewski et al. 2000). For all opsin classes presently studied, the core containing TM domains 1–7 remains largely conserved, which is generally thought to reflect the need to maintain a similar three-dimensional protein structure. Although there is a higher diversity of sequence within the third cytoplasmic domain (Fig. **2.5** and Table **2.1**) that may be the source of the ability for opsins to bind and activate different G proteins (Konig et al. 1989; Strader et al. 1989; Terakita et al. 2002; Yamashita et al. 2000), for example melanopsin coupling to a G_q-type alpha subunit (Bailes and Lucas 2013; Hughes et al. 2012a; Panda et al. 2005; Qiu et al. 2005), it is within the amino- and carboxyl-termini where the greatest degree of diversity is found. The amino-terminus is usually quite short in length, as is commonplace for visual opsin

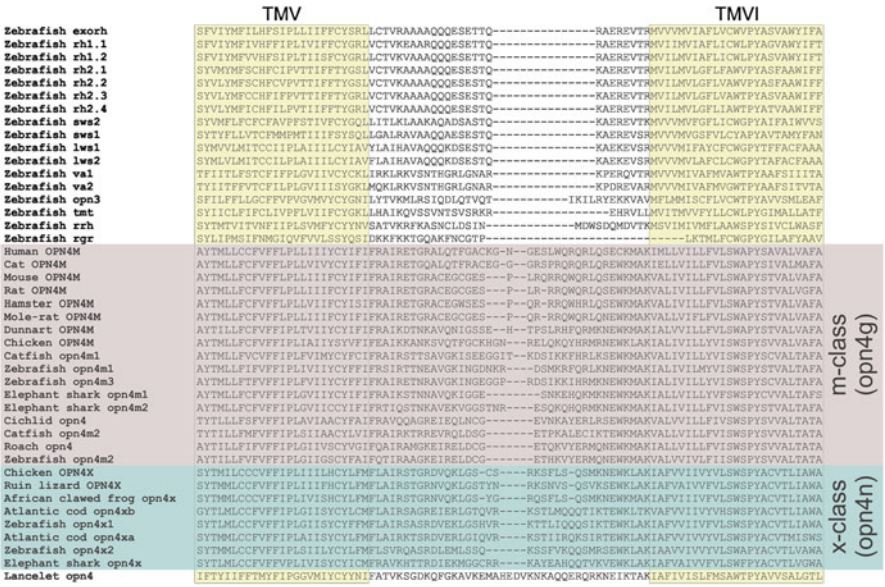

Fig. 2.5 Amino acid alignment of the third cytoplasmic loop (CLIII) of the chordate pigments shown in the phylogenetic tree of Fig. 2.4. Gaps were inserted to maintain a high degree of identity and are indicated by *dashes* (–), whereas *boxed yellow shading* denotes two transmembrane (TM) domains (i.e. TMV and TMVI). Pigment classes include are as follows: (1) extraretinal rod-like opsin (exorh); (2) middle-wavelength-sensitive rhodopsin-like-1 (rod) opsin (rh1); (3) middle-wavelength-sensitive rhodopsin-like-2 (cone) (rh2); (4) short-wavelength-sensitive-2 (sws2); (5) short-wavelength-sensitive-1 (sws1); (6) long-wavelength-sensitive (lws); (7) vertebrate ancient opsin (va); (8) mammal-like/gnathostome melanopsin (opn4m/opn4g or m/g-class) (shaded in *purple*); (9) *Xenopus*-like/non-therapsid melanopsin (opn4x/opn4n or x/n-class) (*shaded in blue*); (10) lancelet (*Branchiostoma belcheri*) melanopsin; (11) retinal G protein-coupled receptor (rgr); (13) retinal pigment epithelium (RPE)-specific rhodopsin homologue (rrh) (peropsin); (13) teleost multiple tissue opsin (tmt); and (14) panopsin/encephalopsin (opn3). The gene nomenclature used follows the guidelines adopted by the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene). In brief, the genes of all terrestrial species are in *uppercase*, except for rodents, where only the *first letter* is *capitalised*. The genes of all aquatic species, including amphibians, are in *lowercase*

proteins, although the melanopsin amino-terminus is about double that of rod pigments (Davies et al. 2010). It is also the site of asparagine (Asn)-dependent or N-linked glycosylation, which is important for the structure and function of photopigments (Kaushal et al. 1994), although evidence suggests that some melanopsins (e.g. human OPN4M and a subset of elephant shark opn4 pigments) are not N-linked glycosylated (Davies et al. 2012b), or at the very least, that it may not be essential for photoactivity even when N-linked glycosylation is present (e.g. rat melanopsin) (Fahrenkrug et al. 2009).

By contrast, the carboxyl-terminus in melanopsin is generally much longer than most other pigment classes (Fig. 2.6); for example, it is up to six times longer than in the visual opsins (Davies et al. 2010). Therefore, the elongated carboxyl-tail may play a significant role in post-translational regulation of melanopsin function, for example

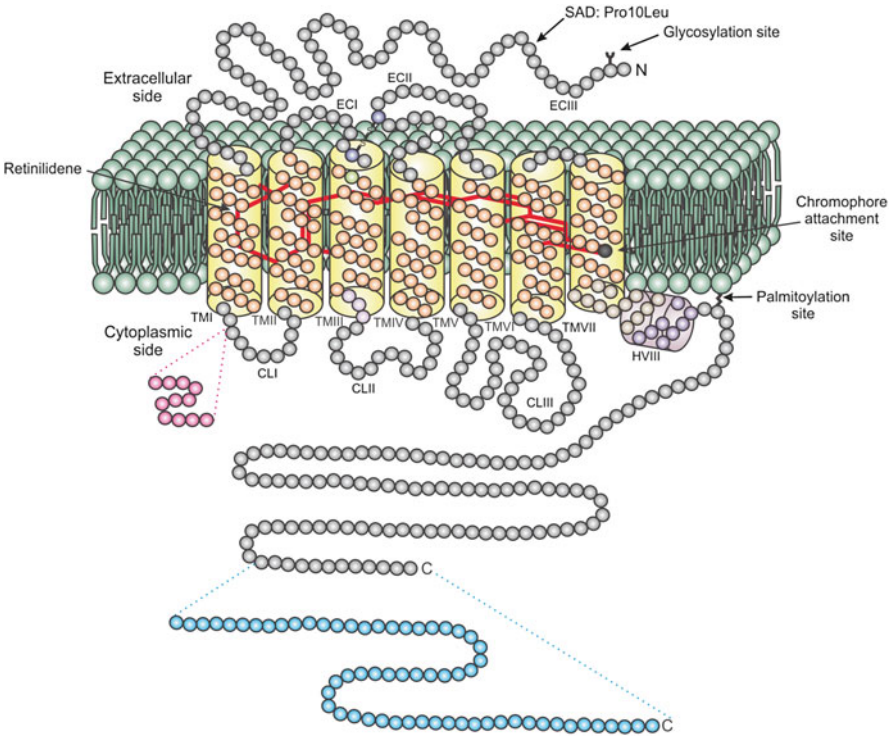

Fig. 2.6 A side-view schematic representation of the human melanopsin pigment showing the presence of seven transmembrane (TM) domains (TMI-VII) (*yellow*), three extracellular loops (ECI-III), three cytoplasmic loops (CLI-III), an amino-terminus (N), a carboxyl-terminus (C), a putative eighth cytoplasmic helix (HVIII) (*purple*) based on homology modelling with the bovine rod opsin crystal structure, and predicted glycosylation and palmitoylation sites. The 11-*cis* retinal chromophore (*red*) is shown attached via a Schiff bass linkage to Lys340 (equivalent to Lys296 using bovine rod opsin numbering) (*black*). The extra 11 amino acids (*pink*) present in CLI of an alternative variant and two different C-terminal isoforms, short (*grey*) and long (*blue*), are indicated. The Pro10Leu mutation associated with an increased risked of seasonal affective disorder (SAD) (Roecklein et al. 2009, 2013) is also highlighted. Modified from Davies et al. (2012d)

via the deactivation of activated pigments by kinases such as G protein-coupled receptor kinase 2 (GRK2) and protein kinase A (PKA) (Blasic et al. 2012a, b). Melanopsin, as well as other non-visual opsins such as VA opsin, is expressed as multiple splice-variants that differ at their 3′-ends and thereby encode polypeptides that possess divergently sized carboxyl-termini with different amino acid sequences that show little phylogenetic conservation (Davies et al. 2010, 2012b; Pires et al. 2009) (Fig. 2.6). Similar to the mechanism for generating different transcripts in other non-visual opsins, such as VA opsin (Davies et al. 2012c; Halford et al. 2009; Kojima et al. 2008; Minamoto and Shimizu 2002), multiple melanopsin variants are generated from a single gene transcript that either undergoes normal exon splicing or shows a failure to remove the last intron (Davies et al. 2012b, d; Hughes et al. 2012b; Pires et al. 2009;

Tomonari et al. 2005; Torii et al. 2007). In the latter case, the retention of the last intron means that translation proceeds through the penultimate exon to the next available termination codon in the adjacent intron, thus producing "long" and "short" carboxyl-terminal variants (Davies et al. 2012b, d; Hughes et al. 2012b; Pires et al. 2009; Tomonari et al. 2005; Torii et al. 2007). Although not extensively studied, different isoforms have been identified in cartilaginous fishes (e.g. elephant shark, but only from the *opn4x* gene) (Davies et al. 2012b), birds (e.g. chicken, where both *OPN4M* and *OPN4X* genes generate "long" and "short" isoforms) (Tomonari et al. 2005; Torii et al. 2007) and mammals (e.g. mouse and human (Fig. 2.6), with two variants that are produced from a single *Opn4m*/*OPN4M* gene) (Davies et al. 2012d; Pires et al. 2009), thereby demonstrating that this mechanism for increasing the repertoire of melanopsin transcripts is evolutionary conserved throughout the gnathostome vertebrates (Davies et al. 2010, 2012b). Only differing in their carboxyl-termini, these variant pigments form proteins that are predicted to be both spectrally and functionally similar (Davies et al. 2012b; Pires et al. 2009; Torii et al. 2007), although other aspects such as deactivation kinetics acting through differential phosphorylation may be dissimilar (Davies et al. 2012b; Pires et al. 2009). In the mouse, isoform-specific immunocytochemical experiments have shown that some RGCs express the "long" isoform only, whilst other cells contain both "long" and "short" variant proteins (Pires et al. 2009). Furthermore, these two isoforms are developmentally regulated (Hughes et al. 2012b). At present the functional relevance of these multiple melanopsin variants is unclear, although in some cases they appear to correlate with the development and maturation of certain pRGC subtypes (e.g. M1 and M2 cells, although five distinct subtypes (M1–M5) have been identified to date (Berson et al. 2010)) that show different biophysical properties, dendritic striations and retinal distribution (Do and Yau 2010; Hughes et al. 2012a, b, 2013; Schmidt et al. 2011a, b; Schmidt and Kofuji 2011). In terms of phylogeny, multiple melanopsin isoforms from a single gene may, at least in mammals, serve to compensate for the loss of the *OPN4X* gene. Interestingly, the human *OPN4M* gene has been shown to transcribe additional variants where the alterative splicing events occur internally within the gene sequence of the protein-coding region and not just at the 3′-end (Davies et al. 2012d). Specifically, these splice-variants differ in the length of the first cytoplasmic loop by 11 amino acids (Fig. 2.6); but the functional significance of these changes remains unknown (Davies et al. 2012d).

2.5 Melanopsin and Retinoid Biochemistry

Any opsin protein is generally rendered photosensitive by the addition of a retinal chromophore (usually 11-*cis* retinal) that resides within the core of the pigment molecule (Figs. 2.1 and 2.6). Much work has been performed using visual opsins, in particular the bovine rod opsin pigment, as model proteins for determining structure-function information (Franke et al. 1988, 1992; Hargrave et al. 1983; Karnik and Khorana 1990; Karnik et al. 1988, 1993; Kaushal et al. 1994; Nathans 1990b;

Palczewski et al. 2000; Sakmar et al. 1991) and it is now clear that specific residues that contour the so-called retinal binding pocket perform a number of important roles (Davies et al. 2012a; Yokoyama 2000). Firstly, Lys296 in the seventh TM domain is critical for the formation of the Schiff base linkage that physically connects the opsin apoprotein to the retinoid molecule (Pepe 1999). Secondly, a relatively small number of residues (spectral "tuning" sites) interact with the electron-dense cloud of the chromophore to determine the overall spectral sensitivity of absorbance, a mechanism that is best illustrated by the examination of pigments that mediate colour vision (Davies et al. 2012a; Yokoyama 2000). Thirdly, the presence of a "counterion" to stabilise the positive charge of the Schiff base upon protonation, thus allowing the pigment to absorb photons within the visible light spectrum (Nathans 1990a; Sakmar et al. 1989; Zhukovsky and Oprian 1989), the exception being many SWS1 pigments that are unprotonated and UV sensitive, although those SWS1 pigments that are spectrally shifted towards longer wavelengths perceived as violet are protonated (Davies et al. 2012a; Hunt et al. 2007, 2009).

Like all opsins, melanopsin is also linked to its chromophore via Lys296 (Provencio et al. 1998b) and appears to be protonated to yield a spectral peak of absorbance (λ_{max}) close to 480 nm (Bailes and Lucas 2013; Davies et al. 2011; Koyanagi et al. 2005; Matsuyama et al. 2012; Qiu et al. 2005; Torii et al. 2007). Visual pigments generally possess a negatively charged Glu113 residue within the retinal binding pocket to counteract the net-positive charge of the protonated Schiff base (Sakmar et al. 1989). By contrast, melanopsin, as well as many other non-visual opsins, has an uncharged tyrosine residue at this site (Tyr113) (Provencio et al. 1998b). By using a site-directed mutagenesis approach, it has been shown that Glu181 may act as a functional counterion (Terakita et al. 2004), with the more familiar role for Glu113 evolving later within the class of visual pigments (Terakita et al. 2004). Thus, it seems that both "tuning" sites and the role of the counterion are important in determining the spectral properties of any given pigment.

For many years, one of the main goals for those that work in *opn4*-related photobiology was to determine the spectral peak of the melanopsin pigment and correlate it to known action spectra for both photosensitive RGCs (pRGCs) (the site of melanopsin expression in the mammalian eye, although the *opn4* gene is also expressed in non-ocular locations in non-mammals) and an array of non-visual tasks, such as photoentrainment, in an attempt to show that melanopsin was the underlying light-sensing molecule. Researchers tackled this problem on two parallel fronts: firstly, the *Opn4* gene was ablated in murine model systems and the effect on physiology observed as discussed below, and secondly, melanopsin was exogenously expressed in cells that were not intrinsically photosensitive. In the latter case, full-length mammalian OPN4 constructs, namely the human (*OPN4M*) and murine (*Opn4m*) orthologues, were transfected into mouse-derived Neuro2A cells (Melyan et al. 2005; Pires et al. 2009), *Xenopus laevis* oocytes (Panda et al. 2005) and human embryonic kidney 293 (HEK293) cells (Qiu et al. 2005) and shown to induce an 11-*cis* or 9-*cis* retinal-dependent endogenous phototransduction cascade upon the application of wavelengths perceived as blue light (420–480 nm). Subsequent electrophysiological studies have yielded similar results when assaying melanopsin orthologues

derived from non-mammalian species including the elephant shark, *C. milii* (Davies et al. 2012b), zebrafish, *D. rerio* (Davies et al. 2011) and chicken, *G. gallus* (Bellingham et al. 2006). In parallel, many melanopsin photopigments have been regenerated in vitro and reconstituted with appropriate retinoids to determine the spectral characteristics of the isolated protein. For non-mammalian orthologues, melanopsin when reconstituted with 11-*cis* retinal in the dark phase yields pigments with λ_{max} values that are close to the predicted spectral peak of 480 nm (e.g. lancelet, 485 nm (Koyanagi et al. 2005), zebrafish, 470–484 nm (Davies et al. 2011) and chicken, 476–484 nm (Torii et al. 2007)) that is important for the light regulation of daily biological rhythms at dusk and dawn when the sun is close to the horizon (Bellingham and Foster 2002; Brown and Robinson 2004; Chen et al. 2011; Davies et al. 2010; Do and Yau 2010; Foster et al. 2007; Hankins et al. 2008; Hannibal and Fahrenkrug 2002; Hattar et al. 2002; Lucas et al. 2012; Markwell et al. 2010; Panda et al. 2002; Peirson et al. 2005, 2009; Schmidt et al. 2011b; Weng et al. 2009). Under these periods of the circadian cycle, the sky is generally enriched with short-wavelengths of light due to the scattering of "blue" light when passing obliquely through the atmosphere (Thorne et al. 2009); thus, it seems logical that any photopigment primarily detecting light at these time points would be spectrally tuned to maximise photon capture at these wavelengths (Davies et al. 2010, 2012a).

Despite this collective evidence, direct determination of the spectral sensitivity of melanopsin in higher mammals, such as mice and humans, remained elusive or inconsistent at best for many years using traditional in vitro protein regeneration and reconstitution techniques. This was most likely due to technical issues that resulted in unstable opsin-retinoid complexes as is commonplace for in vitro non-visual opsin pigment work (Davies et al. 2010) and is the probable cause for melanopsin spectral sensitivity values that were far from those predicted by action spectra, electrophysiology and analysis of OPN4 protein biochemistry in other species (i.e. ~480 nm). For example, the mouse Opn4 photopigment was initially shown to exhibit a λ_{max} between 420 and 440 nm (Newman et al. 2003). More recently, however, this dark-adapted spectral maximum was revised to 467 nm in in vitro experiments where the carboxyl-terminus of the murine Opn4 pigment had been truncated (Matsuyama et al. 2012), an approach that also permitted the spectral peak of melanopsin (λ_{max} = 485 nm) to be successfully determined for the cephalochordate lancelet, *Branchiostoma belcheri* (Koyanagi et al. 2005). Similar investigations with the human melanopsin pigment (using both native and carboxyl-terminal truncated forms) have, however, failed to yield a definitive λ_{max} value using in vitro regeneration techniques alone. Nonetheless, a robust indirect method using an aequorin reporter assay, which measured the Ca^{2+} second-messenger response of HEK293 cells transfected with melanopsin constructs under different wavelengths of light, produced an action spectrum for human melanopsin with a λ_{max} value at 479 nm (Bailes and Lucas 2013). When applied to full-length mouse melanopsin, this approach generated a spectral peak at 484 nm (Bailes and Lucas 2013), which is consistent with spectral sensitivity estimates obtained from studies with mice that lack both cones and rods (Hattar et al. 2003; Panda et al. 2005; Qiu et al. 2005). A *prima facie* comparison between experiments that utilise native versus truncated

forms of the murine Opn4 pigment suggests that the C-terminal tail may affect spectral tuning and hence account for the 17 nm difference in λ_{max} values between direct in vitro regeneration (Matsuyama et al. 2012) and indirect action spectral analyses (Bailes and Lucas 2013). Even though the potential spectral tuning effects of an opsin C-terminal tail have been implicated previously (e.g. in rod opsin, Yokoyama et al. 2007), the ultimate explanation is unclear and may be wholly or partly due to differences in experimental design and analytical methodologies, or critical factors that are absent in heterologous expression systems: such issues continue to plague pigment biochemists.

The difficulty in determining the spectral (and functional) characteristics of melanopsin (especially mammalian orthologues) may also be influenced by the atypical way this pigment appears to interact with its chromophore and its apparent resistance to in vitro and in vivo chemical and photobleaching in some species (Newman et al. 2003; Sexton et al. 2012). Generally, photopigments are classed as being either monostable (i.e. functionally interacts with only the *cis* isomer of the retinal chromophore) or bistable (i.e. functionally interacts with both *cis* and *trans* isomers of the retinal chromophore) (Tsukamoto and Terakita 2010). Traditionally, the pigments expressed in the ciliary photoreceptors of the vertebrate camera-like eye and the rhabdomeres of the invertebrate compound eye have been heralded as archetypal monostable (Davies et al. 2012a; Yokoyama 2000) and bistable (Hillman et al. 1983) pigments, respectively. However, the discovery of melanopsin as a putative bistable pigment (Davies et al. 2011, 2012b; Koyanagi et al. 2005; Matsuyama et al. 2012; Melyan et al. 2005; Mure et al. 2007, 2009; Sexton et al. 2012; Walker et al. 2008), partly based on its close phylogenetic relationship to invertebrate pigments (Borges et al. 2012; Peirson et al. 2009; Provencio and Warthen 2012; Terakita 2005), demonstrated that the classification of pigments based on their chromophore usage was a complex and often misleading distinction. Such a situation exists with the constant comparison and misnaming of vertebrate melanopsin as "invertebrate-like", although there are some similarities in their signalling pathways (as described below) (Isoldi et al. 2005; Panda et al. 2005). Whilst technically difficult to show directly, several lines of evidence demonstrate that melanopsin pigments are likely to be bistable, a property that may allow melanopsin to act partly as an endogenous photoisomerase to regenerate 11-*cis* retinal from all-*trans* retinal (Foster and Bellingham 2002). In a well-established heterologous expression system, human OPN4 has been shown to illicit light-dependent electrophysiological responses under both short-wavelength and long-wavelength light when incubated with 9-*cis* and 11-*cis* retinoids (Melyan et al. 2005) and thus was likely to possess a direct role in cellular photosensitivity in vivo. Another example is that of the lancelet (amphioxus), where the melanopsin orthologue has been shown to form distinct stable photopigments when illuminated with different wavelengths of light: in the dark the amphioxus opn4 pigment forms a complex with 11-*cis* retinal with a λ_{max} value at 485 nm, which "bleaches" under blue light to yield two spectral peaks at 420 and 520 nm, and then reverts to its original spectral state upon further illumination with orange light (Koyanagi et al. 2005). Whole-cell electrophysiological experiments using cells transfected with melanopsin orthologues from other species (e.g. elephant

shark and zebrafish) have been shown to function when either *cis* or *trans* (e.g. all-*trans*) isomers of retinal are used, thus demonstrating that stable interactions are formed between the opn4 protein and each chromophore independently (Davies et al. 2011, 2012b). In these latter studies, melanopsin presents with a higher affinity for 11-*cis* retinal compared to all-*trans* retinal, which appears to contradict the "bistable" photochemical nature of this pigment class. A similar situation has, however, been shown for the confirmed bistable Amphiop1 pigment of the lancelet, *Branchiostoma belcheri*, which exhibits an affinity for 11-*cis* retinal about 50-fold greater than for all-*trans* retinal (Tsukamoto et al. 2005). For many years, it was assumed that melanopsin interacted with only two types of retinoid, 11-*cis* retinal (or 9-*cis* retinal in many in vitro experiments) and all-*trans* retinal, an assumption partly based on the way the chromophore photoisomerises in visual pigments. More recently, however, experiments with the mouse melanopsin orthologue has shown that although a light-dependent equilibrium does exists between photostable products that interact with both 11-*cis* retinal (native melanopsin with a λ_{max} at 467 nm) and all-*trans* retinal (metamelanopsin with a λ_{max} at 476 nm), a third photostable product (extramelanopsin) can form under long-wavelength irradiation that interacts with 7-*cis* retinal to give a λ_{max} at 446 nm, and photoconverts back to metamelanopsin when illuminated with short-wavelength light (Matsuyama et al. 2012).

Despite the many studies that support the bistability of melanopsin, both UV-visible spectrophotometric and electrophysiological techniques have shown that in some non-mammalian species melanopsin forms a monostable pigment that is only able to form stable interactions with *cis* isomers of the retinal chromophore, namely 9-*cis* retinal and 11-*cis* retinal, and thus resembles the retinoid biochemistry of vertebrate visual pigments (Davies et al. 2011, 2012b). In the zebrafish, both opn4x1 and opn4m2 pigments are monostable (Davies et al. 2011) and the opn4x protein isoform is also monostable in the elephant shark (Davies et al. 2012b). Thus, the chromophore valency status of melanopsin photopigments is far more heterogeneous than initially thought, with bistability not being a universal characteristic of chordate melanopsin pigments or limited to a particular class (i.e. m-class versus x-class); nonetheless, opn4 monostability may be a common feature in non-mammalian species, in addition to one or more bistable melanopsin pigments (Davies et al. 2011, 2012b).

2.6 The Melanopsin Signalling Cascade

Subsequent to light-dependent conversion of the retinilidene chromophore, all pigments undergo a conformational change that permits the binding and activation of a G protein trimeric complex. This in turn initiates a series of biochemical steps that terminate in the production of a cellular potential difference. Collectively, it is this so-called phototransduction cascade that converts (and amplifies) a photoresponse into an electrical signal that is ultimately conveyed to an array of cranial processing areas. In classical photoreceptors (i.e. cones and rods), the phototransduction

cascade is well characterised, with each cell-type utilising similar biochemical pathways that involve the activation of transducin (G_i/G_o class), which in turn regulates phosphodiesterase (i.e. PDE6), then guanylyl cyclase, and finally a closure of cyclic nucleotide-gated (CNG) ion channels, resulting in a hyperpolarising membrane potential (Arshavsky et al. 2002; Lamb 2013; Lamb et al. 2009). Although the types of proteins and their activation progression are similar between cones and rods, each cell-type utilises specific isoforms for a number of the steps of phototransduction that derive from orthologous genes that encode subtle functional differences in the properties of these proteins that mediate the cellular differences observed between these two types of outer retinal photoreceptors (Hisatomi and Tokunaga 2002; Kawamura and Tachibanaki 2008; Larhammar et al. 2009; Makino et al. 2003). For example, cone transducin consists of three proteins encoded by the genes guanine nucleotide binding protein (G protein) alpha transducing activity polypeptide 2 (*GNAT2*; α-subunit), G protein beta polypeptide 3 (*GNB3*; β-subunit) and G protein gamma transducing activity polypeptide 2 (*GNGT2*; γ-subunit); whereas rods express *GNAT1*, *GNB1* and *GNGT1* to encode for α-, β- and γ-subunits, respectively (Hisatomi and Tokunaga 2002; Larhammar et al. 2009; Lerea et al. 1986).

Although the phototransduction cascade is initially similar for melanopsin compared to the signalling pathways in cone and rod photoreceptors (i.e. photon absorbance leading to the activation of a G protein cascade), the subsequent steps in the generation of an electrical signal are markedly different especially in causing cellular depolarisation and not hyperpolarisation as is the case with visual photoreception (Hughes et al. 2012a; Panda et al. 2005; Peirson and Foster 2006) (Fig. 2.7). Electrophysiological and pharmacological studies on isolated pRGCs and in vitro expression systems suggested early on that the melanopsin photopigment acted through a $G_{q/11}$ Ca^{2+}-dependent signalling pathway (Graham et al. 2008; Hartwick et al. 2007; Melyan et al. 2005; Panda et al. 2005; Qiu et al. 2005; Warren et al. 2006), where antibodies raised against G_q/G_{11} G proteins were shown to attenuate depolarising responses to light, an effect that could not be replicated by using antibodies raised against $G_{i/o}$ G proteins (i.e. transducin) (Panda et al. 2005). Similarly, in non-photoreceptive cell-lines transfected to express melanopsin, an antagonist of G_q/G_{11} G proteins was shown to block light-dependent responses in the presence of the OPN4 photopigment (Melyan et al. 2005; Qiu et al. 2005). More recently, it has been demonstrated that the melanopsin orthologue identified in the lancelet is able to directly couple to a G_q-type G protein (Terakita et al. 2008). Such experiments, coupled with the evolutionary origins of the *opn4* gene and the bistable nature of its protein product, have indicated that melanopsin resembles invertebrate photoreceptors (e.g. those found in the compound eye of *D. melanogaster*) and, as such, may share many other features of a G_q/G_{11}-type signalling pathway (Do and Yau 2010; Hankins et al. 2008; Panda et al. 2005) (Fig. 2.7). Although much evidence strongly implicates the involvement of $G_{q/11}$ G proteins, which one of the four possible alpha subunit subtypes (i.e. G_q, G_{11}, G_{14} or $G_{15/16}$ (Davignon et al. 1996; Wilkie et al. 1992)) couples to melanopsin is unclear, although since $G_{15/16}$ does not appear to be expressed in the mammalian retina (Peirson et al. 2007) this candidate may be ruled out (Hughes et al. 2012a). Of the three remaining candidates, G_{14} is highly expressed in the retina; nonetheless, it is presently very difficult to assign relative functional

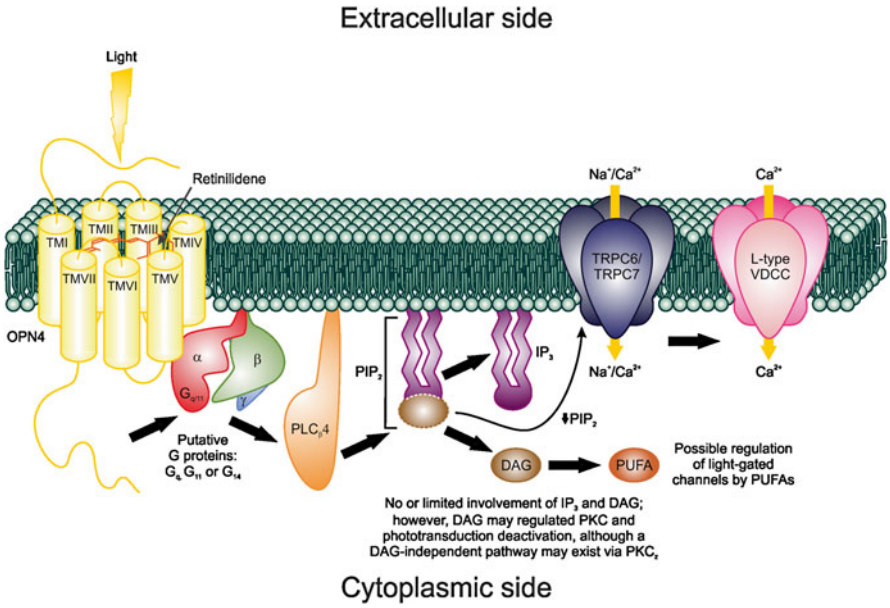

Fig. 2.7 A diagram showing the current hypothesis of how the melanopsin (OPN4) phototransduction cascade generates a depolarising current, thus converting photons into electrical signals. Initially, photostimulation of the retinal chromophore (*orange*) within the melanopsin pigment (*yellow*) results in a conformational change of some of the seven transmembrane (TM) domains (TMI-VII) to permit the binding and activation of a $G_{q/11}$-type G protein, which itself consists of three subunits (alpha, α (*red*); beta, β (*green*); and gamma, γ (*blue*)). This in turn leads to phospholipase C-beta 4 (PLCβ4) (*light orange*) activity, which catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (*purple* and *dark brown* complex) into inositol 1,4,5-trisphosphate (IP_3) (*purple*) and 1,2-diacylglycerol (DAG) (*dark brown*). Although neither IP_3 nor DAG second messengers are thought to play a direct signalling role (except for the putative regulation of light-gated ion channels by polyunsaturated fatty acids (PUFAs) (*light brown*)), it is hypothesised that a reduction in PIP_2 levels modulates two transient receptor potential (TRP)-like (C class) channels (*navy*), namely TRPC6 and TRPC7. As a result, an influx of Ca^{2+} (and possibly Na^+) ions activates a number of other ion channel proteins, including the L-type voltage-dependent calcium channels (L-VDCCs) (*pink*). Despite this overall account, many details that underpin key steps in the signalling pathway of melanopsin-expressing cells (e.g. deactivation, putatively via protein kinase C zeta (PKC_z)) are unclear or unknown altogether. Modified from Hughes et al. (2012a)

significance with regard to the opn4 signalling pathway as they are all present in pRGCs (Graham et al. 2008; Hughes et al. 2012a).

Once activated, the G_q/G_{11} G protein typically interacts with phospholipase C-beta (PLCβ), a complex that has been implicated in the melanopsin-dependent light response of both mammalian and non-mammalian species (Contin et al. 2006; Graham et al. 2008; Isoldi et al. 2005; Nasi and del Pilar Gomez 2009). Although the specific PLCβ isoform is not known, PLCβ4 is highly expressed in the retina, especially in cones and pRGCs (Adamski et al. 1999; Ferreira and Pak 1994; Ferreira et al. 1993; Graham et al. 2008), and pRGC photoresponses are significantly negated in $PLC\beta4^{-/-}$ knockout mice models (Xue et al. 2011). Once activated

PLCβ causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Hubbard and Hepler 2006; Mizuno and Itoh 2009) (Fig. 2.7). Although important for signalling in other sensory systems, the application of analogues to membrane patches or intracellularly for both IP_3 and DAG do not modify pRGC responses, suggesting that they are not involved in the melanopsin phototransduction cascade (Graham et al. 2008; Hartwick et al. 2007; Warren et al. 2006). Nonetheless, the activity of PLCβ results in a decrease in the concentration of PIP_2, an effect that is known to modulate ion channels that are gated by light (Hardie 2003, 2007; Raghu 2006; Suh and Hille 2008). Indeed, the application of wortmannin, which inhibits PIP_2 synthesis, leads to differences in pRGC photosensitivity compared to controls (Graham et al. 2008). Despite the lack of DAG involvement in the activation of melanopsin signalling pathways (although it may be important with regard to the deactivation of phototransduction), studies in *D. melanogaster* have suggested that polyunsaturated fatty acids (PUFAs) that result from the breakdown of DAG may directly influence light-gated ion channels (Chyb et al. 1999b), so it is possible that such a mechanism exists in vertebrate pRGCs (Hughes et al. 2012a) (Fig. 2.7).

Unlike the classical visual photoreceptors that mediate cellular hyperpolarisation by the movement of ions through cyclic guanosine monophosphate (cGMP)-dependent CNG channels (Arshavsky et al. 2002; Lamb 2013; Lamb et al. 2009), pRGC depolarisation is regulated by transient receptor potential (TRP)-like channels that are transiently permeable to Ca^{2+} (and perhaps Na^{+}) ions (Do et al. 2009; Schmidt and Kofuji 2009; Warren et al. 2006) (Fig. 2.7). This is yet another component that the melanopsin signalling pathway shares with the invertebrate phototransduction cascade. In particular, pharmacological and electrophysiological studies suggest that TRP class C (TRPC) channels are the most likely final proponents of pRGC signalling pathways, with TRPC3, TRPC6 or TRPC7 heralded as putative candidates (Hartwick et al. 2007; Sekaran et al. 2007; Warren et al. 2006); however, as TRPC3 is not expressed in pRGCs (Sekaran et al. 2007; Warren et al. 2006) the involvement of this particular TRP channel can be excluded (Hughes et al. 2012a). Despite these findings, more recent work has suggested that neither TRPC3, TRPC6 nor TRPC7 are involved in melanopsin signalling as no effects on pRGC light responses were observed when each gene was obliterated in murine models (Perez-Leighton et al. 2011); this single study does not, however, negate the possible involvement of TRPC channel redundancy or heteromeric channel formation (Hughes et al. 2012a; Schaefer 2005). Indeed, another study demonstrated that knocking out the function of each TRPC channel gene individually did not alter melanopsin-dependent photoresponses in mice (Xue et al. 2011). Nonetheless, all pRGC light-induced currents were abolished in a double knockout model where both *Trpc6* and *Trpc7* genes were ablated (Xue et al. 2011), showing that these two candidates are critical for melanopsin phototransduction, most likely in a biophysically cooperative manner (Hughes et al. 2012a; Xue et al. 2011). Downstream of TRPC activation, the Ca^{2+} ion influx ultimately leads to the stimulation of other ion channels and the generation of an action potential (Graham et al. 2008; Hartwick

et al. 2007; Warren et al. 2006), of which the L-type voltage-dependent calcium channels (L-VDCCs) have received some attention with regard to pRGC light responses (Hartwick et al. 2007). Nonetheless, there is still much to discover regarding the full component of ion channels involved in melanopsin signalling pathways (Hughes et al. 2012a).

Similarly lacking in knowledge are those components of the pRGC phototransduction cascade that are involved in melanopsin deactivation (Hughes et al. 2012a), presumably through protein kinase C (PKC) (or perhaps PKA) activity (Chyb et al. 1999a; Hardie and Raghu 2001; Yau and Hardie 2009) and the binding of arrestin (Hardie 2001; Hardie and Raghu 2001; Panda et al. 2005). It has been suggested that a PKC subclass member, namely PKC_z, might play a central role since the retinal expression of the *Prkc*$_z$ gene was found to be different in mice devoid of visual photoreceptors compared to wild-type controls (with cones and rods) upon photostimulation (Peirson et al. 2007), and characterisation of circadian photoentrainment through the use of pupillometric and behavioural tests in mice lacking the *Prkc*$_z$ gene was almost indistinguishable from *Opn4*$^{-/-}$ animal models (Hughes et al. 2012a; Peirson et al. 2007). Interestingly, PKC_z is atypical of other PKC family members as it lacks both DAG and Ca^{2+}-binding domains (Mellor and Parker 1998), an observation that may underpin the supposed lack of direct DAG involvement in the melanopsin phototransduction cascade (Hughes et al. 2012a).

2.7 Function of the Melanopsin Sensory System

Initially, a mouse model found to be naturally homozygous for the *rodless* or *retinal degeneration* gene (*rd/rd*) was shown to lack all rods and most cones, and as such was essentially blind due to severe degeneration of the retina (Bowes et al. 1990; Keeler 1924). When analysed, these animals retained their ability to photoentrain with a sensitivity that was indistinguishable from wild-type controls that possessed a normal complement of visual photoreceptors, demonstrating that rods were not responsible for regulating circadian rhythms (Foster et al. 1991; Provencio et al. 1994). In response, further murine models (e.g. *rd/rd cl*) were produced that lacked both rods (again via an *rd/rd* genotype, which consists of a mutation in the rod-specific beta subunit of Pde6, namely the *Pde6b* gene (Bowes et al. 1990)) and cones (due to a mutation in the gene that encodes for the cone-specific alpha subunit a retinal CNG channel (i.e. *Cnga3*) (Biel et al. 1999)). Once again, photoentrainment was not significantly altered in these sightless mice, showing that cones were also not involved (Lucas et al. 1999; Semo et al. 2003). Nonetheless, the loss of circadian rhythm regulation in the absence of eyes supported the hypothesis that a third, novel non-cone/non-rod system of photoreception existed within the retina (Freedman et al. 1999), specifically within the RGC layer (Provencio et al. 1998a).

Based on several lines of evidence, melanopsin was initially proposed as a strong photopigment candidate for underpinning circadian regulation in the newly discovered inner retinal photoreceptors, specifically the pRGCs (Hankins et al. 2008). An

early study using fluorescent immunocytochemistry demonstrated that Opn4 mRNA was expressed in about 1 and 2.5 % of RGCs, respectively, in the mouse or rat retina (Hattar et al. 2002). Subsequent retrograde tracing showed that about 75% of neurons from these Opn4-expressing RGCs projected via the retinohypothalamic tract (RHT) to suprachiasmatic nuclei (SCN) in the rat brain, a small paired structure in the anterior hypothalamus (Gooley et al. 2001). Since the SCN region contains a central molecular clock that synchronises with the environmental light/dark cycle, in particular at dusk and dawn, the link was made between melanopsin-positive pRGCs and photoentrainment of the "master" circadian clock. The property of intrinsic photosensitivity in melanopsin-expressing RGCs in the rat was demonstrated by whole-cell recording. This was shown to have a peak absorbance at 480 nm (Berson et al. 2002), a spectral maximum that generally matches the action spectrum for circadian photoentrainment, as discussed above (Berson et al. 2002; Dacey et al. 2005; Hankins and Lucas 2002; Hattar et al. 2003; Lucas et al. 2001), and by the continuation of RGC photoactivity when cell–cell communication was pharmacologically inhibited or RGCs were surgically isolated (Berson et al. 2002). Similar evidence arose from parallel studies using Ca^{2+}-imaging of the retinae of murine models devoid of both rods and cones that demonstrated that a subset of RGCs could differentially depolarise directly after light exposure to generate three different responses, namely cells with repetitive, transient or sustained activities (Sekaran et al. 2003). Collectively, these approaches and many subsequent studies have identified and confirmed that a population of RGCs, which express melanopsin, is able to respond directly to light (Berson et al. 2002; Dacey et al. 2005; Lucas et al. 1999).

Using mice in which their *Opn4* gene locus was replaced by a tau-LacZ reporter gene, it was revealed that β-galactosidase-positive pRGCs also target other central sites involved in the detection of ambient illuminance, sleep regulation and circadian photoentrainment, for example, these include the intergeniculate leaflet, the olivary pretectal nuclei, the ventral subparaventricular zone and the ventrolateral preoptic area (Hattar et al. 2002). From these findings, melanopsin was suggested, therefore, to be associated with divergent non-visual photoresponses (Bailes and Lucas 2010; Davies et al. 2010; Hatori and Panda 2010; Provencio 2011; Rollag et al. 2003). The first important data that melanopsin plays a critical role in the transduction of light information from pRGCs to regulate a multitude of physiological systems came from gene ablation studies. Melanopsin knockout mice (*Opn4*$^{-/-}$) exhibited attenuated phase-shifting and pupillary responses to light, as well as reduced period lengthening in constant light (known as a 12 h/12 h light/light (LL) cycle) (Lucas et al. 2003; Panda et al. 2002; Ruby et al. 2002). However, the critical involvement of melanopsin in non-visual photoreception came from triple-knockout mice that lacked cones, rods and melanopsin-expressing RGCs. These animals were totally unresponsive to light, demonstrating that melanopsin was in some way essential for pRGC photosensitivity (Hattar et al. 2003), but precisely which functions melanopsin were playing were only finally resolved by using direct functional expression studies (Melyan et al. 2005; Panda et al. 2005; Qiu et al. 2005).

By specific targeted cell ablation, it was shown that pRGCs are the exclusive conduits for non-visual light inputs to the mouse brain (Guler et al. 2008). Under

low light conditions, visual and non-visual photic responses appeared to be comparable to wild-type controls, even in *Opn4*$^{-/-}$ murine models; however, as the intensity of light was increased, the loss of melanopsin caused defects in pupil constriction, phase-shifting and photoentrainment, suggesting that this pigment is predominantly functional under bright-light conditions and most likely modulates circadian physiology with synergistic inputs from other photoreceptive systems (Dacey et al. 2005; David-Gray et al. 1998; Hattar et al. 2003; Lall et al. 2010; Lucas et al. 2012; Provencio and Foster 1995). Indeed, recent melanopsin knockout studies, amongst other investigations, have revealed that cones and rods clearly contribute to non-image-forming light responses and in some cases may compensate for the loss of pRGC activity, as well as demonstrating a role for melanopsin in regulating classical vision (Bailes and Lucas 2010; Hankins and Lucas 2002; Lucas et al. 2012; Vandewalle et al. 2007). For example, it has been electrophysiologically demonstrated that primate cones sensitive to short-wavelengths (i.e. S-cones) are able to diminish pRGC firing, whereas pRGC responses are enhanced by the activity of the two remaining cone types (M-cones and L-cones, which are sensitive to middle-wavelengths and long-wavelengths, respectively) and rods (Dacey et al. 2005). Despite these insights, many of the details that underpin the cooperative interaction between visual and non-visual photosensory systems remain unknown; nonetheless, it is undoubtedly becoming a very exciting area of photobiological research.

2.8 Clinical Considerations

Perhaps one of the most clinically relevant observations with regard to the melanopsin sensory system was the reduction in bright-light photoadaptation in animals where either the eye had been removed or the melanopsin gene had been obliterated (Freedman et al. 1999; Hattar et al. 2003; Lucas et al. 2003; Panda et al. 2002; Provencio et al. 1998a; Ruby et al. 2002). This translates directly to patients (Benarroch 2011; Davies et al. 2012d; Hatori and Panda 2010; La Morgia et al. 2011; Pickard and Sollars 2012) as humans that are blind through retinal degeneration, specifically a loss of cones and rods, are able to maintain photoentrainment as pRGCs are usually intact. Unfortunately, in severe congenital and progressive ocular diseases, carcinomas or injury, pRGCs may be damaged or lost subsequent to architectural changes to the retina through the absence of other cell types such as the visual photoreceptors. In some cases, the entire eye may be electively enucleated and replaced with a non-functional prosthetic eye. Whether any residual cells remain in the RGC layer (if enucleation is not performed) or the eye is detached, many patients frequently complain of significant disturbances to their "normal" sleep patterns or suffer from severe insomnia (Davies et al. 2012d).

The importance of pRGCs in human photoentrainment is particularly evident in people suffering from glaucoma, a leading cause of blindness worldwide. In humans (and animal models), glaucoma presents with a slow but progressive loss of RGCs and optic nerve damage, including those that are intrinsically photosensitive (Drouyer

et al. 2008; Jakobs et al. 2005; Wang et al. 2008), although a small number of studies suggest that pRGCs may be spared (Li et al. 2006, 2008), which may ultimately result in circadian rhythm dysfunction (Feigl et al. 2011). Thus, the conflicting studies over RGC loss in glaucoma remains controversial and requires further study.

With the increasing evidence that sleep disruption and circadian disorders are linked to both mental health issues (Lewy 2009; Wulff et al. 2009, 2010), such as mood disorders (e.g. bipolar and unipolar depression) (Jagannath et al. 2013) and schizophrenia (Pritchett et al. 2012; Wulff et al. 2012), and neurodegenerative conditions, such as Parkinson's disease (Archibald et al. 2009; Bodis-Wollner 2009; Willis 2008; Willis et al. 2008) and senile dementia (e.g. Alzheimer disease (Berisha et al. 2007; Hinton et al. 1986; Wu and Swaab 2007), it is vital that research that leads to clinical applications that promote the survival of the retina (e.g. through viral gene therapy (Lin et al. 2008; Lipinski et al. 2013), optogenetic approaches (Garg and Federman 2013) or sophisticated artificial implants (Chuang et al. 2014)), and in particular the melanopsin-expressing pRGCs, is encouraged and prioritised. This is especially important with an ageing society that sleeps less (Cajochen et al. 2006; Carrier et al. 2002), where the occurrence of cataracts is increasing and as a result may cause a decrease in the amount of light reaching the retina (Kessel et al. 2010; Mainster and Turner 2010; Turner et al. 2010), and where a "light hungry" work and entertainment culture is present that is generally unsynchronised from the normal light/dark circadian cycle (e.g. jetlag (Foster et al. 2013)) with photoperiods that habitually extend into the night or are illuminated over an entire 24-h period (Hebert et al. 1998; Jewett et al. 1991). A recent breakthrough in the potential treatment of circadian dysfunction (e.g. sleep) or deregulation of associated light-dependent physiological responses, such as photophobia and a defective pupillary light reflex, has been the discovery that certain sulphonamide compounds (named opsinamides) are able to act as potent synthetic antagonists of melanopsin function in vivo by competing with the endogenous chromophore for the retinal binding site (Jones et al. 2013). Importantly, their effects appear to be specific to melanopsin-expressing RGCs and do not affect image-forming visual responses mediated by cone and rod photoreceptors. Such an insight will invariably be of interest to the pharmaceutical industry and eventually the clinical stage, where newly designed small molecules may be manufactured to manipulate an array of normal physiologically and pathophysiologically photoadaptive behaviours (Jones et al. 2013).

Currently, no naturally occurring mutations in the *OPN4* gene have been identified that lead to a loss of pigment function and subsequent circadian disruption, although this is likely to be due to the lack of serious genetic screening programmes rather than redundancy of function or an insignificant role for melanopsin photoreception. Nonetheless, perturbation of melanopsin signalling has been linked to photophobia and light-exacerbated migraine (Noseda et al. 2010). Additionally, a homozygous polymorphic variant (encoding a proline to leucine substitution; Pro10Leu) (Fig. 2.6) has been identified in the human *OPN4* orthologue that appears to segregate with seasonal affective disorder (SAD) (Roecklein et al. 2009, 2013). Specifically, SAD presents as mild to severe depression during the short days of winter where the amount and duration of daylight is appreciably reduced compared

to the rest of the year. For example, in light-restricted regions such as Finland and the Arctic, the prevalence of SAD is approximately 10% compared to other countries where the annual environmental light regime is less disparate. Although the functional basis for this *OPN4* variant is unknown, the correlation between irradiance detection and depression is quite compelling (Lewy et al. 1987) and suggests that manipulation of the melanopsin-based circadian system, in conjunction with bright-light (usually coloured blue) therapy (Burns et al. 2009; Glickman et al. 2006; Lewy 2009; Lewy et al. 2009), may be beneficial.

2.9 Conclusions

For over a 100 years, the eye was thought to solely mediate light detection for image-forming processes, through a conduit of a duplex retina containing cones and rods for photopic (bright-light and colour) and scotopic (dim-light) vision, respectively. Surprisingly, about two decades ago it was shown that mice with retinal degeneration that eliminated all visual photoreceptors were still able to photoentrain their circadian rhythms, but the complete removal of the eye caused this process to cease. This strongly suggested that the mammalian eye contained a third, novel photoreceptor system and, as such, contained a triplex retina. However, the light-receptive molecules were not known and an intense search in both mammals and non-mammalian species heralded the identification of a number of new non-cone, non-rod photopigments, with pinopsin in birds, VA opsin in teleosts and opn4 in amphibians being amongst the first.

It was not until the discovery that melanopsin, originally identified in the melanocytes of frogs, was expressed in the inner retina of mammals (specifically the RGC layer) and shown to directly respond to light, that this pigment became the forerunner for the photoregulation of circadian rhythms. This finding was especially critical as RGCs had already been implicated in photoentrainment through the discovery that neural networks existed between the "master" circadian clock in the SCN and a subset of RGCs that were intrinsically photosensitive. Proof of this fact derived from murine studies where specifically rendering melanopsin functionless prevented circadian phase-shifting (but not under very bright-light conditions), although the pupil light responses remained attenuated at the highest light intensities tested. With many studies adding to the accumulation of information on the photosensitivity of melanopsin at the molecular, cellular and behavioural levels, there is now overwhelming evidence that melanopsin-expressing pRGCs mediate irradiance detection for an array of physiological processes, including the provision of "time-of-day" cues that regulate circadian entrainment and sleep, pupil constriction and a modulatory effect on the classical image-forming, colour visual system.

It is clear, however, that melanopsin photobiology, especially in non-mammals, is far more complex and presents an interesting evolutionary history with distinct gene lineages, multiple class- and species-specific duplications and the generation of alternatively spliced isoforms. It is also developmentally and diurnally regulated,

exhibits diverse spatial expression patterns in ocular (e.g. retina and iris) and non-ocular (e.g. brain, skin, fin and gill) locations, utilises a multitude of different photoreceptor subtypes with differential signalling and functional roles and is likely to be involved with and influence a plethora of different photosensory tasks (e.g. circadian entrainment; body pigmentation and colouration; orientation; temperature regulation; pupil size; phototaxis; and behavioural arousal and sleep) in many, if not all, craniate species, even those with photoreceptors that perhaps may or may not directly express melanopsin.

With a greater understanding of melanopsin and its associated photoreceptive system, researchers in the near future may be able to manipulate these naturally evolved biological processes to improve animal welfare and conservation, as well as human health, especially with regard to ocular disease, neuropathology, and psychiatric and mental health disorders.

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Chapter 3
The Evolution of Non-visual Photopigments in the Central Nervous System of Vertebrates

Mark W. Hankins, Wayne I.L. Davies, and Russell G. Foster

Abstract In addition to classical image-forming vision, the vertebrates exhibit a range of non-image-forming light detection systems that utilise opsin photopigments. Within the CNS these systems are present in a range of anatomical locations that include both eye and brain. In mammals the eye is both responsible and required for all commonly measured responses to light. By contrast, non-mammalian vertebrates possess a wide range of intrinsically photoreceptive sites. Members of the non-visual opsin family include exorhodopsin, pinopsin, vertebrate ancient opsin (VA), parietopsin, parapinopsin, teleost multiple tissue opsin (TMT), encephalopsin (OPN3), neuropsin (OPN5), peropsin, retinal G protein-coupled receptor (RGR) and melanopsin (OPN4). Opsin-based photopigments have evolved to mediate specific photoreceptive tasks in different light environments, each exhibit functional properties that are tuned to the biological task in which they are involved. Examination of the classes of opsin involved reveals a range of adaptions particularly in spectral sensitivity, chromophore handling and signalling mechanisms. The loss of extraocular light detection in the mammals is associated with an evolutionary reduction in the non-visual opsin representation in the mammalian genome. One clear exception to this is the retention of the melanopsin (OPN4M) gene and the expression of this opsin protein in a single class of mammalian retinal ganglion cell. Exploring the diversity of melanopsin proteins in the lower vertebrates suggests that the property of chromophore biochemistry and bistability does not necessarily define an opsin class and may have evolved more than once.

Keywords Evolution • Opsin • Melanopsin • Non-visual • CNS • Eye • Brain • Irradiance

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3.1 Introduction

Vertebrates respond to light by utilising a wide-ranging array of photosensory systems, with diverse photoreceptor organs expressing a characteristic photopigment. Each pigment consists of an opsin protein that is linked to a light-sensitive retinoid chromophore based on vitamin A. The first stage of light detection originates from a photon-dependent isomerisation of the chromophore that induces a conformational change in the opsin protein. This subsequently permits the binding of a G protein and the activation of a complex biochemical phototransduction cascade. In the eye, the opsin photopigments expressed in both cone and rod photoreceptors have been studied in detail and shown to mediate classical image-forming (IF) vision. By contrast, the molecular and physiological basis for non-image-forming (NIF) and extraocular photoreception is far less understood. NIF light detection occurs in the central nervous system (CNS) of vertebrates, both in the eye and in other regions of the brain. The extensive study of melanopsin-expressing retinal ganglion cells (RGCs) in mammals over the last decade has greatly contributed to our knowledge of NIF photodetection in the vertebrates. That there were indeed cells in the eye utilising opsin photopigments other than the cones and rods represented a major advance in our understanding of the retina, but also marked a conceptual move forward in our understanding of light signalling in the vertebrates. Here we review the extent and sites of non-visual light detection in the vertebrates with an emphasis on the opsin pigments that drive a range of light responses at these locations.

3.2 Critical Aspects of the Opsin Structure

There are currently more than 3,000 identified vertebrate opsin sequences and these have been classified according to several criteria including structure, second messenger coupling and chromophore biochemistry (see Terakita et al. 2012). The vertebrates utilise a subset of these opsins both for classical vision and NIF light detection. In addition to the classical visual opsins (four cone types: LWS, SWS1, SWS2, RH2, and a single rod opsin: RH1), the non-visual opsins utilised by the vertebrates include exorhodopsin, pinopsin, vertebrate ancient (VA) opsin, parapinopsin, parietopsin, encephalopsin/panopsin (OPN3), teleost multiple tissue (TMT) opsin, neuropsin (OPN5), peropsin, retinal G protein-coupled receptor (RGR) opsin and melanopsin (OPN4). The phylogenetic tree showing the evolutionary relationship between these visual and non-visual opsin classes in the gnathostome vertebrates is shown in Figs. 3.1 and 3.2.

In the broadest sense the opsin photopigments can be classified photobiochemically into two types, bleaching photopigments and bistable pigments. Bleaching photopigments are exemplified by the classical cone and rod vertebrate pigments, where the photoproduct of the photopigments is unstable and dissociates from its activated chromophore. In this way the cone and rod pigments of vertebrates are described as bleachable or monostable. By contrast, light activation of the bistable

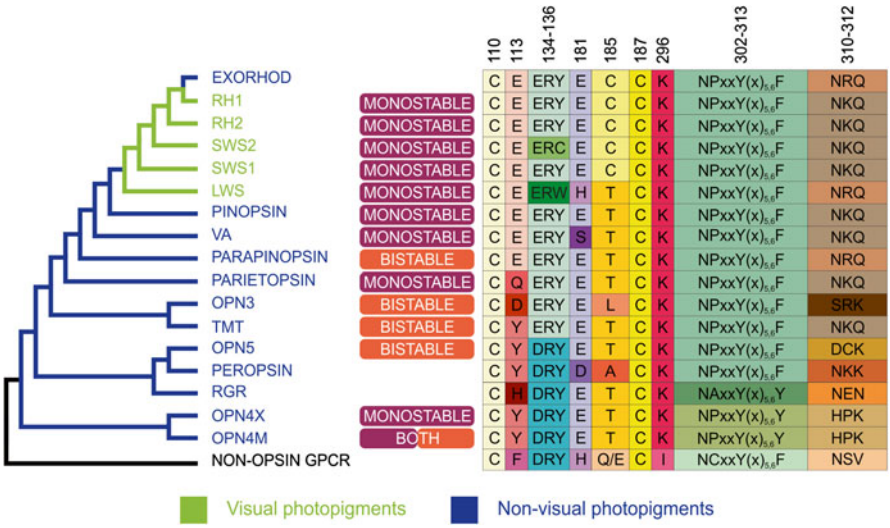

Fig. 3.1 Characterisation of vertebrate photopigments. *Left panel*: a representative phylogenetic tree showing the evolution of known visual (*green*) and non-visual (*blue*) opsin classes in gnathostome (jawed) vertebrates, including "mammalian-like" melanopsin (OPN4M), "*Xenopus*-like" melanopsin (OPN4X), retinal G protein-coupled receptor (RGR), retinal pigment epithelial (RPE)-specific rhodopsin homologue (RRH; also known as peropsin), neuropsin (OPN5), panopsin/encephalopsin (OPN3), teleost multiple tissue (TMT) opsin, parietopsin, parapinopsin, vertebrate ancient (VA) opsin, pineal opsin (pinopsin), long-wavelength-sensitive cone opsin (LWS), short-wavelength-sensitive cone opsin 1 (SWS1), short-wavelength-sensitive cone opsin 2 (SWS2), middle-wavelength-sensitive rhodopsin-like cone opsin 2 (RH2), middle-wavelength-sensitive rhodopsin-like rod opsin 1 (RH1) and extraretinal rod-like opsin (EXORHOD). A non-opsin G protein-coupled receptor (GPCR) is included as an outgroup. *Middle panel*: retinoid-handling status for each pigment as defined in the literature, where "monostable" (*purple*) refers to a stable interaction with one type of chromophore (predominantly 11-*cis* retinal) and "bistable" (*orange*) conveys the ability to stably interact with two distinct retinoid isomers (e.g. 11-*cis* retinal and all-*trans* retinal). The latter is often associated with the capacity of light to convert one form of retinoid into another via photoisomerisation. *Right panel*: consensus sequence of motifs associated with structure and function of different vertebrate pigment classes. These include Cys110, Cys185 (semi-conserved) and Cys187 that are structurally important for disulphide bond formation; visual and non-visual pigment counterions at sites 113 (usually Glu or Tyr) and 181 (usually Glu) to provide negative charges to counterbalance the positive charge of the Schiff base; a conserved Glu/Asp-Arg-Tyr (134–136) motif that assists in stabilising the inactive pigment; Lys296 that is the defining feature of all opsin family members and the site for chromophore linkage via a Schiff base; and an Asn-Lys-Gln motif (310–312) within a conserved Asn-Pro-X-X-Tyr-X-X-X-X-X-(X)-Phe motif (302–313) that is important for maintaining structural integrity of an activated photopigment. Motifs are colour coded to highlight amino acid sequence conservation. All amino acids are numbered according to the bovine rhodopsin protein sequence

pigments generates a stable photoproduct, which can revert to the dark-state by the absorption of a subsequent photon (photoisomerisation). The bistable opsins have been traditionally associated with the invertebrate lineage. However, many of the recently discovered non-visual (NIF) opsins in vertebrates appear to also possess

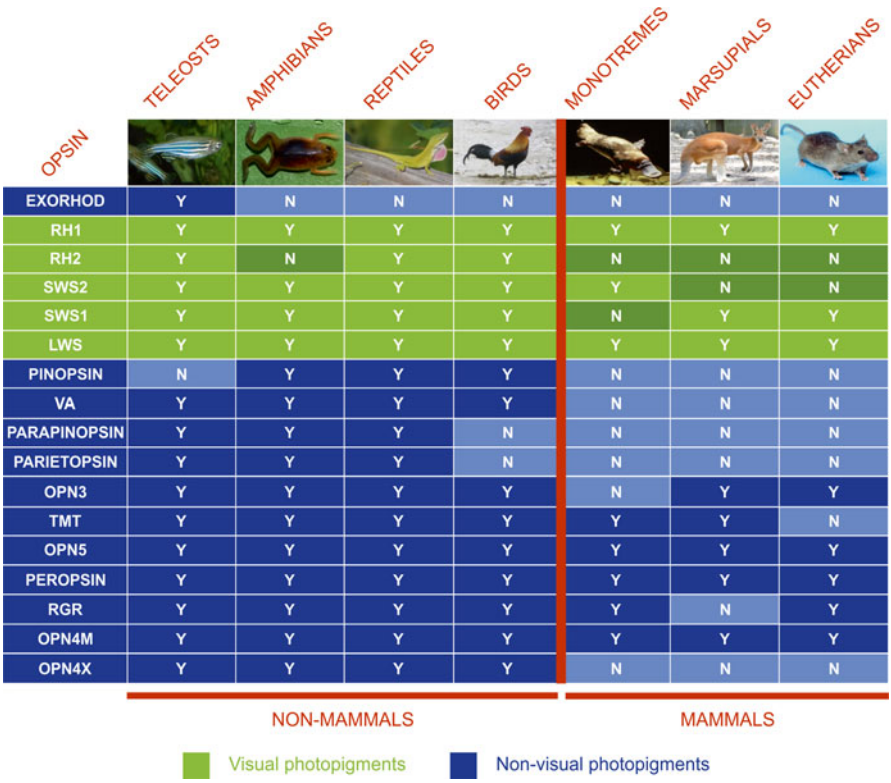

Fig. 3.2 Vertebrate opsin evolution. A summary table showing the presence of visual (*green*) and non-visual (*blue*) opsin orthologues in modern representatives of the main vertebrate classes from bony fishes to mammals. The presence (yes, Y) or absence (no, N) of a particular opsin class is shown, with a *vertical red line* indicating the reptilian-mammalian boundary. The significant loss of opsin genes during this timeline forms part of the "mesopic/nocturnal-bottleneck" hypothesis. Highlighted pigments include "mammalian-like" melanopsin, OPN4M; "*Xenopus*-like" melanopsin, OPN4X; retinal G protein-coupled receptor, RGR; retinal pigment epithelial (RPE)-specific rhodopsin homologue (RRH), peropsin; neuropsin, OPN5; panopsin/encephalopsin, OPN3; teleost multiple tissue opsin, TMT; parapineal gland-expressing opsin, parapinopsin; parietopsin-expressing opsin, parietopsin, vertebrate ancient opsin, VA; pineal gland-specific opsin, pinopsin; long-wavelength-sensitive opsin, LWS; short-wavelength-sensitive opsin 1, SWS1; short-wavelength-sensitive opsin 2, SWS2; middle-wavelength-sensitive rhodopsin 2 (cone), RH2; middle-wavelength-sensitive rhodopsin 1 (rod), RH1; extraretinal rod-like opsin, (exorhodopsin), EXORHOD

the property of bistability. Cone and rod pigments are dependent upon the visual cycle located in the retinal pigment epithelium (RPE) to convert *all*-trans retinal back to *cis*-retinal to replenish the retinal chromophore. In contrast, bistable pigments regenerate their intrinsically bound chromophore isomers directly with light. In the vertebrate non-visual opsins, retaining bistability may be an advantage when chromophore is not plentiful, especially when located away from a regenerating source of retinal.

The retinal chromophore is covalently bound via a Schiff base linkage to a conserved opsin lysine residue (Lys296 based on a bovine rod opsin numbering convention) in the seventh transmembrane helix. In darkness, the 11-*cis* retinal of classical vertebrate opsins acts as an inverse agonist to lock rhodopsin in an inactive state by preventing free opsin from activating the transduction cascade. The counterion is a negatively charged amino acid residue (usually Glu113) that stabilises the positive charge of the protonated Schiff base linkage. Free retinal absorbs maximally in the UV-range (λ_{max} ~380 nm), but when bound to an opsin, the spectral sensitivity of the pigment is shifted into the visible range (i.e. greater than 400 nm). The exception is when the Schiff base is deprotonated (either by a loss of the negative charge at site 113 or the presence of other residues that inhibit protonation such as Phe86 or Cys90 in SWS1 pigments, respectively (Hunt et al. 2009)). Like many other vertebrate pigments, mouse rod and medium-wavelength-sensitive (MWS) cone pigments are protonated with Glu113 acting as a stabilising counterion (Nathans 1990; Palczewski et al. 2000; Zhukovsky and Oprian 1989). By contrast, the mouse short-wavelength-sensitive (SWS1) cone pigment is deprotonated, resulting in a UV-sensitive pigment (Vought et al. 1999). The counterion in all vertebrate rhodopsins is Glu113 (or Asp113) in the third transmembrane helix (only 4.7 Å from C12 of the 11-*cis* retinal) (Palczewski et al. 2000), which has an additional role as an intramolecular switch to activate the G protein efficiently. Rhodopsin activation involves a "counterion switch" mechanism in which Glu181, located in the second extracellular domain that loops back into the opsin to form part of the chromophore binding pocket, transfers a proton to the primary counterion, Glu113 (Yan et al. 2003), during the formation of the major photointermediate—metarhodopsin I (Schertler 2005). In this way Glu181 replaces Glu113 as the counterion to stabilise the protonated Schiff base in the transition stage before its eventual deprotonation. In vertebrate visual opsins and some non-visual opsins (e.g. VA opsin and OPN3), Glu113 or Asp113 are critically conserved residues. However, in most invertebrates and in many of the non-visual vertebrate opsins, the counterion arrangement is rather different. In many bistable non-visual opsins, position 113 is occupied by an uncharged or positively charged amino acid (e.g. tyrosine in OPN4, histidine in RGR and glutamine in parietopsin), with the counterion being displaced to a highly conserved Glu181 instead. It has been suggested that the displacement of the counterion from Glu181 in the ancestral invertebrates to Glu113 occurred in the molecular evolution of the vertebrate visual opsins and effectively promoted the acquisition of the bleaching property of these pigments (Terakita et al. 2004, 2012). However, recent findings with teleost melanopsin (OPN4) suggest that this may have happened more than once during the molecular evolution of vertebrate pigments (Davies et al. 2011). Interestingly, in the case of the vertebrate long-wavelength pigments, Glu181 is replaced by His181, an essential residue for chloride binding and long-wavelength tuning of the spectral peak towards 560 nm. This development could have only occurred after the counterion shift from position 181–113 (Terakita et al. 2012) and this may partly explain the relative paucity of long-wavelength-sensitive bistable pigments. Interestingly, VA opsin uniquely possesses a serine residue at site 181; however, the functional significance of this remains unknown.

3.3 Location of Light Responsive Systems in the Vertebrates

Animals possess an extensive and diverse array of photoreceptors and photopigments that appear to mediate multiple responses to light (Arendt 2008). In the vertebrates, a number of different photoreceptor organs arise from the diencephalon and possess photoreceptor and photopigment systems of varying complexity. These have been classified as: (1) pineal (epiphysis cerebri) and parapineal organs (the pineal complex), which reside within the cranium; (2) extracranial "third eyes", variously called frontal organs (in frogs) and parietal eyes (in lizards); (3) deep-brain photoreceptors; and (4) lateral eyes. In addition to well-characterised cone and rod photoreceptors, lateral eyes also possess intrinsically photosensitive retinal horizontal cells (e.g. in teleosts, Jenkins et al. 2003; Soni et al. 1998), RGCs (e.g. in vertebrates and mammals, Berson et al. 2002; Sekaran et al. 2003) and almost certainly other retinal cell types (Davies et al. 2011; Provencio et al. 1998). In some vertebrates, there are other photodetectors that reside outside of the CNS, such as those present in the skin that are involved in the regulation of body colouration and pigmentation, but are outside the scope of this chapter. A summary of the vertebrate light responsive CNS is illustrated in Fig. 3.3.

Outside of the eye a variety of photoreceptor cell types have also been described in extraocular organs, ranging from the pineal and parapineal organs of lampreys and bony fishes, which possess photoreceptors that closely resemble cones with specialised lamellae membrane structures (Vollrath 1981), to deep-brain photoreceptors with no apparent cone-like or rod-like features (Halford et al. 2009). In addition to the photoreceptors of diencephalic/forebrain origin, many vertebrates possess broad tissue photosensitivity (e.g. in teleosts, Whitmore et al. 2000), dermal/melanophore photoreceptors (e.g. in amphibians, Lythgoe 1985; Rollag et al. 2000) and photosensitive iridocytes within the cornea (e.g. in teleosts, Shand and Lythgoe 1990). The intrinsically photosensitive RGCs (pRGCs) in the vertebrate retina have no cilial outer segment structures like those present in the visual photoreceptors, but express their photopigments on the plasma membrane. For many years, it was assumed that opsin-based light detection required specialised environments and membrane structures; it is now clear that the lack of specialised "visual photoreceptor" structures does not preclude cellular photoreceptive function.

3.3.1 Distinction Between Mammals and Non-mammals

In mammals, enucleation appears to abolish all commonly measured responses to light showing that both visual and non-visual photoreception is ocular (Foster et al. 1991; Nelson and Zucker 1981). By contrast, non-mammalian vertebrates possess a wide range of photoreceptive sites as discussed above (Shand and Foster 1999) (Fig. 3.3). As well as being anatomically diverse, these photoreceptors mediate many different aspects of physiology and behaviour. Identifying the extraretinal opsin photopigments that underlie these responses in non-mammalian photoreceptors has

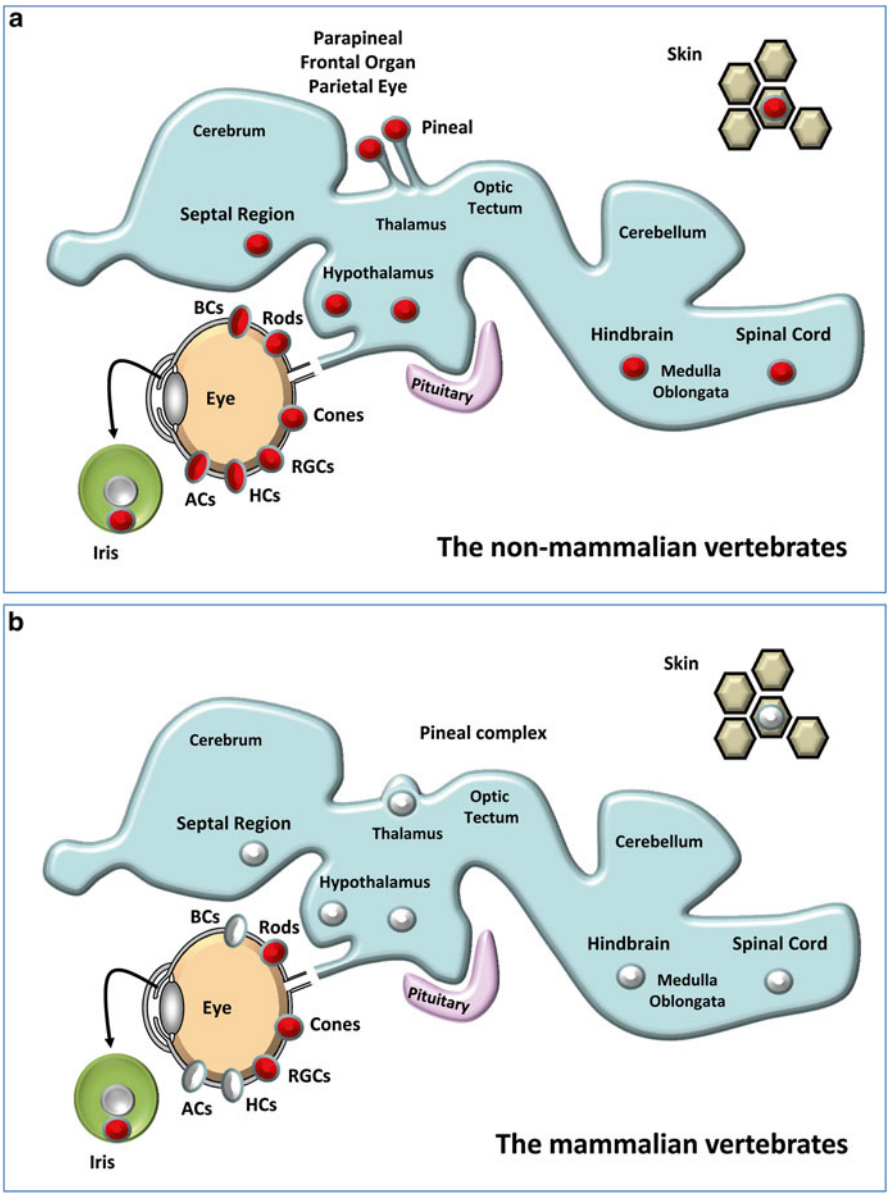

Fig. 3.3 Photoreceptive sites in vertebrates. A summary diagram showing the major regions that are known to be photosensitive (highlighted in *red*) in (**a**) non-mammalian and (**b**) mammalian species (discussed in detail in the main text). In mammals, direct photoreception is limited to the iris and the eye, where it is restricted to the cones, rods and a subset of retinal ganglion cells (RGCs). By contrast, many other regions in non-mammals express photopigments and are intrinsically photosensitive, including ocular tissues similar to those utilised in mammals (i.e. iridocytes, cones, rods and RGCs) with the addition of horizontal cells (HCs), bipolar cells (BCs) and amacrine cells (ACs). Unlike the mammals, the pineal complex and parapineal organs, deep-brain (e.g. septum, hypothalamus, hindbrain), spinal cord and dermis of many non-mammalian species also directly detect light

a long and complex history. Historically, many immunocytochemical studies were undertaken on these tissues, using a wide range of antibodies raised to different retinal or visual pigment preparations (see Shand and Foster 1999). However, as the epitopes and specificities of many of these antibodies were not well defined, it was difficult to make definitive conclusions regarding the molecular identity of the molecules being immunolabelled. After all, these studies were attempting to locate potentially unknown proteins, using a range of available antibodies. The ambiguous terms "cone-like" or "rod-like" were frequently used to describe such immunolabelling. The molecular characterisation of the extraretinal photopigments became further complicated with the discovery of multiple new opsin families, quite different from the classic cone and rod opsins. Parallel studies on mammals also produced results that were initially difficult to interpret. Although it was clear that mammals appeared to lack extraocular photoreceptors (Foster et al. 1991; Nelson and Zucker 1981), mice lacking cone and rod photoreceptors could still regulate multiple physiological responses to light (Freedman et al. 1999; Lucas et al. 1999). Further, these non-visual responses were clearly being mediated by an opsin/vitamin A-based photopigment system (Lucas et al. 2001). Yet until recently the molecular identity of this photoreceptor system remained unknown. In the past decade much new information has emerged regarding the location and function of this and other vertebrate non-cone, non-rod photoreceptor systems.

3.4 Non-visual Light Sensing Tasks

Light-dependent signalling has a clear and well-defined role in vision, with cone and rod photoreceptors providing a processed signal from the lateral eyes to the retinorecipient areas of the primary visual pathway. By contrast the non-visual light sensors have a much more heterogeneous role in physiology and behaviour. In general terms non-cone, non-rod ocular photoreceptors mediate responses to gross changes in environmental light (irradiance) for the regulation of tasks that span "time-of-day" cues for a variety of regulatory mechanisms including the control of sleep and circadian rhythms (Freedman et al. 1999); the levels of behavioural arousal (Lupi et al. 2008); orientation and taxis (Engbretson 1992; Fernandes et al. 2012); body pigmentation and colouration (Bagnara and Hadley 1970); temperature regulation (Tosini and Menaker 1996); pupil size (Lucas et al. 2001); and the regulation of retinal neural circuitry (Hankins and Lucas 2002).

It is of considerable evolutionary interest to explore the mechanisms underlying the loss of extraocular irradiance detectors in mammals and the resulting centralisation of their photoresponsive repertoire. This may be related to the early evolutionary history of mammals and their passage through what has been described as a "nocturnal bottleneck" (Walls 1942). Recently, a closer examination of visual pigment evolution at the reptilian-mammalian boundary has suggested that a prolonged "mesopic bottleneck" may be a more appropriate mechanism, with nocturnality being relevant towards the end of this transition for a number of

derived mammalian species (Davies et al. 2012b). All mammals are derived from nocturnal insectivorous weasel-like therapids (Kemp 2006), with an ancestry that dates back at least 260 million years ago (MYA), with modern mammals first appearing in the fossil record about 225 MYA (Lucas and Luo 1993). The "nocturnal/mesopic bottleneck" proposal suggests that many early mammals were forced to adopt an increasingly dim-light habitat in response to the dominance of the archosaurs in the diurnal realm (Young 1981). During this time, mammals evolved a variety of characteristic traits that appear to be adaptations to a nocturnal/mesopic existence (Walls 1942). Extraretinal photoreceptors would have been adequate for monitoring changes under bright diurnal light conditions but may not have been sufficiently sensitive to discriminate twilight changes in early mammals that spent the day hiding underground and emerging late in twilight. Extraocular photoreceptors and pRGCs were almost certainly both present in the diurnal mammal-like reptiles, but extraocular receptors were probably lost when early mammals began to exploit the twilight and nocturnal realms (Foster and Menaker 1993), when perhaps light levels were too low to adequately penetrate the brain. Indeed the extant mammals retain a nocturnal eye shape irrespective of their diurnal/nocturnal daily patterns of activity (Heesy and Hall 2010).

Interestingly, the loss of extraocular receptors in the mammals is also reflected in the retina. Mammals retain both cone and rod photoreceptors together with the melanopsin-expressing RGCs, but in the non-mammalian vertebrates, intrinsic light sensitivity appears in all the major classes of retinal neuron, including the horizontal (Jenkins et al. 2003), amacrine and bipolar cells (Davies et al. 2011) (Fig. 3.3). Since it is likely that these additional non-visual light responses are involved in the regulation of the retinal circuitry according to retinal irradiance, these too were lost in the mesopic/scotopic environment of the nocturnal/mesopic bottleneck. As well as the development of endothermia and a loss of DNA photoprotective mechanisms, one of the most compelling ways of appreciating the adaptive changes that occurred in the mammals during this prolonged dim-light bottleneck is the loss of opsin-based pigments (recently reviewed in Gerkema et al. 2013). Classically, it is the loss of visual pigments that is held as the epitome of this process (Fig. 3.2) (reviewed in Davies et al. 2012b). Early reptilian-like mammals almost certainly possessed an RH1 (rod) pigment and four cone classes (LWS, SWS1, SWS2 and RH2 and thereby a tetrachromatic colour visual system similar to present-day reptiles and birds) with the potential of sampling light over a spectral range that spanned the entire visible range (UV to "red" wavelengths with λ_{max} values from 360 to 560 nm). However, light-restricted habitats led to the initial loss of the *RH2* gene (generally expressed in a "green" cone) in the ancestor to all extant mammals, leaving only three cone pigments in lineages that led separately to the early trichromatic monotremes (Protheria) and the therian marsupial/eutherian class (Davies et al. 2007). In extant *Monotrema* species (i.e. the platypus, *Ornithorhynchus anatinus*, and the echidna, *Tachyglossus aculeatus*), there has been a further loss of the *SWS1* gene, leaving only the *SWS2* (unique to monotremes) and *LWS* genes (dichromatic colour vision) in addition to a rod pigment (Davies et al. 2007). In the therians, the complement of opsins was also further reduced with the subsequent loss of the *SWS2* gene, leaving

only the *SWS1* and *LWS* genes (and the *RH1* gene expressed in rods), thus forming widespread dichromacy as the therian mammals expanded in number (e.g. Davies et al. 2012b; Cowing et al. 2008). With many mammals adopting diverse niches that were vacant after the significant downfall of the archosaurs, further visual opsin gene losses have occurred (e.g. the loss of *SWS1* in many marine mammals, Fasick et al. 1998) and the loss of all cone pigments in whales (Meredith et al. 2013). Interestingly, many primates have developed trichromacy (i.e. three cone pigments) through a duplication of the *LWS* gene or different allelic variations at the *LWS* gene loci, a process that reflects a return to diurnal habitats (reviewed in Davies et al. 2012b). However, over the past decade, research has shown that the non-visual system has also been significantly affected at the genome level with the loss of many irradiance detectors and the pigments expressed in their photoreceptors (Terakita et al. 2012; Davies et al. 2010). Examples include the absence of pinopsin in teleosts, the loss of VA opsin and one of the two lineages of melanopsin in all mammals, and the genome omission of TMT opsin in therian mammals (Fig. 3.2).

3.4.1 Non-visual Light Detection and the Lateral Eyes

The retinae of the lateral eyes are the most familiar photoreceptive site in vertebrates. The classical photoreceptors of the vertebrate retina consist of the cones and rods. The electrical potentials from these receptors are processed by retinal neurons prior to advanced visual processing in the brain. Light information reaches the visual centres of the brain via retinotopically mapped axons of the RGCs that form the optic nerve. In addition to the "classical" photoreceptors of the outer retina, other retinal cells are now also known to respond to light. A subset of RGCs (approx. 1–2.5 % in the mouse) expresses the photopigment melanopsin (OPN4) (Hattar et al. 2002) and are capable of responding to light directly, responses of which were confirmed in many eutherian mammals including rats (Berson et al. 2002), mice (Sekaran et al. 2003) and primates (Dacey et al. 2005).

In addition to melanopsin-based pRGCs, the teleost retina (and almost certainly amphibian and other non-mammalian retinae) possesses photosensitive horizontal cells. In the cyprinid retina of the roach (*Rutilus rutilus*), a subtype of the horizontal cell, termed "HC-RSD" (horizontal cell-rod secondary depolarisation), expresses both melanopsin and vertebrate ancient (VA) opsin and shows anomalous depolarising responses to light that are maximally sensitive at approximately 477 nm (Jenkins et al. 2003). These cells have longer integration times than cones or rods and maintain their responses when classical photoreceptor inputs are saturated by background light (Jenkins et al. 2003). The studies in the roach were amongst the first non-visual light responsive retinal neurons to be studied at the cellular level and appear to signal local environmental irradiance as well as modulating cone and rod outputs. More recently, these earlier studies were confirmed in the channel catfish (*Ictalurus punctatus*) where horizontal cells were again shown to be directly photosensitive through the expression of both melanopsin and VA (Cheng et al. 2009).

Most recently it has emerged that intrinsic light sensitivity is likely to extend to all the other major classes of retinal neurons in the teleost, with cones, bipolar cells, amacrine cells and RGCs all expressing at least one form of melanopsin in the zebrafish retina (see Davies et al. 2011, 2012a and Chap. 2). It remains to be established which precise roles these potential light-sensitive cells exhibit in the retina, but most probably they are involved in the long-term light regulation of the neural network according to the prevailing irradiance. Indeed there is evidence that such a mechanism persists in mammals, since melanopsin also acts locally to regulate the photopic/cone pathway in both humans according to long-term light exposure (Hankins and Lucas 2002) and in mice (Barnard et al. 2006). Interestingly, it appears that the nocturnal/mesopic bottleneck has also reduced, but not abolished, the diversity of non-image-forming light responsiveness within the retina of mammals (Fig. 3.3).

3.4.2 Light Responsiveness of the Pineal Complex

Perhaps the best characterised photoreceptive site outside of the retina is the pineal organ (epiphysis cerebri). Here the term pineal complex will be used to refer to the intracranial pineal proper as well as the parapineal, although the pigments and possible functional roles between them may be markedly different. Embryologically, the pineal complex is derived from an evagination of the dorsal diencephalon and in non-mammalian vertebrates is located near the surface of the brain (Vollrath 1981). In lampreys (jawless vertebrates) and teleosts (jawed vertebrates), there is commonly a translucent window or area of reduced pigmentation overlying the pineal, allowing approximately 10 % of the incident light to reach the pineal. In amphibians, reptiles and birds, this window is either absent or less transparent. However, large amounts of filtered light still enter the brain, with 0.1–0.3 % of incident light penetrating the pigmented skin and honeycomb skull to reach the pineal (Dodt and Meissl 1982). In animals that lack a pineal window, the spectral quality of the light reaching the pineal (like other regions deep in the brain) is dominated by the absorption characteristics of haemoglobin (Fig. 3.4). As a result, the light environment of most pineal complexes and other deep-brain areas (e.g. the hypothalamus) are relatively enriched in wavelengths beyond 600 nm, although there is a window in light transmission between 460 and 550 nm, with a maxima of transmission around 490 nm (Hartwig and van Veen 1979) (see Fig. 3.4). In all non-mammalian vertebrates, the pineal complex is photoreceptive, with the predominant cell-type being photoreceptor-like in appearance (i.e. the presence of an outer segment). In mammals, the pineal organ expresses many elements of the phototransduction cascade (Korf et al. 1985a, b), but lacks photosensitivity and appears to be exclusively secretory (Foster et al. 1989, 2003). The pineal organ is the primary source of the neurohormone melatonin, which is synthesised in the dark phase of the light/dark cycle, and acts as a signal of darkness to regulate circadian rhythms and photoperiodic responses (Arendt 1998; Korf et al. 1998). Melatonin synthesis is locally regulated

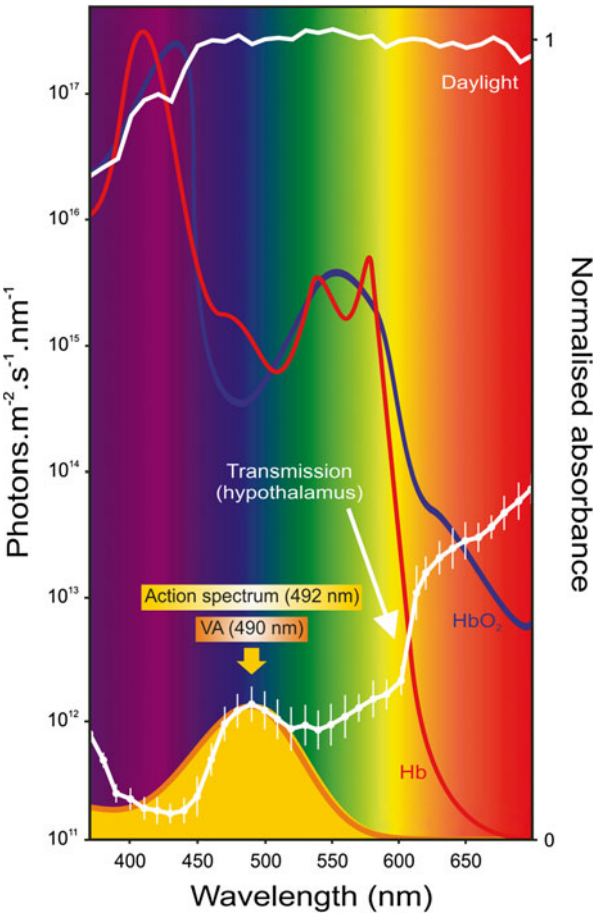

Fig. 3.4 Photoperiodism in birds. A composite diagram showing the change in photic information (wavelength and intensity) from broad-spectrum daylight (~10^{17} photons/m^2/s/nm; *upper white line*) as incident light is transmitted through cranial tissues (i.e. features, bone, blood and soft tissues) that overlay the avian brain. This filtering action (with a reduction of ~10^5 photons/m^2/s/nm) will generate a light environment at the hypothalamus (~10^{12} photons/m^2/s/nm; *lower white line*) that is rich in longer wavelengths (which will not be easily distinguished from thermal noise by an opsin-based photopigment system) and a spectral window of about 450–550 nm that remains after the absorption of short- and long-wavelengths by both oxygenated (HbO_2; *blue line*) and deoxygenated (Hb; *red line*) haemoglobin (shown as normalised absorption profiles). Note that the spectral sensitivity of the avian photoperiodic action spectrum (λ_{max} at ~492 nm; *light orange shading*) overlaps the spectral transmission window and matches the λ_{max} value at 490 nm calculated for the vertebrate ancient (VA) opsin photopigment (*dark orange line*)

by light at the level of the pineal complex in non-mammalian vertebrates, but in mammals, photic information reaches the pineal by way of a multi-synaptic pathway via the retinohypothalamic tract (RHT) and the superior cervical ganglion of the sympathetic nervous system (Korf and Møller 1984; Meissl 1997).

The cytology of the photoreceptor-like cells found in the pineal complex has been reviewed in detail a number of times (e.g. Vigh and Vigh-Teichmann 1988). In summary, fishes and amphibians have an outer segment resembling a retinal cone with multiple invaginations of the photoreceptive membrane. Outer segments appear to contain both cone-like and rod-like opsin proteins. Reptiles may have either cone-like photoreceptors (lizards) or cells with an outer segment with disorganised membrane whorls (snakes). Like the fishes and amphibians, both cone-like and rod-like opsins have been identified within the pineal complexes of lizards. Turtles appear to occupy an intermediate evolutionary position with both types of photoreceptor-like cell (Meissl and Ueck 1980). In birds the photoreceptor-like pinealocytes have either disorganised membrane whorls (as in some snakes) or have an outer segment with few or no membrane structures. The pinealocytes of adult mammals have lost most of their photoreceptor-like morphological characteristics and appear to be exclusively secretory.

A range of opsins has been detected in the pineal complex of vertebrates (Shand and Foster 1999). One of the first extraretinal opsins to be identified, pinopsin (P-opsin), was isolated from the avian pineal (Max et al. 1995; Okano et al. 1994). In the teleost pineal, a range of cone and rod visual opsins, along with VA opsin, were found to be expressed (Philp et al. 2000b). However, it appears that the predominant opsin in the pineal gland of fishes is a rod-like opsin (exorhodopsin/extraretinal rod-like opsin) which differs from that found in the lateral eyes in both protein sequence, gene structure (Mano et al. 1999) and pigment function (Tarttelin et al. 2011). Despite the lack of information about the function of the parapineal organ, a novel opsin photopigment, parapinopsin, has been isolated specifically from the parapineal of the channel catfish (*Ictalarus punctatus*) (Blackshaw and Snyder 1997), as well as from the river lamprey (*Lampetra japonica*) pineal and parapineal organs (Koyanagi et al. 2004).

3.4.3 The Extracranial "Third Eye"

Fossils of primitive bony fishes, early amphibians and ancestral reptiles have a dorsal medial skull socket within which a "third eye" is believed to have been located (Romer 1970). Presently only the Sphenodon (Rhynchocephalia), some lizards (Squamata) and frogs (Anura) possess a dorsal skull socket and an extracranial "third eye". In contrast to the intracranial pineal, parapineal and deep-brain photoreceptors, "third eye" photoreceptors are exposed to largely unfiltered environmental light. In anuran amphibians the pineal complex is the name given to both the extracranial "third eye" (frontal organ or Stirnorgan) and the intracranial pineal proper (epiphysis cerebri).

In lizards, it is unclear whether the pineal complex (pineal organ and parietal eye) develops from a single evagination from the diencephalon or whether these structures are formed from separate evaginations (for review, see Vollrath 1981). The parietal eye of some lizards is the extraocular photoreceptor organ that most

resembles the vertebrate lateral eye, possessing a transparent cornea and a lens. The receptors found within the parietal organ synapse directly on to ganglion cells, the axons of which form a parietal-eye nerve projecting to the brain (for review, see Engbretson 1992). The photoreceptors themselves look like the cones of the lateral eyes, with outer segment membranes that are invaginated and continuous with the extracellular space. Morphological analysis suggests that at least two photoreceptor classes may exist within the parietal eye of some lizards (Engbretson 1992). Functionally, the lizard parietal eye is thought to play a role in thermoregulation, reproduction and the regulation of a range of other behavioural patterns (for reviews, see Engbretson 1992; Quay 1979). Most recently, studies on the parietal eye have identified the expression of two opsins within the same cell, a short-wavelength-sensitive ("blue") pinopsin and a novel MWS ("green") opsin named parietopsin (Su et al. 2006).

3.4.4 Light Responsiveness and Deep Regions of the Brain

Deep-brain photoreceptors were first described in the classical studies by Karl von Frisch in 1911 on blinded and pinealectomised European minnows (*Phoxinus phoxinus*). These fishes still demonstrated colour changes in response to light, leading to the suggestion of "deep diencephalic photoreceptors" (Frisch 1911). Similarly, studies in blinded pinealectomised European eels (*Anguilla anguilla*) showed that deep-brain photoreceptors mediate photoentrainment as well as negative phototaxis (van Veen et al. 1976). Similar conclusions were proposed in recent studies, where zebrafish larva that lack both eyes and pineal glands are still able to perform simple light-seeking behaviour triggered by the loss of illumination (Fernandes et al. 2012). The zebrafish is known to express a number of non-visual opsins in the brain including melanopsin, TMT, VA opsin and OPN5, so future work is required to specify what precise roles these opsins have to play in the brain.

The photoperiodic response in birds, in which gonadal growth is regulated by day length, is also mediated by a deep-brain photoreceptor system rather than by the pineal complex or lateral eyes. Benoit demonstrated that direct illumination of the hypothalamus caused testicular growth in blinded mallards (Benoit 1935a, b). This research was not continued until the 1960s when Menaker and his colleagues began a series of experiments with sparrows, which demonstrated that the regulation of circadian rhythms and the photoperiodic control of testicular growth is mediated by extraretinal and extrapineal photoreceptors (for reviews, see Menaker and Underwood 1976). Action spectroscopy provided a clue as to the molecular identity of these photoreceptors. An absorption-corrected action spectrum for photoperiodic induction in the Japanese quail, where the absorbance of feathers, skin, bone and soft tissues were accounted for, described an opsin/vitamin A-based photopigment with a λ_{max} ~492 nm (Foster et al. 1985). Although this action spectrum inferred the biochemistry of the photopigment, the precise molecular identity still remained unresolved. Attempts to characterise these photoreceptors involved the use of

immunocytochemical techniques that employed antibodies raised specifically against cone and rod photopigments, or other elements of the phototransduction cascade. Such approaches either failed to localise opsins within the avian hypothalamus, or produced ambiguous results owing to the use of unknown epitopes (Silver et al. 1988). However, two candidates have recently emerged as candidate opsins for driving this response, namely VA opsin and neuropsin (OPN5) as discussed in more detail below.

3.4.5 Dermal Photoreception in the Vertebrates

Photoreception by dermal cells mediates colour changes in chromatophores and iridophores. Dermal photoreception has also been linked to the triggering of locomotor activity (Lythgoe 1985; Shand and Foster 1999; Wolken and Mogus 1979). Dermal chromatophores are photosensitive in many vertebrates, regulating the aggregation and dispersal of pigment granules within these cells (Weber 1983). Melanopsin (OPN4), the photopigment of pRGCs across a multitude of vertebrates, was first isolated from *Xenopus laevis* melanophores (Provencio et al. 1998) and appears to be the photopigment mediating the pigment dispersal responses within dermal melanophores (Isoldi et al. 2005), although visual opsins have also been found to be expressed in the chromatophores of some species of fishes (e.g. rod pigment and two types of RH2 cone opsins in the dermal iridophores of the neon tetra, *Paracheirodon innesi*, Kasai and Oshima 2006).

3.5 Photoreceptive Tasks for the Non-visual Photopigments

There is a wide diversity of extraretinal photoreceptors and generalisations regarding their photoreceptive tasks are difficult. However, all seem to monitor overall levels of light in the environment (irradiance) and, with the exception of the extracranial "third eyes" and those species with a pineal window, are incapable of extracting detailed spatial or positional information from the environment. Nonetheless, in some cases roles for extraretinal photoreceptors can be assigned, such as the reporting of environmental irradiance; mediating time-of-day cues for the regulation of the circadian system; providing cues for behavioural orientation kinesis and phototaxis; an involvement with regulating body and/or corneal pigmentation and colouration; directly regulating local neural circuitry (e.g. retina); and providing photic cues for regulating pupil size.

Historically, the study of non-visual opsins began with the investigation of novel extraocular photoresponses and the pigments mediating them were largely unknown. However, as knowledge of the extent and diversity of opsins in the vertebrate genome proliferated, the current challenges have been to assign specific opsins to their physiological role(s). As such, it is imperative to return to the classical criteria

for assigning a photopigment to a particular biological function. Naturally an opsin needs to match the action spectrum for a given biological response, be expressed in the correct anatomical location and be able to act as a photopigment in that environment, coupling to a relevant signalling cascade. Using the non-visual pigment melanopsin as an exemplar, the extensive nature of the work required to assign a pigment to a function becomes apparent. Indeed it took a large body of research to definitively establish that melanopsin was indeed the opsin photopigment expressed in a subset of RGCs that are intrinsically light sensitive. For many other non-visual photopigments, we are far from this level of identity or certainty.

3.6 Characteristics of Non-visual Opsin Photopigments

A comprehensive range of non-visual tasks that are apparently underpinned by a diversity of non-cone, non-rod opsin proteins are outlined above. However, important questions arise as to the nature of the key defining driving forces that led to the evolution of these pigments and their functional diversity. Like visual pigments, both the absolute and relative spectral sensitivities of non-visual pigments should be related to the relevant behavioural ecology of a particular species that expresses them. Thus, it is clearly imperative that an opsin located deep in the brain of a vertebrate should be able to respond to relevant ecological environmental light changes. However, one important difference between visual and non-visual pigments appears to relate to light adaptation. Vertebrate cone and rod pigments and their associated G_t protein (transducin) phototransduction cascades are associated with profound light adaptation, in that background light reduces photoreceptor sensitivity whilst darkness enhances sensitivity. In this way photoreceptors extend their dynamic range and the visual system is dynamic and most responsive to changes in light levels rather than signalling absolute changes in retinal irradiance. The non-visual tasks outlined above are dependent upon reporting absolute levels of irradiance to a range of physiological responses and, therefore, this factor may be an important driving force in non-visual pigment evolution. The majority of these pigments do not appear to be coupled to the transducin G_t protein-coupled cascades of visual pigments: a classic example of this is melanopsin, which appears to be coupled to the $G_{q/11}$ family and induces cellular depolarisation via activation of transient receptor potential (TRP) channels (see review Hughes et al. 2012a). Furthermore, this cascade in pRGCs appears to be able to integrate light stimuli over a time course of many minutes (Sekaran et al. 2003, 2005). It would, perhaps, also be expected that a number of potentially overlapping non-visual pigments may be able to differentially couple to a range of G protein-coupled pathways to permit complex signalling interactions. In this regard, it may be predicted that differential selection pressures would act upon the relevant intracellular G protein interaction domains (e.g. the intracellular domains of photopigments or key residues involved in their structure and function; see Fig. 3.1). Another important factor may be the relative dependence upon retinal chromophore. Classical vertebrate visual photopigments in the eye are dependent upon 11-*cis* retinal that is provided by the RPE as discussed earlier.

However, invertebrate pigments are bistable and able to form a stable association with both the *cis* and all-*trans* isomers of retinoid chromophores and utilise light to interconvert between the *cis* and all-*trans* forms, thereby effectively regenerating themselves. In the early studies of melanopsin, it was often stated, with little direct evidence, that melanopsin was a "bistable" pigment due to a close evolutionary relationship between melanopsin and invertebrate pigments, as well as an assumed similarity in their phototransduction cascades, despite many significant differences between pRGCs and invertebrate photoreceptors. Thus, it is unfortunate that melanopsin is often described as "invertebrate-like", a misnomer that is perhaps incorrect and somewhat confusing. Whilst in some cases it may clearly be advantageous not to rely on exogenous chromophore, it now appears that bistability may not be a unique or defining property of non-visual pigments. Indeed many non-visual pigments (e.g. P-opsin, VA opsin and parietopsin) are monostable (Terakita et al. 2012). Even the epitome of vertebrate bistability, melanopsin, exists in monostable and bistable forms. In the teleost zebrafish (*Danio rerio*), the five distinct melanopsin pigments are split into those that appear bistable in forming stable interactions with both 11-*cis* and all-*trans* retinal (i.e. OPN4M1 and OPN4M3), and the other three pigments (OPN4M2, OPN4X1 and OPN4X2) that are monostable and resemble the classical vertebrate visual opsins in only interacting with 11-*cis* retinal (Davies et al. 2011). More recently, a similar functional divide was observed in the three main melanopsin pigments of the deep-sea elephant shark, *Callorhinchus milii* (Davies et al. 2012c). Coupled with this variability in function, it is difficult to highlight the primary structural characteristics that define a bistable pigment: much has been discussed concerning the proposed functional shift in the position of the counterion in monostable pigments, but this alone is not sufficient to define the retinoid-handling characteristics of monostability versus bistability.

3.7 The Principal Non-visual Opsin Classes in the Vertebrates

In vertebrates there is a large group of non-visual opsins that are involved in extraocular photoreception with non-image-forming functions. These non-visual opsins possess important characteristics of a typical opsin-based photopigment, including the lysine retinal attachment site (Lys296), the presence of a glutamate counterion at site 113 (Glu113) or 181 (Glu181) and two conserved cysteine residues (Cys110 and Cys187) that form a disulphide bridge, but each is unique at both gene and protein levels. The principal classes of vertebrate non-visual pigments and their phylogenetic relationships are summarised in Figs. 3.1 and 3.2.

The diversity of non-visual opsins in the vertebrate lineage has its origins in at least two rounds of whole genome duplications (WGD), one early in the evolution of the vertebrates and a second around the divergence of the teleosts (see Nakatani and Morishita 2008). In broadest terms, WGDs created multiple copies of ancestral opsin genes upon which evolutionary selective pressures are able to act. Interestingly, the evolutionary retention rate of opsin and other GPCR genes following WGD

events in the teleost genome is significantly higher than for other genes in the vertebrate genome (by a factor of 2–3; see Semyonov et al. 2008). This highlights the evolutionary advantage conferred by signalling proteins and opsins in particular. In the zebrafish, for example, five distinct melanopsin genes were discovered that are located on five different chromosomes (Davies et al. 2011). This contrasts with mammals (e.g. mice and humans) that have retained a single copy of this gene that encodes just two splice variants of the melanopsin protein (Pires et al. 2009). All five melanopsin genes in the zebrafish show variations in spectral sensitivity, bistability versus monostability, differential upstream promoters and regulators, and differential tissue localisation (Davies et al. 2011). Thus, it appears that WGDs have provided the substrate for the evolution of a diverse repertoire of opsin proteins. That these were then lost with the evolution of subsequent vertebrate classes (Fig. 3.2) suggests that a change in the dynamics of the light environment in some cases has rendered them unnecessary or non-advantageous. If such a case occurs, these genes would be expected to accumulate mutations, become non-functional through pseudogenisation, and eventually be lost from the genome in subsequent rounds of chromosomal reorganisation.

3.7.1 Exorhodopsin

The presence of opsin immunoreactivity in the teleost pineal was reported in the early 1980s (Vigh-Teichmann et al. 1982, 1983). However, it was not until the independent isolation of a rod-like opsin from the pineals of the zebrafish (Mano et al. 1999), the pufferfish (*Takifugu rubripes*) and Atlantic salmon (*Salmo salar*) (Philp et al. 2000a), that the molecular identity of this opsin was elucidated. Exorhodopsins (also known as exorodopsins) are 74 % identical in sequence to the retinal rod opsin (encoded by the *RH1* gene) from their cognate species, suggesting that they diverged early in the teleost lineage (Philp et al. 2000a) and are indeed restricted to the bony fishes (Fig. 3.2). Another interesting difference concerns their gene structures: whereas exorhodopsin genes contain introns, teleost *RH1* genes do not. Given that other vertebrate rod pigment genes contain introns, it is likely that the processed transcript of the *RH1* gene of bony fishes replaced its intron-containing counterpart during a retrotransposon event in the ancestor to all modern teleosts (Hope et al. 1997; Fitzgibbon et al. 1995). The expression of exorhodopsin appears to be restricted to the pineal gland, although its exact function remains unknown. A recent functional UV-visible (UV-Vis) spectrophotometric and Fourier transform infrared (FTIR) spectroscopy study of pufferfish exorhodopsin revealed that there were no significant differences in its spectral sensitivities ($\lambda_{max} = 501$ nm for rod opsin; $\lambda_{max} = 498$ nm for exorhodopsin), but there were significant differences in its half-life and stability of the photointermediate metarhodopsin II compared to the native rod opsin pigment (Tarttelin et al. 2011). It was suggested that the shortened lifetime at the metarhodopsin II state of exorhodopsin might accelerate its recovery from bleaching, thus contributing to a heightened sensitivity to light. Such differences may reflect the different photic tasks of the rod and pineal forms of this opsin.

3.7.2 Pinopsin

Pinopsin (also called P-opsin) was the first extraretinal opsin to be cloned and was isolated from the pineal gland of the chicken (Max et al. 1995; Okano et al. 1994). It showed 43–48 % amino acid identity to the vertebrate visual opsins. In the chicken, it is expressed exclusively in the pineal. Several groups have reported the in vitro expression and reconstitution of pinopsin with 11-*cis* retinal. All report the formation of a short-wavelength-sensitive pigment, but with slightly different λ_{max} values: 470 nm (Okano et al. 1994); 462 nm (Max et al. 1998); and 460 nm (Nakamura et al. 1999). Pinopsin has also been identified in modern representatives of Amphibia (Yoshikawa et al. 1998) and Reptilia (Kawamura and Yokoyama 1997), with localisation to the anterior preoptic nucleus of the hypothalamus in toads (Yoshikawa et al. 1998) and interestingly in both the retina and pineal gland of a diurnal gecko (*Phelsuma madagascariensis*) (Taniguchi et al. 2001). By contrast, a study on the Ruin lizards (*Podarcis sicula*) suggested that pinopsin expression was restricted to the pineal complex (Frigato et al. 2006). To date pinopsin orthologues have not been isolated from agnathans (see below), cartilaginous and teleost fishes, or mammals (Fig. 3.2).

3.7.3 Vertebrate Ancient Opsin

VA opsin was first described in the Atlantic salmon (*Salmo salar*) (Soni and Foster 1997) and was subsequently isolated from several other teleost fishes including the zebrafish (*Danio rerio*) (Kojima et al. 2000), the common carp (*Cyprinus carpio*) (Moutsaki et al. 2000), the smelt (*Plecoglossus altivelis*) (Minamoto and Shimizu 2002) and the roach (*Rutilus rutilus*) (Jenkins et al. 2003). Sequence comparison analysis shows that VA opsins share 37–41 % identity with the visual opsins and 43 % identity to other non-visual opsins such as pinopsin. Since phylogenetic analysis suggests that VA opsin diverged from a common ancestor at the very beginning of vertebrate evolution, it was given the name "vertebrate ancient" opsin (Soni and Foster 1997). Interestingly, an apparent lamprey (*Petromyzon marinus*) pinopsin orthologue has been identified (Yokoyama and Zhang 1997), which is now considered to be a member of the VA opsin family (Bellingham and Foster 2002; Moutsaki et al. 2000), strengthening the assignment of VA opsin as an ancient opsin orthologue. However, with the identification of a growing number of non-visual opsins, it has been shown that many of these (including parapinopsin, parietopsin, TMT opsin, OPN3, neuropsin, peropsin, RGR and OPN4) arose before VA opsin (Davies et al. 2010) (Fig. 3.1).

Functional studies demonstrated that salmon VA opsin can form a photopigment with a λ_{max} between 460 and 480 nm when expressed in vitro and reconstituted with 11-*cis* retinal (Soni et al. 1998). Significantly, VA opsin was shown to be expressed in a subset of horizontal cells and RGCs (Soni et al. 1998). Subsequently, VA opsin was shown to be expressed within the pineal organ and epithalamic/hypothalamic regions of the teleost brain (Philp et al. 2000b), sites strongly implicated as being

photoreceptive in fishes. Similar findings were reported in the zebrafish (Kojima et al. 2000). Two VA opsin orthologues were isolated in zebrafish, "long" (VAL) and "short" (VAS) forms, which vary in the length of their carboxyl-terminal tails (74 and seven amino acids, respectively). Both isoforms were functionally expressed in human embryonic kidney 293 (HEK293) cells, but only VAL appeared capable of forming a photopigment when reconstituted with 11-*cis* retinal (Kojima et al. 2000). Studies of several other teleosts have confirmed the existence of different isoforms of VA opsins, where the shorter isoform appears to be generated by intron retention at a splice site in all cases. A comparison of the known teleost sequences indicates that they fall into two distinct groups, one consisting of zebrafish, roach and carp, the other of smelt and salmon. This split might be explained by the identification of a second *VA* opsin gene in zebrafish, named VAL-opsin B (Kojima et al. 2008). The functional significance of this gene duplication in the teleost genome remains unclear.

The discovery of VA opsin in teleost fishes prompted the search for orthologues of VA opsin in other vertebrate classes, but until recently attempts have failed. This restricted taxonomic distribution of the VA opsins was puzzling as most other opsin classes span multiple vertebrate taxa. Recently full-length sequences of chicken long and short VA isoforms, VAL and VAS, respectively, were described and shown to form fully functional pigments when heterologously expressed in a mammalian neuronal (Neuro2A) cell-line. Furthermore, their photosensitivities were dependent upon *cis*-forms of retinal chromophore (Halford et al. 2009). Subsequent UV-Vis spectrophotometry of chicken VAL and VAS revealed the peak spectral sensitivity of these pigments to be around 490 nm (Davies et al. 2012a), a wavelength maximum which closely matches the reported λ_{max} of the avian photoperiodic response (Davies et al. 2012a; Foster et al. 1985) (Figs. 3.4 and 3.5). A recent UV-Vis spectrophotometric study of VA opsin in *Xenopus tropicalis* confirmed that VA opsin is not a bistable pigment and forms a bleachable pigment with a λ_{max} at 501 nm (Sato et al. 2011). In addition to the identification of VA opsin orthologues in Agnatha, Neoteleosti and Amphibia, further studies have also shown that VA-like genes are present in Reptilia (e.g. the green anole, *Anolis carolinensis*) and Chondrichthyes (e.g. the elephant shark, *Callorhinchus milii*), but have failed to find any VA orthologues within the mammalian lineage (Davies et al. 2010) (Fig. 3.2). This surprising finding raises important questions as to the possible function of this opsin within vertebrate taxa.

3.7.4 Parietopsin

Recent studies on another photoreceptive structure, the parietal eye of lizards, have identified the expression of two opsins within the same photoreceptor, a short-wavelength-sensitive ("blue") pinopsin and a novel MWS ("green") opsin named parietopsin (Su et al. 2006). These findings are consistent with the observation that the parietal-eye photoreceptors have two antagonistic light-signalling pathways, a hyperpolarising pathway maximally sensitive to blue light and a depolarising pathway maximally sensitive to green light (Solessio and Engbretson 1993). The peak

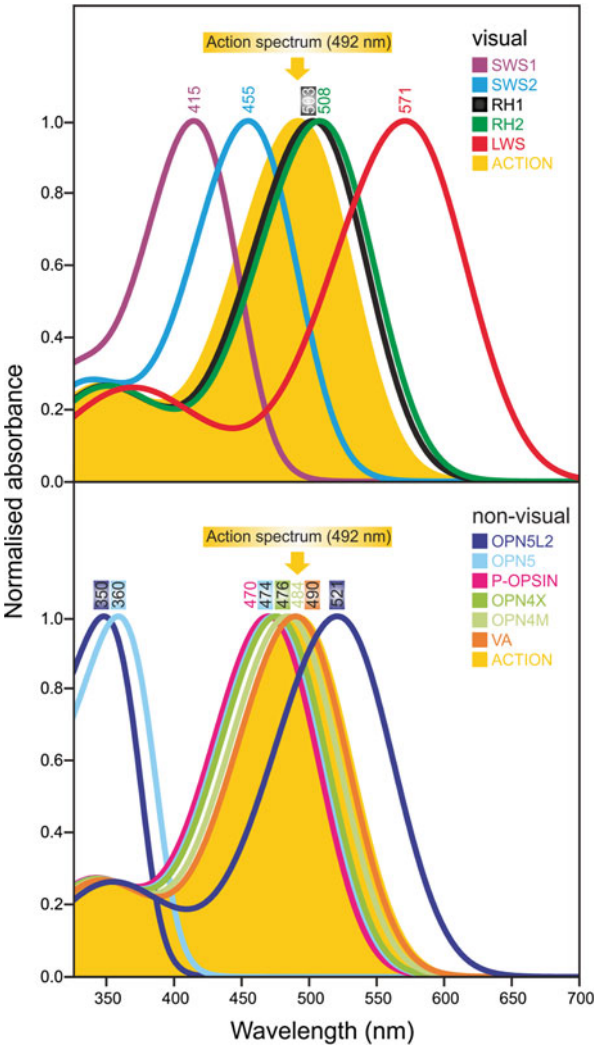

Fig. 3.5 Candidate pigments that may mediate the avian photoperiodic response. A diagram showing typical absorbance templates for an array of visual (*upper panel*) and non-visual (*lower panel*) photopigments found in birds, with each opsin class being labelled with spectral maxima derived from the literature. Many of the pigments are either not expressed in the correct anatomical location to mediate avian photoperiodism or do not possess λ_{max} values that match the action spectrum with a spectral peak at 492 nm (*light orange shading*). Recent studies have proposed a number of putative pigments (e.g. vertebrate ancient (VA) opsin, neuropsin (OPN5) and melanopsin) that may mediate this critically important reproductive function in birds; however, VA opsin specifically is the closest match to the spectral characteristics of both the action spectrum and the hypothalamic light environment and thereby presents itself as the most likely candidate. Normalised avian pigment templates are compared to the photoperiodic action spectrum (*light orange shading*) and are colour coded to denote both visual opsin classes: long-wavelength-sensitive, LWS (*red*); short-wavelength-sensitive 1, SWS1 (*violet*); short-wavelength-sensitive 2, SWS2 (*bright blue*); rod opsin-like 2, RH2 (*dark green*); and rod opsin 1, RH1 (*black*); and many non-visual classes: "mammalian-like" melanopsin, OPN4M (*pale green*); "*Xenopus*-like" melanopsin, OPN4X (*medium green*); neuropsin, OPN5 (*pale blue*) and neuropsin-like 2, OPN5L2 (*dark blue*) pigments; vertebrate ancient opsin, VA (*dark orange*); and pineal gland-specific opsin, pinopsin or P-opsin (*pink*)

spectral sensitivity of lizard (*Uta stansburiana*) parietopsin was reported to be 520 nm (Sakai et al. 2012), which is significantly long-wavelength-shifted when compared to the spectral peaks measured for pinopsin (460–470 nm) (Max et al. 1998; Okano et al. 1994). These spectral absorbencies of pinopsin and parietopsin match directly the "blue" and "green" photosensitivity of the parietal-eye photoreceptor cells, supporting their roles in mediating the observed light responses. Using single cell patch-clamp recording, the depolarising light responses mediated by parietopsin were linked to a G_o-coupled pathway (Su et al. 2006), which resembles the signalling cascade of some invertebrate photoreceptors. In contrast, P-opsin has been shown to couple with gustducin in the parietal eye, a G protein trimeric complex that is closely related to transducin (G_t) in visual photoreceptors (Su et al. 2006). These authors suggested that the G_o-coupled pathway might be more ancient compared to the gustducin pathway, as it can be traced back to the common ancestor of vertebrates and invertebrate molluscs (coelomates). Parietopsin showed the highest degree of amino acid identity (40 %) to parapinopsin (Su et al. 2006) and has been linked to other biological functions that include detecting the spatial orientation of the sun and electromagnetic fields (Foa et al. 2009; Nishimura et al. 2010).

In addition to the identification of parietopsin in teleost fishes and amphibians (Fig. 3.2), a recent study of the orthologue found in the side-blotched lizard (*Uta stansburiana*) revealed that this pigment uses Glu181 as a counterion, together with the photochemistry of the photointermediates (i.e. metarhodopsin I, metarhodopsin II, metarhodopsin III being produced after bathorhodopsin and lumirhodopsin) showing characteristics that are consistent with other vertebrate photopigments (Sakai et al. 2012). This has led to the suggestion that parietopsin represents a functional intermediate between the invertebrate-like and vertebrate-like opsins, however, this remains to be clarified given the confusion over terms like "vertebrate-like" and "invertebrate-like" when parietopsin was identified and is restricted to the vertebrate lineage.

3.7.5 Parapinopsin

Despite the lack of information about the structure and physiological function of the teleost parapineal gland, a novel opsin photopigment, named parapinopsin, has been isolated from this organ. Originally, parapinopsin was isolated from the channel catfish (*Ictalurus punctatus*) and showed a sequence identity of 40 % to other vertebrate opsins, with expression in a majority of parapinealocytes and a subset of pineal and parapineal cells (Blackshaw and Snyder 1997). An orthologue of parapinopsin was isolated from the lamprey pineal complex and appears to form a bistable photopigment, with amino acid sequence identities of 61 % and 71 % to orthologues identified in the rainbow trout and the clawed toad, respectively (Koyanagi et al. 2004). RNA in situ hybridisation showed that lamprey parapinopsin was expressed in the photoreceptor cells located in the dorsal region of the pineal and parapineal organs, and encoded a photopigment with a λ_{max} at 370 nm. Furthermore, upon

illumination with UV light, the 11-*cis* retinal chromophore was photoconverted to all-*trans* retinal to form a stable photoproduct with a λ_{max} at 515 nm (Koyanagi et al. 2004). Recently, a further parapinopsin sequence was isolated from the Green Iguana (*Iguana iguana*) and shown to be UV sensitive (Wada et al. 2012). The authors concluded, therefore, that parapinopsin may be the photopigment that underlies UV-sensitivity in pineal-related organs in species that include lampreys, fishes, amphibians and reptiles (Fig. 3.2).

3.7.6 Teleost Multiple Tissue Opsin

Isolated organs and even cell-lines from zebrafish have been shown to exhibit circadian oscillations in clock gene expression that can be entrained to light (Whitmore et al. 2000). These data provide strong evidence for the existence of one or more photopigments within these cells. TMT opsin was isolated in 2003 as part of a study to identify the photopigment or pigments in these peripheral tissues and shown to share a sequence identity of 33–39 % when compared to other vertebrate opsins (Moutsaki et al. 2003). The full-length sequence of TMT opsin was initially isolated from the pufferfish (*Takifugu rubripes*). It encodes a predicted protein of 402 amino acids, containing all of the essential features of an opsin photopigment including Lys296 for the attachment of a retinoid chromophore. Interestingly the Schiff base counterion at site 113, usually a glutamate, is substituted by a tyrosine in both the pufferfish and zebrafish TMT orthologues. The gene was given its name because it was shown to be expressed in multiple tissues including the liver, kidney, heart, eye and brain, and at that time had only been isolated from teleosts.

TMT opsin is closely related to encephalopsin (OPN3), which also shows multiple tissue expression, but OPN3 was initially thought to be present only in the mammals (Blackshaw and Snyder 1999; Halford et al. 2001; White et al. 2008) and thus a candidate orthologue of TMT in the mammalian lineage. However, TMT and OPN3 are distinct sister gene lineages, although grouped under the same subfamily, sharing ~41 % amino acid sequence identity (Moutsaki et al. 2003). Although OPN3 was confirmed not to be an orthologue of TMT opsin in the mammalian lineage, the latter has been identified in the genomes of monotremes and marsupials (Gerkema et al. 2013) (Fig. 3.2). Both TMT opsin and OPN3 share a partial genomic structure common to the vertebrate visual opsins, with three consistent intron locations (intron 1, 3 and 4) (Moutsaki et al. 2003). However, this gene structure is markedly different from melanopsin. The conservation between OPN3 and TMT opsins suggests that they were likely to have descended from the same ancestral gene that gave rise to both visual and some more recently evolved non-visual opsins (i.e. parapinopsin, parietopsin, VA and pinopsin). Furthermore, the identical genomic structure between these two genes (namely OPN3 and TMT) suggests that they are more closely related to each other than to other opsin groups that flank this monophyletic pigment class (Fig. 3.1) and implies that there may be possible functional similarities between the two classes.

3.7.7 Encephalopsin/Panopsin (OPN3)

OPN3 was originally termed encephalopsin and reported to be an extraocular opsin with strong expression in mouse brain and testis, with lower levels in the heart, liver and kidney (Blackshaw and Snyder 1999). However, a subsequent study demonstrated that OPN3 was in fact expressed in the retina and in all tissues examined; hence, the name "panopsin" was proposed (Halford et al. 2001). A more recent study on human OPN3 confirms the wide tissue distribution and in multiple sites within the retina, including the cones and rods, the outer-plexiform, inner-plexiform and the RGC layers (White et al. 2008). A comparison of OPN3 with the visual opsins shows a low amino acid sequence identity of 30 %. OPN3 contains Lys296; however, the Glu113 counterion has been substituted with Asp113. This does not preclude the formation of a photopigment as an aspartate residue is also present at this position in the violet-sensitive SWS1 photopigment of *Xenopus* sp. (Starace and Knox 1998). Since Asp113 is also negatively charged and functions in a visual pigment to stabilise the protonated Schiff base, it is likely that this residue also acts as a conventional counterion in OPN3 pigments, instead of a counterion shift to site 181 as found in many non-visual pigments that also do not possess Glu113.

Phylogenetic analyses suggest that OPN3 orthologues are present in all the main vertebrate classes from teleost fishes to primates, except for the monotremes (Fig. 3.2). Interestingly genetic linkage analyses have indicated that polymorphisms in the *OPN3* gene located on human chromosome 1q may be associated with atopic and non-atopic asthma. Evidence for this conclusion also arises from expression studies that demonstrate that both OPN3 mRNA and protein levels are high in tissues that are involved in asthma (e.g. bronchiolar epithelium) and the immune system (e.g. macrophages, primary T-cells and dendritic cells) (White et al. 2008).

The function of encephalopsin is further complicated by considerable alternate splicing of transcripts from the human *OPN3* gene (Kasper et al. 2002). Whilst knowledge of OPN3 in the vertebrate lineage remains sparse, a recent study of closely related OPN3 orthologues, as well as pufferfish TMT and mosquito OPN3, has led to the suggestion that they are short wavelength to medium wavelength sensitive bistable pigments that couple to G_i/G_o-signalling pathways that regulate cyclic adenosine monophosphate (cAMP) second messenger levels (Koyanagi et al. 2013). In mammals the role of OPN3 remains of considerable interest and it has been confirmed recently that murine OPN3 protein is abundant in the mouse brain, where it is present in neurons of the cerebral cortex, periventricular area and cerebellar cells (Nissila et al. 2012). As yet, this gene has not been linked directly to a particular photoreceptor function.

3.7.8 Neuropsin (OPN5)

The neuropsin (*OPN5*) gene was identified in 2003 using a bioinformatics approach and found to be present in both murine and human genomes (Tarttelin et al. 2003); its presence has now been extended to all the major vertebrate classes (Fig. 3.2).

OPN5 encodes a predicted protein of 377 and 354 amino acids in mice and humans, respectively, with the mouse pigment possessing a longer carboxyl-terminal tail. All of the expected features of a typical opsin are conserved, but OPN5 shows only 25–30 % sequence identity to other members of the vertebrate opsin superfamily. A reverse-transcription polymerase chain reaction (RT-PCR) analysis suggested that OPN5 was expressed in murine testis, brain and eye tissue and in the retina and brain of humans. Recent studies have now clarified the properties of vertebrate OPN5. Chicken OPN5 (labelled cOPN5M) was shown to be a UV-sensitive bistable pigment that coupled to a G_i α-subunit (Yamashita et al. 2010). When expressed in a HEK293 cell-line, this pigment formed a stable association with both 11-*cis* and all-*trans* retinal, with absorption maxima at 360 nm and 474 nm, respectively. Similarly, mouse neuropsin also formed a bistable pigment with a λ_{max} at 380 nm with 11-*cis* retinal and also activated a G_i α-subunit (Kojima et al. 2011). Heterologous expression of quail (*Cotumix japonica*) pigment in *Xenopus laevis* oöcytes suggested that OPN5 forms a violet-sensitive pigment with a λ_{max} at 420 nm when reconstituted with 11-*cis* retinal (Nakane et al. 2010). Avian OPN5 mRNA has been localised to the paraventricular organ (PVO), leading to the suggestion that it may be the photopigment involved in the photoperiodic response of birds (Nakane et al. 2010). However, OPN5 transcripts have also been found in the avian retina, and are highly expressed in the adrenal glands of birds, leading to the suggestion that they may have dual roles as a photopigment and another accessory chemosensory function in non-light responsive tissues (Ohuchi et al. 2012). Historically, there has been a paucity of candidate opsins for the encephalic photopigment in the avian photoperiodic response. VA opsin (Halford et al. 2009; Davies et al. 2012a), OPN5 (Nakane et al. 2010), and perhaps OPN4 with hypothalamic expression in both the house sparrow, *Passer domesticus* (Wang and Wingfield 2011) and the turkey, *Meleagris gallopavo* (Kang et al. 2010), have now been detected in plausible anatomical deep-brain regions, making them all candidates for the avian photoperiodic response. Nonetheless, based on spectral characteristics for each pigment compared to the action spectrum calculated for the avian photoperiodic response (Foster and Follett 1985; Foster et al. 1985), VA opsin remains the most likely candidate (Halford et al. 2009; Davies et al. 2012a) (Figs. 3.4 and 3.5). Interestingly, the localisation of OPN5 in rat and chicken RGCs raises the possibility of an uncharacterised UV-sensitive pigment in the vertebrate eye (Nieto et al. 2011; Ohuchi et al. 2012). This may be important in non-primate species, but since the corneas and lens of primates, including humans, do not transmit UV light, therefore, the possession of an ocular UV-sensitive pigment in these latter species may not be functionally relevant.

3.7.9 Peropsin/RPE-Derived Rhodopsin Homologue

Peropsin, also called RPE-derived rhodopsin homologue (RRH), was isolated from the RPE of human and mouse eyes in 1997 (Sun et al. 1997). Subsequently, an orthologue of peropsin was found in the eyes of the cephalochordate lancelet

(*Branchiostoma belcheri*) (Koyanagi et al. 2002) and in many invertebrate species, such as the jumping spider, *Hasarius adansoni* (Nagata et al. 2010), as well as in the genomes of representatives from each major class of the vertebrate lineage (Fig. 3.2). Although this opsin shows only a 26 % amino acid sequence identity with other photosensory opsins, phylogenetic evidence suggests that peropsin is closely related to the photoisomerase group that includes retinochrome, RGR (Bellingham et al. 2003; Sun et al. 1997) and neuropsin (OPN5) (Fig. 3.1). Immunocytochemistry and RNA in situ hybridisation showed that peropsin expression is confined to the RPE, specifically localised to the microvilli around the outer segment of photoreceptor cells. The evolutionary position and tissue expression pattern of RRH, when taken together, resulted in the speculation that peropsin may function as a retinal isomerase like RGR, thus maintaining a concentration of 11-*cis* retinal close to visual photoreceptor cells (Bellingham et al. 2003; Koyanagi et al. 2002; Sun et al. 1997). Indeed, it has already been shown that the amphioxus orthologue of peropsin has the ability to bind all-*trans* retinal and convert it into the 11-*cis* retinoid isomer (Koyanagi et al. 2002), thereby further supporting its role in retinal recycling. Despite the close association of RRH to the retinal regeneration function of RPE, there are currently no known mutations in the RRH gene linked to retinitis pigmentosa (Rivolta et al. 2006).

3.7.10 RGR Opsin

RGR opsin was originally isolated by screening a bovine RPE cDNA library (Jiang et al. 1993) and has now been shown to be present in all vertebrate groups, except the marsupials (Fig. 3.2). The expression of RGR was originally thought to be restricted to the RPE and Müller glial cells of vertebrates (Pandey et al. 1994) and thought to play a role as a photoisomerase catalysing the conversion of all-*trans* retinal to 11-*cis* retinal. Like all other opsin classes, this pigment possesses Lys296, but its putative counterion site 113 is occupied by a positively charged histidine residue (Briggs and Spudich 2006). Other opsins usually possess either a negatively charged residue (e.g. glutamate) or a neutral residue (e.g. tyrosine) at site 113. Interestingly, an orthologue of RGR in molluscs (retinochrome) also possesses a positively charged histidine residue at this site, although His113 can also be substituted by an uncharged methionine residue (Terakita et al. 2000), which casts doubt on the role that His113 plays as a counterion. It has been shown that RGR opsin shares 21 % and 24 % sequence identities with the visual opsins and retinochrome, respectively (Hara and Hara 1967). Site-directed mutagenesis in retinochrome demonstrated that substituting His113 does not alter its spectral absorbance, but changing the negatively charged glutamate residue at site 181 causes deprotonation of the Schiff base, leading to a short-wavelength shift in the spectral peak of absorbance (Terakita et al. 2000). This suggests that Glu181 may act as a counterion in both retinochrome and RGR via a counterion switch from site 113 to 181. Spectrally, RGR opsin proteins reconstituted with all-*trans* retinal are short-wavelength-sensitive pigments (λ_{max} at 469 nm) (Hao

and Fong 1996). The presence of RGR contributes to the normal functioning of the retina as mutations in this gene have been associated with retinitis pigmentosa, a common form of inherited blindness (Morimura et al. 1999). It has been speculated that this photopigment functions as a photoisomerase in the RPE for converting all-*trans* to 11-*cis* retinal in a light-dependent manner, which is distinct from the light-independent system of the visual cycle (Radu et al. 2008). However, the role of RGR is complicated as homozygous RGR knockout mice are still capable of light-dependent regeneration of 11-*cis* retinal (Maeda et al. 2003), thus indicating that although RGR acts in the retinoid cycle, its role in chromophore regeneration is not essential.

3.7.11 Melanopsin (OPN4)

An extensive account of the evolution and functional significance of melanopsin is given in Chap. 2 and will only be outlined briefly here. Melanopsin (OPN4) was first discovered in photosensitive dermal melanophores of *Xenopus laevis*, and found subsequently in the eye and brain. Within the retina, melanopsin was detected in non-cone, non-rod cells including horizontal cells, the RPE and the iris (Provencio et al. 1998). In the brain, OPN4 was identified in the ventral part of magnocellular preoptic nucleus and the suprachiasmatic nucleus (SCN), both of which were previously implicated in deep-brain photoreception (Foster et al. 1994). These expression patterns prompted researchers to search for mammalian orthologues of melanopsin, which could potentially have a role in mediating light-dependent regulation of circadian rhythms. Indeed, human and mouse orthologues of melanopsin were subsequently identified (Provencio et al. 2000) and once again localised to pRGCs (Hattar et al. 2002; Sollars et al. 2003). Since its initial discovery in amphibians and eutherian mammals, it is now clear that it exists in two distinct gene lineages, one that is widely expressed in vertebrates, including in an array of mammalian species (named "mammalian-like" or OPN4M), and the other that is absent from mammals (named "*Xenopus*-like" or OPN4X) (Bellingham et al. 2006). Additionally, multiple long and short isoforms have been identified that arise from both *OPN4M* and *OPN4X* genes in a species-specific manner (reviewed in Davies et al. 2010 and in Chap. 2). To date, melanopsin photopigments have also been identified in a broad range of species including cartilaginous fishes (e.g. the elephant shark, *Callorhinchus milii*; both OPN4X (long and short transcripts) and OPN4M forms, Davies et al. 2012c); teleost fishes (e.g. the zebrafish; multiple *OPN4X* and *OPN4M* genes, Bellingham et al. 2002; Davies et al. 2011); reptiles (e.g. the Italian wall lizard; both *OPN4X* and *OPN4M* orthologues, Frigato et al. 2006); birds (e.g. the chicken; again both OPN4 isoforms, with each generating both long and short isoforms, Tomonari et al. 2005); monotremes (e.g. the platypus, *Ornithorhynchus anatinus*; but only *OPN4M* with the loss of *OPN4X*, Bellingham et al. 2006; Davies et al. 2010); marsupials (e.g. the fat-tailed dunnart, *Sminthopsis crassicaudata*; but again only the *OPN4M* isoform, Pires et al. 2007); and in a number of eutherians (but only the *OPN4M* gene, Davies et al. 2010) (Fig. 3.2).

Long and short variants of *OPN4M* are also present in eutherians (Pires et al. 2009), where they are differentially regulated throughout development and expressed in different pRGC subtypes (Hughes et al. 2012b). Interestingly, in humans, a Pro10Leu variant of OPN4M has been reported, which may be associated with seasonal affective disorder (SAD).

Much evidence has been accumulated showing unequivocally that melanopsin is the photopigment that underpins circadian photoentrainment, whereby biological rhythms (e.g. sleep) are synchronised with the external light environment (Ruby et al. 2002; Panda et al. 2002; Lucas et al. 2003; Hattar et al. 2003; Güler et al. 2008 and reviewed in this volume). The property of intrinsic photosensitivity in melanopsin-expressing RGCs in the rat was shown by whole-cell recordings to have a peak absorbance at 480 nm (Berson et al. 2002). Subsequent studies have confirmed that melanopsin, when heterologously expressed in various cell-lines and in *Xenopus laevis* oöcytes, induces retinal-dependent light responses (with either 9-*cis* or 11-*cis* retinal) that activate an endogenous phototransduction cascade (Melyan et al. 2005; Panda et al. 2005; Qiu et al. 2005) with a spectral peak at ~470–480 nm. Furthermore, it has been shown that mammalian melanopsin also functions as a bistable photopigment, with inherent photoisomerase activity similar to many invertebrate opsins (Davies et al. 2011, 2012c; Melyan et al. 2005; Panda et al. 2005). On binding all-*trans* retinal, melanopsin uses long-wavelength light to regenerate 11-*cis* retinal. It has been suggested that this light-driven reversibility allows melanopsin to sustain a prolonged response to light stimulation in pRGCs (Mure et al. 2009; Wong 2012).

Despite the strong evidence for melanopsin signalling through a $G_{q/11}$ cascade, a detailed comparison with invertebrate phototransduction pathways revealed that there are still many unanswered questions in the current model of melanopsin-signalling cascade. One notable area which has yet to be elucidated is the types of protein kinases (PKs) responsible for modulating melanopsin-signalling activity and its desensitisation mechanism (Hughes et al. 2012a). PKA and PKC are the two main classes of protein kinases that have been known to mediate regulation of GPCR-signalling dependent on secondary messengers. The phosphorylation activity of PKA requires the presence of cAMP, whilst that of PKC is activated by a membrane-bound lipid molecule called diacylglycerol (DAG) (Alberts et al. 2002). Since intracellular cAMP is driven by adenylate cyclase (AC), and is typically associated with G_s- and G_i-signalling pathways, it seems unlikely that PKA is employed in melanopsin phototransduction. However, it is now known that certain isoforms of adenylate cyclase can be directly stimulated by the binding of a Gβγ dimer that is released upon activation of the G protein (Diel et al. 2006; Tang and Gilman 1991). Therefore, even though there is a lack of evidence for PKA-cAMP regulation in the melanopsin cascade, its involvement remains a possibility.

The secondary messenger system that activates PKC (i.e. DAG) is closely associated with calcium (Ca^{2+}) signalling, which is a classical response of the $G_{q/11}$ pathway (Melchior and Frangos 2012; Takashima et al. 2006). It has been shown that in invertebrates, PKC activity is linked to light adaptation and desensitisation of the photoreceptor responses (Gu et al. 2005; Yau and Hardie 2009). Light adaptation (also known as background adaptation) occurs when the photoreceptor responds

more quickly to increased intensities of illumination. This adaptation process also reduces sensitivity of the transduction pathway, by lowering the amplitude of each light response (Fain et al. 2001). Interestingly, both light adaptation and desensitisation have been reported in melanopsin-expressing pRGCs, suggesting the potential involvement of PKC (Gamlin et al. 2007; Zhu et al. 2006). Nonetheless, there has yet to be direct evidence showing how and when in the melanopsin-signalling pathway PKC may mediate the observed characteristics of these photoresponses. Interestingly, functional genomics have indicated the involvement of a unique isotype of PKC (PKCz) in the melanopsin cascade, one that does not require Ca^{2+} and DAG for activation (Hughes et al. 2012a; Peirson et al. 2007). It was reported that knockout mice lacking the *PKCz* gene shared strikingly similar phenotypes to those in mice where melanopsin was genetically ablated, with a reduced pupillary light reflex, an attenuated phase-shift circadian rhythm in response to light, decreased period-lengthening under the constant dim-light conditions, and a deficiency in SCN expression of light-induced clock genes (Peirson et al. 2007). However, due to an absence of an association with the secondary messengers of the melanopsin cascade, the involvement of PKCz in this pathway is yet to be proven. Despite the obvious gaps in the current model of the melanopsin phototransduction cascade, it remains the best studied signalling pathway of all of the vertebrate non-visual pigments.

3.8 G Protein-Coupling in the Non-visual Opsins

An important driving force in the evolution of opsin photopigments has been the coupling of the various opsin groups to a range of G protein-signalling cascades, which results from the selective interaction and co-evolution of the opsin and an array of potential cellular Gα subunit-binding partners. Transducin (G_t) is a heterotrimeric G protein with three polypeptide chains: Gα, Gβ and Gγ. Transducin is expressed in vertebrate visual photoreceptors, although cones and rods possess specific α subunits ($G\alpha_t$), as well as separate isoforms for many other molecules involved in the biochemical cascade of the visual system. Briefly, transducin specifically couples the activated photopigment to phosphodiesterase (PDE6), which hydrolyses cyclic guanosine monophosphate (cGMP). This in turn causes the closure of cGMP-gated cation channels in the photoreceptor membrane and hyperpolarises the photoreceptor cell (Arshavsky et al. 2002). The part of the cascade that a specific opsin couples to is defined by the Gα subunit to which the opsin is bound. There are several sites on the opsin protein that interact with the Gα subunit and of these, the third intracellular loop appears to be critical in defining the specificity for Gα binding. Amongst the visual opsins that bind transducin, there is clearly a conserved sequence in the third intracellular loop (Fig. 3.6). This $G\alpha_t$ sequence is also largely conserved in the non-visual opsins, exorhodopsin, VA opsin and pinopsin, that are also able to activate transducin. For other non-visual opsins, there are considerable variations in the sequence of this loop that reflects their coupling to $G_{i/o}$- and $G_{q/11}$-signalling pathways.

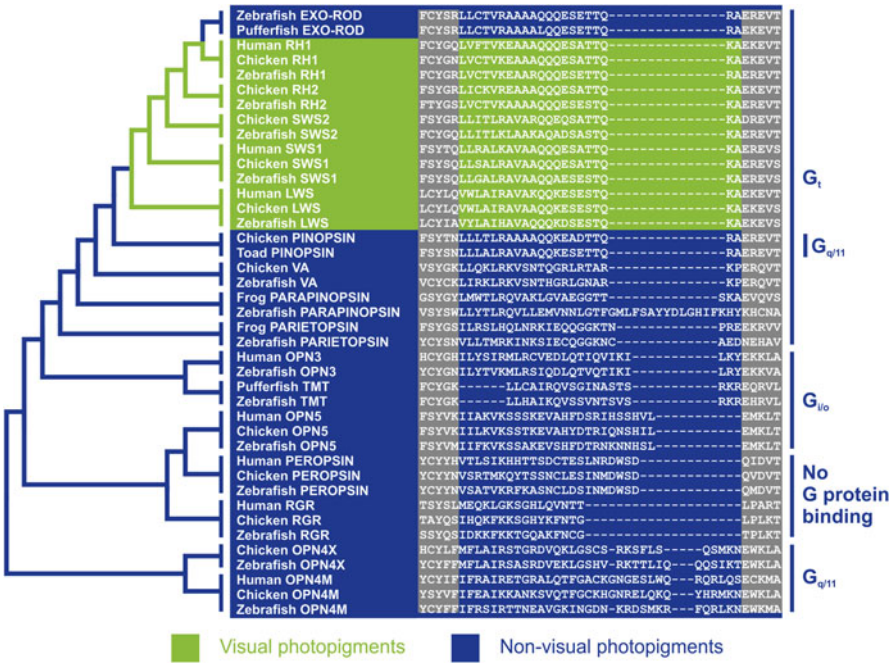

Fig. 3.6 Putative functional diversity through differences in opsin cytoplasmic domains. A codon-matched amino acid alignment of the third cytoplasmic loop present in visual (*green*) and non-visual (*blue*) pigments expressed in representative vertebrates based on the bovine rhodopsin crystal structure. (–) denotes gaps that have been inserted to maintain a high degree of identity. In all cases, grey shading identifies five adjacent residues from the two transmembrane domains (TMV and TMVI) that flank the third cytoplasmic loop. Where known, the class of G protein alpha subunits that interact with the pigment in question is shown. Pigment classes shown above include "mammalian-like" melanopsin, OPN4M; *Xenopus*-like melanopsin, OPN4X; retinal G protein-coupled receptor, RGR; retinal pigment epithelial (RPE)-specific rhodopsin homologue, RRH (peropsin); neuropsin, OPN5; panopsin/encephalopsin, OPN3; teleost multiple tissue opsin, TMT; parietopsin; parapinopsin; vertebrate ancient opsin, VA; pineal opsin, pinopsin; long-wavelength-sensitive cone opsin, LWS; short-wavelength-sensitive cone opsin 1, SWS1; short-wavelength-sensitive cone opsin 2, SWS2; middle-wavelength-sensitive rhodopsin-like cone opsin 2, RH2; middle-wavelength-sensitive rhodopsin-like rod opsin 1, RH1; and extraretinal rod-like opsin, EXO-ROD

3.9 General Conclusions

The role of non-visual light detection in the physiology of the vertebrates has a long history that stretches back to the elucidation of deep-brain photoreception by von Frisch over a century ago. Since then, many studies have continued to add a wealth of information into relating the principal sites of non-visual photoreception to a range of targets in the CNS, including the neural retina itself.

More recently, knowledge of the vertebrate genome has expanded as more species have had their genomes sequenced. As a result, there has been a massive

increase in the number of known opsins in all the main groups of vertebrates. It is now generally accepted that at least two rounds of WGD occurred in the vertebrate lineage, one early in the evolution of the vertebrates and a second around the divergence of the teleost fishes (see Nakatani and Morishita 2008). These evolutionary events have provided the substrate for the molecular evolution of a diverse family of opsin proteins that have evolved to have different functions. Some of the vertebrate opsin gene products evolved as classical photopigments (either monostable or bistable), whilst others became photoisomerases or regulators of retinal signalling. Of the opsin genes that encode for photopigments, a broad range of spectral sensitivities and chromophore-handling variants became conserved in the vertebrates and adapted to perform a wide range of physiological tasks. Now that knowledge of the "opsin genome" is approaching completion within key species, the remaining challenge is to assign a functional role to each pigment class, particularly those expressed in the CNS. This is not a simple task, and begins with opsin localisation with specific antibodies and the matching of pigment spectral sensitivity to the biological action spectrum for a particular physiological response. In many species gene knockout and reporter transgenics are far from routine methodologies. Some of the deep-brain non-visual responses may be mediated by single opsin photopigments, although it is not unlikely that some of these responses may be driven by multiple opsins, each with a distinct role to play. For example, a particular physiological process (e.g. avian photoperiodism) may involve a classical monostable non-visual opsin (e.g. VA opsin) that is reliant upon another opsin protein (e.g. OPN5) to regenerate chromophore that is far from the traditional sites of retinoid isomerisation or photoconversion (e.g. the RPE). Such a biological arrangement may be very difficult to unravel with routine approaches.

Opsin-based photopigments in general have evolved to mediate specific photoreceptive tasks in different light environments (Lythgoe 1979). For example, in environments where the spectral composition and intensity of the light is restricted, such as in deep water, the λ_{max} values of visual photopigments are spectrally tuned to match the maximum available photon flux around 480 nm (Douglas and Partridge 1997; Hope et al. 1997; Hunt et al. 2001). Whether similar spectral tuning arguments can be used to understand the λ_{max} of NIF photopigments remains an intriguing question. Many photoreceptors involved in NIF tasks appear to maximise their spectral peaks close to 480 nm, but with a spread ranging from 360 to 530 nm. In pineal and deep-brain photoreceptors, the light available will be dominated by the transmission of the overlying tissues. This is primarily influenced by two factors. Firstly, short-wavelength light is scattered more than longer-wavelength light, resulting in relatively more light of longer wavelengths penetrating to reach intracranial photoreceptors. Secondly, the spectral quality of incoming light may be significantly modified by light-absorbing molecules before reaching these photoreceptors. The most important biological "spectral filter" in this context is haemoglobin, which exhibits a transmission window between 460 and 540 nm, peaking around 490 nm (Foster and Follett 1985; Hartwig and van Veen 1979). This transmission window may in turn have exerted a strong selection pressure on the spectral tuning of deep-brain and pineal photoreceptors. There are, however,

large numbers of long-wavelength photons reaching the pineal and brain. Also, pigments in the long-wavelength end of the visible spectrum are highly susceptible to thermal noise as a result of their lower excitation energies (Barlow 1957), which may interfere with the fidelity of the light signal. Issues with the location of the counterion may also preclude the spectral tuning of bistable pigments to longer wavelengths, which implies that bistability may be a strong evolutionary driving force in the molecular evolution of many non-visual pigments in the CNS. In contrast photoreceptors located in the vertebrate retina do not suffer such limitations and are able to sample light across the entire visual spectrum.

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Chapter 4
The Evolution of Invertebrate Photopigments and Photoreceptors

Thomas W. Cronin and Megan L. Porter

Abstract The advent of new tools in genetics, labeling, and imaging has led to a revolution in the ability to investigate the genetic and cellular evolution of invertebrate photoreceptor pigments and cells. All opsins, invertebrate and vertebrate, derive from a common ancestral G-protein-coupled receptor, whose descendants form four distinct groups (one of which, the "cnidops," is strictly limited to invertebrates). Today's invertebrate opsins associate with a bewildering assortment of G-proteins and often have unusual properties, including functional bistability and occasionally the capacity to act as photoisomerases for visual pigment chromophores. In this chapter, we review our state of knowledge of how invertebrate opsins—the proteins underlying all visual pigments—have evolved and become functionally specialized as well as how the photoreceptive cells in which they are housed have diversified from a common ancestor—or ancestors—early in animal evolution.

Keywords Opsin • Opsin evolution • Phototransduction • Bistability • Photoisomerase • Spectral tuning • Invertebrate photoreceptors

4.1 Introduction

Several comprehensive reviews of invertebrate vision have been published in recent decades. Of these, the most authoritative are the invertebrate vision volumes in the *Handbook of Sensory Physiology*, Volume 7, Parts 6A, 6B, and 6C, all edited by H. Autrum (1979, 1981a, b); *Photoreception and Vision in Invertebrates* (1984) edited by M.A. Ali, and (much nearer to the present) E. Warrant's and D.E. Nilsson's *Invertebrate Vision* (2006). All these books have abundant content on invertebrate photopigments and photoreceptors, but none considered the evolution of

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invertebrate photoreceptor cells at any length, perhaps because Eakin had masterfully covered the topic of photoreceptor evolution in an earlier volume of the *Handbook of Sensory Physiology* Volume 7, Part 1 (1972). The question of invertebrate visual pigment evolution was entirely ignored, and we begin this review with the photopigments, saving the topic of photoreceptor evolution for later.

With one possible exception, only two classes of photopigments are known to be involved in neural signaling of light's presence in animals: cryptochromes and opsins (the possible exception to this being a gustatory receptor homolog that is thought to modulate phototaxis in the nematode *Caenorhabditis elegans* and in larvae of *Drosophila*; see Liu et al. 2010; Xiang et al. 2010). Not much is known about cryptochrome evolution and function in animals (see Heath-Heckman et al. 2013). Even its signaling pathways are very poorly understood (Partch and Sancar 2005), although the biochemistry of plant cryptochromes is fairly well documented. Light-sensing cryptochromes apparently mediate phototaxis in sponge larvae (Rivera et al. 2012), magnetoreception in birds (Mouritsen et al. 2004), and circadian rhythms in adult *Drosophila* (Stanewsky et al. 1998), the squid *Euprymna scolopes* (Heath-Heckman et al. 2013), and possibly in butterflies (Sauman et al. 2005). These puzzlingly diverse functions certainly hint that light-sensing cryptochromes have many undiscovered roles in animal photoreception; but for now, the "crypto" in their name remains utterly appropriate.

In contrast, opsin—the protein that so far as we know underlies all animal vision (and numerous other photoreceptive functions)—has become a favorite research target, not only of vision scientists but of many researchers interested in the evolution of protein structure, function, and specialization. This level of focus has made the opsins canonical G-protein-coupled receptors (GPCRs) and arguably the most investigated protein group for its evolutionary radiations and diverse functional specializations. Still, opsin's early evolution remains puzzling, and there are many questions throughout its evolutionary history for which we have partial, but tantalizingly incomplete, answers. Obviously the invertebrates, with their astonishing diversity and with evolutionary hints of the most ancient animals in their genomes, functions, and even body plans, offer the best hope of answering many of these fundamental questions. Here, we consider what is known about invertebrate opsin evolution and function, examine how it has become incorporated into photoreceptor cells and into the machinery that provides the intracellular signals that initiate photoreception, and review its functions among the invertebrates.

The photochemistry and spectral properties of visual pigments throughout the invertebrates have been studied for a long time, but the evolution of the opsin proteins forming these pigments has been only a quite recent concern. After all, not a single amino acid sequence of any visual pigment was available until 1982, when the bovine rod opsin protein was sequenced (Ovchinnikov et al. 1982, Hargrave et al. 1983). This permitted Nathans and Hogness (1983) to sequence the gene encoding bovine opsin—an accomplishment that sparked the ever-increasing pace of opsin sequencing that continues to the present. Only a couple of years later, invertebrates joined the family of genetically characterized opsins with the sequencing of Drosophila Rh1 (the main visual opsin) by two teams in 1985 (O'Tousa et al. 1985, Zuker et al. 1985).

Fig. 4.1 Maximum-likelihood tree of 989 genomic and expressed opsin sequences (see Porter et al. 2012 for reconstruction methods). The sequence complement is composed of the dataset from Porter et al. (2012) plus the most recently published invertebrate opsin sequences (e.g. Passamaneck et al. 2011, Henze et al. 2012, Hering et al. 2012, Mason et al. 2012, Schnitzler et al. 2012), and using the *Trichoplax* sequences from Feuda et al. (2012) as outgroups. Invertebrate opsin sequences and associated branches are colored *black*, vertebrate opsin sequences are *light grey*, and outgroup sequences are *dark grey*. The four major opsin sequence groups—C-Type, Group 4, R-Type, and Cnidops—are indicated; additionally, a basal group of cnidarian sequences from the *Nematostella* genome are labeled "N," and the outgroups are indicated by "OGRPS"

The spreading use of the polymerase chain reaction (PCR) in the late 1980s accelerated the rate of sequence documentation, and known opsin sequences accumulated even more rapidly with the onset of genome sequencing in the early twenty-first century. By 2010, nearly a thousand sequences were available for a review of opsin evolution (Porter et al. 2012). Because of the overwhelming focus on vertebrate vision, only about one-third of the sequences in Porter et al.'s (2012) analysis were derived from invertebrates (see Fig. 4.1), and the great majority of those came from arthropods. Cnidarians were also well represented, partly because of recent interest in the early origins of vision. Nevertheless, the fairly irregular sampling across taxa has left us in the dark regarding the photoreceptive abilities of several major animal groups. Intense concentration on some of these outliers has characterized the most recent research. Scarcely a week passes without the publication of an important paper on a previously unstudied (or at least understudied) taxon.

To say the least, this is an exciting time to be a vision scientist—particularly one working on comparative and evolutionary questions.

Looking over the early literature on opsin genetics, one is struck by the excitement present right from the start. The similarity between bovine and fruit fly opsin was striking. Within only a few years it became obvious that there were major subgroups of opsins, and that opsins within these subgroups were more similar across taxa than they were to other subgroups within the same species. The selection of cow and fly for the first sequencing efforts was fortuitous, because increasing numbers of new sequences demonstrated that there was a major division of visual opsins into two realms, each characterized by one of these two founding members of the field. Because membranes of vertebrate photoreceptors, containing the bovine type of opsin, were long known to be derived from ciliary structures, while photoreceptors in flies and other arthropods (as well as mollusks) lacked this ciliary connection and were called "rhabdomeric," it became fashionable to assign the opsins to ciliary and rhabdomeric types, implying that knowing the photoreceptor cell structure foretold the type of opsin that would be present (and vice versa; see Arendt et al. 2004). Naturally, with the accretion of so many new sequences from a proper diversity of species, generalizations like these have become less tenable. Furthermore, opsins are turning up in many cells and cell types that bear little or no resemblance to typical visual photoreceptors, and opsins only distantly related to the classical ciliary type turn up in visual ciliary photoreceptors (Kojima et al. 1997). Here, we aim to provide a current view of the evolution of invertebrate photopigments, attempting to reconcile the various lines of evidence.

4.2 Evolution of Invertebrate Opsin Proteins

The opsins of animals are evolutionarily and functionally quite distinct from two other photoproteins with similar names: the bacteriorhodopsins of halobacters and the channelrhodopsins found in green algae (recently famous for their use in optogenetics). As mentioned, animal opsins in all their diversity are GPCRs (although not all actually activate a G-protein, see below) and all descend from a common ancestor. Like visual opsins, the other two classes of photoproteins bind retinal and are membrane-bound proteins with seven transmembrane helices, but despite these striking similarities, neither is closely related evolutionary to animal opsins nor are they GPCRs. Here, we are concerned only with "true" opsins, and for the rest of the chapter, the term "opsin" refers strictly to the GPCR type found in Eumetazoa.

Opsins arose at about the time of the appearance of the earliest eumetazoans, today represented by cnidarians and ctenophores (see Fig. 4.2 for a diagram of a current view of animal evolution). Of earlier animals, sponges (Porifera) detect and respond to light (Leys et al. 2002), but no opsins have been identified in sponges. Instead, their photoreception—when present—is apparently based on a cryptochrome photopigment (Plachetzki et al. 2007, Rivera et al. 2012). Cryptochromes have survived throughout animal evolution and, as already mentioned, clearly

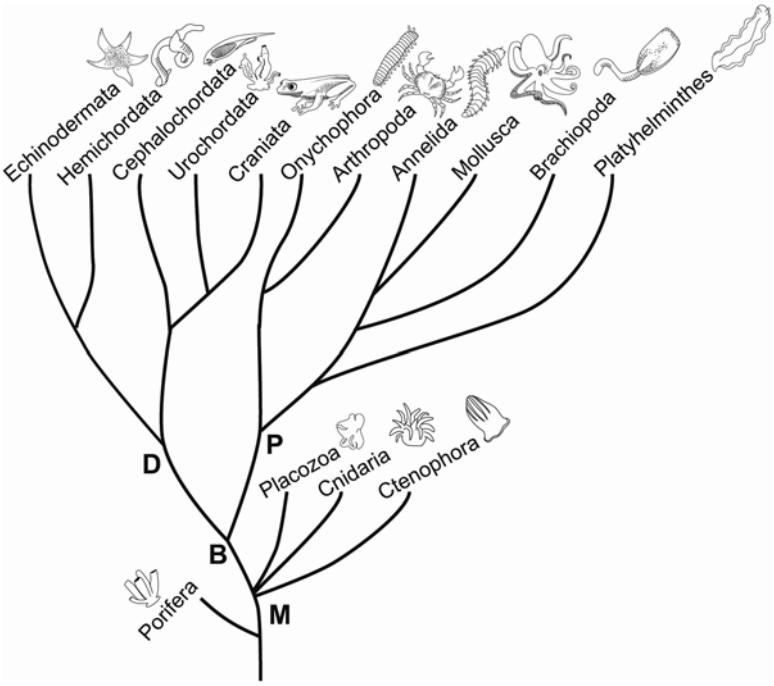

Fig. 4.2 Proposed evolutionary relationships among modern animals showing all groups included in this review plus the craniates (chordates with cartilaginous or bony skulls; for comparison with invertebrates). The *letters* denote major points of evolutionary significance: *M* origin of the Metazoa, *B* origin of Bilateria, *D* origin of Deuterostomes, *P* origin of Protostomes

have important roles in several light-dependent behaviors, including circadian rhythmicity (in insects and squid) and possibly magnetoreception (in birds). Unfortunately, because their evolution and functionality in animals has been neglected (perhaps because they appear not to be light-sensitive in mammals), there is little else to say about them here. Hopefully, future research will help establish their roles in light perception and will also suggest why they lost out so completely to the opsins for higher-level light-sensing functions.

Very likely, opsins started with the huge advantage of being able to couple to established transduction pathways via a G-protein. The ancestral opsin appears to have been allied with a melatonin receptor or similar GPCR, as opsins form a sister group to such GPCRs in the genome of the placozoan *Trichoplax adhaerens* (Srivastava et al. 2008, although a sensory function of the encoded proteins is not established) and to melatonin receptors (Fredriksson et al. 2003, Feuda et al. 2012). Presumably the common ancestor of all true opsins lacked the chromophore-binding lysine at position 296 (bovine rod opsin numbering, used throughout the chapter) but nevertheless interacted with a retinoid ligand to initiate signaling. Making the reasonable assumption that the binding was specific to the conformation of this ligand, the most likely form was an all-*trans* structure (because this is the most

common and stable form of retinoids and also the form present in the chromophore binding pocket when phototransduction is initiated in modern opsins). Modern opsins with the critical lysine mutated to an uncharged residue can be activated by all-*trans* retinoids (Zhukovsky et al. 1991), a property expected in the ancestral pre-opsin. Of course, all opsins devoted to light-sensing today have a lysine residue at this position that forms the Schiff base that covalently binds the chromophore.

Opsins diversified almost immediately after first appearing, forming three undisputed clades: ciliary (or C-type) opsins, rhabdomeric (or R-type) opsins, and Go-RGR (or Group 4) opsins (Feuda et al. 2012, Mason et al. 2012, Porter et al. 2012). A fourth clade discovered by Plachetzki et al. (2007) is composed of a unique group of opsins found only in cnidarians that are sister to C-type opsins (R-type opsins and Group 4 opsins also form sister groups). Although all opsins are classical GPCRs, they vary quite unexpectedly in their favored G-protein, with most members of each clade interacting with a single G-protein family (reviewed in more detail later in the chapter). Thus, C-type opsins generally interact with Gi (or Gt; transducin), R-opsins with Gq, and Group 4 opsins with Go. But these are only generalities. At least one cnidarian opsin activates Gs, and some in Group 4 interact with no G-protein at all. In fact, it is often difficult to ascertain pathways of phototransduction in invertebrates, as they are quite diverse and frequently more complex than those present in rods or cones of vertebrates (Yau and Hardie 2009). Making things worse, many opsins promiscuously activate non-cognate G-proteins in vitro (and possibly even in vivo), so it can be difficult to identify the native pathway. In this review, we are not particularly concerned with the evolution of phototransduction, but since opsin evolution (especially in invertebrates) is associated with the appearance of unexpected phototransductive pathways, we will note cases where the phototransduction mechanism is known and briefly discuss its evolution in a later section. First, we turn to a consideration of opsin evolution within the major invertebrate clades (Fig. 4.3).

4.2.1 Cnidarian Opsins

Cnidarian opsin relationships are illustrated in Fig. 4.3d. As already noted, Plachetzki et al.'s (2007) work with the genomes of cnidarians (specifically, a species of hydrozoan, *Hydra magnipapillata*, and an anthozoan, *Nematostella vectensis*), uncovered a number of sequences that were distinctive from all previously described opsins. Because of these distinctions, they proposed the group name "cnidops." A year later, Alvarez (2008) also described a ciliary-like opsin from *Hydra*, and Suga et al. (2008) published a study of two hydrozoan species probed using PCR, from which they recovered a further 20 opsins. They provided the first comprehensive analysis of cnidarians by adding sequences from genomes of another hydrozoan and an anthozoan, most of which were sister to ciliary opsins. However, a few of their sequences grouped separately from these into two small clusters, one of which

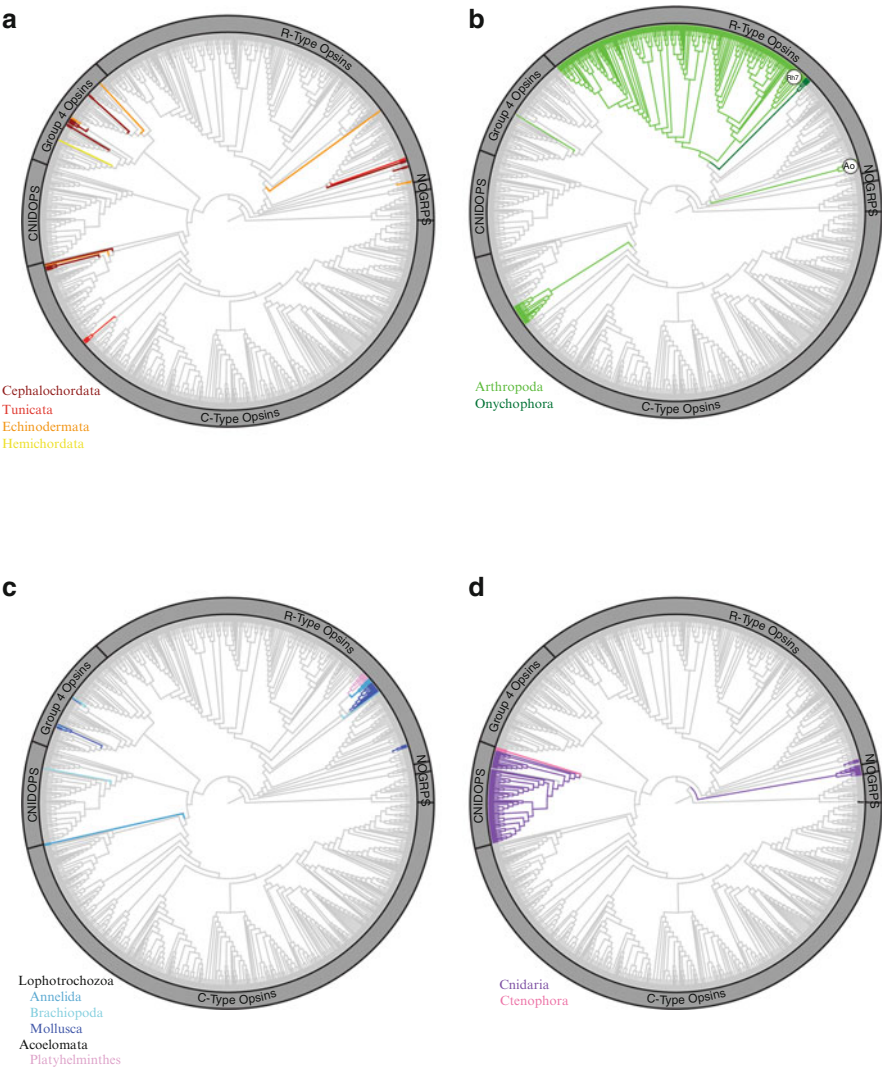

Fig. 4.3 The phylogeny plotted in Fig. 4.1, using the same major opsin group labels, divided into major invertebrate taxonomic groups. Indicated by *colored* branches (see key in each panel) are (**a**) Deuterostome opsin sequences—including cephalochordates, tunicates, echinoderms, and hemichordates; (**b**) Ecdysozoa (Protostome) opsin sequences—including arthropods and onychophorans; the poorly characterized Rh7 (Rh7) and Arthropsin (Ao) groups are indicated; (**c**) Lophotrochozoan (annelid, brachiopod, and mollusk) and acoelomate (platyhelminthes) opsin sequences; and (**d**) Cnidarian and ctenophoran opsin sequences. All non-invertebrate sequences are colored *light grey*

resembled the rhabdomeric opsins. Nordström et al. (2003) had earlier described suspiciously rhabdomeric-like photoreceptors in a cubozoan larva (*Tripedalia cystophora*), indicating that at least some opsins in cnidarians could be rhabdomeric. Porter et al. (2012) found that opsins from a variety of cnidarians (and one

ctenophore species) cluster in a unique clade also sister to the ciliary opsins and termed it "Cnidops" following Plachetzki et al.'s (2007) terminology. Feuda et al. (2012) claimed that at least some cnidarian sequences are sister to each of the other three major opsin groups (i.e. C-opsins, R-opsins, and RGR/Go opsins), but their analyses were based on subjective alignments, so this claim remains tentative. While the point at which the various opsin families originated is still in question, Mason et al.'s (2012) very recent finding that opsins in coral larvae may link to several different transduction mechanisms (including the Gq pathway typical of R-opsins, a novel G-protein they named Gc, and possibly the Gi of C-opsins) is certainly consistent with the view that the major opsin families diverged before the appearance of cnidarians. This question simply demands the inclusion of more species diversity, and also some focus on ctenophore opsins—which themselves may be distinct from cnidarian types (Schnitzler et al. 2012).

Cnidarian opsin evolution and function is of literally fundamental significance for unraveling the tangled history of animal photoreception. When an opsin-like protein was first suggested to be present in *Hydra*, using polyclonal antibodies generated to squid rhodopsin (Musio et al. 2001), it was still unclear whether the protein was photosensitive, or even whether one or more opsin types had been stained. Further genetic work by the same team suggested that at least two opsins are present in this very simple hydrozoan (Santillo et al. 2006), and many opsin classes are known to be expressed in other coelenterates (18 in the hydrozoan jellyfish *Cladonema radiatum* alone; Suga et al. 2008). At present, no opsins have been described from "true" jellyfish (scyphozoans), but the accumulation of sequences in other cnidarians quickly established not only that a large clade of C-opsin like "cnidops" exists across these animals, but that in addition (and somewhat surprisingly) rarer opsin types appear to have other evolutionary homologs. Also unsettling is the finding that the first characterized phototransductive pathway in any cnidarian (in the cubozoan *Carybdea rastonii*; Koyanagi et al. 2008b) turned out to be different from all previously described opsin signaling systems, being initiated by Gs. As already noted, cnidarian sequences have already been associated with opsins from known animals signaling through Gi, Gq, and possibly Go, as well as the strictly cnidarian Gc. All this indicates that all the types of opsins originated before the split between cnidarians and bilaterians, with the cnidarians also evolving some types that are uniquely theirs, and that opsin radiation through gene duplication—so prevalent in later animals—began with the appearance of the first opsins.

Indeed, it can be reasonably argued that everything to do with photosensing and vision was invented before the radiation of the major animal groups. The evolution of photoreceptor types will be covered in a later section, but it is important now to note that photoreceptor cells in coelenterates are extremely diverse and include cells directly involved in vision and in many other functions, often unknown (Martin 2002). Nilsson (2009, 2013) argues convincingly that the evolution of photoreception from simple photoreception to high-quality vision progresses through four functional stages (non-directional photoreception, directional photoreception, low-resolution imaging, and high-resolution vision). Cnidarian examples exist for every step in this sequence, illustrating the deep ancestry of opsin and visual system diversity.

Developing cnidarian eyes even use genetic mechanisms similar to those of higher animals, including vertebrates (Kozmik et al. 2008). Based on what we see in the "simple" cnidarians, it would appear that nearly every important function of opsins most likely originated before the first bilaterian animals appeared, and what followed in subsequent animal evolution arose from modifications to the ancestral collection of "urbilaterian" opsins and their associated mechanisms (see Goldsmith 2013).

4.2.2 Opsin Evolution in Bilateria

After the sponges, placozoans, cnidarians, and ctenophores, all animal phyla present today can be united in a single group, the Bilateria (Fig. 4.2). All (or almost all, depending on where the platyhelminths fall) are either protostomes or deuterostomes, with the separation depending on the fate of the blastopore in early development (in protostomes it becomes the mouth, and in deuterostomes the anus). Protostomes include an astonishing number of minor phyla, but opsins have only been characterized in the Annelida, Mollusca, Onychophora, and Arthropoda. In deuterostomes, containing fewer phyla, opsins from Hemichordata, Echinodermata, and Chordata are described (Figs. 4.1 and 4.3). We will begin with the protostomes, noting in passing that no matter where the platyhelminths (and the phylogenetically problematical acoels, for which no opsins are known) ultimately end up in animal phylogenies, platyhelminth opsins are placed with protostome types (Figs. 4.1 and 4.3; Porter et al. 2012).

4.2.3 Protostome opsins: Annelids and Onychophorans

Annelid opsins (Fig. 4.3c) are represented by sequences from three species—*Platynereis dumerilii* (ragworm, Arendt et al. 2004) and genome sequences from the *Helobdella robusta* (leech, Joint Genome Institute, v1.0, 2007) and *Capitella teleta* (polychaete worm, Joint Genome Institute, v1.0, 2007), but it was investigation of *P. dumerilii* that proved ground-breaking. This research identified one of the first ciliary opsins found in any invertebrate (Arendt et al. 2004), along with a retinal rhabdomeric opsin in simple eyes. This ciliary opsin is apparently present only in the brain, but the rhabdomeric opsin has since turned up in other locations in the adult worm (Backfisch et al. 2013). Not surprisingly, this R-opsin is homologous to those of mollusks and arthropods, as well as vertebrate melanopsins (Arendt et al. 2004; Porter et al. 2012; see Fig. 4.1). The ciliary opsin, given its location in the larval brain, does not appear to have a role in vision. In fact, Terakita (2005) assigned it to a group of odd vertebrate opsins, the encephalopsin/tmt types, of unknown function.

The vaguely annelid-like phylum Onychophora is actually sister to the Arthropoda, likely sharing a common ancestor. Since arthropod opsins are extraordinarily diverse, studying the opsins of the onychophorans (velvet worms; Fig. 4.3b)

has some hope of revealing the roots of the diversity. Velvet worms have tiny ocelli lying laterally on the head that are obviously much simpler than arthropod compound eyes. Thorough analysis of mRNA in several tissues from a variety of species, including both adults and embryos, found only a single type of rhabdomeric opsin in each species (Hering et al. 2012). This "onychopsin" clade is sister to all arthropod opsins, and obviously provides no hint of the forthcoming explosion of opsin paralogs in the arthropods, the phylum to which we now turn.

4.2.4 Protostome Opsins: Arthropods

Arthropod opsins (Fig. 4.3b) are the best characterized of the invertebrate photopigments, from genomic, expression, and protein function studies. Perhaps inspired by the first description of an invertebrate opsin in *Drosophila*, the majority of available arthropod opsin sequences have come from insect species. Relatively fewer species of crustaceans have opsin sequences available. Surprisingly, despite the use of the horseshoe crab, *Limulus polyphemus*, as a model organism for vision studies for over a century, the opsins of few of its fellow chelicerates have been investigated. Finally, with regards to arthropod opsin studies, the myriapods (e.g. centipedes and millipedes) are not yet represented.

When considering arthropod opsin diversity, representatives of the three major evolutionary groups can be found (R-type, C-type, and Group 4 opsins) (Fig. 4.3b). The largest diversity of arthropod opsins is among members of the R-type clade, which are used mainly in high-resolution, image-forming visual systems. The arthropod R-type opsins can be subdivided into four evolutionarily distinct clades. Three of these groups can be described based on the dominant spectral sensitivities of the corresponding visual pigments: long wavelength sensitive (LWS), middle wavelength sensitive (MWS), and short wavelength sensitive (SWS). All three of these clades—LWS, MWS, and SWS—contain opsin sequences from at least two major taxonomic groups (i.e. insect, crustacean, and chelicerate). These data, in conjunction with the presence of a single opsin in Onychophora, suggest that gene duplication and diversification of the major opsin spectral clades occurred in conjunction with the evolution of compound eyes early in the arthropod lineage (Hering et al. 2012). The LWS group contains two sub-clades—one comprised of insect and crustacean sequences, and a second of only chelicerate opsins (e.g. *Limulus* and jumping spiders; Smith et al. 1993; Koyanagi et al. 2008a). The opsin sequences in the SWS group have a similar evolutionary pattern, with a clade of crustacean and insect opsins, and a second group of arachnid sequences (Koyanagi et al. 2008a). A large number of opsin sequences, including the crustacean and arachnid opsin proteins, have been postulated to form visual pigments with ultraviolet sensitivity, although many of these proteins have yet to be functionally tested. In the insects, SWS opsins have diverged into two distinct spectral classes—one group being associated with ultraviolet sensitivity, and a second having evolved sensitivity in the

violet and blue portion of the visible spectrum (Pichaud et al. 1999). Currently, the MWS clade contains only chelicerate (one sequence from *Limulus polyphemus*; Katti et al. 2010) and crustacean sequences, suggesting that this lineage has been lost in insects. The "MWS" designation is still tenuous, as the visual pigment spectral properties have been investigated in only one species, *Hemigrapsus sanguineus* (λ_{max} = 480 nm) (Sakamoto et al. 1996). As very little is known about the spectral diversity, taxonomic diversity, or expression patterns of arthropod MWS opsins, much more work is required to understand this group's evolution and function.

The fourth clade of arthropod R-type opsins is comprised of an enigmatic assortment of sequences called the Rh7 group, after the gene designation in *Drosophila melanogaster*. Rh7 genes have been identified in insect and *Daphnia* genomes and have a unique structure with longer N- and C-termini and a shorter third cytoplasmic loop than other R-type arthropod opsins (Izutsu et al. 2012). Although the in vivo functions are still unclear, available expression data suggest low levels of Rh7 expression in adult *Drosophila melanogaster* eye and brain tissues (Graveley et al. 2011; Chintapalli et al. 2007; Kistenpfennig 2012), and a possible contribution to the *D. melanogaster* circadian clock (Kistenpfennig 2012).

A novel R-type opsin lineage has been described based on sequences from the *Daphnia pulex* genome, termed the "arthropsins" (Colbourne et al. 2011). The arthropsins have undergone gene expansion in the *Daphnia* genome and are a distinct group of opsins, highly diverged from the R-type opsins used in arthropod image-forming visual systems. Structurally, arthropsins have relatively long C-termini and longer cytoplasmic loop 3. Very little is known about the arthropsins, but the absence of this lineage in other genomes suggests that it has been lost many times in other animal groups (Colbourne et al. 2011). As genome resources become available for more non-insect arthropod species, additional representatives of this, and of the poorly characterized arthropod opsin groups, will help clarify the situation.

In addition to the R-type opsins used in high-resolution image formation, arthropod genomes possess opsins from within the C-type group, called the "pteropsins." At present, only insect and crustacean (i.e. *Daphnia pulex*) opsin genome sequences have been identified as clustering with the vertebrate C-type opsin clade (Hill et al. 2002; Velarde et al. 2005; Zhan et al. 2011; Colbourne et al. 2011). Although the expression patterns of most of these genes have not been investigated, a study of the honey bee (*Apis mellifera*) found pteropsin expressed in brain, but not retinal, tissues (Velarde et al. 2005).

Finally, a single arthropod opsin has been characterized from the Group 4 opsins. Nagata et al. (2010) characterized a peropsin in the jumping spider *Hasarius adansoni,* showing that it was expressed in non-visual cells in the distal region of the principal eye retina, and that it has photoisomerase-like characteristics (all-*trans* to 11-*cis* isomerization). The lack of Group 4 opsins from the plethora of available insect genomes suggests that, similar to the R-Type MWS opsins, this lineage has been lost in insects. We suspect that as other arthropod (e.g. crustacean and chelicerate) genomes are sequenced, additional Group 4 opsins will be discovered.

4.2.5 Protostome Opsins: Mollusks

After the flamboyance of the arthropods, the mollusks seem positively ascetic (Fig. 4.3c). Almost all species apparently express a single sensory opsin in their retinas, although recent studies are beginning to suggest that the story is more complex, with some duplication of opsin genes in bivalves (Serb et al. 2013). Nevertheless, members of this group have provided two sets of information critical to understanding the structure and function of opsins. An opsin from the squid *Todarodes pacificus* is the only invertebrate example for which the three-dimensional structure has been solved (Murakami and Kouyama 2008), and another from a scallop, *Pecten irradians*, is the sole member of the Go opsin group for which phototransduction has been described (Kojima et al. 1997; Gomez and Nasi 2000). The Go opsin exists in a ciliary photoreceptor, and its excitation produces hyperpolarization via a cGMP-gated channel, hinting at the presence of a phototransductive pathway similar to what is seen in vertebrate rods and cones (Yau and Hardie 2009, Fain et al. 2010). But research by Gomez and Nasi (2000) uncovered quite a different cascade. On photoexcitation, Goα operates by activating guanylate cyclase, and the resulting increased concentrations of cGMP open a cyclic-nucleotide-gated potassium channel, which produces the hyperpolarization. Yau and Hardie (2009) suggest that this pathway could be the original ciliary phototransductive system, and further propose that it might be widely used in invertebrate ciliary photoreceptors.

Most published examples of molluscan opsin sequences derive from cephalopods, which typically express a single opsin. Evidence for variations in the number of expressed opsins among molluscan species, however, is becoming more common (e.g. scallop—Serb et al. 2013; the firefly squid *Watasenia scintillans*—Michinomae et al. 1994). The described opsins from cephalopod species, all with a single known opsin, include one octopus (Ovchinnikov et al. 1988), four squids (Hall et al. 1991, Morris et al. 1993, Hara-Nishimura et al. 1993, Tong et al. 2009), and a cuttlefish (Bellingham et al. 1998). Their photopigments all have similar spectral absorption, ranging in λ_{max} from 480 to 499 nm, and all contain a very extended C-terminus with many repeated segments (Bellingham et al. 1998). The crystal structure of squid opsin (*Todarodes pacificus*, Murakami and Kouyama 2008) shows that the C-tail has two cytoplasmic α-helices at its start, a previously unknown opsin feature possibly involved in G-protein activation. There is also good evidence that the opsin proteins lie as adjacent pairs in the plasma membrane, interacting with neighboring opsins on parallel microvilli and possibly enabling elevated polarization sensitivity in the receptor as a whole. These features are quite unlike the structure of mammalian rhodopsins (Palczewski et al. 2000), but could be widespread among rhabdomeric opsins activating Gq.

Cephalopods are the only mollusks in which sensory opsins have been identified both in the retina and extraretinally (Fig. 4.4). The sequences are identical (Tong et al. 2009, Mäthger et al. 2010), reemphasizing the unusual conservatism of mollusks regarding opsin diversification. Yet there is reason to think that some

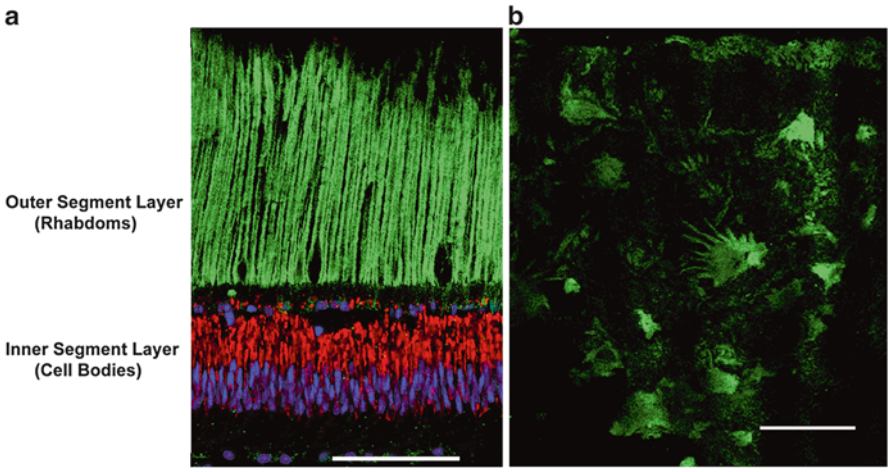

Fig. 4.4 Distribution of rhodopsin and retinochrome in retina and a region of skin in the squid, *Doryteuthis* (*Loligo*) *pealii*. The images show immunohistochemical stains labeling cuttlefish rhodopsin and retinochrome. (**a**) Double staining of a vertical section through the retina, showing regionalization of rhodopsin in the photoreceptor outer segment layer (*green*) and of retinochrome in the inner segment layer (*red*). The rhodopsin is situated in photoreceptive microvilli, while the retinochrome appears to be associated with internal membranes in the cell bodies of photoreceptors. Scale bar: 100 μm. (**b**) Rhodopsin staining of chromatophore cells in the skin. Scale bar: 250 μm. Photographs by Alexandra Kingston

cephalopods contain multiple sensory opsins (in addition to retinochrome, a Group 4 opsin described later that acts as a photoisomerase). The firefly squid, *Watasenia scintillans*, has three spectral classes of photopigments in its retina, all of which appear to be in photoreceptive microvilli (Michinomae et al. 1994). These pigments differ spectrally (peaking at 470, 484, and 500 nm) and—surprisingly—in the chromophores they bind, respectively being the 11-*cis* forms of 4-hydroxyretinal, retinal, and dehydroretinal (Matsui et al. 1988, Seidou et al. 1990). These are very unusual features. No other opsin is known to bind a 4-hydroxyretinal chromophore, nor does dehydroretinal act as a visual pigment chromophore in any other known marine organism. Obviously, these pigments could be based on different proteins as well as different chromophores, but no sequences have been reported for any of them. We also lack wide coverage of mollusks outside the cephalopods, and only four molluscan genomes have been released—the limpet *Lottia gigantea* (Joint Genome Institute, v1.0 2007), the sea hare *Aplysia californica* (Broad Institute v2.0, 2009), and the oysters *Pinctada fucata* (Takeuchi et al. 2012) and *Crassostrea gigas* (Zhang et al. 2012). These genomes suggest the presence of uncharacterized opsin diversity outside of the main molluscan visual pigment clade, particularly in the Group 4 opsins (Fig. 4.1). For all these reasons, the future of opsin research in mollusks bears watching.

4.2.6 Protostome Opsins: Brachiopods

Brachiopods, also known as lampshells, are the most recent group of protostomes to be investigated for opsin diversity (Fig. 4.3c). Similarly to the mollusks, brachiopod larvae express both R-type and several Group 4 opsins (Fig. 4.1; Passamaneck et al. 2011; Passamaneck and Martindale 2013). Larval brachiopods also express what was originally described as a C-type opsin, but it is more closely related to the Cnidops sequences in our analyses (Fig. 4.1, Passamaneck et al. 2011). Regardless of the ultimate phylogenetic relationships of this brachiopod opsin, it appears to be a unique opsin representative and deserves further investigation.

4.2.7 Deuterostome Opsins

In deuterostomes (Fig. 4.3a), far more is known about vertebrate opsins than about those in any invertebrate group, for obvious reasons. Still, with ever-increasing interest in opsin evolution, new data on the opsins of non-vertebrate deuterostomes are starting to accumulate. The interest has been encouraged by the discovery of the R-opsin melanopsin in vertebrate retinas plus the general interest in GPCR function and diversity, not to mention the continually emerging findings of unexpected opsins in all sorts of animals. The discovery of the expression of multiple opsins in amphioxus (also called the lancelet) *Branchiostoma*, a basal chordate often considered to resemble the ancestor of vertebrates, is especially exciting. *Branchiostoma* has at least six opsins spread throughout a variety of cell types, including representatives of the C-opsin, R-opsin, and Group 4 opsin clades (Koyanagi et al. 2002, Gomez et al. 2009, Angueyra et al. 2012, Vopalensky et al. 2012). One set of photoreceptors in amphioxus expresses a variant of the vertebrate R-opsin melanopsin and signals through a Gq cascade, perhaps presaging the vertebrate melanopsin pathway (Gomez et al. 2009; Koyanagi and Terakita 2008). Another opsin variant seems to act as a photoisomerase (Koyanagi et al. 2002). In a particular set of putative photoreceptor cells, Gi co-localizes with C-opsins (Vopalensky et al. 2012). A similar situation exists in the photoreceptive ocelli of larvae of the ascidian *Ciona intestinalis* (Kusakabe et al. 2001, Yoshida et al. 2002), a representative of a chordate taxon grouped perhaps closer than amphioxus to the vertebrates (Fig. 4.2). These observations are strong evidence that vertebrate-type phototransduction, relying on Gt (closely related to Gi), appeared before or about the time of the divergence of the chordates from other deuterostomes.

Besides chordates, the other two relatively successful deuterostome taxa are the hemichordates and the echinoderms. Known hemichordate opsins are currently limited to two Group 4 sequences from the acorn worm *Saccoglossus kowalevskii* (Porter et al. 2012); nothing is known about their function in the animal. This leaves the echinoderms, the phylum that includes starfishes, sea urchins, and their relatives. While these animals have an abundance of light-related behaviors, none of their photoreceptors (or photopigments) was described until quite recently.

In fact, it took whole-genome sequencing of a sea urchin, *Strongylocentrotus purpuratus*, to reveal the first echinoderm opsins (Sodergren et al. 2006). Expression of five of the six (or more) genomic opsin genes was tracked by Raible et al. (2006). All five of these, including R-opsin, C-opsin, and Group 4 opsins (Porter et al. 2012), were detected in various tissues (mostly on or near the body surface) in adult urchins (Raible et al. 2006). Ullrich-Lüter et al. (2011) followed up by looking in detail at expression patterns in the tube feet of the sea urchin. The earlier work by Raible et al. (2006) had detected abundant opsin expression in these tube feet, but of an R-opsin, not the C-opsin expected in a deuterostome. Based on a study integrating molecular, structural, and behavioral data, Ullrich-Lüter's team concluded that this opsin was responsible for photobehavior of *S. purpuratus*. A similar photopigment was apparently found in starfish by staining with an antibody specific to the urchin's R-opsin, encouraging Ullrich-Lüter et al. (2011) to propose that R-opsins could be widely involved in directional light sensitivity in echinoderms. In contrast to these findings, an ortholog of one of the unusual C-opsins, encephalopsin (a vertebrate opsin of unknown function), has been traced to larvae and adults of a second species of sea urchin, *Hemicentrotus pulcherrimus* (Ooka et al. 2010), including the tube feet of adults. In the larvae, this strange opsin appears to direct larval photobehavior (Ooka et al. 2010). The functions of R-opsins and C-opsins generally in deuterostomes thus appear to be complementary, but both serve in a rather general visual sense, since no echinoderm has anything that could be considered an eye. It is not unreasonable to think that only chordates rely on C-opsins for directional vision.

4.3 Phototransduction

As already mentioned, invertebrate phototransduction involves a diverse set of intracellular signaling networks. Starting with a photon of light, phototransduction produces chemical and/or electrical signals that result in a physiological response. Like any GPCR, activated opsin protein signaling occurs via a heterotrimeric G-protein. Upon binding with the opsin, the G-protein alpha subunit dissociates and interacts with downstream second messenger systems to regulate or modulate ion channels. Because the G-protein alpha subunit is the component that transduces photon absorption by the opsin protein to the intracellular cascade, and because G-protein alpha subunits are often associated with similar downstream pathways, GPCR transduction cascades are usually characterized by the subclass of alpha subunit involved in signaling. Although few phototransduction pathways have been studied in enough detail to elucidate all of the interacting components, based on over 40 years work in *D. melanogaster* (Montell 2012) and a growing number of transcriptomic and genomic studies in non-model systems (Rivera et al. 2010; Friedrich et al. 2011; Mason et al. 2012; Schnitzler et al. 2012; Porter et al. 2013; Passamaneck and Martindale 2013), invertebrate opsins bind to a diverse set of G-proteins, and they presumably interact with a correspondingly diverse set of second messenger systems and signaling cascades (Porter et al. 2012).

The most thoroughly studied phototransduction cascade, and the signaling network that serves as the model for most arthropod phototransduction, is in *D. melanogaster* visual photoreceptors (Montell 2012). Unlike vertebrates, work in *D. melanogaster* found all of the phototransduction cascade components assembled in a closely associated protein complex, called the "signalplex," which is linked to the actin cytoskeleton; how broadly this functional structure is found throughout arthropod (or other R-opsin) phototransduction networks is still unknown. Based on analogy to *Drosophila* phototransduction, most arthropod visual systems are thought to utilize a Gq alpha subunit, interacting through a phospholipase C (PLC) second messenger system, and depolarize photoreceptors by opening transient receptor potential (TRP) channels. Additional support for this general signaling cascade in arthropod vision has been found in studies of other insects (Friedrich et al. 2011), chelicerates (Nagata et al. 2010; Dorlöchter et al. 1997; Smith et al. 1995), and crustaceans (Porter et al. 2013; Rivera et al. 2010). Recent work, however, has illustrated that arthropod opsins in other major groups may have diversity in signaling cascades. Expression studies show the mosquito pteropsin/Opn3 protein couples to $G_{i/o}$ alpha subunits (Koyanagi et al. 2013). Further, genomic evolutionary studies show that Pancrustacean phototransduction genes have higher rates of gene duplication, hinting at yet uncharacterized evolutionary plasticity and diversity in arthropod phototransduction cascades (Rivera et al. 2010).

In mollusks, diverse visual cell types have varied phototransduction cascades. Cephalopod and scallop retinal rhabdomeric photoreceptors utilize a Gq-pathway (Nobes et al. 1992; Suzuki et al. 1995; Kojima et al. 1997). Scallop retinas also contain ciliary photoreceptors, which signal via a Go-coupled cascade that involves cGMP and cyclic nucleotide gated (CNG) channels (Gotow et al. 1994; Gomez and Nasi 1997, 2000; Kojima et al. 1997). Interestingly, expressed sequence tag studies of the squid *Euprymna scolopes* light organ found the presence of both Gq- and Gi-mediated phototransduction components, although only R-type opsin was detected (Tong et al. 2009). Cephalopod photoreceptors also contain retinochrome, described in detail later, an opsin protein that does not signal via a G-protein, but instead is involved in chromophore regeneration (Hara and Hara 1965, 1972; Seki 1984).

Genome studies of the amphioxus *Branchiostoma belcheri* identified six opsin genes, with sequences found in the C-type, R-type, and Group 4 Opsins (Koyanagi et al. 2002; Holland et al. 2008). Based on this diversity of opsin sequences, amphioxus potentially has the most diverse set of phototransduction cascades, with different opsins hypothesized to couple with Go, Gq, Gi, or possibly no G-protein subunit at all (Koyanagi et al. 2002, 2005). The most recent work on amphioxus photoreception has confirmed the presence of a Gq-PLC pathway in microvillar membranes (Angueyra et al. 2012; Terakita et al. 2008), opsins that coupled to both Gq and Gi-alpha subunits (Bailes and Lucas 2013), and a Gi-alpha subunit in the frontal eye ciliated cells (Vopalensky et al. 2012).

In contrast to the phototransduction cascades discussed so far, cnidarians signal through a unique visual system pathway. Various studies in box jellyfish and hydra have found either the use of a Gs-coupled cascade that causes a light-dependent increase in cAMP and modulates a CNG channel (Koyanagi et al. 2008a, b;

Plachetzki et al. 2010) or possibly a signaling network utilizing PDE, phosducin, and guanylate cyclase (Kozmik et al. 2008). Even more diversity in phototransduction cascades has been found in corals, where studies of the elkhorn coral *Acropora palmata* found Gq and a new class of cnidarian-specific G alpha subunit, Gc, involved in phototransduction (Mason et al. 2012). This work supports the hypothesis that the Gi/t and Gq pathways diverged prior to the cnidarian–bilaterian split (Schnitzler et al. 2012).

4.4 Evolution of Tuning of Invertebrate Visual Pigments

Relative to vertebrate photopigments, tuning of invertebrate visual pigments is poorly understood. However, it is critical to understanding the evolution of the large diversity of visual pigments found in extant invertebrates (Holland et al. 2008; Colbourne et al. 2011; Porter et al. 2013). This is particularly true in the arthropods, where visual systems often contain unexpected opsin protein diversity (Rajkumar et al. 2010; Porter et al. 2013) hypothetically derived from an ancestral visual system consisting of only one opsin sensitive to blue–green light that duplicated and gave rise to an ultraviolet sensitive pigment early in the lineage (Briscoe and Chittka 2001; Koyanagi et al. 2008a; Hering et al. 2012). Mechanisms of invertebrate spectral tuning include extrinsic (e.g. photoreceptor filters, Cronin et al. 1994; Arikawa et al. 1999a, b) and intrinsic (e.g. gene duplications and amino acid residue replacements, Briscoe 2001; Salcedo et al. 2003, 2009; Frentiu et al. 2007; Kashiyama et al. 2009; Wakakuwa et al. 2010; Henze et al. 2012) mechanisms. It is the intrinsic mechanisms at the molecular level of the opsin protein that we focus on here.

To a large degree, studies of invertebrate spectral tuning have been hampered by the lack of broadly applicable in vitro expression systems. Studies of arthropod opsins suggest that invertebrate opsins in general may require specific chaperones and/or shuttle proteins to form a functional photopigment, making expression systems difficult (Knox et al. 2003; Terakita et al. 2008). Due to these constraints, many studies have used comparative evolutionary methods to identify opsin amino acid sites under selection and identify potential tuning sites in invertebrate opsins (Briscoe 2002; Frentiu et al. 2007; Porter et al. 2007, 2009).

Over the last 15 years, however, several laboratories have successfully expressed invertebrate opsins in either mammalian cell expression systems (honey bee ultraviolet (UV) and blue visual pigments and amphioxus melanopsin, Terakita et al. 2008; butterfly blue and violet opsins, Wakakuwa et al. 2010; mosquito pteropsin/Opn3, Koyanagi et al. 2013) or by ectopic expression in "blind" *ninaE* mutant *Drosophila melanogaster* lines (*Drosophila* Rh5 and Rh6, Salcedo et al. 1999; honey bee UV and blue opsins, Townson et al. 1998). Studies using these systems have shown that arthropod visual pigments can be spectrally tuned using either the same (Salcedo et al. 2003, 2009) or novel (Wakakuwa et al. 2010) amino acid sites compared to previously identified residues known to be important in vertebrate spectral tuning.

Perhaps the most thoroughly studied tuning in invertebrate opsins, somewhat surprisingly, is of ultraviolet (UV)-sensitive pigments. UV sensitivity has been reported in the majority of arthropod species that have been investigated (Briscoe and Chittka 2001; Kashiyama et al. 2009). Studies using site-directed mutagenesis have found that mutations leading to opsins with a deprotonated Schiff base shift visual pigment absorption into the UV (Salcedo et al. 2003; Hunt et al. 2001).

Most of the described studies have been done with arthropod visual systems employing R-type opsins. Although a number of other invertebrate opsin types have been studied using expression systems, including squid retinochrome (Terakita et al. 2000) and amphioxus melanopsin, peropsin, and Go-opsins (Koyanagi et al. 2002, 2005; Terakita et al. 2008), few have looked specifically at spectral tuning. As studies of invertebrate spectral tuning become more common, it will be interesting to look at the underlying visual pigment function across all of invertebrate opsin diversity.

4.5 Visual Pigment Bistability and Chromophore Regeneration

Work with vertebrate visual pigments over the years showed that their absorption of light invariably led to "bleaching," producing a colorless product. The bleaching is caused by a release of the all-*trans* chromophore from the opsin immediately after photoisomerization, and the process was viewed as a general property of visual pigments. It was a surprise, then, when Wald and Hubbard (1957) found that a visual pigment extracted from lobster eyes formed a thermally stable photoproduct, which they called metarhodopsin (the term already referred to the far less stable photoproduct of vertebrate visual pigments). A year later, Hubbard and St. George (1958) discovered that squid rhodopsin similarly forms a stable metarhodopsin, and furthermore, that this state of the visual pigment can be reisomerized to rhodopsin if exposed to light in the appropriate part of the spectrum.

With more research, it became clear that visual pigments formed from R-opsins had the general property of converting to thermally stable all-*trans* bound photoproducts, now generally known as metarhodopsins, and the pigments became known as bistable visual pigments. Examples of the spectral properties of rhodopsins and metarhodopsins of selected bistable visual pigments from arthropods and cephalopods are illustrated in Fig. 4.5. Besides the R-opsins, a few members of other opsin families also form bistable pigments, but except for the photoisomerases (e.g. RGR, retinochrome) little is known of their function (Tsukamoto and Terakita 2010). One general property of bistable pigments is that the counterion to the Schiff base is at position 181 (in the bleachable vertebrate pigments it is at position 113), and that their rate of G-protein activation is substantially lower than that of bleachable opsins (Tsukamoto and Terakita 2010).

Bistability simplifies the problem of restoring the active state of the chromophore after the initial photoisomerization to the all-*trans* form. The 11-*cis* isomer is

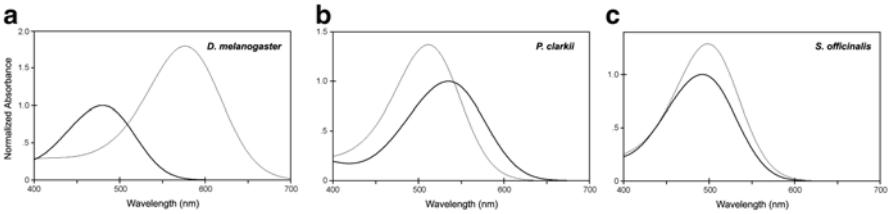

Fig. 4.5 Examples of bistable rhodopsin:metarhodopsin photosystems in arthropods and a cephalopod. In each panel, the spectrum of the rhodopsin (11-*cis* chromophore) is plotted as a thick line and that of the metarhodopsin (all-*trans* chromophore) as a *thin line*. (**a**) Fruit fly, *Drosophila melanogaster* (data from Stavenga, 2010); (**b**) Crayfish, *Procambarus clarkii* (data from Cronin and Goldsmith, 1982); (**c**) Cuttlefish, *Sepia officinalis* (data from Brown and Brown, 1958)

regenerated in the binding pocket as long as light is present, obviating the need for a biochemical visual cycle to supply a continuous stream of new or reisomerized chromophore. This suggests the attractive hypothesis that the earliest visual opsins were bistable, although it seems very unlikely that they were members of the R-opsin family. The bleachable opsins would have followed, perhaps after effective chromophore recycling systems were available. The spectral positions of metarhodopsins are not obviously correlated with those of the corresponding rhodopsin forms (Fig. 4.5), but since they invariably absorb in the visible spectral range, the Schiff base must remain protonated. Presumably the spectral shifts are due to changes in the shape of the chromophore binding pocket. Metarhodopsins are usually far more effective absorbers of light than the rhodopsins, perhaps because the all-*trans* chromophore has an extinction coefficient nearly twice that of the 11-*cis* form. An elevated extinction somewhat offsets the relatively higher quantum efficiency of conversion from 11-*cis* to all-*trans* over the reverse photoconversion; in crayfish the *cis*-to-*trans* quantum efficiency is 0.69, while the *trans*-to-*cis* efficiency is only 0.49 (Cronin and Goldsmith 1982). Photosensitivity for photoisomerization is the product of absorbance and quantum efficiency, so the transition from metarhodopsin to rhodopsin is about equally likely as the original conversion of rhodopsin to the meta state.

Some metarhodopsins, however, have unexpected spectral properties. Dipteran flies have rather unusual photosystems (Fig. 4.5, left), where the metarhodopsin has a λ_{max} placed almost 100 nm to longer wavelengths than the corresponding rhodopsin, and the extinction ratio is ~1.8 times as high (Stavenga 2010). The pigment system is almost certainly adapted for high photoconversion efficiency, because the metarhodopsin's spectral location permits it to absorb long-wavelength light transmitted by red screening pigments in fly eyes (think of the typical red-eyed *Drosophila*) to which the blue-absorbing rhodopsin is not sensitive. Rhodopsin is not immortal, however, and when fly metarhodopsin binds an arrestin during inactivation it becomes susceptible to internalization by endocytosis (Satoh and Ready 2005) and therefore must be renewed. In crayfishes, metarhodopsin survives at least overnight, and occasionally for several days (Cronin and Goldsmith 1984).

Here, replacement appears to be mainly via membrane turnover. In contrast, butterfly metarhodopsins can be quite short-lived, disappearing within minutes in some species; the rhodopsin is replaced comparatively rapidly, implying the activity of a chromophore regeneration system, or at least a ready reserve of chromophore (Bernard 1983). This has led to a search for an isomerase to regenerate chromophore in the dark (e.g. Wang et al. 2010), which has thus far been unsuccessful (unlike the situation in vertebrate retinas, where at least two dark isomerases act; see Goldsmith 2013).

The apparent absence of a dark isomerase has not deterred invertebrates from renewing chromophores; they just use photoisomerases instead. Surely, the most unusual example of this is the employment of an actual opsin as a photoisomerase. Retinochrome, first identified in a squid (Hara and Hara 1965), is one of the Group 4 opsins. Hara and Hara (1968) suggested that it acts solely as a photoisomerase, regenerating 11-*cis* retinal from all-*trans* to restore opsin to the active state, and indeed there is no evidence that retinochrome interacts with any type of G-protein. The details of the mechanism of chromophore exchange are obscure, but a retinal-binding protein appears to mediate the transfer of chromophore between the visual pigment and the photoisomerase (Terakita et al. 1989). Some sort of shuttle mechanism is required, because retinochrome is located primarily in the inner segments of photoreceptor cells, perhaps associated with internal membranes, while the rhodopsin is in the microvilli of outer segments of cephalopod photoreceptor cells (Fig. 4.4; see Hara and Hara 1976). A photoregeneration system with similar organization and components has also been found in the eyes of two species of marine gastropods (Ozaki et al. 1986, Katagiri et al. 2001), implying that the rhodopsin:retinochrome system could be quite common among mollusks. As in the squid retina, gastropod retinochrome is found in the cell body, separated from the photoreceptive microvilli and apparently associated with internal membranes.

Photoisomerases also keep chromophore supplied in arthropods, but in these cases they are more conventional molecules (i.e. not members of the opsin family). The first evidence for such a photoisomerase came from insects (blowflies and honey bees; Pepe and Cugnoli 1980, Schwemer 1984; Schwemer et al. 1984). In both insects the protein apparently bound all-*trans* retinal, which when exposed to blue light was then photoisomerized to 11-*cis*. Unlike retinochrome, the insect photoisomerase is water-soluble (Pepe and Cugnoli 1980). The honey bee photoenzyme was further studied by Smith and Goldsmith (1991a, b), who localized it to pigment cells surrounding the photoreceptors in bee ommatidia. Both the water solubility and the separation from photoreceptor membrane strongly imply some sort of transport mechanism, particularly since retinal itself is water-insoluble. A retinol dehydrogenase is present in pigment cells of *Drosophila*, interconverting retinal and retinol (Wang et al. 2010). Retinol is commonly shuttled among cells in vertebrate retinas via binding proteins, and a similar system probably acts in arthropods. Oddly, although a photoisomerase exists in the compound eyes of *Limulus* (Smith et al. 1992), no example from a crustacean has been reported.

4.6 Evolution of the Photoreceptor Cells of Invertebrates

Ever since Eakin's work on photoreceptor morphology and diversity, it has been fashionable to assign photoreceptor cells to one of two types, ciliary and rhabdomeric. In a beautifully illustrated chapter published over 40 years ago, Eakin (1972) included dozens of extraordinary electron micrographs of invertebrate photoreceptors documenting his proposal that all photoreceptors could be placed in one of these two classes. The fundamental division is based on the observation that some photoreceptors are clearly derived from cells with sensory cilia, having their photosensitive membranes directly associated with the cilium (or its remnant) and expanded into lamellae, disks, flattened sacs, or microvilli. The others lack a cilium; invariably these project microvilli as their membrane expansions. The division was consistent with phylogeny, since all known photoreceptors of vertebrates, but only a few in invertebrates, contained a cilium; while the huge majority of invertebrates, and particularly arthropods and mollusks, have photoreceptors with enormous masses of microvilli—often highly ordered—and no sign that a cilium was ever present in the cell.

The discovery some 20–30 years later that there was also a fundamental division between the opsins of ciliary and rhabdomeric photoreceptors seemed to seal the story. Examples of microvilli on photosensory cilia in hydrozoan cnidarians and a few other animals were assumed to be exceptional, and the fact that almost the entire classification system was based on only three phyla of relatively large-bodied creatures (vertebrates, arthropods, and mollusks) was discounted. To be fair to Eakin, he did include many "minor" phyla in his survey, such as cnidarians, ctenophores, rotifers, echinoderms, tunicates, cephalochordates, and literally almost anything he and his colleagues could get their hands on; it was many of these examples that provided the strange receptor sets that sometimes had cilia and sometimes did not. He recognized the problem with a delightful quote, "The erection of a system of pigeon holes usually presents a problem of what to do with the pigeon that does not fit any of the holes" (Eakin 1972, p. 653). However, a system with only two pigeon holes cannot really fail—either the cell has a cilium or it does not; thus, ciliary or rhabdomeric. Eakin's odd bird was a protist, where the organism, cell, and receptor are the same thing.

Sensory cilia are universal among animals, so it is not surprising that many photoreceptors contain such structures, nor that the ciliary remains could be lost over time as the expanded membranes took over the role of sensation and transduction. Indeed, Eakin (1979) had to face down a challenge by Salvini-Plawen and Mayr (1972), who proposed that photoreceptors appeared independently dozens of times, while simultaneously deflecting Valfleteren's and Coomans' (1976) proposal that a single ancestral cell could account for all types. Unfortunately, this was bound to be a losing battle, as the ever-expanding family of opsins began to turn up almost everywhere in one species or another, from gonads to skin cells to the center of the brain (Fig. 4.6). More significantly, they are being found in cells that definitely have

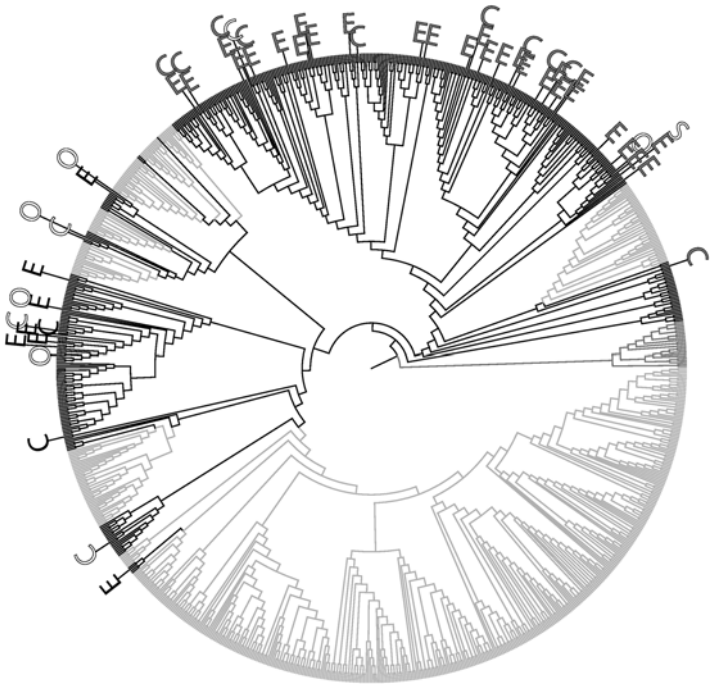

Fig. 4.6 The Fig. 4.1 phylogeny, with the cell type and tissue of expression indicated for opsin sequences, where known (data from Porter et al. 2012). The tissue of expression is indicated by the letter associated with a tip branch: *E* eye, *C* central nervous system (CNS), *S* skin, *O* other. The *gray* level of each letter indicates the photoreceptor cell type: ciliary = *black* letters, rhabdomeric = *grey* letters, and other cell types = *white* letters. Branches in the phylogeny are denoted as in Fig. 4.1: invertebrate opsin sequences and associated branches are in black, vertebrate opsin sequences in light grey, and outgroups in *dark grey*

other jobs than photoreception alone, such as relay neurons in the retina (intrinsically photosensitive retinal ganglion cells, or ipRGCs of vertebrates) or chromatophores of fishes and cephalopods. A strong case can be made that photoreceptor cells probably did not evolve dozens of times; instead the number is hundreds or thousands of times, as opsins became expressed in tissues whenever photosensing became useful (see Ramirez et al. 2011; also Fig. 4.6). Complicating the issue, in leeches a type of photosensory cell known as a phaosomal photoreceptor cell exists. Phaosomes are photosensitive structures resembling vacuoles in supporting cells, and are found both in pigmented forms in simple eyes and unpigmented in extraocular locations. Döring et al. (2013) traced their evolution by comparative genetic analysis, finding that these weird photoreceptors are actually derived from rhabdomeric ancestors—an example of a rather poorly differentiated light-sensing cell evolving from a classic photoreceptor type.

This argument is not entirely fair to Eakin's research group or to most of the others who have grappled with the problem of photoreceptor cell evolution, because

they were concerned with strictly visual photoreceptors, or at least those specially designed to have an elevated sensitivity to light (the ones with all that expansion of membrane). The first photoreceptors were surely cells that were already involved in other functions, and even now the greatest diversity of photoreceptor cells is in non-visual organs, or at least organs that serve some generalized light-sensing for a variety of functions. Thus, opsins have been located in neurons, cells of the ovary, chromatophores, and numerous other cell types, mostly but not always of epidermal origin (Fig. 4.6). See Westfall and Kinnamon (1978) for an early description of such a sensory cell in the epidermis of *Hydra*, and more recent descriptions of various non-visual photoreceptors in cnidaria (Plachetzki et al. 2012, Suga et al. 2008), a ctenophore (Schnitzler et al. 2012), an annelid (Backfisch et al. 2013), and echinoderms (Ooka et al. 2010, Ullrich-Lüter et al. 2011). In Nilsson's view of visual evolution, non-directional light sensing requires no membrane specializations for function throughout the range of natural irradiances; it is only when photoreceptors become specialized for directional vision that membrane proliferation becomes important (Nilsson 2009, 2013).

Thus, the question turns to the origins of photoreceptor cells with membrane expansions: were the ancestral types derived from proliferation of ciliary membranes or other membranous systems? This matter is likely to remain unresolved for some time, since the common ancestor of animals as simple as cnidarians and the putative urbilaterian already possessed a diversity of opsin types probably expressed in an equally diverse assortment of photoreceptors. Vanfleteren and Coomans (1976) and Vanfleteren (1982) argued that photoreceptor membranes were originally produced in association with sensory cilia and that modern differences among photoreceptors largely reflect modifications of this photoreceptor form. On the other hand, in our search of the literature, we could find no example of an R-opsin expressed in a cell with even the remnant of a sensory cilium present, suggesting that microvillar receptors have always lacked a cilium; any ancestor that would argue to the contrary is surely long gone. Even when opsins are co-expressed in a single cell, all cases described to date involve sets of R-opsins or C-opsins (examples: insect, Arikawa et al. 2003; crustaceans, Sakamoto et al. 1996, Rajkumar et al. 2010; *Limulus*, Katti et al. 2010; lizard, Su et al. 2006; mammal, Applebury et al. 2000). All of these co-expressing cells have the classic cellular morphology of rhabdomeric or ciliary photoreceptors. Nevertheless, it is certainly possible that one of the unusual receptor types, for instance photosensitive neurons or ovarian cells, could co-express members of different rhodopsin families.

Early photoreceptors involved in directional sensing of light were already advanced over the simple irradiance detectors that were undoubtedly the primal light-sensing cells. They needed extended membrane surfaces to be able to capture light from a more limited angular field than that necessary for irradiance detection and also to provide the ability to sense changes in light intensity in shorter time periods (Nilsson 2009, 2013). An additional requirement is the need for a pigment screen to prevent light from reaching the photosensitive membranes from the "wrong" direction. Nilsson (2009, 2013) agrees with Arendt et al. (2009), who proposed that early photoreceptors incorporated the pigment into the same cell as the

membranes, as seen today in some cnidarian photoreceptors (Westfall and Kinnamon 1978; Nordström et al. 2003). Arendt's team proposed a "division of labor" sequence for further elaboration, whereby the screening functions were moved to specialized cells (Arendt et al. 2009; see also Arendt and Wittbrodt 2001; Arendt 2003). The pairing of a single pigmentary cell with a rhabdomeric photoreceptor cell is already sufficient to orient phototaxis in marine zooplanktonic larvae (Jékely et al. 2008). With the availability of specialized photoreceptor cells, simple optics, and adaptive behavior, the underpinnings of all future visual evolution were in place.

4.7 Summary and Conclusions

The origin of opsin-based photopigments is still shrouded in antiquity, but intense recent interest in the problem has brought some promise that this puzzle will be solved. If so, it will be the range and variety of invertebrate opsins that leads to the solution. Although the current situation has uncovered a previously unexpected diversity of opsins, the continual addition of sequences from new, perhaps less prominent, taxa will surely provide a thorough picture of their origin and diversification with time.

Opsins probably were favored in their early evolution because their ancestor, a GPCR, efficiently activated a primal phototransductive cascade. Currently, four major groupings of opsins are thought to exist. One of these, the cnidops, is found only in invertebrates (indeed, only in cnidarians and possibly brachiopods), while the other three groups are shared with vertebrates. Cnidarians themselves might possess examples drawn from all four groups, suggesting that visual evolution was carried quite far in these seemingly simple organisms. The fundamental opsin groups differ consistently in sequence and also routinely interact with different, generally group-specific, G-proteins, again concealing the original activation pathway. Indeed, some members of Group 4 have secondarily lost their ability to couple to a G-protein and have acquired a photoisomerase function.

The most successful invertebrate opsins appear to be the R-opsins, which dominate in visual receptors of arthropods and mollusks. However, these two groups have diversified their R-opsins along fundamentally different pathways. The arthropods have proliferated their opsin types, expressing numerous variants in a single retina (e.g. Porter et al. 2009). Mollusks, on the other hand, commonly express only a single opsin variant, even expressing their retinal sensory opsin class in photosensory cells elsewhere in their bodies. R-opsins are thermally bistable, hinting that the original photoreceptive pigment itself might have been bistable, relying only on light to regenerate a functional photopigment. Today, invertebrates use photosiomerases shaped from opsin itself (retinochromes) or other molecules. Unexpectedly, no recognizable dark isomerase has yet been uncovered in an invertebrate retina.

Until recently, photoreceptor cells have been sorted into rhabdomeric vs. ciliary types. This convenient system has gradually broken down, leading to opposing suggestions that all current photoreceptors descended from a common ancestor or that

they arose independently many times. Given that photosensory cells exist in many locations outside eyes in both vertebrates and invertebrates, it seems likely that photoreceptors suitable for building a retina could repeatedly have emerged independently. However, a counterargument can be made that a common set of transcription factors specifies the development of most—or all—complex eyes, so there must have been a single ancestral cell type to be put to use in vision. As with the opsins themselves, it will be fascinating to learn what future research will reveal.

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Chapter 5
Insect Photopigments: Photoreceptor Spectral Sensitivities and Visual Adaptations

Kentaro Arikawa and Doekele G. Stavenga

Abstract The spectral sensitivity of photoreceptors is primarily determined by the expressed rhodopsins. After a brief introduction to the photochemistry of insect rhodopsins, the relatively simple case of bee visual pigments and photoreceptors is described, followed by the more complicated cases of butterflies and flies. Although the main focus is on the properties of visual pigments, considerable attention is also given to other photostable filter pigments that importantly modify the spectral properties of the photoreceptors. The sexual dimorphism of the filter pigments results in the sexual dimorphism of photoreceptor spectral sensitivities.

Keywords Color vision • Compound eye • Ommatidium • Photoreceptor • Rhabdom • Spectral sensitivity • Sensitizing pigment • Filter pigment • Fluorescent pigment • Sexual dimorphism

Abbreviations

S	Short wavelength-absorbing
M	Middle wavelength-absorbing
L	Long wavelength-absorbing
R1–R9	Photoreceptor 1–9

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5.1 Introduction

Visual pigment diversity is under evolutionary pressure because the visual pigments are the essential elements that determine the spectral sensitivity of the photoreceptors. A photoreceptor set with different spectral sensitivities serves as the physiological basis of color vision. The spectral environment and visual tasks thus have to be considered in close connection with the visual pigments' spectral properties.

The study of insect visual pigments essentially started about a hundred years ago when Karl von Frisch published his pioneering work on the color vision of the European honeybee, *Apis mellifera* (Frisch 1914). Since then insect color vision has become a central topic in biology, making von Frisch in 1973, together with Konrad Lorenz and Nikolaas Tinbergen, a Nobel laureate for fundamental contributions to the study of animal behavior.

Because of the influence of von Frisch, honeybees have been intensely studied ever since. Fifty years after the initial color vision studies, Autrum and von Zwehl, applying intracellular electrophysiology to insect photoreceptors for the first time, reported four distinct spectral sensitivities of single photoreceptors in the worker honeybee (Autrum and von Zwehl 1964). The number of spectrally different photoreceptors were later adjusted to three, with sensitivities peaking at around 350 nm (ultraviolet, UV), 440 nm (blue, B) and 540 nm (green, G) (Menzel and Blakers 1976). These three spectral receptor classes provide the physiological basis for the trichromatic system of honeybees (Daumer 1956; von Helversen 1972). It is strikingly similar to the human trichromatic system with a notion that the visible light range of insects is shifted about 100 nm toward the shorter wavelengths.

Due to the emphasis on honeybee research, studies on other insect species were initially largely ignored (Ilse 1941). Extensive comparative research started in the late 1960s (Bennett et al. 1967; Menzel 1979), and flourished in the 1980s. The outcome of those studies later established that insect photoreceptors can have quite variable spectral sensitivities, depending on the species (Eguchi et al. 1982, 1984; Matic 1983; Arikawa et al. 1987). Stimulated by the early electrophysiological studies, in vitro and in situ spectroscopy was applied in the 1970s to understand the physiological nature of visual pigments (Hamdorf 1979).

Subsequently, in the 1980s, molecular biology became widely applicable. The primary structures of some mammalian visual pigment opsins were first identified (Hargrave et al. 1983; Nathans and Hogness 1983; Nathans et al. 1986), rapidly followed by the analysis of the visual pigments of the fruitfly *Drosophila melanogaster* (Zuker et al. 1985, 1987). Next, three opsins of honeybees were cloned in the 1990s (Chang et al. 1996; Bellingham et al. 1997; Townson et al. 1998), which was followed by the sequence data of opsins from an increasing number of insect orders, totaling to date more than 2,000 visual pigment molecules. The data clearly indicate that insect opsins collectively form three clades: short wavelength (S) or UV-absorbing, middle wavelength (M) or blue-absorbing, and long wavelength (L) or green-absorbing visual pigments (Fig. 5.1).

The accumulated information about visual pigments provides a broadly coherent view about their molecular and photochemical properties. Furthermore, it has

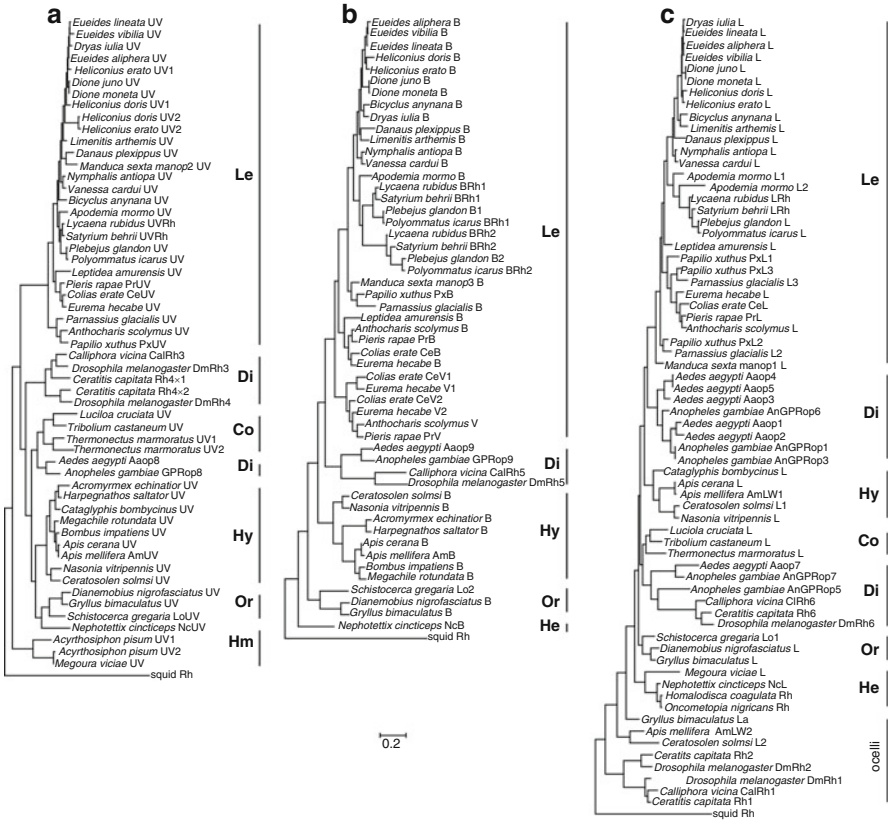


Fig. 5.1 Molecular phylogeny of the three clades of insect opsins. (**a**) Short wavelength (S) or UV-absorbing type. (**b**) Middle wavelength (M) or blue-absorbing. (**c**) Long wavelength (L) or green-absorbing

promoted an understanding of the physiological mechanisms underlying photoreceptor spectral sensitivities. Insect compound eyes thus have become to be a rich source for studies of visual specializations. For instance, compound eyes often show a distinct regionalization as well as sexual dimorphism, affecting the expression of the visual pigments and consequently the photoreceptor spectral sensitivities. Non-visual, so-called screening pigments may also play a distinct role in modifying the spectral sensitivities.

The spectral sensitivity of photoreceptors is primarily determined by the expressed visual pigments. After a brief introduction to the photochemistry of insect visual pigments, the relatively simple case of bees (order Hymenoptera) will be presented, followed by more complicated cases, particularly those of butterflies (order Lepidoptera) and flies (Diptera). Although the main focus will be on the properties of visual pigments, considerable attention will be given to other pigments that importantly modify the spectral properties of the photoreceptors.

5.2 Photochemistry of Insect Visual Pigments

A visual pigment molecule consists of two components, an opsin protein and a vitamin A aldehyde attached to opsin as the chromophore. Commonly in vertebrates the chromophore is 11-*cis* retinal, but many insects employ 11-*cis* 3-hydroxy-retinal (Vogt and Kirschfeld 1983). Visual pigments with different chromophore molecules are sometimes given different names, but for simplicity we here use the term rhodopsin for all visual pigments, independent of the type of chromophore.

When a rhodopsin molecule absorbs a photon, the chromophore is isomerized into the all-*trans* form. The quantum efficiency of the conversion has not been measured for any insect rhodopsin, but it may be similar to that determined for bovine rhodopsin, which is about two-thirds (Dartnall 1972). The isomerization of the chromophore subsequently causes the transformation of the whole pigment molecule, via a few thermally unstable intermediates, to metarhodopsin. In the case of vertebrate visual pigments, metarhodopsin further decays, but in invertebrates it is thermally stable (Gärtner 2000).

The metarhodopsins of insects have their own distinct absorption spectra, so that photon absorption by metarhodopsin can re-isomerize the all-*trans* chromophore back into the 11-*cis* form. Prolonged exposure of insect visual pigments to monochromatic light hence creates a photosteady state that depends on the ratio of the absorption coefficients of the two states, rhodopsin and metarhodopsin.

The photochemical steps are often accompanied by both shifts of the absorption peak wavelength and distinct changes in peak absorption. By measuring absorbance difference spectra, the photochemical processes can be studied even in turbid or complex tissues containing other pigments, and even in the living eye (Hamdorf 1979).

5.3 The UV Pigment of the Owlfly *Libelloides macaronius*

The intermediate states in the photochemical cycle of both vertebrate and invertebrate visual pigments are thermostable below a certain critical temperature, and they can thus be studied sequentially by photoconversion of rhodopsin at appropriately chosen temperatures. These low temperature studies are preferentially performed on visual pigment extracts. As an example, a few photochemical steps are shown for the UV-rhodopsin of the owlfly *Libelloides* (formerly *Ascalaphus*) *macaronius* (Neuroptera), the first lucid example of an UV-absorbing visual pigment (Fig. 5.2; Belušič et al. 2013). Upon illumination the pigment can attain various states, as witnessed by their different absorbance spectra. The owlfly rhodopsin absorbs maximally at 345 nm (Hamdorf 1979). Absorption of a photon by rhodopsin (R) at −50 °C yields lumirhodopsin (L). In this state the visual pigment absorbs much stronger than the native rhodopsin, indicative of the 11-*cis* all-*trans* isomerization of the chromophore, and the peak wavelength is shifted to about 375 nm. The lumirhodopsin can be photoconverted back to rhodopsin at −50 °C, but it is unstable

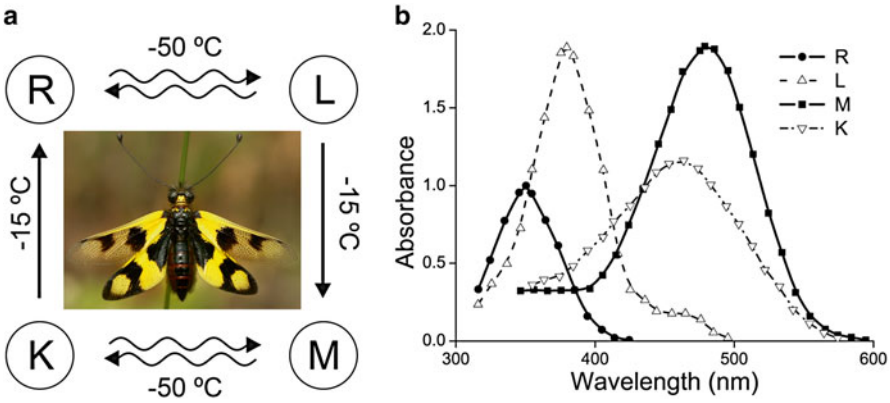


Fig. 5.2 Photochemical cycle and spectral characteristics of the UV-absorbing rhodopsin and its photoproducts in the owlfly *Libelloidus macaronius*. (**a**) At low temperatures (−50 °C) photon absorption results in conversion of rhodopsin, R, to lumirhodopsin, L, which can be photoconverted back into rhodopsin. Upon warming (−15 °C) lumirhodopsin transforms to metarhodopsin, M. At low temperatures this is photointerconvertible with an intermediate, K, which above −15 °C decays to the rhodopsin state. The chromophores in R and K take the 11-*cis* configuration, and the chromophores in L and M are all-*trans*. (**b**) Absorbance spectra normalized to the rhodopsin peak absorbance of the various visual pigment states (modified from Hamdorf 1979)

at −15 °C. The end photoproduct, metarhodopsin (M), then results (Fig. 5.2). The strong bathochromic shift of the peak wavelength indicates distinct intramolecular conformation changes. As in lumirhodopsin, the peak absorbance coefficient of metarhodopsin is about 1.8 times that of rhodopsin.

Irradiation of metarhodopsin at −50 °C yields an intermediate K (Fig. 5.2), which is photointerconvertible with metarhodopsin. The strongly reduced absorbance coefficient signifies the all-*trans* to 11-*cis* isomerization, but the peak wavelength shift to 460 nm suggests relatively minor changes in the conformation of the whole protein. At −15 °C intermediate K decays thermally to rhodopsin.

Low temperature spectroscopy of visual pigments and their intermediates has been performed in several vertebrates and a few invertebrates (Yoshizawa 1972; Hamdorf 1979; Vought et al. 2000). The decay scheme appears to follow a rather uniform temporal pattern, at least in the pathway of vertebrates, where rhodopsin transforms via bathorhodopsin, lumirhodopsin, and metarhodopsin to retinal and opsin. The photoconversion of the intermediates, demonstrated to occur at low temperatures, occurs much more rapidly at physiological temperatures. However, due to the brief lifetimes of the intermediates, it then requires extreme irradiation intensities to cause noticeable photoconversions, and the chance of photoconverting intermediates is negligible at light fluxes existing under normal, physiological conditions. For all general spectral considerations, it is therefore adequate to consider insect visual pigments to exist either in the rhodopsin or the metarhodopsin configurations.

5.4 Bee Visual Pigments

The compound eyes of honeybees consist of about 6,000 ommatidia, each containing nine photoreceptor cells, R1–R9. Eight of them (R1–R8) are elongated cells that extend over the full retinal layer of the ommatidium, while R9 is a small photoreceptor at the base of the ommatidium. Each photoreceptor extends microvilli that form a visual pigment-containing rhabdomere. The rhabdomeres of a honeybee ommatidium are closely apposed, forming a fused rhabdom that acts as a single optical waveguide (Fig. 5.3b).

As suggested by the phylogeny of visual pigment opsins (Fig. 5.1), the eye of the honeybee has three photoreceptor classes, UV, B and G (Fig. 5.3c), each expressing a specific opsin: AmUV (*Apis mellifera* UV), AmB, and AmL (Fig. 5.3d–f). The spectral sensitivities of honeybee photoreceptors thus can be explained by the different absorption spectra of the three visual pigments. The absorption spectra of the honeybee visual pigments were first estimated from electrophysiological recordings, but they were more accurately assessed by ectopically expressing the opsins in *Drosophila* (Townson et al. 1998).

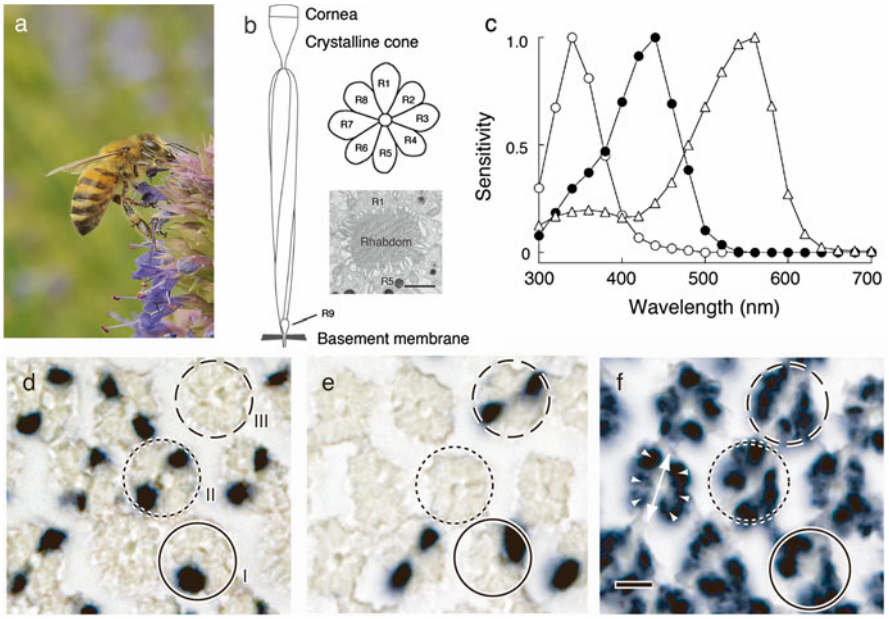


Fig. 5.3 Spectral receptors of honeybees. (**a**) The honeybee *Apis mellifera* (courtesy of Keram Pfeiffer). (**b**) Diagram of an ommatidium with the twisted photoreceptor bundle, and an electron micrograph of a transverse section of a rhabdom (bar: 1 μm). R1–R9, photoreceptor numbers. (**c**) Spectral sensitivities of UV, B and G photoreceptors. (**d–f**) Three consecutive transverse sections of the retinal layer, showing in situ hybridization of mRNAs encoding opsins of UV (**d**), blue (**e**), and green (**f**) absorbing visual pigments. The three types of ommatidia are indicated by *closed* (type I), *dotted* (type II), and *dashed* (type III) circles. The G probe (**f**) labeled six photoreceptors in all ommatidia (*white arrowheads*) (bar **d–f**: 5 μm)

The shape of a visual pigment's absorption spectrum is determined by the chromophore present but is otherwise generally well described by a template formula, with the peak wavelength value as the only variable (Govardovskii et al. 2000; Stavenga 2010). It thus was shown that AmUV is a visual pigment absorbing maximally at 353 nm (R353) and AmB is an R439 (Townson et al. 1998), matching the measured spectral sensitivities of the photoreceptor classes (Fig. 5.3c).

The question relating to which photoreceptors the individual visual pigments are expressed in was first studied histologically after monochromatic stimulation (Gribakin 1969), and then by a combination of single cell electrophysiology and intracellular dye injection (Menzel and Blakers 1976). These first results indicated that each ommatidium contains three UV (R1, R5, and R9), two B (R2, R6), and four G (R3,4,7,8) receptors. This view has been widely accepted, but a recent in situ hybridization study of opsin mRNAs in the retina indicated that some adjustments are necessary (Wakakuwa et al. 2005). The in situ hybridization revealed that the R1 and R5 photoreceptors are either UV or B receptors, while R2–4 and R6–9 are G receptors (Wakakuwa et al. 2005). A particularly interesting finding was that there are three ommatidial types. According to the mRNAs contained in R1 and R5, type I ommatidia have one UV and one B receptor, while type II and III have two UV and two B receptors, respectively: the ommatidia are themselves spectrally heterogeneous.

The three types of ommatidia are distributed somewhat randomly in an otherwise hexagonal pattern, but some regionalization exists. The dorsal region contains more type II ommatidia, with two UV receptors, while the ventral region contains more type III, with two B receptors (Wakakuwa et al. 2005). The concentration of B receptors in the ventral region of the eye is probably related to a better contrast detection by B receptors of terrestrial targets (Giurfa et al. 1999). Behavioral observations indicate that the dorsal rim area, which is crucial for polarization-based navigation, contains exclusively UV receptors (Helversen and Edrich 1974), but this has not yet been confirmed at the molecular level.

The retinal organization found in honeybees seems very similar to that of the bumblebee *Bombus impatiens* (Spaethe and Briscoe 2005). Its eyes are also furnished with a set of UV, B and G receptors (Skorupski and Chittka 2010). Peitsch et al. (1992) measured photoreceptor spectral sensitivities of 43 hymenopteran species from 14 families of bees and wasps, most of which appeared to have a set of three spectral receptors. In some species UV and/or B receptors were not found, but this may have been due to incomplete electrophysiological measurements (Peitsch et al. 1992). Four species were found to have photoreceptors with spectral sensitivities peaking at around 590–600 nm. The opsins expressed in these "red (R)" receptors have not yet been identified, but the unusual spectra may well be caused by spectral filtering (see below). The presently available data nevertheless clearly show that hymenopteran species share very similar sets of spectral photoreceptors.

The spectral sensitivities can be modified by the anatomical characteristics of the rhabdom. In the fused rhabdom of bees, the rhabdomeres of different classes of spectral receptors are tightly packed together (Fig. 5.3b). Because the fused rhabdom acts as an optical waveguide, the visual pigments in the various rhabdomeres forming the fused rhabdom mutually act as spectral filters. This effect is called lateral filtering (Snyder et al. 1973). For example, when the rhabdomeres of B and G

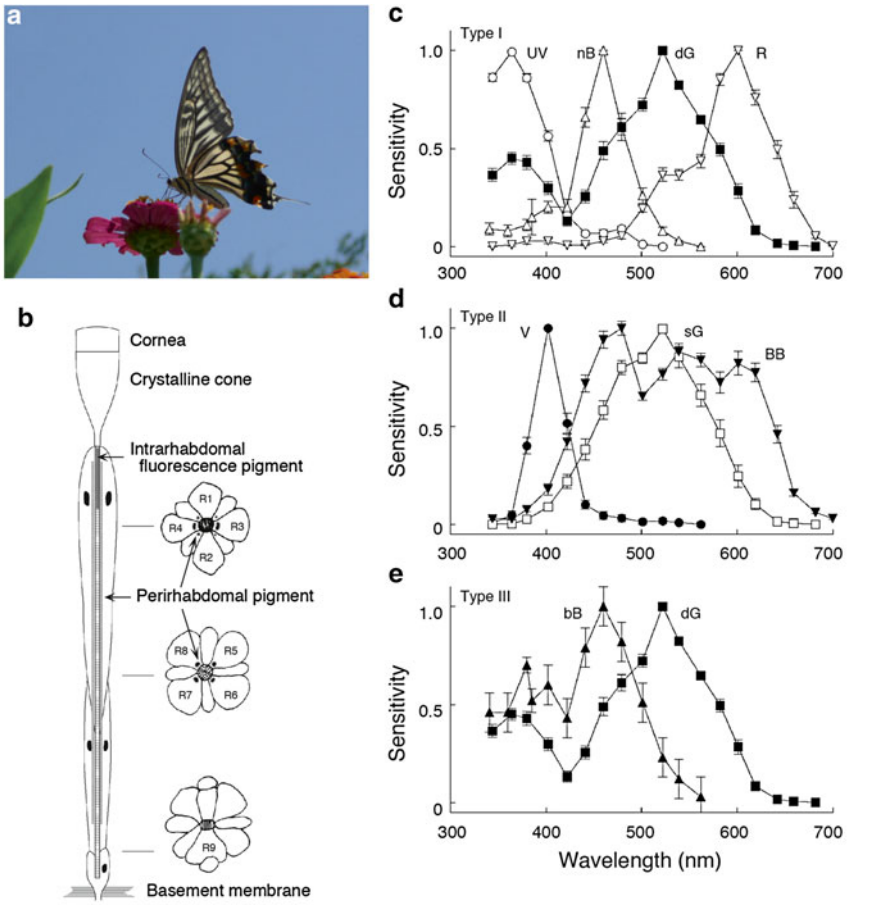
receptors coexist in the rhabdom, lateral filtering shifts the sensitivity peak of the B receptors hypsochromically (toward shorter wavelengths), while it shifts the peak of the G receptors bathochromically (toward longer wavelengths). Furthermore, when UV, B and G receptors coexist, the sensitivity profile of especially the B receptor becomes narrower. Another possible effect modifying spectral sensitivities is self-screening. This will occur in very long rhabdomeres or rhabdoms, because then the upper visual pigment layers act as spectral filters for the lower layers. Self-screening predicts broadening of the spectral sensitivity, but actual electrophysiological measurements do not provide clear examples.

5.5 Visual Pigments of Butterflies—The Exemplary Case: *Papilio xuthus*

Color vision of colorful butterflies has attracted researchers for quite some time (Ilse 1928; Swihart 1969; Bernard 1979). In recent years, several novel and important phenomena related to the mechanisms underlying photoreceptor spectral sensitivities as well as color vision in this group of insects have been revealed.

The most extensively studied species in this respect is the Japanese yellow swallowtail, *Papilio xuthus* (Papilionidae, Fig. 5.4a). The eye of *Papilio* is furnished with at least six classes of photoreceptors, peaking in the UV (360 nm), violet (V, 400 nm), B (460 nm), G (540 nm), and R (600 nm) wavelength regions, or having a broad-band (BB) sensitivity. The B and G receptor classes each have two subclasses: the spectral sensitivities of the B receptors can be narrow (nB) and broad (bB), while those of the G class can be double-peaked (dG) or single-peaked (sG) (Fig. 5.4c–e). On the other hand, *Papilio* eyes express only five visual pigment opsins: one UV-absorbing (PxUV, for *Papilio xuthus* UV), one B-absorbing (PxB), and three L-absorbing (PxL1, PxL2, and PxL3) (Fig. 5.1; Table in Fig. 5.4). The existence of multiple L opsins is due to gene duplication events (Briscoe 2000).

An ommatidium of *Papilio* contains nine photoreceptors, R1–9 (Fig. 5.4b). Unlike in bees, the rhabdom of *Papilio* has three tiers and is not twisted (Arikawa and Uchiyama 1996). The distal two-thirds of the rhabdom consist of the rhabdomeres of four distal photoreceptors, R1–4, while the proximal one-third is made up of the rhabdomeres of four proximal photoreceptors, R5–8. The basal photoreceptor, R9, forms a small, third tier at the base of the rhabdom. In transverse section, each rhabdom is surrounded by four clusters of red or yellow pigment. These perirhabdomal pigments form an absorbing layer around the rhabdom. They thus act as red or yellow filters for the boundary wave of light that propagates immediately outside the rhabdom. Interestingly, a subset of red-pigmented ommatidia distinctly fluoresces under UV excitation. The fluorescent pigment exists in the distal portion of the rhabdom and is most likely 3-hydroxy-retinol (Arikawa et al. 1999a). In summary, the compound eyes of *Papilio* comprise a collection of three types of ommatidia: I, red; II, red and fluorescent; and III, yellow (Table in Fig. 5.4).



Pigment	Type I red		Type II red		Type III yellow	
Fluorescence	no		yes		no	
Photoreceptors	opsin	S(λ)	opsin	S(λ)	opsin	S(λ)
R1	PxUV	UV	PxUV	V	PxB	bB
R2	PxB	nB				
R3,4	PxL1, PxL2	dG	PxL1, PxL2	sG	PxL1, PxL2	dG
R5-8	PxL3	R	PxL2, PxL3	BB	PxL2	dG

Fig. 5.4 Spectral organization of the retina of *Papilio xuthus*. (**a**) The Japanese yellow swallowtail, *Papilio xuthus*. (**b**) Schematic diagram of a *Papilio* ommatidium. The rhabdomeres of the R1–R9 photoreceptors form together a fused rhabdom, which is surrounded by clusters of red (type I and II ommatidia) or yellow (type III) perirhabdomal pigment. The rhabdom of type II ommatidia contains a fluorescent pigment. (**c**) Spectral sensitivities of the receptors of type I ommatidia. *UV* ultraviolet, *nB* narrow blue, *dG* double-peaked green, *R* red. (**d**) Spectral receptors in type II ommatidia. *V* violet, *sG* single-peaked green, *BB* broad-band. (**e**) Spectral receptors in type III ommatidia. *bB* broad blue. The table summarizes the spectral organization of the three types of ommatidia, with the localization of five opsins

Intracellular electrophysiology coupled with dye injection demonstrated that the location of all six classes of spectral receptors (eight when counting the B and G subclasses) could be unambiguously identified in the array of the three different types of ommatidia (Fig. 5.4c–e). This has now been combined with in situ hybridization studies of the five opsin mRNAs, to give a full understanding of the spectral organization of the *Papilio* eye (Arikawa 2003). R1 and R2 are either UV, V or B receptors, but their combination varies between the ommatidial types. Type I ommatidia have a UV and a narrow blue (nB) receptor, while type II have two violet (V) receptors and type III two broad-blue (bB) receptors. R3 and R4 are G receptors in all ommatidia (double-peaked green, dG, in type I and III ommatidia, and single-peaked green, sG, in type II). R5–8 are R receptors in type I, BB receptors in type II and dG receptors in type III ommatidia (Table in Fig. 5.4).

In general, individual photoreceptors express a single type of visual pigment, the so-called one rhodopsin per receptor rule (Stavenga and Arikawa 2008). Although the majority of photoreceptors follow this rule, it may not be universal, because opsin protein and/or its mRNA are found to be coexpressed in some photoreceptors of both invertebrates (Sakamoto et al. 1996; Rajkumar et al. 2010) and vertebrates (Roehlich et al. 1994; Makino and Dodd 1996; Lyubarsky et al. 1999; Applebury et al. 2000; Glosmann and Ahnelt 2002; Parry and Bowmaker 2002). An obvious question is whether these visual pigments participate in the phototransduction process. Some electrophysiological measurements to investigate this have been performed (Makino and Dodd 1996; Lyubarsky et al. 1999), but such attempts have remained rather rare. *Papilio* eyes have provided an early and conclusive proof. First it was reported that the R3 and R4 photoreceptors coexpress PxL1 and PxL2 mRNAs (Kitamoto et al. 1998). Because all R3 and R4 are G receptors throughout the eye, both PxL1 and L2 must be green-absorbing visual pigments. Furthermore, the R5–8 of type I ommatidia are R receptors and express PxL3 mRNA, indicating that the PxL3 visual pigment must be a red-absorbing visual pigment. Interestingly, the R5–8 of type II ommatidia coexpress PxL2 and PxL3 mRNA, and the spectral sensitivity of these photoreceptors is found to be broad, stretching from 400 to 650 nm (that is, a half-bandwidth of almost 210 nm compared with the usual 100 nm). The combined molecular and electrophysiological evidence clearly indicates that the PxL2 (green) and PxL3 (red) visual pigments are both functional in the broad-band (BB) receptors (Fig. 5.4d; Arikawa et al. 2003).

Papilio eyes yielded another unexpected result, namely that photoreceptors expressing the same opsin can have different spectral sensitivities. Both the UV receptors of type I ommatidia and the V receptors in the type II ommatidia express PxUV, which is a UV-absorbing visual pigment (Fig. 5.4d, Table in Fig. 5.4). The reason why PxUV-containing photoreceptors become V sensitive is the presence in type II ommatidia of a pigment, which acts as a UV-absorbing filter (Arikawa et al. 1999a). The perirhabdomal yellow and red pigments act also as spectral filters, but they only slightly shift the spectral sensitivities of the proximal R5–8 photoreceptors (Arikawa et al. 1999b).

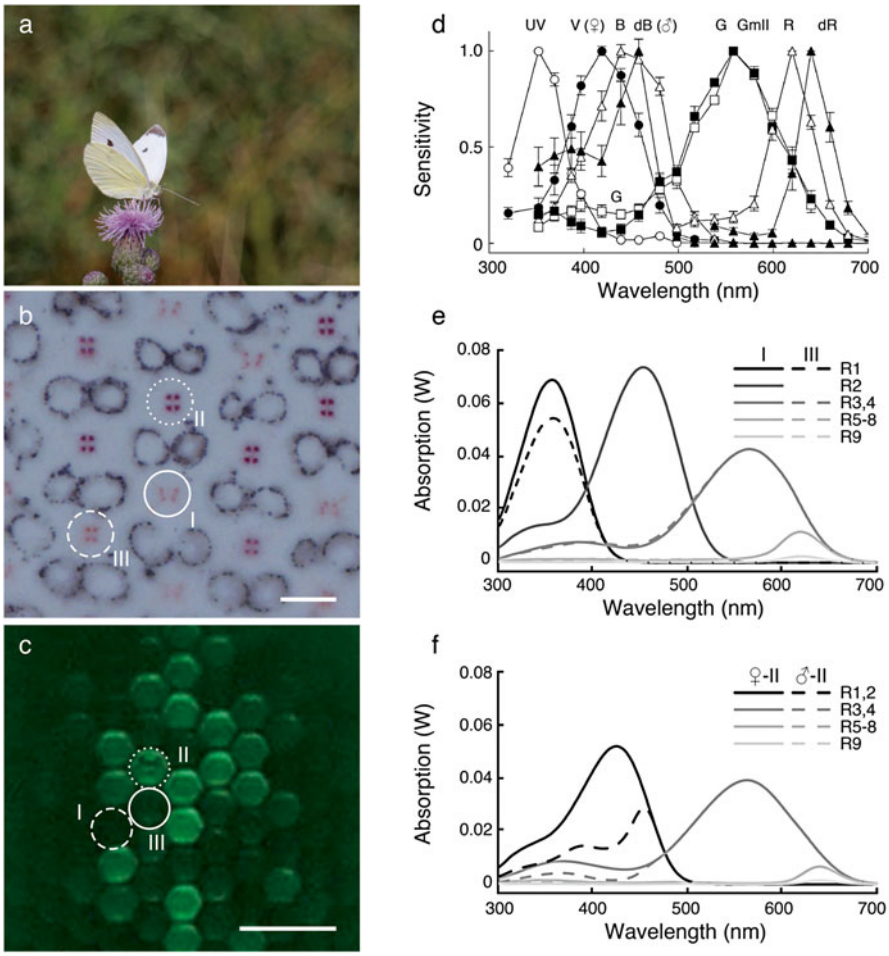
5.6 Other Papilionid Species

As Briscoe et al. (2003) stated, "not all butterfly eyes are created equal." This is in fact true even within the same family. The Glacial Apollo, *Parnassius glacialis*, is a member of the subfamily Parnassiinae in the family Papilionidae (http://tolweb.org/Papilionidae/12177). Their eyes express four visual pigments: one UV (PgUV, *Parnassius glacialis* UV), one B (PgB), and two L (PgL2 and L3). This indicates that in *Parnassius*, L opsin duplication happened only once, whereas in *Papilio*, a member of the tribe Papilionini, duplication must have happened twice (Fig. 5.1). The expression pattern of opsin mRNA is also quite different: in *Parnassius* type II and III, PgUV and PgB are coexpressed in R1 and R2. The R3 and R4 photoreceptors of type I and II express PgL2, presumably a green-absorbing pigment, while the R3 and R4 of type III express PgL3, which is probably red-absorbing (Matsushita et al. 2012).

A survey of opsin mRNAs in four tribes (Zerynthini, Troidini, Luehdorfini, and Leptocircini) in the subfamily Papilioninae revealed that all of the tested species have either two (Zerynthini and Troidini) or three (Luehdorfini and Leptocircini) L opsins, one B opsin, and one UV opsin. Evidently, in the lineage of Papilionidae, duplication of L opsins has occurred repeatedly, probably to acquire red receptors. Duplication appears to be absent in UV and B opsin clades, but nevertheless polymorphic short-wavelength receptors are present. This occurred in type II ommatidia of *Papilio* by the acquisition of a UV-absorbing, fluorescent pigment. Fluorescing ommatidia have been found in all papilionid species tested, indicating that the mechanism exists universally in papilionids (Matsushita et al. 2012; Chen et al. 2013). The eyes of the Common Bluebottle, *Graphium sarpedon* (Leptocircini), even show a further diversification: their ommatidia are either strongly, weakly or non-fluorescent. Because of this variation, they have at least four subclasses of B receptors (unpublished observation).

5.7 *Pieris rapae*: Effect of Perirhabdomal Filters

Although the optical filter effect of the red and yellow pigments is minor in Papilionidae, it is particularly strong in Pieridae (Fig. 5.5). The cellular arrangement of the ommatidia of the Small White butterfly, *Pieris rapae crucivora*, is similar to that of *Papilio*. In both cases, nine photoreceptors construct a tiered rhabdom: four distal (R1–4), four proximal (R5–8), and one basal (R9). However, in *Pieris* the trachea creates a tapetum proximal to each rhabdom. Light entering the facet propagates along the rhabdom until it is absorbed by the visual pigments or the perirhabdomal pigments. However, part of the light reaches the proximal end of the rhabdom without having been absorbed and is then reflected by the tapetum back into the rhabdom. A minor fraction of light escapes absorption even during the second trip and thus leaves the eye. This can be observed with epi-illumination microscopy and



Pigment	Type I Trapezoid, red		Type II Square, wine-red			Type III Rectangular, red	
Fluorescence	No		♂Yes, ♀No			No	
Photoreceptors	opsin	S(λ)	opsin	♂S(λ)	♀S(λ)	opsin	S(λ)
R1	PrUV	UV	PrV	dB	V	PrB	B
R2	PrB	B					
R3,4	PrL	G	PrL	GmII	G	PrL	G
R5-8		R		dR	dR		R

Fig. 5.5 Spectral organization of the retina of *Pieris rapae*. (**a**) Small white, *Pieris rapae*. (**b**) Unstained transverse section of an eye of *Pieris*. The color and arrangement of the perirhabdomal pigment identify the ommatidial types I, II and III; bar: 10 μm. (**c**) Violet-induced green fluorescence of the same eye region shown in (**b**). Type II ommatidia are strongly fluorescent; bar: 50 μm. (**d**) Spectral sensitivity functions recorded from single photoreceptors of *Pieris rapae. UV* ultraviolet; *V* violet, in female type II; *B* blue; *dG* double-peaked blue; *G* green; *GmII* green in male type II; *R* red; *dR* dark red. (**e**, **f**) Absorption spectra of R1–9 photoreceptors calculated with a wave-optics model for type I and III ommatidia (**e**) and the sexually dimorphic type II ommatidia (**f**) (Stavenga and Arikawa 2011). The table summarizes the spectral organization of the three types of ommatidia, with the localization of the four opsins

is called "eyeshine." The eyeshine color depends on the light absorption and tapetal reflection of the ommatidial types. In the ventral two-thirds of the *Pieris* eye, the rhabdoms are surrounded by either orange-red or wine-red colored screening pigments, so that with white-light illumination, a red or dark-red colored eyeshine results, with reflectance spectra of the different ommatidia peaking at 635 or 675 nm (Qiu et al. 2002).

The color and the spatial arrangement of the perirhabdomal pigments demonstrate that the eyes of *Pieris* consist of three ommatidial types. Transverse sections show that in all types, four clusters of pigment surround the rhabdom. The pigment clusters are arranged in a trapezoidal (type I), square (type II), or rectangular (type III) pattern (Fig. 5.5b). Type I and III ommatidia contain the orange-red pigment, while in type II the pigment is wine-red. In addition, the distal part of the rhabdom of type II ommatidia contains a fluorescent pigment that functions as an optical filter (green emission under violet excitation, Fig. 5.5c), but the fluorescent pigment exists only in males. The eyes of *Pieris* thus are sexually dimorphic (Arikawa et al. 2005).

The eyes of *Pieris* express four opsins, which belong to UV- (PrUV, *Pieris rapae* UV), violet- (PrV), blue- (PrB) and long wavelength-absorbing (PrL) classes (Fig. 5.1). Unlike in *Papilio*, a gene duplication appears not to have happened in *Pieris* in the L opsin clade, but rather in the M opsin clade, creating distinct B- and V-opsins. In situ hybridization revealed that although the distal R1 and R2 photoreceptors express one visual pigment, they do so in three combinations: PrUV in R1 and PrB in R2, or vice versa (type I), PrV in both R1 and R2 (type II), or PrUV in both R1 and R2 (type III). The distal R3–4 and the proximal R5–8 all express the PrL opsin (Wakakuwa et al. 2005).

The spectral sensitivities of the distal R1 and R2 photoreceptors are rather simple, because the PrUV- and PrB-expressing photoreceptors straightforwardly correspond to UV and B receptors. The PrV-expressing receptors in type II ommatidia have a peak sensitivity at 420 nm in females, but those of males are maximally sensitive at 460 nm. The latter appears to be caused by a fluorescent pigment in the type II ommatidia of males, which acts as a spectral filter. The consequence of the sexual dimorphism of the filter pigment thus is that it produces sexually dimorphic spectral sensitivities (Fig. 5.5d, Table in Fig. 5.5).

In order to address the question how the duplicated opsins have acquired different spectral absorption spectra, accurate spectroscopic analyses are required. In vitro expression of invertebrate visual pigments in cultured cells has been successful only in a few cases (Terakita et al. 2008; Nagata et al. 2012) including *Pieris rapae* (Wakakuwa et al. 2010). In vitro reconstitution of the duplicated opsins PrB and PrV has revealed that the absorption spectra of these pigments peak at 450 nm and 420 nm, respectively. Among 24 amino acid residues located within 5 Å from any carbon of the chromophore, the amino acids at positions 116 and 177 were found to be crucial for the spectral tuning (the numbering according to squid rhodopsin). The amino acids of these sites are Ser (116) and Phe (177) in PrB, while they are Ala and Tyr in PrV. Because most lepidopteran B opsins so far identified have Ser and Phe at these sites, which is also the case in PrB, the amino acid pair

(Ser/Phe) must be ancestral in the B clade. Substituting Ser116 by Ala in PrB resulted in a 13 nm short-wavelength shift, from 450 to 437 nm, and substituting Phe117 by Tyr resulted in a 4 nm short-wavelength shift, to 446 nm in the mutant molecule. This tuning mechanism appears to be shared, at least in part, by the pigments of other pierid and lycaenid butterfly species (Wakakuwa et al. 2010).

The spectral sensitivities of the PrL-expressing photoreceptors cannot be understood without considering the contribution of the perirhabdomal pigments (Fig. 5.5b). In the distal tier, where the effect of the pigments is negligible, the spectral sensitivity of the PrL-containing R3 and R4 photoreceptors well match the absorption spectrum of a visual pigment maximally absorbing at 563 nm, indicating that the PrL is an R563 pigment. In the proximal tier, the spectral sensitivity of R5–8 in type I and III ommatidia, where the perirhabdomal pigment appears orange-red, peaks at 620 nm (R receptor). The sensitivity of the R5–8 in type II ommatidia with wine-red pigment peaks at 640 nm (DR receptor). Evidently, the large sensitivity shifts, from 563 to 620 and 640 nm, are caused by the orange-red and wine-red pigments acting as spectral filters (Wakakuwa et al. 2004).

It should be noted that the above descriptions only hold for the ventral two-thirds of the *Pieris* eye. The situation is somewhat different in the dorsal one-third, because the *Pieris* eye exhibits a distinct degree of regionalization, a phenomenon generally encountered in compound eyes. Regionalization can be directly observed in butterfly eyes with tapeta, since the ventral and dorsal eye often exhibit quite different eyeshines (Stavenga et al. 2001). Whereas the eyeshine is reddish ventrally, it is yellow in the dorsal region of the *Pieris* eye. The latter is due to the absence of perirhabdomal pigments. The dorsal region also contains three types of ommatidia, but the R1 and R2 of the type II ommatidia in the dorsal region express PrB but not PrV. Close examination of the eyeshine in *Pieris rapae* with monochromatic light has revealed a transitional zone with six rows of ommatidia between the ventral and dorsal regions. In the transitional zone, these photoreceptors coexpress both PrB and PrV. The coexpression may be due to the overlapping of regulatory factors determining the ventral and dorsal regions during the developmental process.

The multitude of visual and screening pigments, together with sexual dimorphism and regionalization, makes the *Pieris* eye extremely complex. Nevertheless, the combination of anatomy, intracellular electrophysiology, in situ hybridization, microspectrophotometry, and optical observations using the eyeshine effect has produced a sufficiently comprehensive knowledge to allow quantitative optical modeling of the spectral sensitivities of all photoreceptors (Stavenga and Arikawa 2011). Specifically, the modeling revealed that the absolute sensitivities of the proximal R5–8 photoreceptors are significantly reduced due to the strong screening effect of the perirhabdomal pigments (Fig. 5.5e, f) (Stavenga and Arikawa 2011). The modeling indicated that the small basal photoreceptor, R9, for it to have any functional light sensitivity, must express PrL. Even then the light sensitivity is very low. Presumably therefore, PrL functions specifically in bright light conditions.

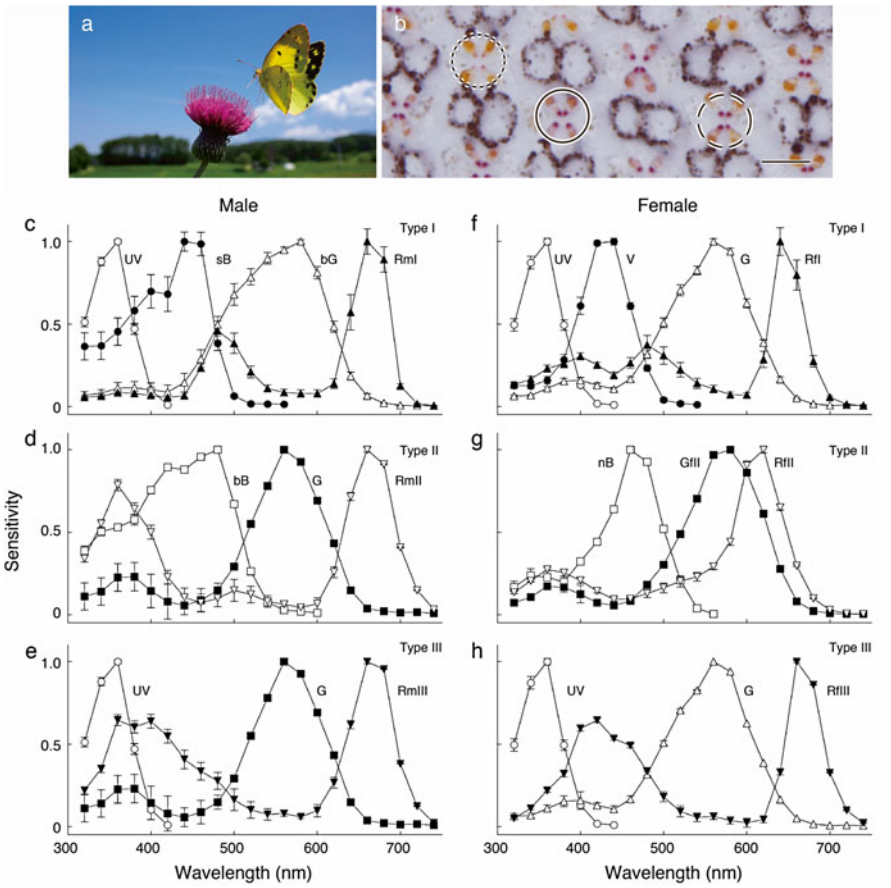
5.8 *Colias erate*, Sexual Dimorphism

Sexual dimorphism of the retina is even more pronounced in the Eastern Clouded yellow butterfly, *Colias erate*, a member of the subfamily Coliadinae in the family Pieridae. The wings of males are yellow, but most females are white; some females (less than 15 %) have a male-like yellowish color (Watanabe and Nakanishi 1996). The larvae of this species feed on clover leaves, and mated females select high quality leaves to lay eggs on. As in *Pieris rapae*, the retina of *Colias* is composed of three types of ommatidia with the perirhabdomal pigments arranged trapezoidally (type I), square (type II), and rectangular (type III) (Fig. 5.6b). Although belonging to the same family, it differs from *Pieris* in that both sexes have fluorescing ommatidia, namely the type I ommatidia in males and type II in females. In addition, the color of the perirhabdomal pigment in female type II ommatidia is paler. These rather subtle differences contribute to a clear sexual dimorphism (Ogawa et al. 2012, 2013).

As shown in Fig. 5.1, the eyes of *Colias erate* express five visual pigment opsins: CeUV (*Colias erate* UV), CeB, CeV1, CeV2, and CeL (Awata et al. 2009; Ogawa et al. 2012). The expression pattern of the mRNAs is summarized in Fig. 5.6. R1 and R2 express S (CeUV) and M (CeB, CeV1, and CeV2) opsins, while the R3–8 photoreceptors express the L opsin (CeL), as in other species. The most conspicuous feature here is the colocalization of M opsins. The mRNAs of CeV1 and CeV2 are always colocalized, but in type II ommatidia of the ventral region they are coexpressed together with CeB. The UV opsin, CeUV, is expressed in type I and III ommatidia. No sexual dimorphism has been detected in the opsin expression pattern (Fig. 5.6, Table).

The reason why CeV1 and CeV2 are always expressed together is not known, but presumably the genes are localized in tandem downstream of a common promotor. At the present stage, the two visual pigments have clearly not yet been subjected to subfunctionalization. In fact, the spectral sensitivity of photoreceptors expressing both CeV1 and CeV2 in non-fluorescing ommatidia (female type I, Fig. 5.6f) closely matches the absorption spectrum of an R430 pigment, suggesting that the absorption spectra of CeV1 and CeV2 are very similar. The corresponding photoreceptors in males exist in fluorescing ommatidia and have a narrower spectral sensitivity peaking at 440 nm, with a kink at 420 nm (Fig. 5.7c). The spectral shift is evidently caused by the fluorescent pigment acting as a spectral filter.

The situation in type II ommatidia is reversed, because in these ommatidia, the fluorescent pigment exists only in females. The R1 and R2 photoreceptors in type II ommatidia of both males and females express three M opsins, CeB, CeV1 and CeV2. In males, these photoreceptors have a broad spectral sensitivity, with half bandwidth about 150 nm and peak wavelength 460 nm (Fig. 5.7d). The bB spectral sensitivity can be understood from the colocalized visual pigments R430 (CeV1 plus CeV2) and R460 (CeB) (Ogawa et al. 2012). In females, the R1 and R2 photoreceptors of type II ommatidia become nB receptors, peaking at 460 nm (Fig. 5.7g), due to the presence of a fluorescent pigment acting as a spectral filter.



Pigment	Type I Trapezoid, red			Type II Square, ♂red, ♀orange			Type III Rectangular, red		
Fluorescence	♂Yes, ♀No			♂No, ♀Yes			No		
Photoreceptors	opsin	♂S(λ)	♀S(λ)	opsin	♂S(λ)	♀S(λ)	opsin	♂S(λ)	♀S(λ)
R1	CeUV	UV	UV	CeB					
R2	CeV1 CeV2	sB	V	CeV1 CeV2	bB	nB	CeUV	UV	UV
R3,4	CeL	bG	G	CeL	G	Gf-II	CeL	G	G
R5-8		Rm-I	Rf-I		Rm-II	Rf-II		Rf-III	Rf-III

Fig. 5.6 Spectral organization of the retina of *Colias erate*. (**a**) A male Eastern clouded yellow, *Colias erate* (courtesy of Kazuo Unno). (**b**) Unstained transverse section of the female retina. Three types of ommatidia are indicated by *closed* (type I), *dotted* (type II), and *dashed* (type III) circles (bar: 10 μm). (**c**) Spectral receptors in male type I ommatidia. *UV* ultraviolet, *sB* sharp blue, *bG* broad green, *RmI* red of male type I. (**d**) Male type II. *bB* broad blue, *G* green, *RmII* red of male type II. (**e**) Male type III. *RmIII* red of male type III. (**f**) Female type I. *V* violet, *RfI* red of female type I. (**g**) Female type II. *nB* narrow blue, *GfII* green of female type II, *RfII* red of female type II. (**h**) Female type III. *RfIII* red of female type III. The table summarizes the spectral organization of three types of ommatidium, with the localization of five opsins

The most prominent sexual dimorphism is found in the long wavelength receptors in *Colias*, which all express CeL. In the distal tier, the CeL-expressing R3 and R4 photoreceptors are all green-sensitive, with peak sensitivity at 560 nm. The spectral sensitivity can be reasonably well explained with the spectrum expected for an R565 visual pigment (Ogawa et al. 2012). In the proximal tier, however, the R5–8 of all ommatidial types in males are red-sensitive peaking at 660 nm (Fig. 5.7c–e). This shift of about 100 nm is readily explained by the filtering effect of the perirhabdomal red pigment. The red filter effect is probably enhanced by the strong constriction of the rhabdoms occurring between the distal and proximal tiers (Arikawa et al. 2009). In females, the spectral sensitivity of the R5–8 differs among the ommatidial types. They peak at either 650 (type I ommatidia), 610 (type II), or 660 nm (type III). The differences in the sensitivity peak shifts, from 565 to 650, 610 and 660 nm, respectively, is due to the differences in the spectral and spatial properties of the screening pigments (Fig. 5.7f–h). Notably the type II ommatidia of females contain a pale-orange screening pigment, which causes a much smaller spectral shift than the red pigment in the other ommatidial types (Ogawa et al. 2013).

A set of photoreceptors with different spectral sensitivities provides an animal with the ability to discriminate light of different wavelengths (von Helversen 1972; Koshitaka et al. 2008). Pierid butterflies appear to have an amazingly pronounced sexual dimorphism in the spectral sensitivities of their photoreceptors. Most likely, the male and female butterflies view the colored world quite differently, especially in the red wavelength region. This ability may be crucial for females to judge the quality of leaves on which to lay eggs (Ogawa et al. 2013).

5.9 *Lycaenidae*, Sexual Dimorphism in Opsin Expression

The expression pattern of the visual pigment opsins in Pieridae is identical in both sexes, and hence the sexual dimorphism in the photoreceptor spectral sensitivities is due to differences in screening pigments. Lycaenidae appear to have followed another strategy to create sexual dimorphism in the photoreceptor spectral sensitivities by changing the opsin expression pattern between sexes: males and females express opsins differently (Sison-Mangus et al. 2006).

As in Pieridae, several species of Lycaenidae have duplicated M opsins, in addition to one S and one L opsin (Fig. 5.1). In the Ruddy Copper butterfly, *Lycaena rubidus*, the absorption spectra of their UVRh, BRh1, BRh2, and LWRh (Sison-Mangus et al. 2006) have peak wavelengths at 360 nm, 437 nm, 500 nm and 568 nm, respectively. The UVRh, BRh1 and BRh2 opsins are complementarily expressed in R1 and R2 photoreceptors in six fixed combinations (UV-UV, B1-B1, B2-B2, UV-B1, UV-B2, B1-B2) throughout the eye, while other butterfly species typically have three ommatidial types. This spectral variety in the eyes of *Lycaena rubidus* suggests that, with appropriate neuronal wiring, they may have a better spectral resolution, particularly in the blue wavelength region (Sison-Mangus et al. 2006). The possible enhancement of color vision in the blue region has been considered to

be useful for conspecific visual communication in these blue butterflies (Sison-Mangus et al. 2006). Upon searching for flowers that provide nectar, the duplicated B opsins appear also to be useful to discriminate greenish colors in other lycaenid species, *Polyommatus icarus* (Sison-Mangus et al. 2008). However, electrophysiological evidence demonstrating that the various photoreceptor classes have different spectral sensitivities is not yet available.

Sexual dimorphism is observed in photoreceptors R3–8 of Lycaenidae. In the ventral region of the eye these photoreceptors all express the L opsin. In the dorsal region of male eyes, R3–8 exclusively express the B1 opsin mRNA, while in females the R3–8 coexpress the B1 and L opsin mRNAs (Sison-Mangus et al. 2006). The latter photoreceptors most likely therefore have an extremely broad spectral sensitivity, as in the *Papilio* BB receptors that coexpress the green-absorbing PxL2 and red-absorbing PxL3 (Fig. 5.4).

5.10 Dipterans: *Drosophila* and Other Flies

A considerable part of our present understanding of insect vision has been gained by research on the visual systems of flies, that is, the higher Diptera. Specifically the fruitfly, *Drosophila melanogaster*, has played a key role in the unraveling of the molecular properties of insect visual pigments, because this is one of the most important model organisms in biology and most of the modern genetic and molecular tools are available in this species (Fig. 5.7). The compound eye of *Drosophila* consists of about 800 ommatidia, each containing eight photoreceptors, R1–8. The rhabdomeres of R1–6 are spatially separate and surround the rhabdomeres from R7 and R8, which are positioned in tandem (Fig. 5.7a). The crystalline anatomy of fly eyes has been useful for unraveling retinal properties, but this has been supported and extended by the existence of many relevant gene mutations.

The complete set of six visual pigments of *Drosophila* (Rh1–Rh6) was identified by measuring difference spectra of retinal extracts in two extreme photosteady states (Salcedo et al. 1999) (Fig. 5.7b). An analysis of the difference spectra with visual pigment template formulae (Stavenga et al. 1993) yielded the rhodopsin and metarhodopsin spectra for each visual pigment type. The derived peak wavelengths are given in Fig. 5.7b; e.g. the rhodopsin of Rh1, R486, absorbs maximally at 486 nm and its metarhodopsin, M566, at 566 nm. The amplitude of the absorbance coefficient of the metarhodopsins relative to that of their rhodopsin varies between 1.4 and 1.7.

The blue–green absorbing Rh1 visual pigment is present in all R1–6 photoreceptors. The blue-absorbing Rh2 was found to be the visual pigment of the ocelli. The exclusively UV-absorbing Rh3 occurs in the R7 photoreceptors of 30 % of the ommatidia, randomly distributed throughout the eye. These ommatidia are called p-type, based on their similarity to the UV-absorbing rhodopsin in the R7 of the p-type ommatidia of larger flies (Hardie 1985). Rh4, which also absorbs UV, is found in the complementary y-type ommatidia, which makes up the remaining 70 % of R7 photoreceptors. The blue-absorbing Rh5 opsin is expressed in all R8s of the p-type

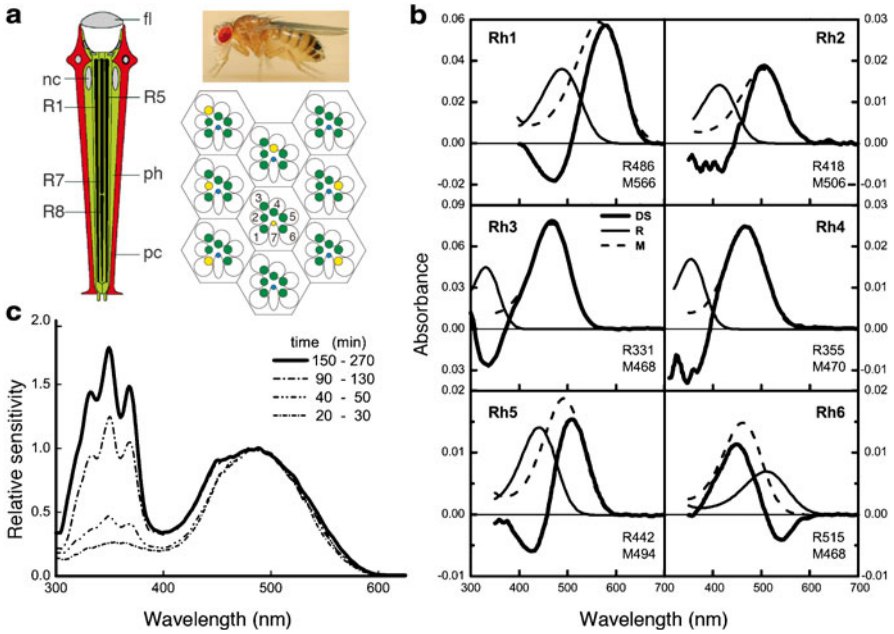


Fig. 5.7 *Drosophila* and blowfly. (**a**) The wild type fruitfly eye is red due to pigments in the cells that surround the pseudocone, the structure proximal to the facet lens (fl). The red pigment optically isolates the ommatidia from each other. Each fly ommatidium contains eight photoreceptor cells, R1–8. The six large, outer or peripheral photoreceptors, R1–6, have long and fat rhabdomeres, and the two slender, inner or central photoreceptors, R7 and R8, have thinner rhabdomeres, arranged in tandem, with R7 distal and R8 proximal. The photoreceptor cells (ph), with distal nucleus (nc), are surrounded by screening pigment cells (pc). The cross-section diagram shows that the rhabdomeres of R1–6 (green) have a characteristic trapezoidal pattern, with central the R7,8 rhabdomeres. The light-colored rhabdomeres mark photoreceptors that share the same visual direction and that project their axons onto the same higher order neurons. (**b**) Spectral characteristics of the visual pigments Rh1–Rh6 of the fruitfly *Drosophila*. Difference spectra (DS) were measured from eye extracts and fitted with calculated absorbance spectra of visual pigments using template formulae. The peak wavelengths (in nm) of the concluded rhodopsin (R) and metarhodopsin (M) spectra are indicated by the numbers (modified from Salcedo et al. 1999). (**c**) Incorporation of a sensitizing pigment in the photoreceptors of vitamin-A-deprived blowflies, resulting in an enhanced sensitivity in the UV with respect to that in the blue–green. The enhancement is due to binding of a 3-hydroxy-retinol to rhodopsin. UV-light absorbed by the 3-hydroxy-retinol then results in transfer of energy to the chromophore of rhodopsin, 3-hydroxy-retinal. The fine structure emerging in the UV is interpreted to be caused by a rigid binding of the 3-hydroxy-retinol. The spectra were measured at the indicated time after supplying all-*trans* retinal to the eye (modified from Hamdorf et al. 1992)

ommatidia; the green-absorbing Rh6 opsin is expressed in the R8s of the y-type ommatidia. The 70 % y-type ommatidia are non-homogeneous, with 60 % expressing only Rh4, while 10 % coexpress Rh3 and Rh4; the latter localize in the dorsal third of the *Drosophila* eye (Mazzoni et al. 2008). As the double expression of the UV rhodopsins occurs in the R7s in the dorsal third of the compound eye, Mazzoni et al. (2008) hypothesized that these R7 photoreceptors, together with the underlying

R8s, function in analyzing the UV light in the sky, specifically to detect differences in the solar and non-solar parts of the sky—that is, sky near the sun and away from it—which can differ considerably in short-wavelength light content. This skylight-discriminating ability may serve to help the fly orient for navigational purposes.

The absorption spectrum of visual pigments generally consist of different absorption bands that are called α, β, γ, etc. (Stavenga and van Barneveld 1975). Figure 5.7b shows the α-bands of the fruitfly visual pigment states, rhodopsin and metarhodopsin, but the β-bands were not determined. For all visual pigments studied in extracts, the amplitude of the β-band is much smaller than the amplitude of the α-band and restricted to the UV wavelength range. Surprisingly, early electrophysiological studies of R1–6 fly photoreceptors commonly yielded spectral sensitivities in the UV that were as large as the sensitivity at around 500 nm, the presumed α-band range (Hardie 1979). Extensive research by Kirschfeld and co-workers has revealed that the high UV-sensitivity is created by a 3-hydroxy-retinol molecule that is additionally bound to the opsin and acts as a sensitizing pigment (Kirschfeld et al. 1977). The 3-hydroxy-retinol absorbs UV light and transfers the energy to the native chromophore, 11-*cis* 3-hydroxy-retinal, resulting in activation of the rhodopsin molecule. The multiple peaks in the UV wavelength region in the spectral sensitivity (Fig. 5.7c) are attributed to the absorption spectrum of the 3-hydroxy-retinol molecule.

The action of the sensitizing pigment was directly demonstrated in a series of experiments in the blowfly *Calliphora vicina* reared on different vitamin-A diets (Hamdorf et al. 1992). Photoreceptors of blowflies reared on a vitamin-A-deprived diet have a low absolute light sensitivity, due to the necessity of vitamin A for producing rhodopsin. Supplying retinoids results in an increased visual pigment concentration, as witnessed by an increased sensitivity. Figure 5.7c presents the sensitivity spectra, normalized to the sensitivity peak of the α-band, measured after application of all-*trans*-retinal to the eye. The initial spectral sensitivity closely resembles a classical rhodopsin spectrum, with a low sensitivity band in the UV. Within a few hours this band had risen considerably and then featured a prominent vibronic fine structure, with peaks at 333, 350 and 369 nm. These peaks prove the presence of 3-hydroxy-retinol. Evidently, this derivative was enzymatically produced from the administered all-*trans*-retinal.

5.10.1 Spectral Characteristics of Insect Visual Pigments

Figure 5.7b shows that in addition to the difference in absorbance, the peak wavelengths of the two photostable visual pigment states often differ considerably. Interestingly, the absorption peak wavelengths of rhodopsin and metarhodopsin have characteristic relationships for the visual pigments of *Drosophila* as well as other insects (Fig. 5.8). The peak shift of the S and M (UV and B) visual pigments is always bathochromic, while for the L (G) visual pigments the peak shift is hypsochromic. The spectral shift for the S visual pigment is positive and generally large (about 130 nm). For the M-opsins, the shift is much smaller (50–80 nm), whereas

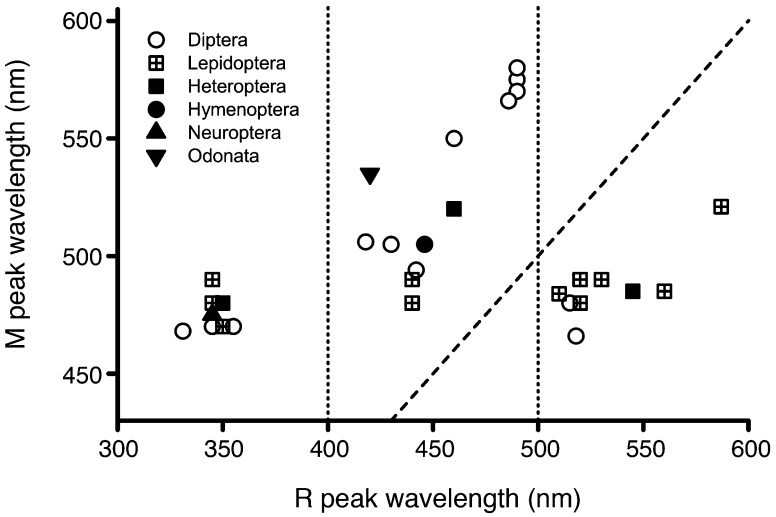


Fig. 5.8 The absorption peak wavelength of metarhodopsin as a function of the absorption peak wavelength of its rhodopsin for a number of insect orders. The three visual pigment types, UV-, blue, and long wavelength-absorbing are roughly separated by the *dotted vertical lines*. At the oblique *dashed line* the peak wavelengths of rhodopsin and metarhodopsin are identical. The S-(UV-) and M-(blue) rhodopsins have bathochromic-shifted metarhodopsins, but the L-(long-wavelength-absorbing) rhodopsins have a hypsochromic-shifted metarhodopsin

the spectral shift for the L-opsins is negative, ~40 nm. No interpretation in molecular terms has yet been formulated, but the differences in spectral shift have important consequences for the regeneration processes of the visual pigments in the eyes of different insect species (Stavenga 1992).

An important consequence of the bistability of insect visual pigments is that under bright daylight conditions, every visual pigment molecule of an insect eye regularly shuttles back and forth from the rhodopsin to the metarhodopsin state. When unprotected by a light-controlling pupil mechanism, the conversion rates in fly photoreceptors in natural light conditions are of the order of seconds (Stavenga and Hardie 2011), but with a closed pupil each visual pigment molecules will flip-flop about once in every minute.

Fly eyes contain, like butterflies, red screening pigments, but their location and function is very different. The red screening pigments are not located in the photoreceptors, but in separate screening pigment cells (Fig. 5.7a). These cells surround the photoreceptors and thus protect them for activation by off-axis stray light. Yet, with incident broad-band white light, the high transmittance of the screening pigment cells in the longer wavelength range results in a considerable amount of red stray light, which evades the photoreceptor layer. This is unimportant for the R486 pigment that is concentrated in the rhabdomeres of the R1–6 photoreceptors, because it predominately absorbs only at rather short wavelengths. However its metarhodopsin form, M566, readily absorbs red light, and thus the red stray light

can favorably convert the metarhodopsin into its rhodopsin state. The function of the red-transmittant screening pigment cells is thus to support photoregeneration of the visual pigment (Stavenga and Hardie 2011).

This elegant method of visual pigment regeneration does not work for green-absorbing rhodopsins, because red stray light will be preferably absorbed by the rhodopsin molecules where it will cause unwanted background noise. Most insect eyes therefore have strongly absorbing, brown–black screening pigments because the majority of their photoreceptors rely on green-absorbing rhodopsins, as shown above for the cases of bees and butterflies (see Figs. 5.5b and 5.6b). The regeneration of rhodopsin from metarhodopsin then must occur through a complicated, enzymatic renewal cycle, involving the degradation of metarhodopsin and renewal of rhodopsin, requiring numerous cellular components of the retina (Schwemer 1984, 1989; Smith and Goldsmith 1991; Wang et al. 2010). The speed of decay is faster than that of the regeneration, and both strongly depend on temperature (Bernard 1983). Under bright light conditions, the green-absorbing visual pigments are "bleached," that is, their concentration is reduced. Interestingly, this expands the intensity range where the photoreceptors can function, just as occurs with human cone photoreceptors (Stavenga and Hardie 2011).

5.11 Concluding Remark

Insects are particularly interesting for studying the evolution of visual pigments because of their phylogenetic variety, different lifestyles (diurnal vs. nocturnal) as well as the variety of compound eye structures (apposition vs. superposition). Here, we focused on the mechanisms underlying the spectral sensitivities of insect photoreceptors. The main player is of course the visual pigment. In order to produce photoreceptors of different spectral sensitivities, duplication of opsin genes is therefore the most straightforward mechanism. In fact, opsin duplication appears to happen repeatedly in a variety of animal lineages. Duplicated visual pigments are sometimes coexpressed in single photoreceptors, making their spectral sensitivities abnormally broad when the absorption spectra of visual pigments have diverged.

In addition to the visual pigments, various other photostable pigments act as spectral filters that significantly modify the absorption spectra of the visual pigments in situ, and thus enact a crucial function for the final shaping of the spectral sensitivities. The photostable pigments include the sensitizing pigment and the perirhabdomal and intrarhabdomal (fluorescent) filter pigments. The sensitizing pigment is found only in higher flies, but filter pigments are widely encountered among various insects, including butterflies and hymenopterans, and even can produce sexual dimorphism in the spectral sensitivities of the photoreceptors.

Information about spectral tuning at the level of opsin molecules is rather sparse in insects. This is because any stable technique for expressing insect visual pigments in vitro is lacking. Some pigments in honeybees (Terakita et al. 2008), the small white butterfly (Wakakuwa et al. 2010), and the Adanson jumping spider (Nagata et al. 2012) have been expressed in human embryonic kidney (HEK) cells.

However these examples are exceptional, and it has been still quite difficult to express and reconstruct insect rhodopsins in vitro. Overcoming this technical barrier will considerably stimulate the study of insect rhodopsins and will possibly uncover the evolutionary background of their amazing adaptation to a variety of light environments.

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Chapter 6
The Evolution of Photoreceptors and Visual Photopigments in Vertebrates

David M. Hunt and Shaun P. Collin

Abstract All classical vertebrate retinal photoreceptors have an evolutionary origin from ciliary cells and retain a modified cilium that links the inner and outer segments. Vertebrate visual pigments fall into five classes, a rod pigment that is found in rod photoreceptors that are responsible for dim-light vision and four spectral classes of cone photoreceptors that are responsible for vision in daylight and provide for the sensation of color if two or more classes of cone are present. The underlying mechanisms that determine the overall sensitivity of individual photoreceptors to different light levels and hence whether they are functional in dim or bright light however extend beyond the type of visual pigment present into the processes of phototransduction. The kinetics of phototransduction is determined by the different rod and cone isoforms that comprise many of its component processes, combined with the concentration of certain key components. The spectral tuning of visual pigment is determined by the type of chromophore present and its interaction with the opsin protein. The amino acid present at key sites within the opsin protein determines spectral tuning; these sites are limited in number and tend to be shared across different pigment classes. Other changes include the protonation of the Schiff base for short wavelength-sensitive type 1 (SWS1) pigments and the binding of chloride ions for long wavelength-sensitive (LWS) pigments. Opsin gene loss and/or duplication is common across the different vertebrate taxa, with certain classes of cone opsins absent in certain lineages. These changes can be related in many cases to differential expression during development or to evolutionary changes in lifestyle and the light environment.

Keywords Visual opsins • Spectral tuning • Phototransduction • Photoreceptors • Retina • Color vision • Ultraviolet sensitivity

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6.1 Origin of Retinal Photoreceptors

Sensitivity to light is a key requirement for survival in most animal species and this is achieved by the presence of photoreceptors that contain light-sensitive photopigments. Photoreceptors can be divided into two distinct types depending on their evolutionary origin: rhabdomeric that derive from apical villi, and ciliary that possess membrane systems that extend from a modified cilium with a 9 + 0 microtubule organization (Lamb et al. 2007). Rhabdomeric photoreceptors undergo depolarization in response to light, whereas ciliary photoreceptors hyperpolarize. Both rhabdomeric and ciliary photoreceptors co-exist in a variety of organisms that include the chordate *Amphioxus* sp. (Lacalli 2004) and the polychaete worm *Platynereis* sp. (Arendt et al. 2004). Moreover, they may also co-exist in vertebrates where, based on the type of photopigment and G-protein they contain, it has been proposed that vertebrate ganglion cells may be modified rhabdomeric photoreceptors (Arendt 2003; Arendt et al. 2004; Arendt and Wittbrodt 2001). However, the two distinct classes of vertebrate photoreceptors, the highly sensitive rods that are responsible for scotopic or dim light vision, and the less sensitive cones that are responsible for vision at high light levels such as daylight, that form a duplex retina are both ciliary in origin. They comprise an outer segment that contains the photosensitive visual pigments and other key components involved in the phototransduction cascade, a connecting cilium, an inner segment containing mitochondria, ribosomes and membranes and a cell body containing the nucleus. Rod photoreceptors generally have a rod-like or cylindrical outer segment, whereas the outer segments of cones are generally shorter, tapered and cone-like in shape. Outer segments comprise numerous membranous disks in which the visual pigments are embedded; in rods, there is a limiting (plasma) membrane surrounding the disks but in cones, the disk membranes remain continuous. For both photoreceptor types, a modified cilium is retained at the base of the outer segment.

Cones are also responsible for high acuity vision and color vision. High acuity vision depends on high densities of cones in certain regions of the retina. For example, in humans, high acuity central vision is achieved by a peak cone density at the fovea of around 100,000–200,000 cones/mm^2 (Curcio et al. 1990), increasing to over a million cones/mm^2 in the fovea of some birds of prey. Cones also provide a mechanism for the sensation of color, which derives from sampling light at different wavelengths by spectrally distinct cone types.

The light-sensitive molecule present in all photoreceptor classes comprises an opsin protein with a light-sensitive chromophore attached via a Schiff base (SB) linkage to a lysine residue. In vertebrates, the chromophore is derived either from vitamin A_1 or vitamin A_2 to give rhodopsin and porphyropsin pigments, respectively. When the vitamin A_1-derived chromophore (retinal) is present, the peak sensitivities (λ_{max}) of the four cone pigments range from 360 nm (ultraviolet, UV) to 450 nm (violet) for the short wavelength-sensitive type 1 (SWS1) class, through 400–470 nm for the short wavelength-sensitive type 2 (SWS2) class, 480–530 nm for the middle wavelength-sensitive (MWS or RH2) class, to a maximum of around

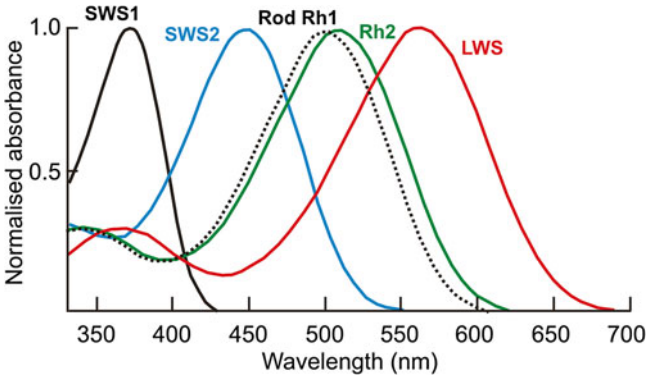


Fig. 6.1 Example absorbance spectra for the single rod (Rh1) and four cone classes of visual pigment; short wavelength-sensitive type 1 (SWS1), short wavelength-sensitive type 2 (SWS2), middle wavelength-sensitive (MWS or Rh2) and long wavelength-sensitive (LWS). An ultraviolet-sensitive (UVS) SWS1 pigment is depicted

570 nm for the longwave-sensitive (LWS) class (Fig. 6.1). In contrast, there is generally just a single rod or RH1 pigment with a λ_{max} value around 500 nm. The RH1 opsin gene most likely arose from a duplication of the RH2 cone opsin gene with which it shows the closest sequence similarity (Okano et al. 1992). With the vitamin A_2-derived chromophore (3,4-didehydroretinal), the λ_{max} values for both cone and rod pigments are pushed to longer wavelengths, especially for the LWS cone pigments, which can have a peak sensitivity of >600 nm. With the important exception of the UV-sensitive (UVS) SWS1 pigments, all vertebrate visual pigments have an SB that is protonated in the resting state, with a negatively charged residue at site 113 (bovine rod opsin numbering), usually glutamate, acting as a counterion to stabilize the proton of the SB.

6.2 Visual Pigment Classes

The vertebrate radiation split into two distinct lineages around 500 mya: the jawless agnathans and the jawed gnathostomes (Hardisty 1982) (Fig. 6.2). Lampreys and hagfishes are the sole survivors of the Agnatha and therefore provide a window into the visual system of the last common ancestors prior to the split. The eyes of adult hagfishes lie under an opaque patch of skin and the retina is relatively undifferentiated (Fernholm and Holmberg 1975; Lockett and Jorgensen 1998). In contrast, the eyes of adult lampreys are prominent and contain a well-differentiated retina (Dickson and Collard 1979; Collin et al. 1999; Lockett and Jorgensen 1998; Rubinson 1990; Rubinson and Cain 1989), with up to five morphologically distinct cone-like classes of photoreceptors in the retina of the southern hemisphere lamprey, *Geotria australis* (Collin et al. 2003a, 2009; Govardovskii and Lychakov

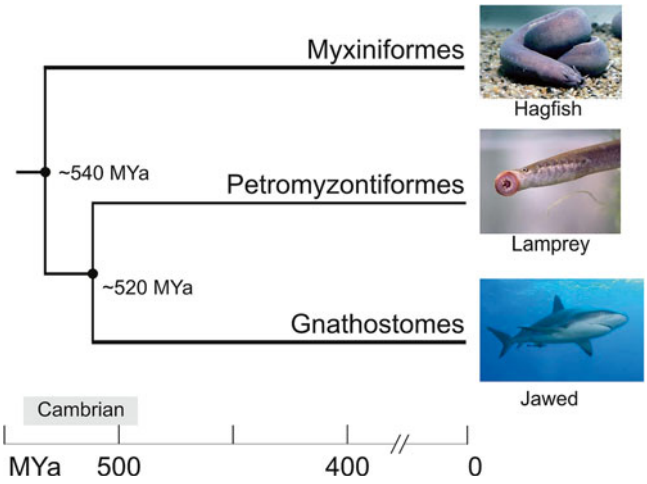


Fig. 6.2 The evolution of jawed vertebrates is illustrated against an approximate time-scale of millions of years ago (mya). Jawless craniates (agnathans) were present in the early Cambrian, by 525 mya, and a time of 530 mya has been indicated for their presumed first appearance. Numerous lines of jawless fish evolved between 500 and 430 mya ago, but none have survived to the present day. The first jawed (gnathostomes) vertebrates arose around 430 mya, and this line is represented today by cartilagenous fishes, bony fishes and tetrapods. Redrawn from Lamb et al. (2007)

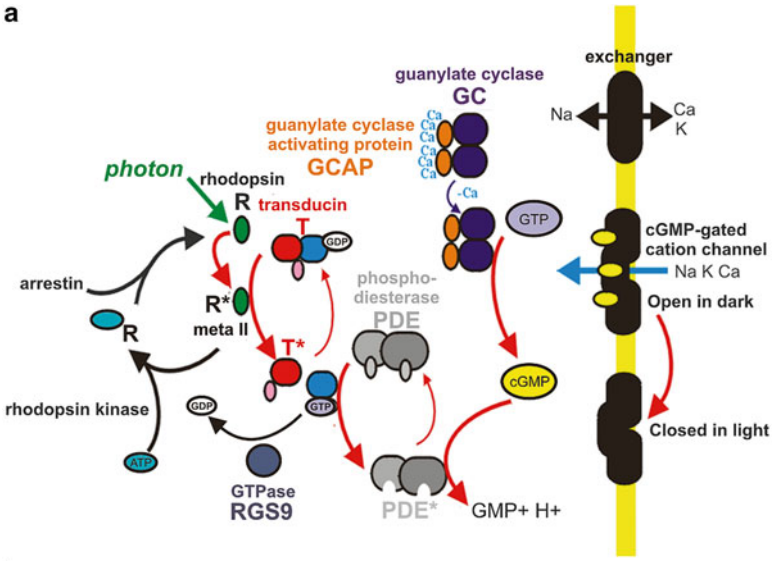
1984; Collin and Trezise 2004). *G. australis* also expresses five different visual pigments that correspond to the SWS1, SWS2 and LWS cone classes found in the jawed gnathostomes, plus two forms of an *RH*-like opsin (named *RHA* and *RHB*) (Collin et al. 2003b; Davies et al. 2007b). The *RHA* and *RHB* pigments are spectrally very similar with peak absorbances at 492 nm and 497 nm, respectively, and show a similar pattern of opsin expression at different developmental stages of the protracted lifecycle, i.e. downstream and upstream migrant phases (Davies et al. 2007b). Phylogenetic analysis of these two pigments initially identified them both as cone-like (Collin et al. 2003b; Collin and Trezise 2004), with the origin of the *RH* gene in the lamprey being quite separate to the duplication event that gave rise to the cone *RH2* and the "true" rod *RH1* gene lineages in the gnathostome lineage (Collin et al. 2003b). True rod-based scotopic vision may not therefore be present in the Agnatha. More recent analyses using a larger cohort of opsin sequences and different phylogenetic algorithms have suggested, however, that *RHA* and *RHB* genes may be the forerunners of the *RH1* and *RH2* gene classes of the jawed vertebrates, respectively (Pisani et al. 2006; Davies et al. 2009a). Therefore, it may be that dim-light vision did arise prior to the separation of the agnathan and gnathosome lineages (Pisani et al. 2006), although the presence of an *RH1* ortholog is not, in itself, sufficient evidence for the presence of true dim-light photoreception. As discussed later (see Sect. 6.3), rod and cone photoreceptors of jawed vertebrates express, respectively, rod- and cone-specific isoforms of many of the components of phototransduction and these contribute to the overall kinetics of the photoresponse in these two photoreceptor types. It is presently unknown whether such isoforms are also present in the lamprey retina.

The presence of the orthologs of the four cone pigment classes found in the gnathostomes in *G. australis* implies that the blueprint for vertebrate color vision was first set down before the agnathan/gnathostome split, more than 540 mya (Shu et al. 2003; Xian-guang et al. 2002). Therefore, depending on the functional classification of the RHA photoreceptor in *G. australis*, this species (and by extrapolation the ancestral lamprey) possesses the potential for either tetra- or pentachromatic vision; if pentachromacy is present it would be unique amongst vertebrates (Collin et al. 2003b).

6.3 Phototransduction

Scotopic and photopic vision arises in gnathostomes from differences in the sensitivity of rods and cones to light. In general, rod photoreceptors are more sensitive than cones, respond with slower kinetics, have less "dark noise," and adapt over a much narrower range of light intensities (Ebrey and Koutalos 2001; Miller et al. 1994; Pugh et al. 1999; Yau 1994). In cones, spontaneous thermal isomerizations of the chromophore are much more frequent (Baylor et al. 1980), with a substantial proportion of opsin lacking bound chromophore. This apo-pigment is able to activate phototransduction and may explain, in part, why cones show a faster and larger response than rods (Kefalov et al. 2005; Travis 2005). Adaptation to a dim light environment may also involve key amino acid substitutions in the opsin protein. The residues present at two sites, 122 and 189, have been implicated in distinguishing between rod and cone photopigments (Kuwayama et al. 2002, 2005; Imai et al. 1997); rod opsins generally have Glu122 and Ile189, and site-directed mutagenesis has shown that these residues contribute toward the increased thermal stability, slower decay of photointermediates and slower regeneration of the pigment in the dark (Kuwayama et al. 2005).

The presence of a particular class of opsin however makes only a minor contribution to the determination of the kinetics of the photoresponse. This is exemplified by the photoreceptors of the nocturnal Tokay gecko, *Gecko gecko*, that have rod-like morphology (Underwood 1951) and rod-like photokinetics (Kleinschmidt and Dowling 1975), yet contain only cone opsins (Kojima et al. 1992). The fundamental differences between rods and cones with respect to sensitivity to light are not therefore simply dependent on whether a rod or cone visual pigment is present. The proteins involved in phototransduction have now been largely identified (Fig. 6.3) and a striking feature is the number of rod and cone-specific isoforms that underlie many of the component processes. Absorption of light causes the isomerization of the chromophore, 11-*cis*-retinal, to the all-*trans* form in a photobleaching sequence with consequent conformational changes in the opsin protein, leading to the activation of the G protein transducin by the activated form of the visual pigment, metarhodopsin II (meta II). Meta II activates the heterotrimeric GTP-binding protein transducin, which is composed of α, β and γ subunits. GDP bound to transducin is replaced by GTP and the GTP-α-subunit conjugate dissociates from the βγ component. Different isoforms for all three of these subunits are present in rods and cones. Phosphodiesterase (PDE) in rods is composed of catalytic α and β subunits



b

Gene	Subunit	Rod	Cone
Visual pigment		RH1 opsin	SWS1, SWS2, RH2, LWS
Transducin	Alpha Beta Gamma	GNAT1 GNB1 GNGT1	GNAT2 GNB3 GNGT2
Phosphodiesterase	Catalytic Inhibitory	PDE6A, PDE6B PDE6G	PDE6C PDE6H
Guanylate cyclase activating protein		GCAP1 GCAP2	GCAP1 GCAP2
Cyclic nucleotide gated channel	Alpha Beta	CNGA1 CNGB1	CNGA3 CNGB3
Na-K/Ca exchanger		NCKX1	NCKX2
Rhodopsin kinase		GRK1	GRK7
Arrestin		SAG	ARR3

Fig. 6.3 Phototransduction cascade. (**a**) Schematic diagram showing role of component processes in activation and inactivation of the cascade. (**b**) Component process that are encoded by different genes in rods and cones

and two inhibitory γ subunits, whereas the cone form is composed of two identical α′ subunits and two inhibitory γ subunits. Activation of PDE involves the interaction with GTP-α-transducin and the dissociation of the inhibitory γ subunits. This activation results in the breakdown of cGMP and the closing of the cGMP-gated (CNG) channels, which also differ between rod and cones, leading to reduced levels of intracellular Ca^{2+}. The rod β subunit of the CNG channel protein includes a N-terminal region rich in Gly and Arg residues (GARP) that is thought to interact with another photoreceptor protein, peripherin/rds (Poetsch et al. 2001), in the morphogenesis and maintenance of rod disk outer segments (Vos et al. 2010).

Rod and cone channels are modulated by Ca^{2+}-calmodulin (Weitz et al. 1998; Peng et al. 2003); cone channels are generally more permeable to Ca^{2+} than rod channels, and this may underlie, in part, the more rapid and larger light-dependent changes in Ca^{2+} concentration in cone cells (Frings et al. 1995).

The restoration of the cGMP and Ca^{2+} resting states is achieved by the activation of retinal-specific guanylate cyclases (GCs) by the Ca^{2+}-binding guanylate cyclase-activating proteins (GCAPs) (Hunt et al. 2009a). At the low levels of Ca^{2+} that exist after light exposure and the closure of the CNG channels, GCAPs activate the cyclase function of GC and thereby the production of cGMP. As Ca^{2+} (and cGMP) levels rise, binding of Ca^{2+} to GCAP via EF hands results in the progressive inhibition of cyclase activity. Two isoforms of GC have been identified; GC1 is clearly the more important enzyme for phototransduction in mammals since null mutations cause the severe blinding disease of Leber Congenital Amaurosis in humans (Perrault et al. 1996) and altered photoreceptor survival in gene knock-out mutant mice (Yang et al. 1999). It is present in both rods and cones, although the level of expression is higher in the latter (Dizhoor et al. 1994). Multiple isoforms of retinal GCAPs have also been identified, with up to eight forms in the zebrafish retina (Imanishi et al. 2004), although only three forms are found in mammals (Dizhoor et al. 1995; Haeseleer et al. 1999; Palczewski et al. 1994). GCAP1 and GCAP2 appear to be localized to rods or cones in a species-specific manner, with GCAP3 expressed in all cone types (Imanishi et al. 2002). In the zebrafish retina, the expression of three of the newly identified forms is restricted to cone photoreceptors. Activation differences between different GC/GCAP combinations are largely unknown.

The inactivation of the phototransduction cascade is necessary for recovery from a photoresponse. The deactivation of meta II occurs as a two-step process, involving phosphorylation targeted to serine residues in the carboxy terminus of the opsin protein by two retinal-specific rhodopsin kinases, GRK1 (Palczewski et al. 1993; Zhao et al. 1997) and GRK7 (Hisatomi et al. 1998b; Weiss et al. 1998), followed by binding of the inhibitory protein arrestin (Brannock et al. 1999). These two kinases appear to be expressed in the rods and cones of mammals in a species-specific manner (Weiss et al. 2001). GRK7, which is preferentially present in human cones, shows a higher efficiency, leading to a decreased lifetime of activated cone meta II (Tachibanaki et al. 2001). Some modulation by Ca^{2+} via recoverin is also present (Chen 2002), providing a link with the fluctuating Ca^{2+} levels present in photoreceptors that arise from the sequential closing and opening of the cGMP-gated ion channels in the final step of phototransduction. Recoverin is a member of the neuronal Ca^{2+} sensor family of proteins that regulates the activity of GRK1 by forming a complex with rhodopsin to prevent phosphorylation (Komolov et al. 2009; Zernii et al. 2011). In doing so, it acts to modulate the rate of kinase-dependent rhodopsin inactivation by light (Chen et al. 2010a). It also acts along with GRK1 to modulate the rate of decay of the light-activated phosphodiesterase (PDE) and thereby increases the temporal resolution of rods during light adaptation (Chen et al. 2010b, 2012). Phosducin, a small protein found abundantly in photoreceptors (Schulz et al. 1996; Lee et al. 1987, 1988), regulates the expression of transducin βγ subunits in order to maintain normal levels but does not contribute to adaptation (Krispel et al. 2007). Inactivation of meta II is completed by the binding of arrestin to the

phosphorylated opsin; two isoforms of arrestin are present, S-antigen in rods, and C- or X-arrestin in cones (Craft and Whitmore 1995).

An integral part of the deactivation of the photoresponse is the inactivation of the GTP-α-subunit. This requires GTPase activity that is provided by a complex of proteins that consists of RGS9 (Regulator of G-protein Signaling 9), R9AP (RGS9 anchoring protein) and Gβ5L, the long splice variant of the type 5 G-protein β-subunit (Cowan et al. 1998; Hu and Wensel 2004; Makino et al. 1999b). Confirmation of the role of these proteins in phototransduction comes from studies with RGS9–/– or R9AP–/– knock-out mice; rod and cone photoreceptors both show normal responses to flashes of light but recover much more slowly than normal photoreceptors (Chen et al. 2000; Hermann et al. 2011; Lyubarsky et al. 2001). The hydrolysis of GTP by the GTP-ase activity of the RGS9 complex is the rate-limiting step in the deactivation of phototransduction (Burns and Pugh 2009) and the role of this complex in determining the differential sensitivities of rods and cones has been clearly demonstrated by Tachibanaki et al. (2012) in a study of the rate of GTP hydrolysis in the carp retina. Transducin inactivation was around 25 times higher in cones than in rods and this correlated with an approximate 20-fold higher concentration of RGS9 in cones than in rods. In the Tokay gecko with its rod-only retina, relatively slow recovery times are found, as expected for rod photoreceptors (Zhang et al. 2006), despite these rod photoreceptors expressing cone visual pigments and only the cone isoforms of the component processes of phototransduction. There must be, therefore, another mechanism that slows recovery and it is significant that RGS9-1 levels are lower in these photoreceptors than in mammalian cones or rods. Cones typically show a tenfold higher concentration than rods (Zhang et al. 2003), so this may explain the slower recovery of gecko rods. The higher levels of RGS9-1 and GAP in cones are likely therefore to be a major contributor to the rapid photoresponses of cones.

Phototransduction is confined to the outer segment of the photoreceptor and the proteins involved in this process are transferred from the cell body where they are synthesized, to the outer segment through the connecting cilium. This transfer is achieved either by diffusion or by active transport by the intraflagellar transport (IFT) system. The visual opsins and GC1 are examples of proteins that use IFT (Insinna and Besharse 2008; Marszalek et al. 2000; Pazour et al. 2002). These proteins only show anterograde movement but three important components of the phototransduction process, arrestin, transducin and recoverin, show movement in both directions (Fig. 6.4). Anterograde and retrograde movement of these three proteins has been the subject of a number of studies and recent results favor the hypothesis that this occurs by intracellular diffusion (summarized by Calvert et al. 2006). In the dark, rod outer segments contain very little arrestin but this is reversed by the rapid movement of arrestin into outer segments when light intensity reaches the upper limit at which rods can still signal differences in light levels (Strissel et al. 2006; Baylor et al. 1984; Nakatani et al. 1991; Krispel et al. 2003). When light levels fall, the process is reversed with the return of arrestin molecules to the inner segment. The subunits of transducin show a converse response with movement out of the outer segments at higher light levels, causing a reduction in light sensitivity and thereby allowing rods to operate under lighting conditions that would otherwise

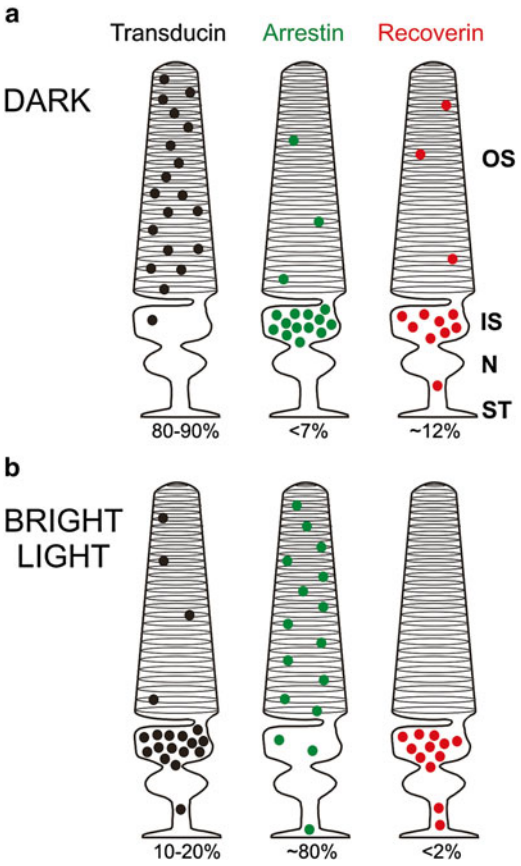


Fig. 6.4 The distribution of transducin, arrestin, and recoverin in rod photoreceptors. Dark-adapted (**a**) and light-adapted (**b**) rod photoreceptors. The *numbers* below each photoreceptor represent the percentage of the proteins found in the outer segments. *OS* outer segment, *IS* inner segment, *N* nucleus, *ST* synaptic terminal. Re-drawn from Calvert et al. (2006)

saturate the photoresponse (Sokolov et al. 2002). Movement of arrestin would also be expected to affect the visual cycle by reducing the amplitude of response or accelerating recovery as levels rise in outer segments. Finally, recoverin moves from the outer to the inner segments in response to bright light; this would serve to affect the lifetime of activated rhodopsin and hence the amplitude of response and rate of recovery. In contrast, in cones, transducin movement can be triggered when activation exceeds a critical level where the photoresponse is saturated; in the normal retina this level is never achieved so retrograde movement of transducin does not occur (Lobanova et al. 2010).

6.4 Opsin Co-expression in Photoreceptor Development

Opsin co-expression is frequently present during retinal development, arising from an SWS1 to LWS developmental switch in opsin gene expression. This results in the transient co-expression of both opsins in the cones of some mammals (Rohlich et al. 1994; Lukats et al. 2005; Szél et al. 1996), and has led to the proposal that S

cone (defined as cones expressing SWS1 opsin) development is the default pathway. Cone and rod differentiation occur in partly overlapping waves; in mammals, cone genesis starts before rod genesis, and in cones, SWS1 opsin expression precedes that of LWS opsin expression (Nasonkin et al. 2010; Swaroop et al. 2010). Co-expression is, however, quite variable between species; in the development of the rat and gerbil retina, all cones initially express the SWS1 opsin, the majority then switch to LWS opsin expression within about 2 weeks, with co-expression occurring during the transition (Szel et al. 1994). In contrast, in the human retina, most cones exclusively express their adult opsin throughout development, although there is a small fraction of cones that transiently co-express both SWS1 and LWS opsins (Xiao and Hendrickson 2000). In the tree shrew, most developing cones also express their adult opsin, with only about 5 % of the cones showing a transient co-expression of both opsins (Lukats et al. 2005). These observations imply that either opsin switching occurs very rapidly such that co-expression is generally missed, or that only a minority of L cones (defined as expressing LWS opsin) go through this transition. Interestingly, the density of cones expressing SWS1 in either the human or tree shrew retina is insufficient at any time point to encompass all future LWS cones.

The developmental control of this switch in opsin gene expression involves the thyroid hormone system. Thyroid hormone (TH) and the cone-specific TH receptor TRβ2, a nuclear transcription factor encoded by the *thrb* gene, control the relative expression of SWS1 and LWS opsins during cone development and maturation (Applebury et al. 2007; Ng et al. 2009); in mice with a deletion of *thrb*, there is a selective loss of L cones with a concomitant increase in adult S cones (Ng et al. 2001), demonstrating that the *thrb* gene is involved in directing cone development from the default S cone pathway to form L cones. Indeed, in mice with congenital hypothyroidism, all cones develop to dominantly express SWS1 opsin and largely (or even completely) repress LWS opsin expression (Lu et al. 2009; Pessoa et al. 2008). Unexpectedly, TH continues to control opsin gene expression in mature cones in the adult rodent retina (Glaschke et al. 2011). Co-expression is not, however, limited to development but occurs throughout life in many mammalian species (Fig. 6.5), which include humans, mice, guinea pigs, rabbits, European moles, subterranean mole-rats and bats (Ahnelt and Kolb 2000; Rohlich et al. 1994; Applebury et al. 2000; Xiao and Hendrickson 2000; Lukats et al. 2005; Muller et al. 2009; Szél et al. 1996; Glosmann et al. 2008; Peichl et al. 2004). In many cases, this co-expression either shows a dorso-ventral gradient or a distinct partition (Fig. 6.5). In the mouse, the dorsal retina contains single pigment LWS and SWS1 cones, with a majority of dual pigment cones found in the ventral retina (Rohlich et al. 1994; Glosmann and Ahnelt 1998; Applebury et al. 2000), whereas in the rabbit, the region of co-expression is restricted to the ventral retinal periphery (Juliusson et al. 1994; Famiglietti and Sharpe 1995; Rohlich et al. 1994). Precise demarcation of co-expression is seen in Campbell's dwarf hamster, *Phodopus campbelli*, where it is present in the ventral retina, but not in the dorsal retina where SWS1 pigment expression is totally lacking (Huber et al. 2010). Conversely, the subterranean pocket gopher, *Thomomys bottae*, shows substantial opsin co-expression in the

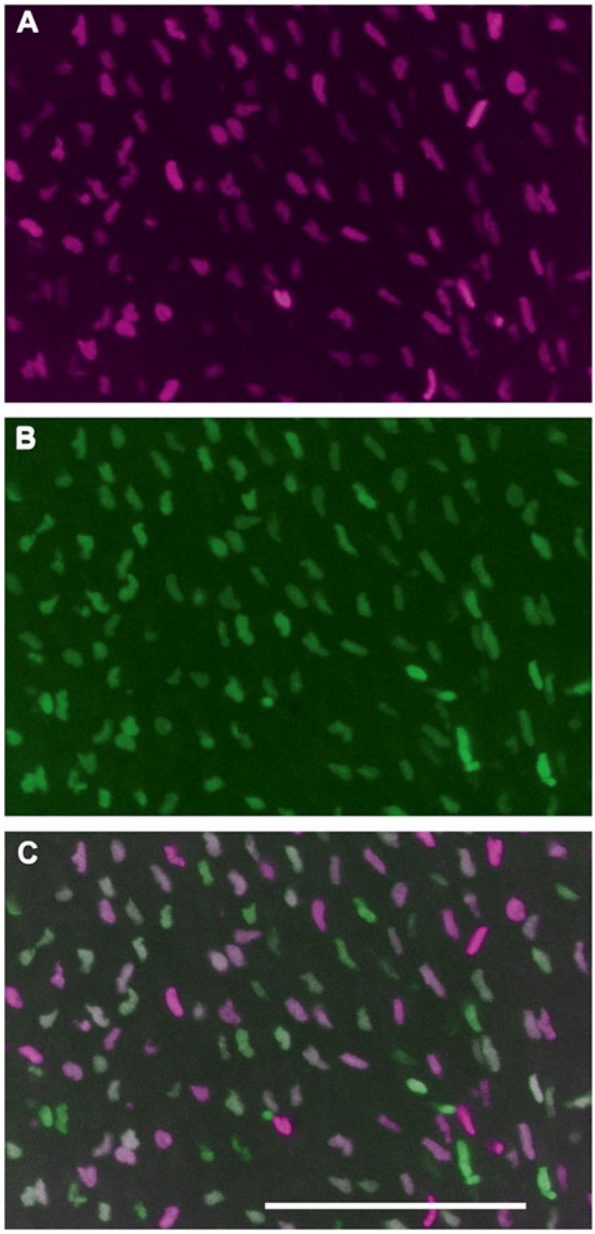


Fig. 6.5 Co-expression of SWS1 and LWS opsins in the cones of the guinea pig, *Cavia porcellus*. In mid-retina, there is a transition zone (shown here **a**–**c**) where most cones co-express SWS1 and LWS opsins. Immunofluorescence labeling for the SWS1 opsin (**a**) is *magenta* and for the LWS opsin (**b**) is *green*. In (**c**), the (**a**) and (**b**) images have been merged to show co-expression in individual cells. Scale bar, 50 **μ**m. Hunt and Peichl (2014) with permission

dorsal retina, but with very little LWS pigment in the ventral retina. Finally, in the mouse retina, there is a sparse population of S cones that do not co-express LWS opsin, which suggests that the dual pigment cones are really L cones that have failed to fully turn off SWS1 opsin expression.

6.5 Spectral Tuning of Visual Pigments

6.5.1 Amino Acid Substitutions

Visual adaptation of a species to its environment largely involves change in visual sensitivity. This can occur either at the receptor level by varying the number of spectral classes of cone, at the pigment level by altering the spectral sensitivity of individual visual pigments (a process called spectral tuning) or by combinations of both mechanisms. The peak spectral sensitivity of a particular visual pigment depends on interactions between the opsin protein and the chromophore. The crystal structure of bovine rod opsin (Palczewski et al. 2000) places the chromophore into a pocket formed by the seven transmembrane domains of the protein. It is the amino acid residues that line this pocket and thereby interact directly with the chromophore, that are mainly involved in the tuning mechanism. In many cases, the particular amino acid substitutions involved in the spectral tuning of particular pigments are unique to those pigments. There are, however, several changes that are replicated in many species and serve to identify examples of convergent evolution in the spectral tuning mechanism.

The role of amino acid substitutions in spectral tuning was first established for the LWS pigments of primates. The *LWS* gene exists in two spectral forms in Old World primates that encode the L and M variants (Ibbotson et al. 1992; Nathans et al. 1986b) with spectral peaks in humans and other Old World primates at around 563 nm and 535 nm, respectively. Differences at only three sites were found to be responsible for most of the spectral shift between these pigments (Neitz et al. 1991), i.e. at sites 180 in TM4, and 277 and 285 in TM6, with polar residues Ser, Tyr and Thr in the L form and non-polar residues Ala, Phe and Ala in the M form, respectively. Substitutions at these sites act in an essentially additive fashion to tune the spectral sensitivity of the pigment (Asenjo et al. 1994). As shown in Fig. 6.6, in each case the residue is adjacent to the chromophore. The "five-sites" rule proposed by Yokoyama and Radlwimmer (1998) includes the above sites and two additional sites at positions 197 and 308. As described in Sect. 6.5.3, sites 197 and 308 are involved in the binding of chloride ions in LWS pigments (Wang et al. 1993; Davies et al. 2009a).

6.5.2 Protonation of the Schiff Base

All visual pigments possess a Lys residue at site 296/312 (bovine rod/human LWS opsin numbering) in the seventh transmembrane domain that is covalently linked to the chromophore via a SB. The crystal structure of bovine rod opsin shows that site 113/129 (bovine rod/human LWS opsin numbering) is located in the vicinity of the SB (Palczewski et al. 2000). In vertebrate pigments with λ_{max} values >380 nm, the SB is protonated with the negatively charged residue at site 113 (usually Glu) acting as a counterion to stabilize the proton of the SB (Nathans 1990).

The loss of protonation of the SB in SWS1 pigments results in a short wavelength (SW) shift in peak sensitivity into the UV. The effect of protonation was

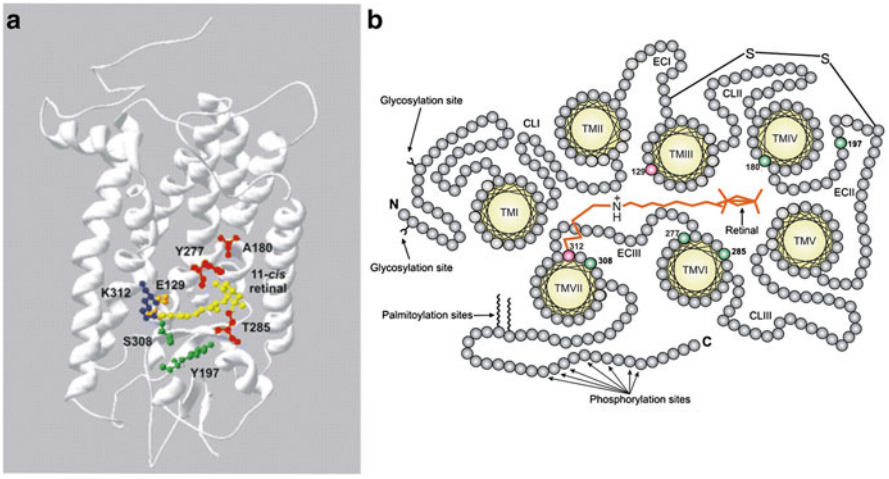


Fig. 6.6 Tuning of LWS pigments. (**a**) Putative structural model of a LWS pigment generated by homology modeling with the crystal structure of bovine rhodopsin (Okada et al. 2004; Palczewski et al. 2000) modified to show the relative position of the five key tuning sites (A180, Y197, Y277, T285 and S308). (**b**) Schematic of a LWS visual pigment, showing the arrangement of the seven transmembrane domains (*yellow*), in relation to the chromophore (*orange*). The retinal attachment site for a LWS pigment (Lys312) and counterion (Glu125) to the Schiff base are shown. The five key tuning sites cluster around either the Schiff base or ionone ring of the retinal chromophore. *TM* transmembrane, *CL* cytoplasmic loop, *EC* extracellular loop

demonstrated experimentally by Fasick et al. (2002) in a SWS1 pigment with a peak sensitivity at 424 nm. Replacement of charged Glu113 with uncharged Gln yielded a pigment with a λ_{max} that was pH-sensitive, switching from UV at 351 nm to violet at 424 nm as pH was raised. This change in spectral peak resulted from the recruitment of a chloride counterion from solution to generate a protonated pigment. In native UVS SWS1 pigments, the SB is unprotonated (Babu et al. 2001; Fasick et al. 2002; Shi et al. 2001; Mooney et al. 2012) but violet-sensitive (VS) SWS1 pigments have a protonated SB; therefore, the protonation state of SWS1 pigments is linked directly to spectral sensitivity. Within the pigment, a single amino acid substitution appears sufficient to alter the state of protonation. Ancestral SWS1 pigments are UVS (Hunt et al. 2004), but the shift to violet sensitivity has occurred many times in vertebrate evolution and, in all cases described so far, it is the replacement of Phe at site 86 (usually with the polar residues Tyr or Ser), that is responsible (Cowing et al. 2002b; Fasick et al. 2002). A feature of UVS SWS1 pigments is that their peak sensitivities fall within a narrow range from 355 to 370 nm, so other residue changes would appear to have little or no impact. In contrast, VS pigments show a much wider range from 406 nm to around 460 nm, with spectral tuning occurring over a 54 nm range.

With the possible exception of the fishes, both UVS and VS SWS1 pigments are found in all vertebrate classes. In many birds, however, UVS pigments are not ancestral but have arisen subsequent to the loss of UV-sensitivity by a Phe86Ser substitution that occurred at the base of the avian lineage (Carvalho et al. 2007). Uniquely in avian UVS pigments, the shift back to UV-sensitivity has been achieved by substitution

at site 90, where Ser is replaced by Cys (Wilkie et al. 2000). This substitution represents an alternative method of generating a UVS pigment, and presumably also results in an unprotonated SB, although this has yet to be demonstrated experimentally.

In rod pigments, protonation is stabilized by a hydrogen bond network around the SB. The residues involved in this SB network include those at sites 94, 113, 181, 186, 192, and 268, with a water molecule contributing to the complex counterion of the SB (Janz and Farrens 2004). Interestingly, only site 94 differs in SWS1 pigments, with Thr present in rod pigments replaced by Val in SWS1 pigments. There are, therefore, no differences at these sites between UVS and VS SWS1 pigments, so it would seem unlikely that these sites contribute to the mechanism that determines whether or not the SB is protonated.

6.5.3 Chloride Binding Site

An anion sensitivity, which is unique to the LWS class of cone visual pigments (Crescitelli 1977; Crescitelli and Karvaly 1991; Fager et al. 1979; Kleinschmidt and Harosi 1992; Shichida et al. 1990), arises from the interaction of chloride ions with a His residue at site 197 in the second extracellular loop of the opsin protein (Wang et al. 1993). The effect of this interaction is to long-wavelength (LW) shift the λ_{max} of the pigment by around 30 nm (Wang et al. 1993). The LWS pigments of the mouse, rat, and rabbit have a natural mutation at site 197 that replaces His with Tyr (Radlwimmer and Yokoyama 1998; Sun et al. 1997); the effect of this change is to SW shift the λ_{max} of the pigment to around 510 nm. However, only part of this shift is the result of the presence of Tyr197, the remainder being due to the replacement of Ala308 with Ser, as demonstrated by a Ser308Ala substitution in the mouse pigment, which LW shifts the peak to between 526 and 531 nm (Sun et al. 1997; Davies et al. 2012b). Ser308 is also found in the LWS pigment of the bottlenose dolphin (*Tursiops truncates*), the Pilot whale (*Globicephala melas*) and the Harbor porpoise (*Phocoena phocoena*) (Newman and Robinson 2005; Fasick et al. 1998), and even though the chloride-binding site is intact in these species, the λ_{max} of the LWS pigment is SW-shifted from a 552 to 524 nm (Fasick and Robsinson 1998). Interaction between the chloride-binding site and site 308 has also been implicated in the tuning of the LWS pigments of the elephant shark, *Callorhinchus milii*, a cartilaginous fish belonging to the subclass Holocephali. In this species, two *LWS* pigment genes are present that encode pigments with λ_{max} values at 499 nm (LWS1) and 548 nm (LWS2) (Davies et al. 2009a). Both pigments retain a chloride-binding site but LWS1 possesses Ser308, whilst the LWS2 pigment has Ala308. Based on the five-sites rule detailed above, the predicted λ_{max} for the LWS1 pigment is 521 nm, not 499 nm as experimentally determined (Davies et al. 2009a). In this pigment, however, the chloride-binding site would appear to be totally inactivated as a His181Tyr substitution did not further SW-shift the λ_{max} of the mutant pigment (Davies et al. 2009a).

The three-dimensional structure of the chloride binding site derived from crystallography studies (Okada et al. 2004; Palczewski et al. 2000) indicates that sites 197 and 308 form a triform binding pocket with the SB. When a His197/Ala308 combination

is present, a chloride ion can be accommodated but changes at either or both sites that introduce residues with larger side chains will sterically hinder access of chloride ions to the binding site (Davies et al. 2012b). In the elephant shark LWS1 pigment, Ser308 appears to completely eliminate chloride sensitivity but in the mouse LWS pigment, the intermolecular interactions would appear to be somewhat different. It has been proposed, therefore, that in the mouse, rat and rabbit pigments, a chloride ion can also act indirectly to stabilize the pigment when Ser308 sterically inhibits the binding of a chloride ion to His197, resulting in a smaller SW shift (Davies et al. 2012b).

6.6 Visual Pigment Variation in Aquatic Vertebrates

6.6.1 Agnathan Fishes

The eyes of hagfishes are buried under unpigmented skin and lack a lens, iris, cornea and ocular muscles (Locket and Jorgensen 1998). The photoreceptors in the retina have a relatively simple structure with poorly organized outer segments. In this regard, they resemble those found in the pineal organ of many non-mammalian vertebrates (Vigh et al. 1998; Ekstrom and Meissl 2003). Behaviorally, hagfishes show only a weak response to light that is not affected by the removal of its eyes so it is likely that they are responsible for non-image-forming tasks such as circadian entrainment and phototaxis, similar in function therefore to the pineal organ of jawed vertebrates (Lamb et al. 2007). In contrast, although the larval forms of lampreys are effectively blind and have eyes that are similar to those of hagfishes, the eyes of adult lampreys are well developed and, as described above, the anadromous southern hemisphere lamprey, *G. australis*, possesses five photoreceptor types, and five distinct visual pigments (Govardovskii and Lychakov 1984; Collin and Trezise 2004; Collin et al. 2003a, b) that give the potential for tetrachromatic (or even pentachromatic) color vision. The visual system of *G. australis* would appear to be well adapted therefore to the brightly lit regions of the upper water column (1–200 m) of oceanic waters that it inhabits during its marine phase (Potter et al. 1979). The retina of the Japanese river lamprey, *Lampetra japonica*, contains two distinct types of photoreceptors identified as long and short cells (Ishikawa et al. 1987); these two photoreceptor types have subsequently been shown to stain differentially with antibodies raised to rod opsin and cone visinin and may correspond therefore to the rods and cones of jawed vertebrates (Negishi et al. 1987). However, it is only in the northern hemisphere sea lamprey, *Petromyzon marinus*, that the corresponding pigments, RH1 and LWS, have been identified (Zhang and Yokoyama 1997; Davies et al. 2009b), confirming that the complement of visual pigments has been reduced to only two. In its marine phase, *P. marinus* occupies the dimly lit and spectrally restricted light environment of the deep ocean (Froese and Pauly 2009); the RH1 and LWS pigments would be expected to provide monochromatic vision at depth and in shallower waters, respectively, but it is unlikely that *P. marinus* possesses true color vision. It is possible however that limited color vision may arise from rod-cone opponency at mesopic light levels but this has yet to be investigated fully.

6.6.2 Cartilaginous Fishes

The Chondrichthyes or cartilaginous fishes reside at the base of the gnathostome lineage as the oldest, extant, jawed vertebrate group, where they share a common ancestor (dating back at least 450 mya) with all other jawed vertebrates (i.e. teleosts and tetrapods) (Sansom et al. 1996). They are divided into two lineages, the holocephalans (chimaeras) and the elasmobranchs (batoids, sharks, and skates), with the separation of these two lineages occurring about 370 mya (Cappetta et al. 1993). Evidence that all four vertebrate cone classes survived into this lineage is, so far, lacking. The elephant shark, *Callorhinchus milii*, a chimaera that inhabits the continental shelf at 200–500 m (Last and Stevens 1994), possesses only two cone opsin gene classes, *RH2* and *LWS*, but has a duplication of the *LWS* gene to give two spectrally different LWS pigments (Davies et al. 2009a). The elephant shark spawns in the more brightly lit waters of estuaries and shallow bays (6–30 m), so the presence of cones expressing LWS and RH2 pigments would provide for photopic color vision in this environment. The second copy of the *LWS* gene encodes a pigment that is spectrally tuned to shorter wavelengths. These three pigments provide the possibility for trichromatic vision if they are expressed in separate cones (Davies et al. 2009a), but this is as yet unknown.

Although skates are reported to possess a rod-only retina (Dowling and Ripps 1970; Szamier and Ripps 1983), many sharks and rays possess both cones and rods in varying proportions (Hart et al. 2004; Theiss et al. 2007). The retinae of three species of ray, *Rhinobatos typus*, *Aptychotrema rostrata*, and *Dasyatis kuhlii*, have been shown to contain three spectral classes of cones with λ_{max} values for each class between 459–477 nm, 492–502 nm and 552–561 nm, respectively (Hart et al. 2004; Theiss et al. 2007), thereby giving the potential for trichromatic color vision. In contrast, sharks appear to have just a single cone class with λ_{max} values ranging from 531 to 560 nm (Hart et al. 2011). These species are L cone monochromats and, except for the possibility of color opponency via the rods that may be present under mesopic conditions, are completely color blind (Hart et al. 2011). Rather less is known about the molecular identity of the cone opsin genes in sharks and rays, where just a single study in wobbegong sharks (*Orectolobus maculatus* and *O. ornatus*) has shown the presence of a retina-expressed *LWS* opsin gene (Theiss et al. 2012).

6.6.3 Teleost Fishes

Unlike cartilaginous fishes, the archetypal cone classes of opsins that first appeared in the agnathans have passed without deletion into the Osteichthyes (bony fishes) radiation, which began in the Cretaceous around 150 mya. Many actinopterygian or ray-finned fishes possess multiple copies of opsin genes, which is likely to have originated from a whole genome duplication event that occurred in this lineage (Amores et al. 1998; Jaillon et al. 2004) subsequent to the separation of the

ray-finned fishes from the sarcopterygian or lobe-finned fishes. The individual opsin gene duplications that follow from a whole genome duplication may have provided the basis for the evolution of additional spectrally distinct pigments, as found in many teleosts that possess multiple duplicated opsin genes in their genomes.

The full complement of opsin genes has now been determined for a number of species and, in many cases, single orthologs of the four cone opsin genes are present (Johnson et al. 1993; Shand et al. 2008; Hisatomi et al. 1997). However, with the exception of the *SWS1* gene, where there is only one report of gene duplication in the smelt, *Plecaglossus altivelis* (Minamoto and Shimizu 2005) (a member of the order Osmeriformes), duplicate copies of these genes are not uncommon in teleost fishes. The zebrafish, *Danio rerio*, a cyprinid and member of the superorder Ostariophysi, possesses a total of eight cone opsin genes (Fig. 6.7), two *LWS* genes, four *RH2* genes, but only a single *SWS2* and *SWS1* gene (Chinen et al. 2003; Takechi and Kawamura 2005). It also possesses two RH1 genes. The *LWS* genes are both syntenic with the *SWS2* gene, whereas the *RH2* genes are located in tandem gene clusters elsewhere in the genome (Chinen et al. 2003). The peak sensitivities of the LWS1 and LWS2 pigments occur at 558 nm and 548 nm, respectively, and the peak spectral sensitivities of the four RH2 pigments range from 467 to 505 nm (Chinen et al. 2003). The genes show major differences in expression, with the two *LWS* genes and three of the four *RH2* genes showing significantly lower expression levels than the *SWS1* and *SWS2* genes. For both the *LWS* and *RH2* classes, the longer wavelength subtypes are expressed later in development, where they are confined to the peripheral and ventro-nasal regions of the retina, whereas the shorter wavelength subtypes are expressed earlier and confined largely to the central region of the retina (Takechi and Kawamura 2005).

Extensive *LWS* gene duplications (Fig. 6.7) are found in the guppy, *Poecilia* spp., with four genomic copies that includes an intronless version (presumably an inserted processed sequence) identified by Ward et al. (2008), that show variation in retinal expression (Archer et al. 1987; Rennison et al. 2011). All except the processed gene are syntenic to the *SWS2* gene, with each encoding a pigment which differs at one or more of the key tuning sites, 180, 277 and 285 (Watson et al. 2011; Weadick and Chang 2007) to give predicted λ_{max} values of around 530 nm, 551–557 nm and 556–565 nm, respectively (Davies et al. 2012b). The three spectrally distinct types of

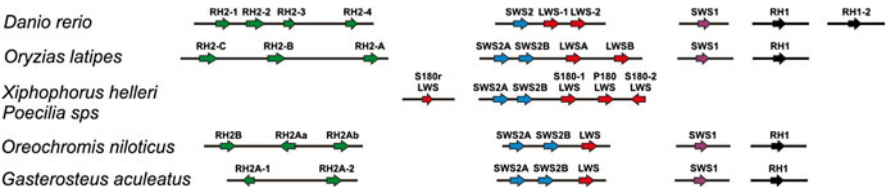


Fig. 6.7 Opsin gene duplications in teleost fishes showing syntenic relationships between classes. *Arrows* indicate direction of transcription. Modified from Rennison et al. (2011). Note that only the SWS2 and LWS genes have been fully characterized for *Xiphophorus helleri* and *Poecilia* spp. so the other genes are not shown. S180r LWS refers to a processed gene that lacks introns

L cones present in the guppy retina have sensitivity peaks at 533, 543 and 572 nm (Archer and Lythgoe 1990), indicating that the intermediate 543 nm type may express a mixture of the 530 nm variant with one or other (or both) of the two longer wavelength pigments. In the closely related species, *Xiphophorus helleri*, only two types of L cone are present with peak spectral sensitivities at 534 and 568 nm (Watson et al. 2010). Other species in which the genomic localizations for RH2, SWS2 and LWS gene duplications have been determined include the medaka, *Oryzias latipes*, the Nile tilapia, *Oreochromis niloticus*, and the three-spine stickleback, *Gasterosteus aculeatus* (Watson et al. 2011).

The complement of visual opsins has been studied extensively in cichlid fishes. Species flocks of cichlids inhabit the East African Rift Lakes with up to 500 different species thought to be endemic to Lake Malawi (Kornfield and Smith 2000). These fish are ecologically diverse and color vision clearly plays an important role, as many species are sexually dimorphic for color patterning. The four cone opsin classes are generally present (Carleton et al. 2000; Carleton and Kocher 2001), but in many cases, duplications have occurred to give additional spectral variants (reviewed in Carleton 2009). For example, in a group of four species of cichlids from Lake Malawi, three copies of the *RH2* and two copies of the *SWS2* genes are present to give a total of seven opsin genes. Individual species express only three of the seven genes, with each species selecting different combinations to give a unique overall spectral sensitivity (Parry et al. 2005). In other species, the different isoforms show differential ontogenetic expression and this may explain why multiple copies of these genes are retained (Spady et al. 2006).

Duplicate copies of the *RH1* gene are much less common and have been found so far only in the zebrafish and other species of this genus (Davies et al. 2012a; Morrow et al. 2011), in the common carp, *Cyprinus carpio* (Lim et al. 1997), in the European eel, *Anguilla anguilla* and Japanese eel, *A. japonica* (order Anguilliformes) (Cottrill et al. 2009; Hope et al. 1998; Beatty 1975; Carlisle and Denton 1959) and in the deep-sea pearl-eye, *Scopelarchus analis* (Pointer et al. 2007), a member of the order Aulopiformes.

6.6.3.1 Opsin Gene Switching

Opsin gene duplication plays an important role in the complex life cycles of European and Japanese eels as the duplicate copies of the RH1 gene encode spectrally distinct pigments that are expressed at different developmental stages (Berry et al. 1972; Tesch 1977) in habitats that exposes them to very different photic environments. Both are catadromous species that spend most of their lives in freshwater but migrate to the sea to breed. For the European eel, this occurs in the Sargasso Sea (Schmidt 1923; van Ginneken and Maes 2005) and for the Japanese eel, in an area west of the Mariana Islands in the North-West Pacific Ocean (Tsukamoto 1992), with spawning at depths of around 200 m. After hatching, the leptocephalus larvae of the European eel spend 1–2 years drifting with the Gulf Stream into the North Atlantic, travelling around 6,000 km to the European continental shelf. Here, they

metamorphose into glass eels, initially as elvers or pigmented glass eels but then as the larger yellow eels, before travelling up European rivers to spend 6–20 years in a freshwater environment, where they grow and mature as a freshwater species. Mature eels must then cross the Atlantic or Pacific Oceans to spawn. Just prior to and during this migration, they undergo a pubertal change (Rousseau et al. 2009) called "silvering" (Aroua et al. 2005), to become sexually mature adult fish. During this part of the life cycle, the photopic environment of the eel changes markedly from the yellow/brown of shallow freshwater rivers to the green of coastal waters and then to the blue of the deep ocean. During this transition, the rod photoreceptors undergo commensurate changes in spectral sensitivity from a λ_{max} at around 523 nm to around 482 nm (Fig. 6.8). This is achieved firstly by the replacement of a vitamin-based A_2 chromophore with a vitamin A_1-based form (Beatty 1975; Carlisle and Denton 1959) to switch from a porphyropsin to a rhodopsin pigment. This is then extended by a change in *RH1* gene expression, which is seen in individual rod photoreceptor outer segments as a progressive replacement of the "freshwater" form by the SW-shifted "deep-sea" form (Wood and Partridge 1993; Hope et al. 1998).

A similar switch-over in opsin expression is also seen in the retinae of Pacific salmon and trout. At hatching, all single cones express a UVS SWS1 opsin but these cones later switch to expressing a SWS2 pigment with a λ_{max} at approximately 434 nm (Cheng and Flamarique 2007) and, as for the eel rods, cones undergoing the switch-over exhibit two spectral absorbance profiles, initially with the SWS2 peak limited to the base of the outer segment (Cheng et al. 2006, 2007). In other cones, although the UV pigment becomes progressively reduced from tip to base, there is no sign of any blue absorbance so it would appear that these cones are undergoing apoptosis. Both events occur at the same time and show a ventral to dorsal progression (Cheng et al. 2006).

6.6.3.2 Adaptation to Deep Water Environments

Although many fishes occupy brightly lit environments and possess complex eyes that express a multitude of spectrally tuned visual pigments, others have colonized the more dimly lit environments of deep lakes and the deep ocean. The effect on the transmission of light through a body of water is not only an attenuation of intensity with a maximum limit for vision at about 1,000 m in the clearest tropical oceans (Jerlov 1976; Denton 1990), but also a change in spectral composition, with maximum penetration occurring in the blue region of the spectrum around 480 nm. In deep water, the ambient light is, therefore, composed of dim blue downwelling sunlight (Marshall 1979), but below around 1,000 m, vision depends on the frequency and intensity of bioluminescence (Herring 1983; Nicol 1969).

In many species occupying the deep ocean, cone photoreceptors have been completely lost and hence all sensation of color (Partridge et al. 1988, 1989). Other adaptations that serve to increase sensitivity in a dim-light environment include large rod outer segments, multiple banks of photoreceptors, a reflective tapetum, the formation of bundled groups of rods and the summation of multiple rods on to

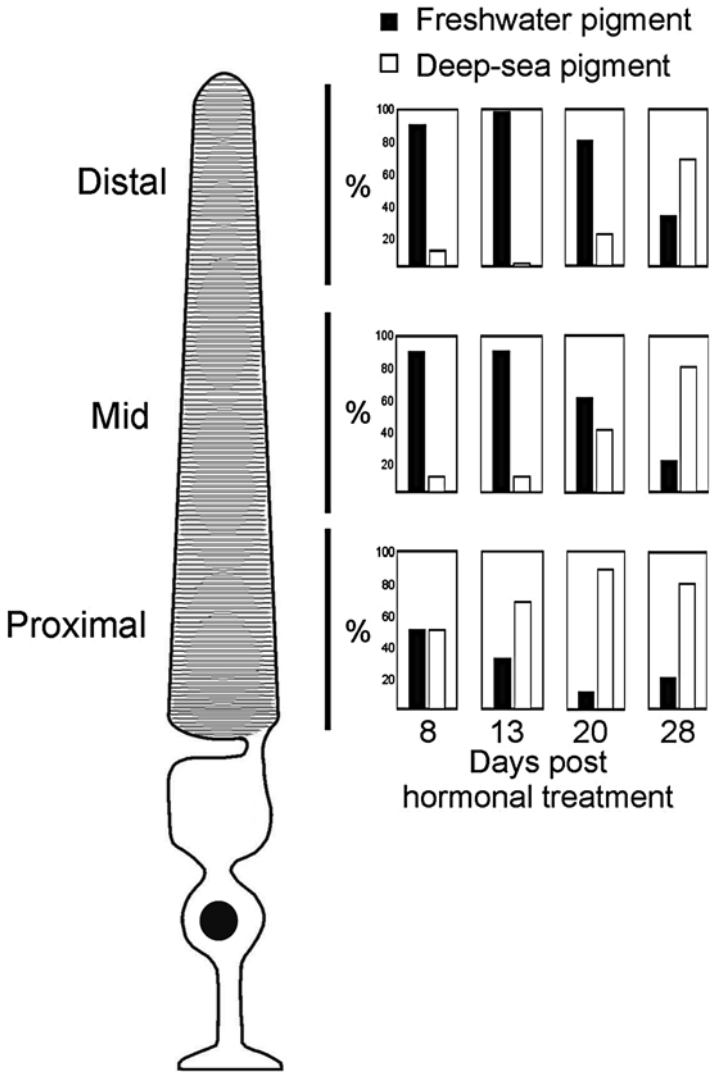


Fig. 6.8 Pigment changes in eel rod photoreceptors during hormone-induced metamorphosis. Note progressive changes in the proportions of deep-sea and freshwater RH1 opsins along rod outer segments at four different intervals post hormonal treatment. Redrawn from Hunt et al. (2013)

single ganglion cells (reviewed in Warrant et al. 2003). In many cases, the rod pigment is tuned to a maximum spectral sensitivity that approximates to the peak wavelength of the available light, whether downwelling or bioluminescent (Partridge et al. 1988, 1989; Hunt et al. 2001). Studies of the RH1 pigment in numerous deep-sea species from a number of the major Orders have identified the precise molecular changes responsible for the SW shifts. These differ between species (Hunt et al. 2001; Hope et al. 1997), although all use one or more substitutions at just eight sites,

indicating that the generation of SW shifts in spectral sensitivity can only be achieved by substitution at a limited number of sites.

Dragon fish from the genera *Aristostomias*, *Pachystomias* and *Malacosteus* (Order Stomiiformes) are unusual in possessing suborbital photophores that produce far-red bioluminescence at around 700 nm, in addition to the more usual blue bioluminescence-emitting postorbital light organs (Denton et al. 1970; Widder et al. 1984). The peak sensitivities of their rod photoreceptors are LW-shifted to around 520 nm, most likely to enhance perception of prey under the red light illumination of the suborbital photophores. The residue change that generates this spectral shift is a Phe261Tyr substitution (Douglas et al. 1999; Hunt et al. 2001) that is also responsible for the tuning of LWS cone pigments to longer wavelengths in other species. *Malacosteus niger* shows a further refinement with the possession of a photosensitizer pigment in the retina. This pigment, which has been characterized as a mixture of defarnesylated and demetallated derivatives of bacteriochlorophylls *c* and *d*, spectrally shifts light to shorter wavelengths and thereby increases the perception of red bioluminescent light (Douglas et al. 1999).

Cone photoreceptors have been retained in only a few species that occupy the deep ocean. Examples are the lantern fishes, *Lampanyctus crocodilus*, *Benthosema glaciale*, and *Myctophum punctatum* (members of the Superorder Scopelomorpha) that possess a rod-dominated retina but retain a few cones mainly distributed in central retina (Bozzano et al. 2007). The underlying visual pigments in these cones remain, however, unknown. The only deep-sea species in which the corresponding cone opsin gene has been identified is the pearl-eye, *Scopelarchus analis*, which has retained an expressed *RH2* gene in addition to duplicated copies of the *RH1* gene (Pointer et al. 2007), even though it occupies depths in excess of 500 m.

Changes in the retention and tuning of pigments are also seen in freshwater fishes occupying deep water habitats (Fig. 6.9). Lake Baikal in Eastern Siberia is the world's deepest freshwater lake, with depths in excess of 1,600 m. Endemic to the Lake and surrounding rivers is a species flock of cottoid fishes (Order Scorpaeniformes), with different members of the flock occupying different depth habitats (Bowmaker et al. 1994; Hunt et al. 1996). Surface dwelling species have retained L cones but these are lost in species inhabiting deeper water. As depth increases, the peak sensitivity of the rod photoreceptors shows progressive SW shifts, which can be accounted for at the molecular level by sequential substitutions at only three tuning sites (83, 261, and 292) within the RH1 opsin (Hunt et al. 1996). A similar SW shift is seen for the middlewave and shortwave cones, and for the SWS2 pigment, where the key substitutions have again been identified (Cowing et al. 2002a).

6.6.4 Lobe-Finned Fishes

Lungfishes (Dipnoi) have long been considered to be the link between aquatic and terrestrial vertebrates (Yokobori et al. 1994). The visual system of lungfishes more closely resembles that of terrestrial vertebrates than that of another sarcopterygian

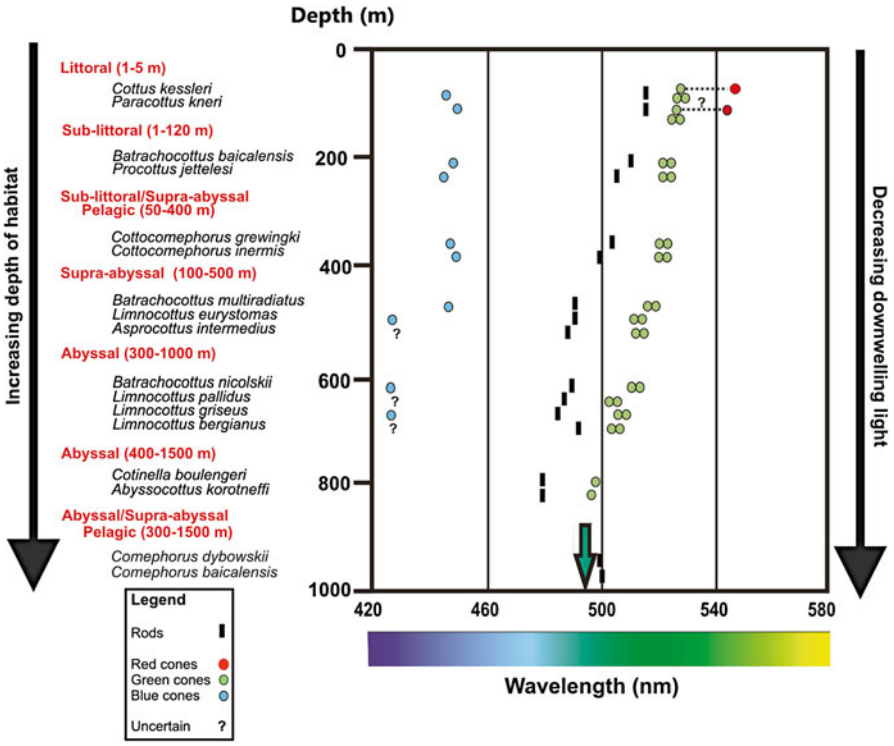


Fig. 6.9 Depth distribution of different cottoid fish species in Lake Baikal and the spectral location of their rod and cone pigments. The maximum penetration of downwelling daylight is 1,000 m. At this depth, the spectral composition is restricted to blue–green light at around 475 nm, as indicated by the *green arrow*. Note the loss of *red*-sensitive cones in all but the two littoral species, and the shift in peak absorbance of rod, and *green*- and *blue*-sensitive cones to shorter wavelengths with increasing depth. Data from Bowmaker et al. (1994)

fish, the coelacanth, *Latimeria* spp., which supports the notion that lungfishes, rather than coelacanths, are the closest living relatives of the tetrapods. There are three extant species of lungfishes, located in Australia, South America and Africa, respectively. The Australian species, *Neoceratodus forsteri*, possesses several classes of cones that are distinguished by brightly colored oil droplets and myoidal pigment inclusions (Bailes et al. 2006; Robinson 1994). The oil droplets contain carotenoid pigments that are matched to the spectral sensitivity of the particular cone class and act as cut off filters to increase spectral discrimination. At the molecular level, a rod RH1 opsin plus orthologs of all four classes of cone opsin gene are present (Bailes et al. 2007). In contrast, the retina of the coelacanth, *Latimeria chalumnae*, is rod-dominated with only a single cone opsin (RH2) present (Yokoyama et al. 1999). The loss of all but one cone class is consistent with the photon-limited deep-sea environment of between 100 and 400 m that the coelacanth inhabits (Locket 1973; Millot and Carasso 1955).

6.7 Visual Pigment Variation in Amphibians

Amphibians are grouped into the limbless, burrowing caecilians (Order Gymnophiona), the urodeles, salamanders and newts (Order Caudata) and the frogs and toads (Order Anura) (Tudge 2000). Caecilians have small eyes and rod-only retinae (Mohun et al. 2010), whereas duplex retinae are found in all other amphibians. Uniquely, anurans and some salamanders possess two spectral classes of rod photoreceptor, a majority class (90–95 %) that expresses a rod RH1 pigment with a λ_{max} at ~500 nm (termed "red" rods), and a minority class that expresses a blue-sensitive SWS2 cone pigment (termed "green" rods) (Darden et al. 2003; Hisatomi et al. 1999; Ma et al. 2001a). Cones are also present; double cones contain a LWS pigment (Makino et al. 1999a; Rohlich and Szel 2000; Sherry et al. 1998), with single cones containing either a UVS SWS1 pigment or the SWS2 pigment that is found in the "green" rods (Deutschlander and Phillips 1995; Harosi 1975; Hisatomi et al. 1998a; Ma et al. 2001a, b; Takahashi et al. 2001). A surprising finding is that the SWS2 cone pigment is paired in "green" rods with the rod isoforms of the G-protein transducin (Ma et al. 2001b). The RH2 cone class is consistently missing from amphibians, with the SWS2 pigment LW-shifted to 474 nm in the newt (Takahashi and Ebrey 2003) to spectrally resemble an RH2 pigment.

6.8 Visual Pigment Variation in Reptiles

Amongst the reptiles, the anoline lizards are perhaps the best studied. Anoline lizards are reported to have a pure cone retina but retain a low level of retinal expression of a rod *RH1* gene (McDevitt et al. 1993; Kawamura and Yokoyama 1997), which encodes a pigment with a λ_{max} at 491 nm (Loew et al. 2002; Kawamura and Yokoyama 1997, 1998; Provencio et al. 1992). This finding raises the possibility that either a small population of rods is present or the *RH1* opsin gene is expressed in cones alongside a cone pigment. Double and single cones are present and each photoreceptor type contains colored oil droplets matched to the spectral sensitivity of the particular cone class that acts to increase spectral discrimination. In the green anole, *Anolis carolinensis*, there are four spectrally distinct cone classes with λ_{max} values at 365, 455, 495 and 564 nm, thereby providing the potential for tetrachromatic color vision (Loew et al. 2002; Kawamura and Yokoyama 1997, 1998; Provencio et al. 1992). Colored oil droplets are also present in turtles, in the four spectral classes of single cone that are thought to support tetrachromacy, but only in the principal member of the double cones. Both members of double cones contain a LWS visual pigment but the presence of oil droplets only in the principal member means that they are spectrally distinct (Lipetz 1984; Loew and Govardovskii 2001; Ohtsuka and Kawamata 1990; Ohtsuka 1985a, b). The retinae of other reptilian species lack oil droplets. The crocodilian reptiles have a rod-dominated retina with single and double cones; the Mississippi alligator, *Alligator mississippiensis*, has four spectral classes of cone, with double cones that contain pigments with peak

spectral sensitivities at 566 and 503 nm and two single cone classes with peak spectral sensitivities at 535 and 443 nm (Sillman et al. 1991). The spectacled caiman, *Caiman crocodiles*, has single cones that are spectrally similar to those of the alligator, and double cones that contain either a 535 nm pigment in both members or a 506 nm/535 nm pigment combination (Govardovskii et al. 1988).

Lifestyles amongst reptiles varies from diurnal to crepuscular and nocturnal and it is thought that during evolution, some species have transitioned from a diurnal lifestyle to nocturnality and then back to diurnality, resulting in the transformation of cones into rods, and then back into cones, as proposed by Walls in his transmutation theory (Walls 1934, 1942). This theory would appear to be applicable to the geckos (Gekkonidae), which evolved from ancestral pure-cone diurnal lizards through a nocturnal phase and then back to diurnality. However, although the photoreceptors of nocturnal geckos are very rod-like, they nevertheless exhibit ultrastructural characteristics of cones (Roll 2000), and this is supported by the presence of visual pigments belonging to three of the four cone classes, LWS, RH2 and SWS1 (Kojima et al. 1992; Yokoyama and Blow 2001) in diurnal geckos (Ellingson et al. 1995; Taniguchi et al. 1999).

Snakes are another group where transmutation of cones into rods has been proposed (Walls 1934, 1942). Primitive snakes such as boas and pythons belonging to the superfamily Henophidia have a rod-dominated retina with two classes of single cones with spectral sensitivity peaks at 360 and around 550 nm (Sillman et al. 1999, 2001). They have, therefore, retained the capacity for dichromatic color vision even though they hunt for prey under mesopic and nocturnal conditions. Molecular analysis of the pigments present in these two cone classes in the python, *Python regius*, and the related sunbeam snake, *Xenopeltis unicolor* (Davies et al. 2009c), has identified a UVS SWS1 pigment with a λ_{max} at 361 nm and a LWS pigment with a λ_{max} at 550 nm. By contrast, the retinae of some more "advanced" snakes from the family Colubridae, such as the diurnal garter snake, *Thamnophis sirtalis*, completely lack rods (Jacobs et al. 1992; Sillman et al. 1997); LWS-expressing double and large single cones are present with peak sensitivities at about 554 nm, plus small single cones with λ_{max} values at 482 and 360 nm. Rods are also absent from two species of sea snakes, the spine-bellied, *Lapemis curtus*, and the horned, *Acalyptophis peronii* (Hart et al. 2012). In both species, three types of single cones are present with λ_{max} values at 428–430, 496 and 555–559 nm, plus double cones in which both members express a pigment with a peak sensitivity at 555–559 nm. The molecular identities of the corresponding pigments have yet to be determined, but if a transmutation event has occurred, then one of the cone classes in the colubrid snakes may express an RH1 pigment.

6.9 Visual Pigment Variation in Birds

Color vision is thought to have played a particularly important role in the evolution of the complex networks of mate selection seen in highly colored bird species (Doutrelant et al. 2012; Eikenaar et al. 2011; Laczi et al. 2011; MacDougall and Montgomerie 2003; Pryke and Griffith 2007; Bennett et al. 1997; Berg and Bennett 2010).

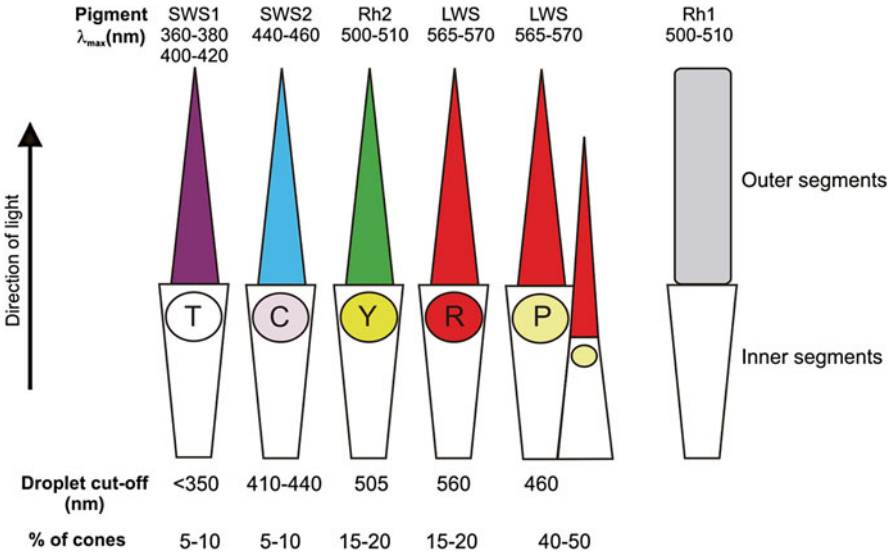


Fig. 6.10 Schematic representation of the complement of single and double cones and rod photoreceptors in the avian retina. Depending on the species, the SWS1 pigments have λ_{max} values either in the UV (360–380 nm) or in the violet (400–420 nm) region of the spectrum. From Hunt et al. (2009b)

A consistent feature of the avian retina is the presence of colored oil droplets in the inner segments of cone photoreceptors (Fig. 6.10), a feature they share with reptiles and marsupials. The principal member of double cones contains a large pale yellow, P-type droplet that functions as a cut-off filter to wavelengths shorter than 460 nm, and the accessory member contains either a small discrete droplet or diffused pigment in the inner segment. In most diurnal species of birds, four classes of single cone are present, each expressing a different cone opsin, and thereby providing the potential for tetrachromacy. Unlike reptiles, however, the LWS pigment of diurnal birds is present in both members of double cones (Hart and Hunt 2007). The LWS single cones contain a red R-type droplet that cuts off at about 560 nm, while single cones with the RH2 pigment have a yellow Y-type droplet that cuts off at about 505 nm, and single cones with an SWS2 pigment have a clear C-type droplet that cuts off at 410–440 nm (Hart and Hunt 2007; Bowmaker 2008). Finally, cones expressing an SWS1 pigment possess a transparent T-type droplet that shows no significant absorbance above 350 nm (Bowmaker et al. 1997; Hart et al. 2000).

6.9.1 Tuning of Avian Visual Pigments

Except for the SWS1 pigments, which vary from UVS to VS, the peak sensitivities of avian pigments are generally conserved, with notable exceptions in the LWS pigment of the Humboldt penguin, *Spheniscus humboldti*, which peaks at 543 nm

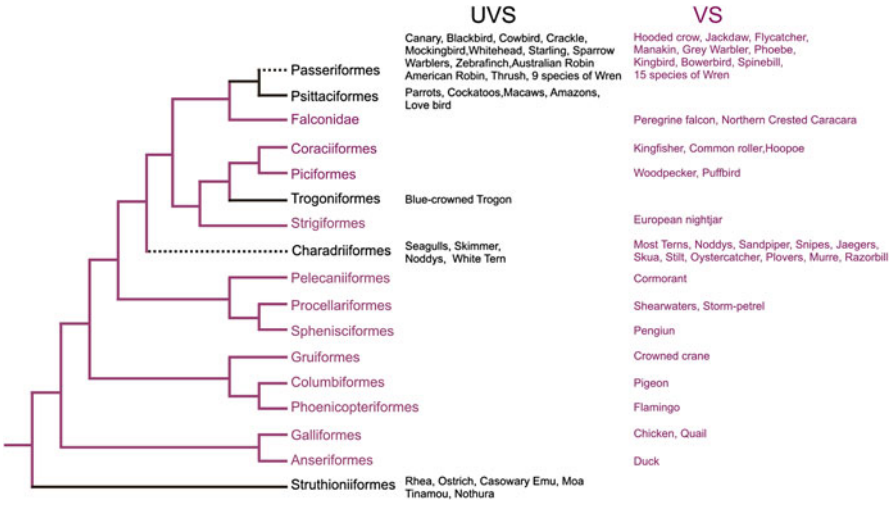


Fig. 6.11 Evolution of SWS1 pigments in birds showing the presence of VS and UVS pigments in the different avian Orders. Example species with UVS and VS pigments are listed alongside their respective Orders. *Black lines* indicate UVS pigments only, *violet lines* indicate VS pigments only, and *dotted lines* indicate both UVS and VS pigments. From Hunt and Peichl (2014) with permission

(Bowmaker and Martin 1985), and the tawny owl, *Strixa luco*, which peaks at 555 nm (Bowmaker and Martin 1978), compared with peaks of around 565 nm in most other species. The ancestral avian SWS1 pigment was most likely a VS pigment (reviewed in Hunt and Peichl 2014) that arose at the base of the avian lineage by a single amino acid substitution of Phe by Ser at site 86 (Carvalho et al. 2007). Subsequently, UVS pigments arose from a Ser to Cys substitution at site 90 (Wilkie et al. 2000; Yokoyama et al. 2000), which contrasts with the UVS pigments of other vertebrates where Phe86 and Ser90 are retained (Cowing et al. 2002b; Hunt et al. 2004). Cys90 is found in the UVS pigments of a number of species distributed across several avian orders (Odeen and Hastad 2003) and their phylogenetic relationships (Fig. 6.11) indicate that the Ser90Cys substitution occurred on a number of separate occasions in the generation of UVS pigments (Hastad et al. 2005; Odeen and Hastad 2010; Odeen et al. 2010, 2011, 2012).

Our knowledge of the cone complement of nocturnal birds is restricted to just a few species. As might be expected, a rod-dominated retina is present (Bowmaker and Martin 1978; Rojas et al. 2004). Cone spectral sensitivities have been studied in only one species, the tawny owl, *Strixa luco*; three classes are present with λ_{max} values at 555, 503 and 463 nm which most likely correspond to LWS, RH2 and SWS2 pigments (Bowmaker and Martin 1978), but whether this complement is common to other nocturnal species, including the absence of an SWS1 pigment, remains to be established.

6.10 Visual Pigment Variation in Mammals

6.10.1 Nocturnal and Fossorial Adaptations

With the single exception of total cone loss in deeper diving whales (Meredith et al. 2013), all mammalian retinae possess both rods and cones. The proportion of cones is highly correlated with lifestyle, varying from around 1 % or less in nocturnal species, to 2–10 % in crepuscular and arrhythmic species, and to 5–30 % in diurnal species (Ahnelt and Kolb 2000; Peichl 2005), with a few strictly diurnal species such as ground squirrels and tree shrews possessing more cones than rods (Kryger et al. 1998; Muller and Peichl 1989). A nocturnal phase is thought to mark the early evolution of the mammals around 150–200 mya and may be responsible for the reduction in the number of cone visual pigment genes to only two classes to give dichromatic color vision. The genes retained, however, differ between the egg-laying monotremes on the one hand and the marsupials and placental mammals on the other. In marsupial and placental mammals, the *LWS* and *SWS1* genes are retained, with the loss of the *SWS2* and *RH2* genes, whereas in the two extant species of monotremes, the platypus, *Ornithorhynchus anatinus*, and echidna, *Tachyglossus aculeatus*, the *LWS* gene is again retained but this is paired with the *SWS2* gene, with the loss of *SWS1* and *RH2* genes (Davies et al. 2007a; Wakefield et al. 2008). This means that the ancestral mammal, prior to the protherian/therian split, would have the potential for trichromacy arising from the retention of the *SWS1* gene, most likely encoding a UVS pigment, the *SWS2* gene encoding a blue-sensitive pigment, and the *LWS* gene encoding a yellow-sensitive pigment.

With the exception of primates, placental mammals are at best dichromats with S and L cones that contain respectively SWS1 and LWS pigments. There is, however, evidence that some Australian marsupials have a class of middlewave-sensitive cones in addition to S and L cones, which may provide for trichromacy (Arrese et al. 2006). This was first reported for the fat-tailed dunnart, *Sminthopsis crassicaudata*, and the honey possum, *Tarsipes rostratus* (Arrese et al. 2002), and subsequently extended to the quokka, *Setonix brachyurus*, and quenda or bandicoot, *Isoodon obesulus* (Arrese et al. 2005). Attempts, however, to identify this third cone pigment gene have failed, despite extensive efforts by two laboratories (Cowing et al. 2008; Strachan et al. 2004), so it is probable that trichromacy arising from three cone pigment classes is not present in these animals. A second rod *RH1* pigment gene was identified in the genome of the fat-tailed dunnart by Cowing et al. (2008) and these authors have advanced the hypothesis that MWS cones may express a rod pigment.

The shift in the λ_{max} of the SWS1 pigment from UVS to VS has occurred several times in mammalian evolution (Fig. 6.12), and in each case, the single replacement of Phe at site 86 appears sufficient to shift the peak from UV at around 360 to >400 nm (Cowing et al. 2002b; Davies et al. 2012a; Hunt and Peichl 2014). Indeed, amongst placental mammals, it is only species from the Orders Rodentia, Chiroptera (bats) (Wang et al. 2004) and Insectivora (Glosmann et al. 2008) that have retained

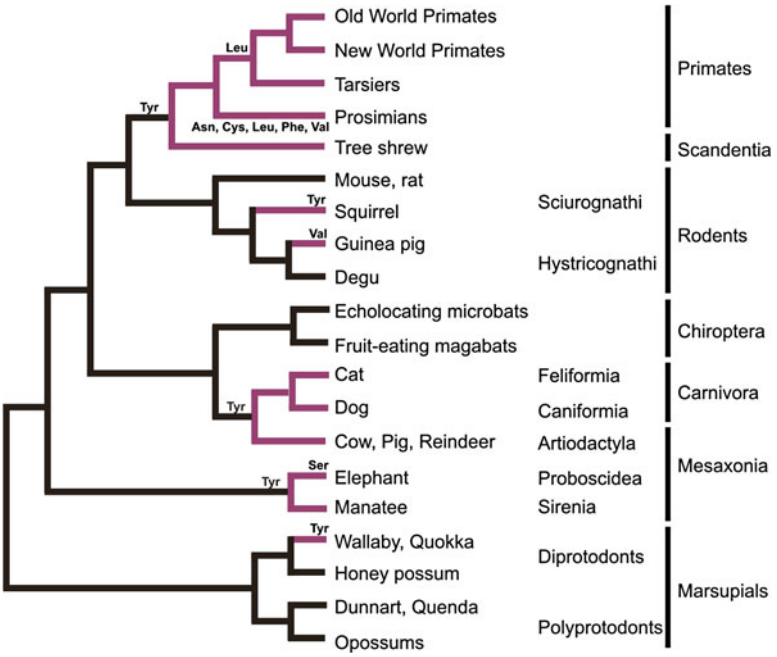


Fig. 6.12 Amino acid substitutions at site 86 in the spectral tuning of SWS1 pigments in mammals. The ancestral gene encoded Phe86 to generate a UVS pigment (*black lines*). The evolutionary occurrence of substitutions at this site (shown on the branches) generated VS pigments (*violet lines*). The phylogeny is based on Song et al. (2012)

UVS pigments. In bats, Phe86 is retained in three species of Macrochiroptera and two species of Microchiroptera (Muller et al. 2009; Wang et al. 2004), with UV sensitivity demonstrated in the latter group by electroretinography (ERG) (Muller et al. 2009). Amongst the two rodent suborders (the Sciurognathi and the Hystricognathi), mouse and rat members of the sciurognathians have UVS pigments with Phe86 (Shi et al. 2001), whereas other species such as the gray squirrel, *Sciurus carolinensis*, have VS pigments with Tyr86 (Carvalho et al. 2006). Members of the Hystricognathi also show a mixture of UVS and VS pigments, with UVS pigments with Phe86 present in the diurnal degu, *Octodon degus*, and two subterranean species of tuco-tuco, *Ctenomys talarum* and *Ctenomys magellanicus* (Schleich et al. 2010), whereas the guinea pig, *Cavia porcellus*, has a VS pigment with Val86 (Parry et al. 2004).

In the remaining Orders of placental mammals, only VS pigments are found. In the Order Artiodactyla, Tyr86 is present in the VS pigments of the cow, *Bos taurus*, and pig, *Sus scrofa* (Cowing et al. 2002b; Fasick et al. 2002) and in the aquatic West Indian manatee, *Trichechus manatus* (Newman and Robinson 2005), whereas in the elephants (*Loxodonta africana* and *Elephas maximus*), Ser86 is present, a change that is known to shift the peak spectral sensitivity into the violet (Yokoyama et al. 2005). This latter change must have occurred within the Proboscidea lineage, most

likely following a Phe86Tyr substitution that occurred in the common ancestor to the Artiodactyla. Tyr86 is also found in the SWS1 pigments of two species of carnivores, the dog, *Canis lupus familiaris*, and cat, *Felis catus* (Hunt et al. 2009b).

The retention of UVS pigments is more common amongst marsupials than placental mammals. In the two major Orders, the Diprotodontia and the Polyprotodontia, that form the Marsupialia, members of the Diprotodontia have either VS pigments with Tyr86, as found in the Tammar wallaby, *Macropus eugenii*, and quokka, *Setonix brachyurus* (Arrese et al. 2005; Deeb et al. 2003), or UVS pigments with Phe86, as found in the honey possum, *Tarsipes rostratus* (Arrese et al. 2002; Cowing et al. 2008). In contrast, members of the Polyprotodontia, such as the fat-tailed dunnart, *Sminthopsis crassicaudata* (Cowing et al. 2008), the South American big-eared opossum, *Didelphis aurita* (Hunt et al. 2009c) and the quenda, *Isoodon obesulus* (Arrese et al. 2005), all have UVS pigments with Phe86. The absence of VS pigments, therefore, amongst polyprotodonts indicates that the Phe86Tyr substitution seen in the Tammar wallaby and quokka occurred within the diprotodont marsupial lineage.

6.10.2 Primates

The trichromacy found in primates does not arise from the retention of a third cone opsin pigment gene from the ancestral trichromatic mammal but has been newly acquired from changes in the *LWS* gene. The evolutionary drive behind the acquisition of trichromacy is thought to be improved color discrimination in the red/green region of the spectrum for the detection and evaluation of ripe fruits (Mollon 1989; Osorio and Vorobyev 1996; Sumner and Mollon 2000; Regan et al. 2001) and young nutritious leaves (Dominy and Lucas 2001) against the green foliage of the rainforest. This has been achieved in different ways within the two major primate groups, the Old World primates or catarrhines from Africa and Asia, and New World primates or platyrrhines from Central and South America. In Old World primates, trichromacy has arisen from a duplication of the X-linked *LWS* gene; with the single exception of the megabat, *Haplonycteris fischeri*, where a recent duplication of the *LWS* gene is also present (Wang et al. 2004), the duplication of the *LWS* gene is unique to primates. This occurred at the base of the catarrhine lineage (Nathans et al. 1986b; Hunt et al. 1998) to give two adjacent genes, the upstream copy encoding a long wavelength-sensitive (L) pigment with λ_{max} around 563 nm and the downstream copy encoding a middle wavelength-sensitive (M) pigment with λ_{max} around 535 nm. These are expressed in separate L and M cone classes alongside S cones expressing the autosomal *SWS1* gene.

The original duplication in catarrhines involved the insertion of a chromosomal segment that includes the entire *LWS* gene plus an almost complete copy of another gene, *TEX28* (Hanna et al. 1997), to give a tandem repeat (Dulai et al. 1999). A direct consequence of the close proximity and high level of sequence homology of the duplicate copies of the gene is mispairing during meiosis, and when this is

followed by crossing-over, has led to an expansion in the number of copies of the downstream M gene to form a gene array (Drummond-Borg et al. 1989; Nathans et al. 1986a; Feil et al. 1990; Jorgensen et al. 1990). A further consequence of mispairing within the array is the deletion of gene copies and the creation of hybrid genes, and it is these events that underlie the high frequency (approaching 10 %) of red–green color vision deficiencies of full dichromacy and anomalous trichromacy, respectively, in humans (Nathans et al. 1986b; Neitz and Neitz 2000). In contrast, however, such defects in color vision are extremely rare in non-human catarrhines, with just two reports of similar genetic defects (Jacobs and Williams 2001; Verrelli et al. 2008). In the first study carried out in a colony of long-tailed macaques, *Macaca fascicularis*, three male animals were found to have a hybrid L/M gene amongst 744 males screened (Onishi et al. 1999), a frequency of anomalous trichromacy of 0.4 %, and in the second study, an L/M hybrid was identified in a single male chimpanzee, *Pan troglodytes*, amongst a group of 58 males examined (Terao et al. 2005). Nevertheless, evidence for gene conversion, a process whereby sequences of related genes become homogenized, is found within the L/M opsin gene arrays in both human and non-human primates. Conversion has resulted in the coding and non-coding regions of the L and M opsin genes being more like each other within species than the same regions of either the L or M orthologs are between species (Ibbotson et al. 1992; Balding et al. 1992; Shyue et al. 1994, 1995). Gene conversion involves exchanges between genes; for the L and M opsin genes, the generation of hybrids by mispairing and crossing-over would appear to be the most likely mechanism. This implies that this is ongoing within non-human primates, which nevertheless show a much lower frequency of hybrid genes and gene deletions than in humans. A recent study of the L/M gene array in gibbons (Hiwatashi et al. 2011) identified conversion in introns and purifying selection in exons to maintain the spectral differences between pigments, together with a significant incidence of multiple M genes (an average rate of 23.5 % in males across all species studied). The corollary of mispairing and crossing-over to gain additional genes within the array is gene loss, but this was not seen, nor were any hybrid genes present, in the 152 individual animals studied. This would appear to be strong evidence that red–green color vision deficiencies are selectively detrimental to survival within non-human primate communities and are removed by natural selection.

The genetic mechanism underlying trichromacy in New World primates and in a few species of prosimians (the lorises and galagos from Africa and Asia, the lemurs from Madagascar and the tarsiers from Southeast Asia) is, with one exception, based on a polymorphic *LWS* gene (Neitz et al. 1991; Williams et al. 1992), with the different allelic copies that specify pigments with λ_{max} values ranging from 535 to 565 nm (Table 6.1). Since only females have two X chromosomes, full trichromacy is limited to heterozygous females with different allelic forms of the gene. Homozygous females as well as all males are therefore dichromats (Mollon et al. 1984). A polymorphic *LWS* gene is also found in three diurnal prosimian species, the red ruffed lemur, *Varecia variegata rubra*, Coquerel's sifaka, *Propithecus verreauxi coquereli*, and the blue-eyed black lemur, *Eulemur macaco flavifrons*, and in a nocturnal species, the greater dwarf lemur, *Cheirogaleus major*

Table 6.1 The allelic variation found in the *LWS*/*MWS* (L/M cone opsin) genes of New World monkeys and prosimians

	Family	Genus	Common name	Number of L/M genes	Variants per gene	λ_{max} (nm)
Platyrrhini	Atelidae	*Alouatta*	Howler monkey	2	1	558, 530
		Ateles	Spider monkey	1	2	563, 550
		Lagothrix	Woolly monkey	1	3	562, 550, 530
	Pitheciidae	*Callicebus*	Titi monkey	1	5	562, 550, 542, 535, 530
		Pithecia	Saki monkey	1	3	
	Cebidae	*Cebus*	Capuchin monkey	1	3	563, 549, 535
		Samiri	Squirrel monkey	1	3	564, 550, 536
		Aoutus	Owl monkey	1	1	545
		Leontopithecus	Lion tamarin	1	3	563, 555, 543
			Saddle back tamarin	1	3	563, 557, 545
		Callithrix	Marmoset	1	3	565, 559, 543
Prosimians	Lemuroidea	*Varecia*	Red-ruffed lemur	1	2	558, 543
		Eulemur	Blue-eyed black lemur	1	2	558, 543
	Cheirogaleidae	*Cheirogaleus*	Greater dwarf lemur	1	2	558, 543
	Indriidae	*Propithecus*	Coquerel's sifaka	1	2	558, 543

Data taken from Jacobs and Deegan (2001, 2003a, b, 2005); Jacobs et al. (2002); Mollon et al. (1984); Saito et al. (2004); Tan and Li (1999); Travis et al. (1988); Talebi et al. (2006); Veilleux and Bolnick (2009)

(Jacobs et al. 2002; Tan and Li 1999; Veilleux and Bolnick 2009); in all cases, the two *LWS* alleles encode a 543 nm M pigment and a 558 nm L pigment, respectively. It is possible, however, that trichromacy is more common amongst prosimians as many of the studies have been restricted to small cohorts of animals. An *LWS* gene polymorphism may therefore have been missed (Tan and Li 1999). The exception mentioned above is found in the howler monkey, *Alouatta* spp., where a duplication of the *LWS* gene similar to that in Old World primates has occurred (Dulai et al. 1999; Jacobs et al. 1996a) to give separate *L* and *M* genes that encode pigments with λ_{max} values of 530 nm and 558 nm, respectively (Saito et al. 2004), and thereby confer full trichromacy in both sexes.

The spectral shifts between primate LWS pigments is largely due to substitutions at only three sites, 180, 277 and 285 (Neitz et al. 1991). Amongst the catarrhines,

the L variants of the LWS pigment have the polar residues Ser, Tyr and Thr at these sites, respectively, whereas the M variants have the non-polar residues Ala, Phe and Ala, respectively (Ibbotson et al. 1992; Nathans et al. 1986b). As shown by site-directed mutagenesis and in vitro expression studies, the spectral effects of substitutions at these sites are approximately additive (Asenjo et al. 1994; Merbs and Nathans 1993; 1992). Site 180 is polymorphic in humans; Ser180 is the more common residue but Ala180 is present at a significant frequency to give a SW-shifted L pigment (Sanocki et al. 1993).

Substitutions at the same three sites are also used to spectrally tune the LWS pigments of platyrrhine monkeys (Neitz et al. 1991; Williams et al. 1992). In the howler monkey, the residues at these three sites encoded by the separate *L* and *M* variants of the *LWS* gene parallel those encoded by the *L* and *M* variants of the *LWS* gene of catarrhine primates to give pigments that peak at 558 nm and 530 nm, respectively (Saito et al. 2004), and substitutions at the same sites are also responsible for the spectral shifts between the pigments encoded by the allelic variants of the *LWS* gene in platyrrhine monkeys (Neitz et al. 1991; Williams et al. 1992). The use of these sites differs, however, in members of the two major families of New World monkeys; in the family Cebidae, which includes the capuchin and squirrel monkeys, all three sites vary amongst the different alleles to give pigments that differ by a maximum of around 30 nm, whereas in the family Callitrichidae, which includes the marmosets and tamarins, site 277 is not polymorphic, so the L and M pigments differ by a maximum of only 19–20 nm. In many cases however, the spectral shift(s) between pigments is less since different combinations of residues across the three sites produce three or more different spectral variants with intermediate λ_{max} values.

Polymorphism is also the mechanism of trichromacy in prosimians (Tan and Li 1999) but, in this case, the spectral shifts between pigments arise from substitution at just site 285, with Thr in the L pigment and Ala in the M pigment. Polymorphism is also limited to just four species although, as mentioned above, this may be an underestimate of the number of trichromatic species. In the majority of non-polymorphic species, an M pigment (with Ala285) is present, with L pigments (with Thr285) present in only three species; the existence of L and M genes in different present-day species of tarsiers and strepsirhines may indicate therefore that trichromacy was present in the common ancestor but subsequently lost.

It has been argued that the maintenance of the L/M gene polymorphism in platyrrhines is due to balancing selection operating to maintain a heterozygous advantage for trichromatic females (Boissinot et al. 1998; Surridge and Mundy 2002). Sequence analysis of the different alleles in two platyrrhine species, the spider monkey, *Ateles geoffroyi*, and the white-faced capuchin monkey, *Cebus capucinus*, has provided evidence for gene conversion in the intronic and flanking regions of the LWS gene and for balancing selection in the retention of differences within exons 3–5, which encode the key tuning sites (Hiwatashi et al. 2010). What remains unclear is exactly which behavior pattern is responsible for the retention of the *LWS* gene polymorphism and the maintenance of trichromacy, as different studies have yielded contradictory results in the ability of males and females to locate feeding sites (Smith et al. 2003; Dominy et al. 2003), in the time spent by dichromats and trichromats in foraging for food (Vogel and Janson 2007) and in their efficiency in finding fruits (Hiramatsu et al. 2008).

6.10.2.1 Tuning of the SWS1 Pigment in Primates

Ubiquitously within the primates, the *SWS1* gene encodes a VS pigment to give S cones with peak sensitivities of >400 nm (Bowmaker et al. 1991; Hunt et al. 1995). A switch from ancestral UVS to VS is therefore ancestral to modern-day primates. Based on a comparison of mouse UVS and human VS pigments, it has been proposed that this spectral shift required the simultaneous replacement of residues at three sites (Phe86Leu, Thr93Pro, and Ser118Thr) (Shi et al. 2001; Yokoyama and Shi 2000). Other residue changes at these sites are, however, found amongst New World primates and prosimians (Hunt and Peichl 2014). Significantly, site 86 is occupied by Leu in Old and New World primates and tarsiers, but is much more variable in prosimians with Cys, Ser, Leu, Val, Tyr and Asn present in at least one species. Even Phe86, which has been shown to generate a UVS pigment in other animal groups (Hunt et al. 2004), is found in the VS pigment of one species (Carvalho et al. 2012), the aye-aye, *Daubentonia madagascariensis*, a strictly nocturnal and highly endangered primate endemic only to Madagascar. The switch to VS pigments would appear therefore to be more complicated in primates than in other groups. As shown in Table 6.2, the only residue consistently present across all putative tuning sites of the SWS1 pigments is Pro93. Pro93 is also present in the VS pigment of the clawed frog, *Xenopus laevis* (Starace and Knox 1998), so this may be the key change in generating a VS pigment in primates. Consistent with this, the replacement of Pro93 with Thr in the aye-aye shifts the λ_{max} of the pigment into the UV (Carvalho et al. 2012). However, substitutions at site 86 may also be important; with the singular exception of the aye-aye pigment, Phe86 is replaced in all primate SWS1 pigments with either Cys, Leu, Ser or Val, and in two close relatives of primates, the Sunda colugo, *Galeopterus variegatus*, and the tree shrew, *Tupaia glis*, Tyr86 is present (Moritz et al. 2013; Carvalho et al. 2012; Petry and Harosi 1990; Hunt et al. 2009b). It is possible therefore that the shift in primate SWS1 pigments from UVS to VS originally arose from a Phe86Tyr (Fig. 6.13), but the subsequent acquisition of a Thr93Pro substitution provided a secondary mechanism for the maintenance of violet-sensitivity and thereby removed the constraint on site 86 (Carvalho et al. 2012). Under this scenario, the presence of Phe86 in the aye-aye pigment would be a back mutation.

6.10.3 S Cone Loss in Aquatic Mammals

Most aquatic mammals fall into two major groups, the Cetacea comprising the whales and dolphins, and the Pinnipedia comprising the seals, sea-lions and walruses. Cetaceans are closely related to the Artiodactyla but have a quite separate evolutionary origin from the Pinnipedia, which arose from the Carnivora. Both groups lack a functional SWS1 pigment and thereby conventional color vision (Peichl and Moutairou 1998; Levenson and Dizon 2003; Levenson et al. 2006; Newman and Robinson 2005). The absence of S cones in the retinae of these species

Table 6.2 Residues present at three sites in transmembrane helices II and III of the VS pigments of primates compared to the UVS pigments of two marsupials, the fat-tailed dunnart (GenBank acc. AY7724723) and honey possum (GenBank acc. AY772472), and two rodents, the mouse (GenBank acc. NM_007538) and rat (NM_031015)

Residues	UVS		VS																	
			Simian				Prosimian													
	Dunnart/Honey possum	Mouse/rat	Great apes	Old world monkey	New world monkey	Tarsier	Aye-aye	Brown lemur	Ring-tailed lemur	Sportive lemur	Coquerel's sifaka	Woolly lemur	Coquerel's mouse lemur	Gray mouse lemur	*Fat-tailed dwarf lemur*	*Greater dwarf lemur*	*Lesser galago*	*Small-eared galago*	*Greater galago*	*Pygmy slow loris*	*Slow loris*
86	F	F	L	L	L	L	F	C	C	S	L	V	S	S	S	S	Y	N	N	C	Y
93	T	T	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
			Haplorrhini				Strepsirrhini														

Species in *italics* possess a *SWS1* pseudogene

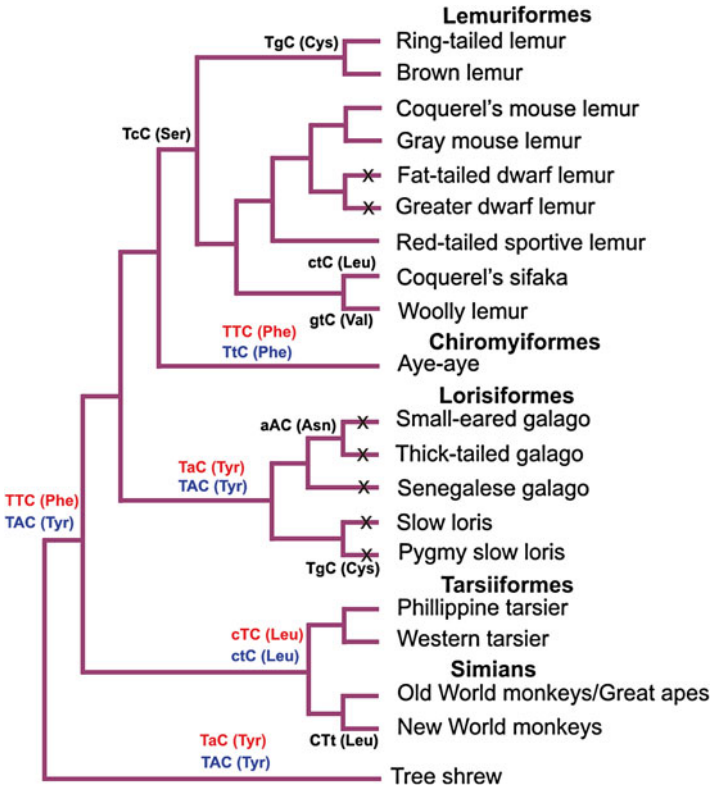


Fig. 6.13 Phylogenetic relationships of different primate species showing the different residues found at site 86 in SWS1 opsins, together with the corresponding codon sequences. The effects on nucleotide substitution of the alternative hypotheses of ancestral Phe86 or Tyr86 are shown in *red* and *blue*, respectively, with *black* used where both hypotheses are equivalent. *Lower case* is used to indicate required substitutions in codon 86 under each hypothesis. *Crosses* on lineages indicate species where SWS1 pseudogenes are present. From Hunt and Peichl (2014) with permission

has been established by a range of techniques that include immunohistochemistry, electrophysiology and/or gene sequencing (Jacobs 2013; Hunt and Peichl 2014). Genomic studies on several whale and seal species have shown the presence of *SWS1* opsin pseudogenes with deleterious mutations that preclude the production of a functional pigment. Therefore, it would appear likely that the dimly lit habitat of the deep ocean frequented by these aquatic mammals resulted in the relaxation of selection pressure to maintain color vision, leading to the accumulation of mutations in the *SWS1* gene; the retention of the LWS pigment in preference to the SWS1 pigment may reflect the relative paucity of S cones compared to L cones in the mammalian retina. In many deeper diving whales, however, even L cones have been lost (Meredith et al. 2013) to give rod monochromatic vision. This has major implications for the mechanism of signal processing within the retina. Rods utilize cone

bipolar cells to connect to the ganglion cells (Wassle 2004), so it remains to be established how the retinal circuitries have been modified in these whales that (uniquely amongst mammals) lack functional cones.

The Sirenia form a third group of aquatic mammals, represented by the manatees and dugongs. As members of the Mesaxonia, their closest terrestrial relatives are the elephants. Manatees have been shown to be dichromats (Cohen et al. 1982; Griebel and Schmid 1996) with SWS1 and LWS pigments (Newman and Robinson 2006), consistent with their more shallow water habitat of coastal and estuarine areas, where they feed on submerged seagrasses. S and L cones are also retained by other aquatic or semi-aquatic mammals such as the sea otter (*Enhydra lutris*), river otter (*Lontra canadensis*), polar bear (*Ursus maritimus*), and pygmy hippopotamus (*Choeropsis liberiensis*) (Ahnelt and Kolb 2000; Griebel and Schmid 1996; Peichl et al. 2001, 2005b; Levenson et al. 2006; Newman and Robinson 2006).

6.10.4 Nocturnal and Fossorial Adaptations

Although many mammals are nocturnal, both S and L cones are frequently retained in a rod-dominant retina. The loss of color vision is not unexpected in nocturnal species, where activity occurs at light levels largely below the sensitivity of cones. However, where one of the two cone classes has been lost, it is the S cone class that is absent to give L cone monochromacy. Examples are found in two nocturnal carnivores, the raccoon *Procyon lotor* and *P. cancrivorous*, and in the kinkajou, *Potos flavus* (Jacobs and Deegan 1992; Peichl and Pohl 2000). Amongst the rodents, most members of the squirrel family are strictly diurnal with cone-rich retinae (Long and Fisher 1983; Blakeslee et al. 1988; Kryger et al. 1998). However, flying squirrels are nocturnal, with the *SWS1* gene only retained as a pseudogene with multiple deletions (Carvalho et al. 2006). Many other rodents are nocturnal but most appear to have retained two functional cone pigment genes. Examples are the rat (*Rattus norvegicus*), pocket gopher (*Thomomys bottae*), gerbil (*Meriones unguiculatus*), cururo (*Spacopus cyanus*), and degu (*Octodon degus*) (Govardovskii et al. 1992; Jacobs and Deegan 1994; Jacobs et al. 2003; Chavez et al. 2003; Peichl et al. 2005a; Williams et al. 2005; Deegan and Jacobs 1993). In Syrian hamsters, the situation is a little more complex, with S and L cones present in the Siberian dwarf hamster, *Phodopus sungorus*, but only L cones present in the Syrian golden hamster, *Mesocricetus auratus* (Calderone and Jacobs 1999).

Fossorial (subterranean) rodents also show a loss of cone classes. In the blind mole rat (*Spalax ehrenbergi*), S cones and a functional *SWS1* gene are absent (David-Gray et al. 2002), but even though the eyes are atrophied and located below a layer of skin and fur (Sanyal et al. 1990; Cernuda-Cernuda et al. 2002), both rods and L cones are present (David-Gray et al. 1998; Janssen et al. 2000). These animals lack the ability to respond to visual images (Haim et al. 1983; Rado et al. 1992; Cooper et al. 1993) but retain the ability to entrain their activity to diurnal changes via the ocular perception of environmental light (Rado et al. 1991; Rado and Terkel

1989; David-Gray et al. 1998; Pevet et al. 1984). Not all subterranean rodents, however, have lost S cones. Both cone classes are present in the retinae of the European mole, *Talpa europaea*, a subterranean insectivore with small eyes, and in the Chilean cururo, *Spalacopus cyanus*, although in this species, S cones constitute up to 20 % of all cones in the ventral retina (Peichl et al. 2005a). Both *SWS1* and *LWS* opsin genes are also expressed in the bathyergid mole rats, *Cryptomys anseelli*, *Cryptomys mechowi* and *Heterocephalus* glaber; the SWS1 opsin is expressed in most cones whereas the LWS opsin is co-expressed in only a subset of these cones, and, where present, at a lower level than SWS1 opsin (Peichl et al. 2004).

The nocturnal owl monkey, *Aotus*, a platyrrhine primate, lacks S cones (Wikler and Rakic 1990; Levenson et al. 2007) and retains the *SWS1* gene only as a pseudogene, again with deleterious mutations (Jacobs et al. 1996b). *SWS1* pseudogenes are also present in many species of prosimians, notably in all members of the Lorisiformes, and in some species of the Cheirogaleidae family which forms part of the Lemuriformes group (Kawamura and Kubotera 2004; Tachibanaki et al. 2000; Tan et al. 2005). This latter group includes the fat-tailed dwarf lemur, *Cheirogaleus adipicaudatus*, and greater dwarf lemur, *Cheirogaleus major*, but their close relatives, the gray lemur, *Microcebus murinus*, and Coquerel's mouse lemur, *Mirza coquereli*, have both retained a functional gene. SWS1 and LWS pigments are also found in the strictly nocturnal aye-aye. In this species, the opsin gene would appear to be fully functional with evidence for purifying or stabilizing selection (Perry et al. 2007), indicating that dichromacy remains advantageous for the aye-aye despite its strictly nocturnal activity pattern.

The two major groups of bats, the Megachiroptera and Microchiroptera, differ in their diurnal activity. The former are crepuscular (primarily active at dawn and dusk) and have a well-developed visual system, while the latter are nocturnal and rely on acoustic orientation or echolocation more than vision. It is not surprising, therefore, to find that most megabats have retained S and L cones (Wang et al. 2004), although there appear to be exceptions, with three species, *Rousettus madagascaries*, *Eidolon dupreanum* and *Epomophorus gambianus*, having L but no S cones (Muller et al. 2007). The unexpected finding is that the nocturnal microbats (Wang et al. 2004) also express both SWS1 and LWS pigments.

6.10.5 Nocturnal Bottleneck

From the above, it is clear that the loss of a functional *SWS1* opsin gene has occurred on several occasions in mammalian evolution. In nocturnal species which depend largely on rod-mediated vision, a loss of color vision arising from the loss of the SWS1 pigment may not be detrimental, but in general across nocturnal species, there is no compelling evidence that the loss of SWS1 opsin is adaptive, i.e. conveys an advantage to the respective species (Jacobs 2013). It should also be noted that with the singular exception of the bathyergid mole rats, L cones are generally more numerous than S cones and provide the dominant input to the luminosity and visual

acuity channels at photopic and mesopic light levels. The loss of L cones would severely impact on visual performance, whereas the loss of S cones would likely only affect color vision (Jacobs 2013; Mollon 1989). In fact, limited color discrimination is also possible with just one spectral cone type, through interactions with rod photoreceptors at mesopic light levels, where both the cones and the rods are in their working range, as demonstrated in the owl monkey (Jacobs et al. 1993) and in a human cone monochromat (Reitner et al. 1991). The impact of adaptation to mesopic light levels on the evolution of the mammalian visual system is discussed in depth in Davies et al. (2012a).

6.11 Conclusion

A feature of vertebrate evolution is the diversity in the frequency of rod and cone photoreceptors in the retina, the loss of certain classes of cone visual pigments in certain lineages, and the spectral shifts in peak absorbance of visual pigments achieved largely by changes at key amino acid sites within the opsin protein. The effect of such changes is to vary the overall spectral sensitivity of the visual system, and in most cases, this can be directly linked to evolutionary adaptations occurring in response to changes in the quality and quantity of environmental light. So species living in dim-light environments tend to have fewer classes of cone photoreceptors and rod photoreceptor-dominated retinas, whereas species that are largely active in bright light tend to retain cone classes, albeit within the limits of the particular lineage, and thereby possess color vision.

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Chapter 7
Diversity and Functional Properties of Bistable Photopigments

Hisao Tsukamoto

Abstract Rhodopsin and rhodopsin-like photopigments function in visual and non-visual photoreceptor cells of various animals. Since the 1950s, photopigments having bistable nature have been found in invertebrate visual photoreceptor cells. The bistable photopigments are characterized by high thermal stability of the active photoproduct as well as photo-interconvertibility between inactive (dark) and active (light) forms. Recent studies have revealed that bistable photopigments are present in the non-visual photoreceptor cells of vertebrates including mammals. This observation means that an understanding of the molecular properties of bistable pigments is critical to the understanding of photoreception not only in invertebrates but also in vertebrates. In this chapter, the molecular characteristics, diversity and structure–function relationships of bistable photopigments are summarized. Recent research progress and future directions of bistable pigment study are also overviewed and discussed.

Keywords Photoreceptor • Rhodopsin • Vision • Visual pigment • Optogenetics • Molecular evolution • Membrane protein • G protein-coupled receptor

Abbreviations

cAMP	Cyclic adenosine monophosphate
GPCR	G protein-coupled receptor
ipRGC	Intrinsically photosensitive retinal ganglion cell
meta-II	Metarhodopsin II

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7.1 Introduction

In various visual and non-visual photoreceptor cells, rhodopsins and rhodopsin-like photopigments receive external light signals and transmit them to intracellular signaling cascades via light-dependent activation of trimeric G proteins (Terakita 2005; Yau and Hardie 2009). These photopigments are typical G protein-coupled receptors (GPCRs) with seven transmembrane α-helices and they possess 11-*cis*-retinal as a chromophore in the transmembrane region (Fig. 7.1) (Hofmann et al. 2009). Different characteristics of the pigments lead to different cellular photoresponses. For example, absorption spectral properties of the photopigments are responsible for the "color" which the photoreceptor cells detect (Nathans 1989; Stavenga and Schwemer 1984).

The rhodopsin-like photopigments can be divided into two types based on their properties of photoproduct (Terakita 2005, 2010). When vertebrate visual pigments in rod or cone visual cells absorb light, the chromophore 11-*cis*-retinal is isomerized to all-*trans* form, resulting in the formation of active photoproduct metarhodopsin II (meta-II) (Hofmann et al. 2009; Wald 1968) (Fig. 7.2b). Meta-II binds and activates G proteins (Emeis et al. 1982), and spontaneously dissociates into free

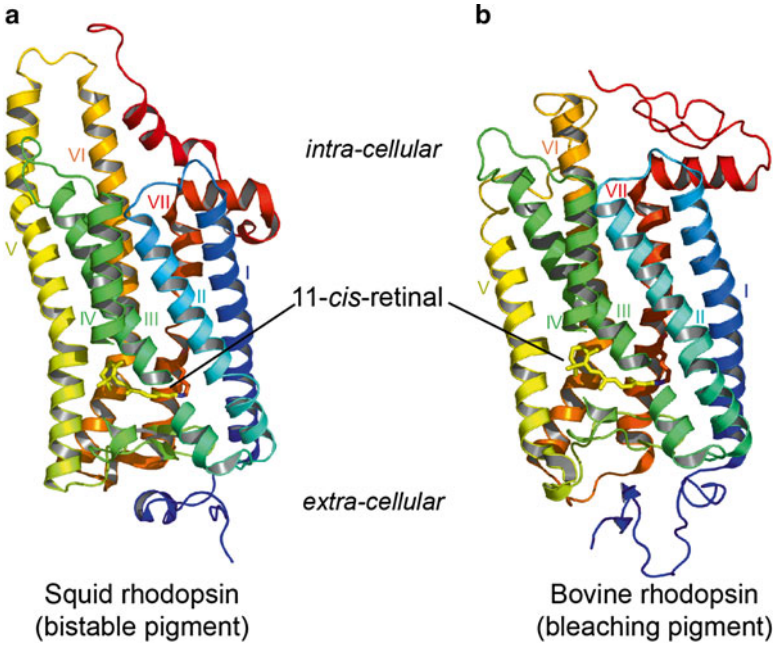

Fig. 7.1 Structures of bistable and bleaching photopigments. Crystal structures of the dark states of a bistable pigment (**a**, squid rhodopsin, PDB ID 2Z73) (Murakami and Kouyama 2008) and a bleaching pigment (**b**, bovine rhodopsin, PDB ID 1U19) (Okada et al. 2004) are shown. The chromophore 11-*cis*-retinal and the numbering of transmembrane helices are indicated. The models based on crystal structures were prepared using the software PyMOL (http://www.pymol.org/)

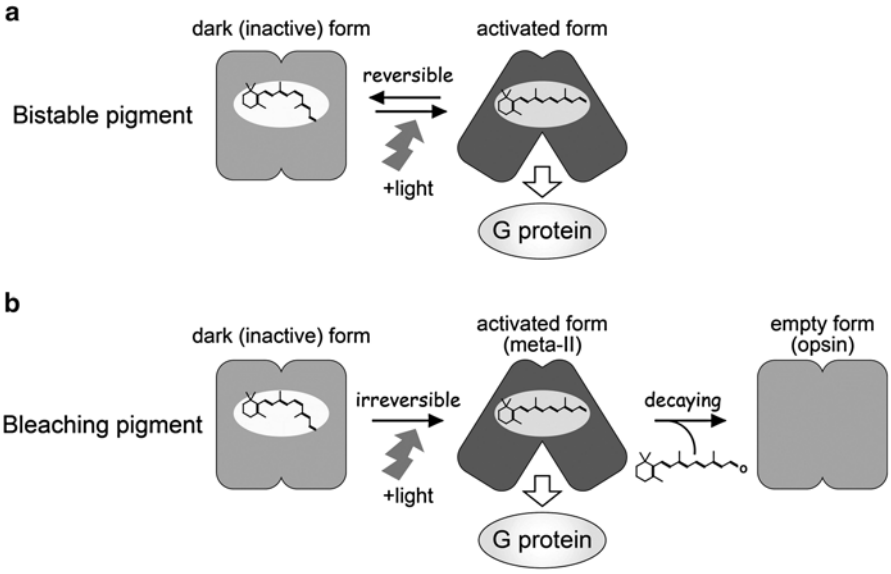

Fig. 7.2 Photo and thermal reactions of bistable and bleaching photopigments. For bistable pigments, the dark (inactive) form with 11-*cis*-retinal and the photoactivated form with all-t*rans*-retinal are inter-convertible by irradiation (**a**). The activated form is thermally stable. For bleaching pigments, photo-conversion from the dark form to meta-II, the photo-activated form, is unidirectional (**b**). Also, the meta-II state is thermally unstable and dissociates to the empty form opsin and free all-*trans*-retinal

(all-*trans*) retinal and a protein moiety opsin (Wald 1968) (Fig. 7.2b). Notably, irradiation of meta-II does not cause re-isomerization of the retinal back to 11-*cis* configuration (Bartl et al. 2001) (Fig. 7.2b). Such photopigments are called as "bleaching pigments" or "bleaching rhodopsins," because meta-II and opsin do not absorb visible light (Terakita 2010; Tsukamoto and Terakita 2010). On the other hand, studies of squid (*Todarodes pacificus*) and octopus (*Enteroctopus dofleini*) rhodopsins in the 1950s revealed the existence of photopigments with different properties in invertebrate visual cells (Hubbard and St George 1958; Kropf et al. 1959). Irradiation of the invertebrate visual pigments also causes isomerization of the retinal from 11-*cis* to all-*trans* form, and the active photoproduct activates G proteins. However, unlike meta-II, the photoproduct is thermally stable (Hillman et al. 1983; Hubbard and St George 1958) (Fig. 7.2a). Furthermore, the photoproduct can be reconverted back to the original (dark) form by subsequent photo-absorption (Hubbard and St George 1958; Ostrovsky and Weetall 1998) (Fig. 7.2a). The invertebrate rhodopsins are therefore called "bistable photopigments" or "bistable rhodopsins" (Hillman et al. 1983; Terakita 2005, 2010; Tsukamoto and Terakita 2010). Classically, it had been thought that invertebrate rhodopsins (and rhodopsin-like photopigments) are bistable and vertebrate pigments are bleaching (Hillman et al. 1983; Stavenga and Schwemer 1984), but it is now clear that in various vertebrate non-visual

photoreceptor cells, bistable photopigments act as photoreceptive proteins (Koyanagi et al. 2004; Peirson and Foster 2006; Terakita 2010). For example, in lamprey (*Lethenteron camtschaticum*) pineal and parapineal organs, the UV-absorbing bistable photopigment, parapinopsin, is present (Chap. 1) (Koyanagi et al. 2004), and in the intrinsically photosensitive retinal ganglion cells (ipRGCs) of the mammalian retina, including human one, melanopsin, which has very similar amino acid sequence to invertebrate visual rhodopsins (Provencio et al. 1998, 2000), is found (Sect. 7.3.2 and Chap. 2) (Berson et al. 2002; Hattar et al. 2002). This means that an understanding of the diversity and functional properties of bistable photopigments is essential for the understanding of photoreception not only in invertebrates but also in vertebrates. In this chapter, the biochemical and spectroscopic properties of bistable photopigments, the functional variety of bistable pigments, and the molecular mechanisms underlying the bistability will be discussed and summarized.

7.2 Biochemical and Spectroscopic Properties of Bistable Photopigments

As mentioned above, bistable pigments are characterized by stable photoproducts and photo-interconvertibility between 11-*cis* and all-*trans* retinal bound forms (Fig. 7.2a). The active photoproduct meta-II of bleaching pigment has quite different properties. In addition, there are other spectroscopic and biochemical differences between bistable and bleaching pigments.

Meta-II absorbs UV light at around 380 nm (Wald 1968), but most of the active photoproducts of bistable pigments absorb visible light (Hillman et al. 1983; Stavenga and Schwemer 1984). This is because the Schiff base linkage between the retinal and the lysine residue in opsin [Lys-296$^{7.43}$ in bovine (*Bos Taurus*) rod opsin, the superscript gives the Ballesteros–Weinstein numbering (Ballesteros and Weinstein 1995)] (Findlay and Pappin 1986; Hargrave et al. 1983; Pitt et al. 1955) is deprotonated in meta-II, but protonated in the active photoproduct of bistable pigment. The difference in the protonation state of the photoproducts is highly correlated with the position of the counterion (Terakita et al. 2000, 2004), which stabilizes the proton on Schiff base. The counterion of bistable pigments is a glutamic acid at position 181 (Glu-181) (Terakita et al. 2000, 2004) (Fig. 7.3a) and that of bleaching ones is Glu-113$^{3.28}$ (Sakmar et al. 1989; Zhukovsky and Oprian 1989) (Fig. 7.3b). In meta-II, the proton on the Schiff base cannot be stabilized by the counterion Glu-113$^{3.28}$ (Jager et al. 1994), leading to deprotonation of the Schiff base (Fig. 7.3b), but in the active photoproduct of bistable pigments, the proton is stabilized by Glu-181 (Fig. 7.3a) (Terakita et al. 2004). The difference in counterion position and protonation state of the Schiff base should be responsible for differences in the photoproduct properties. Furthermore, the difference in counterion is responsible for the difference in G protein activation by bistable and bleaching pigments. When bistable and bleaching pigments are reconstituted with G protein in vitro, bleaching

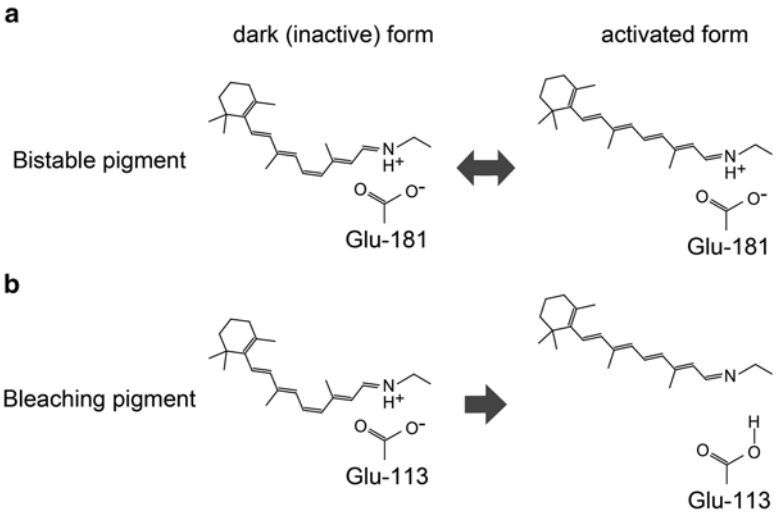

Fig. 7.3 Schiff base and counterion interactions in bistable and bleaching photopigments. For bistable pigments, Glu-181 stabilizes a protonated Schiff base between retinal and the opsin protein in both the dark (inactive) and the activated forms (**a**). For bleaching pigments, Glu-113$^{3.28}$ stabilizes a protonated Schiff base in the dark form, but in the photoactivated form (meta-II), the proton is transferred to Glu-113$^{3.28}$ resulting in formation of a deprotonated Schiff base, which absorbs UV light at around 380 nm (**b**). Note that in some bistable and bleaching pigments, the protonated Schiff base may be stabilized indirectly by the counterion (Glu-113$^{3.28}$ or Glu-181) via the mediation of water molecules and/or other amino acid residues

pigments can activate G protein about 20–50-fold more efficiently than bistable pigments (Terakita et al. 2004). Interestingly, the different G protein activation efficiency is not due to differences in the amino acid sequence of G protein interaction site between both pigments (Terakita et al. 2002; Tsukamoto et al. 2009); a comparative fluorescence labeling study on bistable and bleaching pigments revealed rather that the difference is due to different "amplitudes" of conformational changes upon photo-absorption (Sect. 7.4) (Tsukamoto et al. 2009). In addition, there is another important difference between bistable and bleaching pigments. As already mentioned, the active photoproducts of bistable and bleaching pigments possess all-*trans*-retinal. Interestingly, when exogenous all-*trans*-retinal is added directly to "empty" protein opsin (Fig. 7.4), for some bistable pigments, an active product, which couples with G protein, is formed (Ashida et al. 2004; Tsukamoto et al. 2005; Yamashita et al. 2010), whereas the addition of all-*trans*-retinal to opsin of bleaching pigments does not cause the formation of meta-II (Jager et al. 1996). The ability to form a stable association with all-*trans*-retinal may be general characteristics of bistable pigments, but the direct binding of all-*trans*-retinal is not observed in all bistable pigments. This means that bistable pigments do not necessarily require specific isomerase enzymes to produce 11-*cis*-retinal (Montell 2012), whereas such enzymes are necessary in vertebrate visual cells in order to supply 11-*cis*-retinal for bleached photopigments (Kiser et al. 2012).

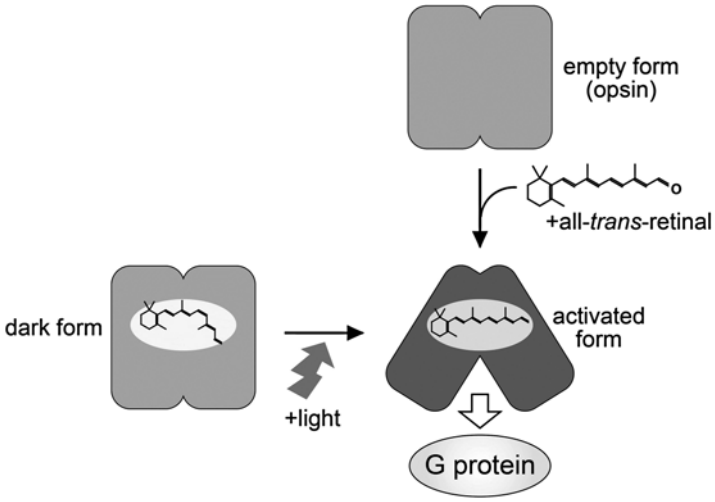

Fig. 7.4 A model for direct binding of all-*trans*-retinal to ligand-free opsin. In some bistable pigments, exogenous all-*trans*-retinal can bind directly to ligand-free opsin, resulting in the formation of the same state as the photoactivated state having photo-isomerized all-*trans*-retinal. In contrast, such a direct binding of all-*trans*-retinal does not occur in bleaching pigments (Jager et al. 1996)

7.3 Diversity of Bistable Photopigments

So far, more than 2,000 kinds of rhodopsin and rhodopsin-like photopigments have been identified from various animal species (Terakita 2010). They are referred to as an "opsin family," and the family can be classified into at least ten groups based on amino acid sequence similarity and functional properties (Terakita 2010). A schematic phylogenetic relationship of the respective groups in the opsin family is shown in Fig. 7.5. Out of the ten groups, the vertebrate visual pigment group contains bleaching pigments including rod and cone opsins (Yokoyama 2000) (Chap. 6). Each group has its particular molecular characteristics.

7.3.1 Gq-Coupled Visual Pigment Group

This group contains invertebrate visual pigments including squid, octopus and *Drosophila* rhodopsins, all of which were established as bistable pigments several decades ago (Hillman et al. 1983; Hubbard and St George 1958). These pigments function in invertebrate visual photoreceptor cells and are coupled to Gq-type G proteins (Koyanagi and Terakita 2008). Spectroscopic properties of various invertebrate visual cells have been analyzed, and it is known that members of this group absorb across a remarkably wide spectrum of wavelength light from UV (~350 nm) to red (~600 nm) (Chaps. 4 and 5) (Hillman et al. 1983; Stavenga and Schwemer 1984).

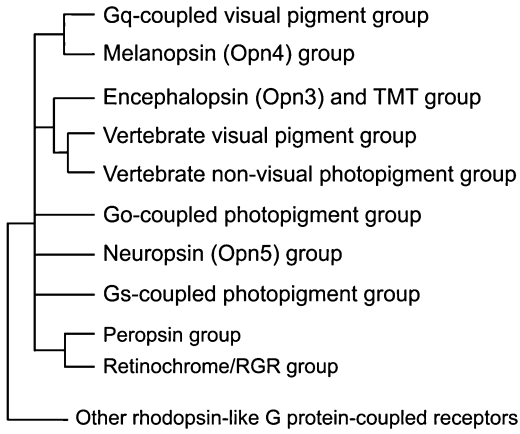

Fig. 7.5 Phylogenetic relationships of the different photopigment groups within the opsin family. Phylogenetic relationships of ten groups within the opsin family, as well as other rhodopsin-like GPCRs, are shown schematically. Various bistable (like) pigments are classified into their respective groups (text for details). See Koyanagi and Terakita (2008) and Terakita (2005) for more detailed phylogenetic trees

Squid and octopus rhodopsins can be purified from native tissue (Kropf et al. 1959), and a *Drosophila* rhodopsin (Rh1) can be purified from transgenic flies (Vought et al. 2000). These purified rhodopsins exhibit stable photoproduct and photo-interconvertibility, although the photoproduct of *Drosophila* rhodopsin is a little destabilized after purification (Vought et al. 2000). In addition, octopus opsin can bind exogenous all-*trans*-retinal directly to form the same product as the photoproduct (Ashida et al. 2004). It should be noted that in squid, octopus and *Drosophila* eyes, there are enzymes producing 11-*cis*-retinal in light-dependent and/or independent manner (Hara and Hara 1967; Hara et al. 1967; Montell 2012). Thus, their inactive (11-*cis*-retinal bound) forms are probably formed not only by photo-conversion from the active photoproduct but also by the enzymatic pathways that form the "visual cycle" (Montell 2012).

Heterologous expression of Gq-coupled visual pigments in cultured cells has been very difficult (Knox et al. 2003), and this has hampered progress in the understanding of the structure–function relationships of these pigments. However, recently honeybee (*Apis cerana japonica*) and jumping spider (*Hasarius adansoni*) visual pigments were successfully expressed in HEK-293 cells and characterized spectroscopically and biochemically (Nagata et al. 2012; Terakita et al. 2008); it is anticipated that mutational studies will be possible with these Gq-coupled pigments and that this will enable further progress to be made in the understanding of structure–function relationships.

7.3.2 Melanopsin (Opn4) Group

Melanopsin (or Opn4) (Provencio et al. 1998, 2000) is a photopigment that works in ipRGCs of mammals (Chap. 2) (Berson et al. 2002; Hattar et al. 2002). Non-mammalian vertebrates and some invertebrates also possess melanopsins

(Bellingham et al. 2006; Koyanagi and Terakita 2008). Interestingly, the melanopsin group can be divided into two subgroups, named as Opn4x and Opn4m, respectively (Bellingham et al. 2006; Koyanagi and Terakita 2008). Mammals retain only Opn4m type melanopsin, and many non-mammalian vertebrates have both Opn4x and Opn4m types (Bellingham et al. 2006; Koyanagi and Terakita 2008). Most of melanopsins including Opn4m and Opn4x are reported to absorb blue light around ~480 nm (Davies et al. 2011; Isoldi et al. 2005; Koyanagi et al. 2005; Matsuyama et al. 2012; Terakita et al. 2008; Torii et al. 2007), which is consistent with an action spectrum of ipRGCs (Berson et al. 2002). In this regard, melanopsin differs from other Gq-coupled visual pigments, as members of this group show wide variety of spectral sensitivities from UV-absorbing to red-absorbing (Hillman et al. 1983; Stavenga and Schwemer 1984).

Because the amino acid sequence of melanopsin is very similar to that of Gq-coupled invertebrate visual pigments (Provencio et al. 1998), melanopsin is considered to be bistable and coupled to Gq. Melanopsin from amphioxus (*Branchiostoma belcheri*), a chordate closely related to vertebrates, is clearly bistable and Gq-coupled (Gomez Mdel et al. 2009; Koyanagi et al. 2005; Terakita et al. 2008). Also, using cultured cells, it is reported that mouse and human melanopsins can couple with and activate Gq (Fu et al. 2005; Melyan et al. 2005; Panda et al. 2005; Qiu et al. 2005). Recently, purified mouse melanopsin expressed in HEK-293 cells as a recombinant protein was shown to be bistable (Matsuyama et al. 2012), although a previous study had suggested that photoreaction of mouse melanopsin purified from ipRGCs in transgenic mice is complex and probably not bistable (Walker et al. 2008). This discrepancy suggests that the photoproduct properties of mouse melanopsin are highly sensitive to the surrounding environment (detergents, lipids, and so on). The bistable nature of mouse melanopsin is controversial at the level of cellular responses; one study (Mure et al. 2007) reported that photo-interconvertibility between inactive and active forms of melanopsin can modulate melanopsin-mediated cellular responses such as pupillary light reflex, but another study challenged this observation (Mawad and Van Gelder 2008). In contrast, zebrafish melanopsins clearly show variability; of the five different melanopsins identified, two are bistable and three are monostable (Davies et al. 2011). Taken together, melanopsin group may contain some photopigments that are not bistable.

Direct binding of all-*trans*-retinal to an opsin moiety of melanopsin is also controversial. Melanopsin possess 11-*cis*-retinal as its chromophore, and photoreception results in isomerization to all-*trans*-retinal (Matsuyama et al. 2012; Walker et al. 2008). Several studies showed that melanopsin becomes photosensitive after incubation with all-*trans*-retinal as well as 11-*cis* or 9-*cis* retinal in the dark (Fu et al. 2005; Melyan et al. 2005; Panda et al. 2005; Peirson and Foster 2006). If so, in melanopsin proteins, all-*trans*-retinal should be isomerized to 11-*cis* retinal in a light-independent rather than a light-dependent manner (Sexton et al. 2012). The mechanism for the light-independent isomerization of retinal in melanopsin is not fully understood yet. Thus, further analyses are required to determine unequivocally whether melanopsin can bind exogenous all-*trans*-retinal or not.

7.3.3 Encephalopsin (Opn3) and TMT Group

Encephalopsin (or Opn3) was originally found in the brain as well as other tissues of mouse and human (Blackshaw and Snyder 1999), and homologs of encephalopsin have been found in other vertebrates and invertebrates. Encephalopsin shows relatively high sequence similarity to vertebrate visual (bleaching) pigments (Terakita 2005). Since encephalopsin-like pigments of teleost fishes exist in various tissues, these are named as TMT (Teleost-Multiple-Tissue) opsins (Moutsaki et al. 2003). The photochemistry and photoreaction of mammalian encephalopsin is as yet unknown, but recent studies on encephalopsin (or TMT) orthologs from puffer fish (*Takifugu rubripes*) and mosquito (*Anopheles stephensi*) showed that these pigments are bistable (Koyanagi et al. 2013). These orthologs also activate Gi/o in light-dependent manner (Koyanagi et al. 2013). It is very intriguing if encephalopsins in the mammalian brain are photosensitive, since the demonstration of photoreception in mammalian brains has so far proved elusive (Chap. 3).

7.3.4 Vertebrate Non-visual Photopigment Group

This group includes parapinopsin (Blackshaw and Snyder 1997) and VA-opsin (Soni et al. 1998), two pigments that are more like vertebrate visual pigment than encephalopsins. Parapinopsin was originally identified in the parapineal organ of catfish (*Ictalurus punctatus*) (Blackshaw and Snyder 1997). In the lamprey, parapinopsin functions in both the parapineal and pineal organs (Koyanagi et al. 2004). Lamprey parapinopsin absorbs UV light and shows the typical behavior of a bistable pigment (Koyanagi et al. 2004). The lamprey parapinopsin was the first bistable pigment found in vertebrates. VA-opsin was originally identified in salmon (*Salmo salaar*) (Soni et al. 1998) and subsequently in zebrafish (*Danio rerio*) (Kojima et al. 2000). Recently, spectroscopic properties of a frog (*Xenopus tropicalis*) VA-opsin were revealed (Sato et al. 2011). Interestingly, the frog VA-opsin is converted to a stable photoproduct upon irradiation, but the photoproduct is photo-insensitive and cannot be reconverted back to the original (dark) state (Sato et al. 2011). Thus, the frog VA-opsin has an intermediate property between bistable and bleaching photopigments. It should be noted that pinopsin, which was discovered in chicken (*Gallus gallus*) pineal organ as a non-visual rhodopsin-like photopigment (Okano et al. 1994), has even higher amino acid sequence identity to vertebrate visual pigments, and the photoproduct shows a typical bleaching behavior (Nakamura et al. 1999).

7.3.5 Go-Coupled Photopigment Group

A Go-coupled photopigment was originally found in scallop (*Mizuhopecten yessoensis*) (Kojima et al. 1997), but whether it is bistable is as unknown. Subsequently, it was reported that amphioxus possesses an ortholog of Go-coupled pigment and

that this pigment can be expressed efficiently in HEK-293 cells (Koyanagi et al. 2002). Further characterization of the amphioxus pigment clearly showed that it is bistable and can bind exogenous all-*trans*-retinal directly to form the same product as the native photoproduct having photo-isomerized all-*trans*-retinal (Tsukamoto et al. 2005). Thus, current evidence strongly suggests that Go-coupled photopigments are typical bistable pigments.

7.3.6 Neuropsin (Opn5) Group

Neuropsin (or Opn5, Opsin 5) was identified in human and mouse neural tissue (Tarttelin et al. 2003), but its function is still unknown. Recently, neuropsins from quail (*Coturnix japonica*) (Nakane et al. 2010) and chicken (*Gallus gallus*) (Yamashita et al. 2010) were characterized in detail. Quail neuropsin is violet-sensitive (Nakane et al. 2010) and chicken neuropsin is UV-sensitive (Yamashita et al. 2010). Interestingly, the quail neuropsin is shown to be Gq-coupled (Nakane et al. 2010), whereas the chicken form is reported to be Gi-coupled (Yamashita et al. 2010); this discrepancy may reflect a true species difference or a difference in experimental methods. The chicken neuropsin exhibits the typical behavior of a bistable pigment and binds all-*trans*-retinal directly to form the equivalent photoproduct (Yamashita et al. 2010). Mouse and human neuropsins are also UV-sensitive bistable pigments (Kojima et al. 2011). In the mouse, neuropsin has been shown to be Gi/o-coupled and is localized in eye, brain and ear tissues (Kojima et al. 2011).

7.3.7 Gs-Coupled Photopigment Group

Rhodopsin-like pigments have been identified in Cnidarians, and they make up one group of the opsin family (Koyanagi et al. 2008; Kozmik et al. 2008; Suga et al. 2008). Among these pigments, a jellyfish (*Carybdea rastonii*) opsin is the most characterized. It absorbs maximally at around 500 nm, and forms a stable photoproduct upon irradiation (Koyanagi et al. 2008). However, the reverse photoreaction back to the original state was not observed, suggesting that this pigment is not a typical bistable pigment. It co-localizes with Gs in the visual cells and causes an elevation of intracellular cAMP level in a light-dependent manner, indicating that the pigment is Gs-coupled (Koyanagi et al. 2008).

7.3.8 Peropsin Group and Retinochrome/RGR Group

Peropsin (Sun et al. 1997), retinochrome (Hara and Hara 1967; Hara et al. 1967) and RGR (Shen et al. 1994) are not described here in detail because, unlike conventional rhodopsin-like pigments, these pigments possess all-*trans*-retinal in the dark

and the retinal is converted to 11-*cis* form in a light-dependent manner. They are therefore thought to act as photoisomerases (probably not GPCRs) that supply 11-c*is*-retinal for other (conventional) photopigments. It should be noted that the peropsin from the jumping spider shows bistable-like behavior as it is photo-interconvertible between all-*trans*-retinal bound (dark) and 11-*cis*-retinal bound (light) forms (Nagata et al. 2010).

7.4 Structural Features of Bistable Photopigments

In previous sections, I have described the functional properties of diversified bistable photopigments, and those properties were compared with those of bleaching pigments. In this section, structure–function relationships of bistable and bleaching pigments are compared, and the molecular mechanisms underlying the behavior of bistable pigments are discussed.

The structural features of bovine rod opsin, a typical bleaching pigment, have been extensively studied, because it can be easily obtained from retinal tissue and recombinant mutant proteins can be highly expressed in mammalian cultured cells. Currently, crystal structures of the dark state (11-*cis*-retinal bound) and meta-II (all-*trans*-retinal bound) of the bovine pigment have been solved (Choe et al. 2011; Li et al. 2004; Okada et al. 2004; Palczewski et al. 2000; Standfuss et al. 2011) (Figs. 7.1 and 7.6), and the dynamic activation process has been illuminated through the application of various biophysical techniques including site-directed labeling and NMR (Ahuja and Smith 2009; Farrens 2010; Hubbell et al. 2003). This abundant structural information has now enabled the question as to how meta-II is formed subsequently by photoreception to be addressed. Photo-absorption causes isomerization of the retinal to the all-*trans* form. The β-ionone ring moiety of the isomerized retinal directly interacts with transmembrane helix (helix VI) (Ahuja et al. 2009), and the proton on the Schiff base is transferred to the counterion Glu-113$^{3.28}$ (Fig. 7.3b) (Jager et al. 1994). The steric interaction of the retinal with helix VI in the transmembrane region and the changes in protonation states around the retinal leads to a rearrangement of the hydrogen-bonding network around the cytoplasmic end of helix VI, resulting in a dramatic outward movement of the cytoplasmic region to interact with the G protein (Farrens et al. 1996; Sheikh et al. 1996) (Fig. 7.6). Recent progress in the crystallographic analyses of GPCRs other than rhodopsin has revealed that a similar rearrangement of the hydrogen-bonding network and the movement of helix VI are involved in agonist-induced GPCR activation (Katritch et al. 2012; Lebon et al. 2011; Rasmussen et al. 2011a, b). It might be expected then that, in the formation of an active photoproduct for the bistable pigment, similar conformational changes are involved. Biochemical and spectroscopic properties are however quite different between bistable and bleaching pigments (Sect. 7.2). For example, proton transfer from Schiff base to counterion (Jager et al. 1994) would not be expected in activation of the bistable pigment, because in the active photoproduct of bistable pigments, the Schiff base is still protonated (Sect. 7.2 and Fig. 7.3a).

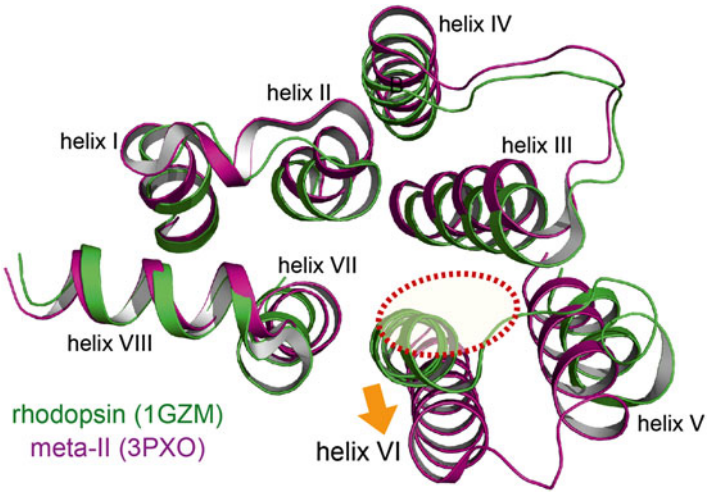

Fig. 7.6 Outward movement of the cytoplasmic end of the sixth transmembrane helix upon activation of rhodopsin. Cytoplasmic regions of crystal structures of the dark state (*green*, PDB ID 1GZM) (Li et al. 2004) and the photo-activated state (meta-II) (*magenta*, PDB ID 3PXO) (Choe et al. 2011) of bovine rhodopsin are shown. A major difference between the two states is the outward movement of helix VI (*orange arrow*). The binding site of the C-terminus of the **α**-subunit of the G protein is shown as a *broken red circle*. This G protein-binding site is not accessible in the dark (inactive) form. The molecular models based on crystal structures were prepared using the software PyMOL (http://www.pymol.org/)

Compared with the abundant structural information for bleaching pigments, structural studies of bistable pigments are limited, although there has been some recent progress. The only available crystal structure for a bistable pigment at atomic resolution is for the dark state (11-*cis*-retinal bound) of squid rhodopsin (Murakami and Kouyama 2008) (Fig. 7.1a), which is a member of Gq-coupled visual pigment group. The crystal structure is very similar to that of the dark state of bovine rhodopsin especially in the transmembrane regions (Schertler 2008) (Fig. 7.1). This is not entirely surprising as the structure of transmembrane regions in inactive states is highly conserved not only among rhodopsins but also in other rhodopsin-like GPCRs (Katritch et al. 2012). So, structural differences underlying the different properties of bistable and bleaching pigments may be largely restricted to the active photoproducts.

A study using site-directed fluorescence labeling provides some clues for the structural differences between bistable and bleaching pigments. The study compared conformational changes in bistable lamprey parapinopsin (Sect. 7.3.4) and bleaching bovine rhodopsin arising from the formation of the active photoproducts (Tsukamoto et al. 2009). In both pigments, a light-induced outward movement of the cytoplasmic end of helix VI was observed through a fluorescent probe attached on the helix. Interestingly, the observed light-induced movement of helix VI was less in the bistable pigment than in the bleaching one. In other words, the “amplitude” of the

conformational change was much larger in the formation of meta-II. Originally (in 2009), this result was interpreted as reflecting a different rearrangement of helix VI on activation of bistable and bleaching pigments (Tsukamoto et al. 2009).

In 2011, the crystal structures of the β-adrenergic receptor, a member of rhodopsin-like GPCR family, with its G protein or its mimetic were reported (Rasmussen et al. 2011a, b) as a major breakthrough in the study of GPCRs (Buchen 2011). These crystal structures clearly show that the arrangement of transmembrane helices is also conserved in the active conformation of GPCRs (Steyaert and Kobilka 2011), strongly suggesting that a similar arrangement helices exists in the active photoproduct of bistable pigments. Why then was a smaller amplitude of helix VI movement observed in the photoactivation of the bistable pigment? The different amplitudes of conformational changes can be interpreted as follows. When a bleaching pigment absorbs light, helix VI moves to a new position in the active conformation of meta-II (Fig. 7.7a), whereas in the photoactivation of a bistable pigment, helix VI again moves but the photoproduct remains in dynamic equilibrium between inactive and active conformations (Fig. 7.7b). Since the fluorescence labeling analysis measured an average movement of the helix, the apparent amplitude is therefore smaller in bistable pigment. This interpretation is supported by two facts. Firstly, the difference in G protein activity between bistable and bleaching pigments. As mentioned in Sect. 7.2, the G protein activation efficiency of bistable pigments

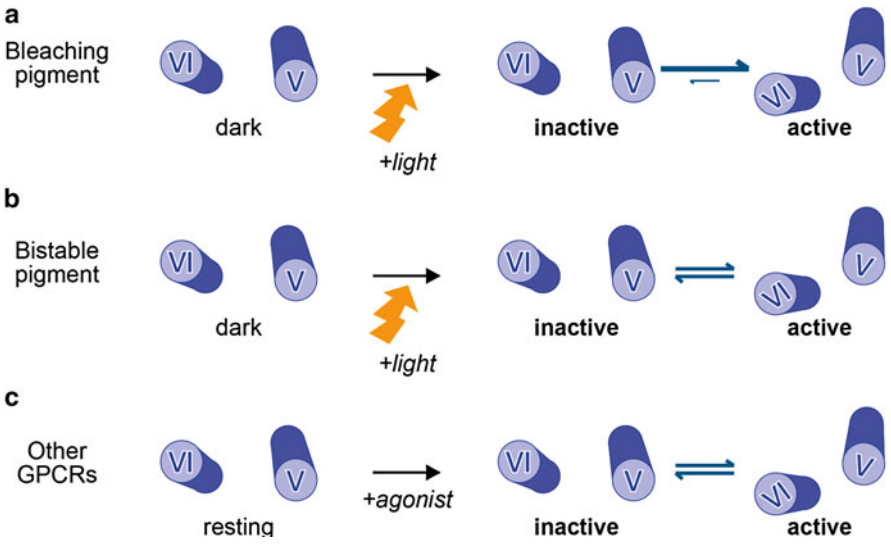

Fig. 7.7 A model for the conformational dynamics of activated bleaching and bistable photopigments, and for typical agonist-activated GPCRs. In these cartoons, the relative orientations of the cytoplasmic ends of helices V and VI in bleaching and bistable pigments as well as other rhodopsin-like GPCRs before and after activation are shown schematically. As mentioned in the text, in the activated state, the equilibrium between inactive and active conformations for bleaching pigments (meta-II) is highly constrained to the active one (**a**). In contrast, in the activated bistable pigment (**b**) and other GPCRs (**c**), the inactive and active conformations are in a more dynamic equilibrium

is 20–50-fold lower than that of bleaching pigments (Terakita et al. 2004). This observation is consistent with the interpretation that meta-II is in a "fully" activated state, whereas the activated bistable pigment is a "partially" activated state (Fig. 7.7a, b). Secondly, the crystal structure of meta-II shows complete movement of helix VI even in the absence of G protein or its mimetics (Choe et al. 2011), whereas the crystal structures of some GPCRs including the β-adrenergic receptor with agonists but without G protein mimetics show almost no rearrangement of helix VI (Rosenbaum et al. 2011; Warne et al. 2011), although biophysical studies of the β-adrenergic receptor detected significant agonist-induced movement of helix VI (Yao et al. 2006). This suggests that in these GPCRs, the agonist-bound state is in equilibrium between inactive and active conformations (Fig. 7.7c) and the equilibrium is shifted toward the inactive form in the crystallized state. That idea is confirmed by a recent study of the dynamics of the β-adrenergic receptor using NMR and molecular dynamics simulation (Nygaard et al. 2013). In a similar way, and unlike meta-II, the active photoproducts of bistable pigments would be also in equilibrium between inactive and active conformations (Fig. 7.7b); this dynamic nature of the photo-activated bistable pigment may enable a back reaction to the original dark state upon a second photo-absorption, because even after photoactivation, some proportion retains an "inactive" conformation that is very similar to the dark state (Fig. 7.7b). Further progress in crystallographic and biophysical studies on various bistable pigments is needed to clarify how the structural features of bistable pigments related to their functional characteristics. In particular, the molecular mechanisms within bistable pigment underlying interaction with G proteins, photointerconvertibility and direct binding to all-*trans*-retinal need to be understood in more detail for bistable pigments.

7.5 Application of Bistable Photopigments for optogenetics

As already described, bistable pigments have very interesting characteristics and show functional diversity. In particular, there are bistable pigments that are coupled to Gq, Gi/o or Gs-type G proteins (Terakita 2010), although a jellyfish Gs-coupled opsin may not be strictly bistable (Koyanagi et al. 2008). Also, some bistable pigments can bind all-*trans*-retinal directly, unlike bleaching photopigments. These features of bistable pigments raise the possibility of their use as a tool for optogenetics (Koyanagi and Terakita 2014).

Optogenetics is a method whereby the activities of specific neural cells can be stimulated (or suppressed) by light irradiation (Bernstein et al. 2012; Boyden et al. 2005; Zhang et al. 2007). Currently, most of optogenetics studies use channelrhodopsins and its derivatives, which are light-gated cation channels (Hegemann and Nagel 2013; Inagaki et al. 2014; Nagel et al. 2003). It should be noted however that channelrhodopsins are phylogenetically close to the bacterial rhodopsins that include the light-driven proton pump bacteriorhodopsin (Hou et al. 2012) and show little amino acid sequence identity to animal rhodopsins (bleaching and bistable photopigments).

If the channelrhodopsins are expressed in specific cells and stimulated by light, ions will flow into the cells and the cells will be excited. Optogenetics using channelrhodopsins has been substantially developed and expanded, but there are some limitations. For example, light-dependent manipulation of intra-cellular Ca^{2+} concentrations in the important process of Ca^{2+} signaling is currently difficult using channelrhodopsins. Also, there are signaling cascades that are not initiated by ion permeation. The application of bistable photopigments as new tools may overcome at least some of the limitations in current optogenetics.

Invertebrate visual pigments and melanopsins are Gq-coupled, and activation of Gq-type G protein results in elevation of intracellular Ca^{2+} concentration via phospholipase C. This fact suggests that these Gq-coupled pigments can be used as an optogenetics tool for controlling intracellular Ca^{2+} levels by light (Zemelman et al. 2002). One study has already shown that intracellular glucose levels can be controlled in a light-dependent manner via Ca^{2+} level elevation by melanopsin expressed in specific cells (Ye et al. 2011). Most melanopsins absorb blue light at around 480 nm (Sect. 7.3.2), but members of Gq-coupled visual pigment group show a wide variety of spectral properties. So, if Gq-coupled visual pigments can be used for optogenetics, Ca^{2+} signaling in different sets of cells could be manipulated simultaneously or separately using different Gq-coupled pigments absorbing different colored light.

In addition, jellyfish Gs-coupled opsin was shown to be a potential tool for inducing light-dependent increases of cAMP levels in specific cells (Bailes et al. 2012; Karunarathne et al. 2013). In contrast, pufferfish and mosquito Opn3 (Gi/o--coupled) could be used as a tool for decreasing intercellular cAMP levels in a light-dependent manner (Koyanagi et al. 2013). Taken together, by using bistable pigments as optogenetics tools, the possibility exists to control several signaling cascades by light irradiation. Bistable pigments have further potential advantage for optogenetics. As described above, bistable pigments are interconvertible between inactive (11-*cis*-retinal bound) and active (all-*trans*-retinal bound) states. As the two states of some bistable pigments absorb very different wavelengths of light [e.g. parapinopsin (Koyanagi et al. 2004) or Opn5 (Kojima et al. 2011; Yamashita et al. 2010)], then the conversion between "ON" and "OFF" states becomes an all-or-none process by irradiation with different colored light. Thus, optogenetics using such bistable pigments could modulate "ON" processes as well as "OFF" processes in some intracellular signaling cascades.

There is one potential limitation for optogenetics using bistable pigments; they use 11-*cis*-retinal as the chromophore. So, specific enzymes may be required within the target cells for chromophore supply. This limitation may not apply however, to the bistable pigments that can bind exogenous all-*trans*-retinal directly, as the isomer is thought to exist in various cell types, at least in mammals. The evidence for this is that channelrhodopsins, which require all-*trans*-retinal as the chromophore, can function in a variety of cells without the supplementation of the retinal. Also pragmatically, melanopsin-induced (Ye et al. 2011) and Opn3-induced (Koyanagi et al. 2013) cellular responses are observed without supplement of retinal [although jellyfish Gs-coupled opsin requires exogenous retinal for significant amounts of

light-dependent cAMP production (Bailes et al. 2012)]. Alternatively, co-expression of retinochrome, which is a photoisomerase making 11-*cis*-retinal from all-t*rans*-retinal in light-dependent manner (Sect. 7.3.8), could solve the supply problem of 11-*cis*-retinal. Taken together, bistable photopigments are a promising new tool for a next-generation optogenetics, and such studies are expected to develop in the future.

7.6 Conclusions

In this chapter, the molecular characteristics and diversity of bistable photopigments have been reviewed. In addition, structural features underlying the characteristics of bistable pigments have been discussed, and the potential application of bistable pigments as a tool for optogenetics has been outlined. All aspects described here clearly indicate that bistable pigments are very interesting and useful molecules for the wide range of biological studies that include crystallography, spectroscopy, molecular evolution, physiology, and optogenetics. It is confidently expected that the studies of bistable pigments will substantially extend the understanding of photo-reception and neural activities in various animal species.

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Chapter 8
Visual Photopigment Evolution in Speciation

Karen L. Carleton

Abstract Visual pigment sensitivities are known to vary across organisms and habitats. The sensory drive theory was formulated over 20 years ago to help explain how such sensory variation could contribute to divergent selection and speciation. Since then, there have been only a few examples that support the idea that visual pigment evolution contributes to speciation. Here, I discuss what is required to demonstrate that evolution of visual pigments (and visual sensitivities) play a role speciation. I then identify systems where visual pigments are unlikely to have a role, where they might play a role, and where they likely have driven speciation. This review concludes that more examples are needed to identify instances where visual pigment evolution contributes to speciation and to determine how frequently sensory drive is at work.

Keywords Visual pigment • Opsin • Speciation • Sensory drive • Divergence

Abbreviations

A1	Vitamin A_1-derived 11-*cis* retinal
A2	Vitamin A_2-derived 3,4-didehydroretinal
M/LWS	Medium to long wavelength-sensitive
MSP	Microspectrophotometry
RH1	Rod opsin
RH2	Rod opsin like 2
SWS	Short wavelength-sensitive
UV	Ultraviolet

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8.1 Introduction

Speciation is the process by which one ancestral species gives rise to two (or more) descendent species (Coyne and Orr 2004). This immediately raises the question of what is a species. Although species are a fundamental concept in biology, their definition has been elusive if not contentious. Dobzhansky was one of the first to assemble a variety of species definitions and biological luminaries have discussed whether species are real or arbitrary constructs and how they might be defined (Dobzhansky 1935, 1937; Mayr 1942, 1982; Haldane 1956). The biological species concept of Ernst Mayr is one of the most intuitive, defining species as biological entities, which are reproductively isolated from other groups. This can be at odds with other species concepts, where a species is defined because it occupies a particular ecological niche, maintains mate recognition, or is phylogenetically distinct (de Queiroz 2005). These varied concepts can be reconciled by recognizing species as metapopulations or lineages that interbreed (de Queiroz 2005). This latter definition differs from that of Ernst Mayr in that it defines species by what they do (interbreed within their group) rather than what they do not do (interbreed with other groups). By either definition, individuals must breed more frequently with like (conspecific) than with unlike (heterospecific) individuals, for species to survive. Therefore, questions of mate choice and reproductive isolation remain closely linked in discussions of speciation.

Different evolutionary mechanisms contribute to reproductive isolation, and therefore to the creation or maintenance of species (Coyne and Orr 2004). The action of these mechanisms can be broken down by the life stage at which they act and include three critical times (Fig. 8.1): before individuals mate (pre-mating), after they mate but before the zygote forms (post-mating/pre-zygotic), and after the zygote has formed (post-mating/post-zygotic). Post-mating isolation mechanisms can be quite strong in some species and often have a genetic basis. Post-mating pre-zygotic isolation can result from non-competitive processes such as gamete incompatibility (sperm binding proteins do not recognize heterospecific eggs), or from competitive processes (conspecific sperm outcompete heterospecific sperm). Post-mating post-zygotic isolation can result when hybrids are developmentally or ecologically inviable, or sterile.

Since vision is unlikely to be important for post-mating isolation, its most likely role is its potential effect on pre-mating isolation. Pre-mating isolation may occur through differences in ecology, where species prefer to mate in particular habitats or at particular times (Coyne and Orr 2004). Habitat choice can be influenced by visual sensitivities, and might lead to a divergence in visual pigment peak absorbance. The other key mechanism for pre-mating isolation is the result of behavior, where either male/male competition or female/male mate choice determines which individuals breed. Variation in visual sensitivities may alter the interpretation of sensory cues used to choose whether and with whom to mate or with whom to fight. Different visual sensitivities could cause a change in mating success and lead to divergence within a population.

In this review, I will discuss ways in which vision might affect speciation. I first outline some mechanisms by which visual sensitivities and visual pigments can be

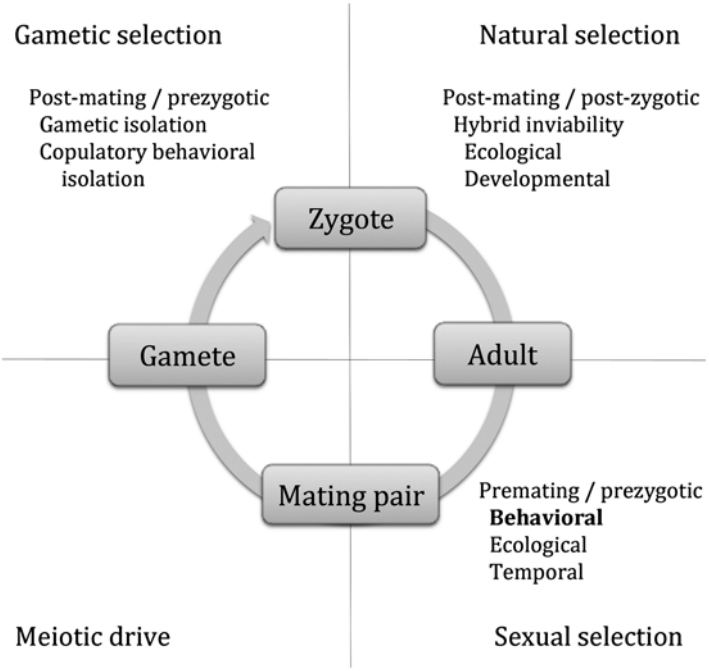

Fig. 8.1 Different forms of selection can act throughout the life cycle, altering phenotypes and causing speciation. Vision will play the biggest role in pre-mating isolation, when adults are choosing with whom to mate. Variation in visual sensitivities may increase and strengthen behavioral isolation between species by altering sexual selection through male–male completion or female mate choice. It might also influence ecological isolation if organisms adapt to different habitats

tuned. Next, I discuss theoretical models for how visual sensitivities might play a role in sexual selection and speciation. Then, I discuss several categories of systems including those where visual sensitivities are not likely to cause speciation, those where vision varies but it is unclear if this causes speciation, and then a few cases where visual evolution is likely to drive speciation. I finish with a discussion of what we have yet to learn about vision's role in speciation.

8.2 Visual Sensitivities and Visual Pigments

Several models propose that if visual sensitivities differ, these differences may help drive speciation (Sect. 8.3). Such differences might arise within an interbreeding population and lead to reduced mating between different parts of the population, thereby reducing gene flow and increasing reproductive isolation and the potential for divergence. While differences in higher order visual processing or visual preferences might contribute to the discrimination of visual signals, these are not yet well enough understood for us to speculate on their role. I will therefore confine this

discussion to differences in visual sensitivities, with an emphasis on the absorption spectra of the visual pigments. I will briefly summarize some different tuning mechanisms for changing visual sensitivities, although this is discussed more completely in Chaps. 4, 5 and 6 of this book.

A visual pigment is composed of an opsin protein bound to a chromophore, most typically 11-*cis* retinal (Wald 1968). The chromophore's absorption spectrum is shifted by interactions with certain amino acids of the opsin protein to produce visual pigments with peak sensitivities (λ_{max}) that vary across the spectrum from ultraviolet to red wavelengths. Five main classes of opsin genes arose early in vertebrate evolution (Yokoyama 2000; Ebrey and Koutalos 2001; Collin et al. 2003). These include the RH1 class found in rods, and four cone opsin classes: the very short wavelength-sensitive (SWS1) and short wavelength-sensitive (SWS2) classes, the medium wavelength-sensitive rod opsin-like (RH2) class and the long wavelength-sensitive class (LWS). Proteins from each class, in combination with a chromophore, produce visual pigments that absorb light in distinct parts of the spectrum. Invertebrates also have multiple opsin classes, referred to as UV, blue, and LWS (Briscoe and Chittka 2001; Wakakuwa et al. 2007; Briscoe 2008). In many invertebrates, the long class is green-sensitive, but in some species for example butterflies, pigment sensitivities can extend to red wavelengths.

There are several mechanisms by which the λ_{max} of a visual pigment can be varied. First, the amino acid sequence of a given opsin gene can change. Numerous studies have shown that key amino acids close to the retinal binding pocket seem to have the most significant effect, particularly if they change polarity (Chang et al. 1995; Yokoyama 2000; Hunt et al. 2007, 2009). Changes to λ_{max} are typically small (3–10 nm; Asenjo et al. 1994; Takahashi and Ebrey 2003; Yokoyama 2008), though there are a few sites in the very shortest-sensitive opsins, which can have large effect (60–70 nm; Wilkie et al. 2000; Yokoyama et al. 2000; Cowing et al. 2002; Fasick et al. 2002). Second, the class of opsin gene that is expressed in a particular photoreceptor type can be changed (Bowmaker 1995; Carleton and Kocher 2001; Fuller et al. 2005; Hofmann and Carleton 2009). A change in the expressed opsin class typically causes large spectral shifts (30–100 nm) as each class is sensitive to a different part of the spectrum (Hofmann et al. 2009). This mechanism has been found most commonly in fishes where different opsin genes are expressed at different developmental stages, although there are species where adult expression patterns differ between closely related species (Carleton et al. 2008; Shand et al. 2008; Hofmann et al. 2009). The third mechanism for tuning visual pigment λ_{max} is to change the chromophore bound to the opsin protein (Loew and Dartnall 1976; Munz and McFarland 1977; Beatty 1984). In both vertebrates and invertebrates, the vitamin A_1-derived 11-*cis* retinal (A1) can be replaced with the vitamin A_2-derived 3,4-didehydroretinal (A2). This mechanism occurs in fishes and reptiles, and produces smaller spectral shifts (10 nm) for short wavelength opsin classes and larger shifts (20–30 nm for RH2, 30–60 nm for LWS) for longer wavelength opsin classes (Harosi 1994; Parry and Bowmaker 2000). A1/A2 chromophore shifts also occur in freshwater invertebrates with comparably large shifts for the longer wavelength-sensitive opsins (Briscoe and Chittka 2001; Cronin and Hariyama 2002; Cronin 2006). The last mechanism that is important for spectral tuning is gene duplication, followed by

differential gene expression and modification. Gene duplication has been demonstrated to occur relatively often in aquatic vertebrates such as fish (Hofmann and Carleton 2009; Rennison et al. 2012), but also in invertebrates including jumping spiders (Koyanagi et al. 2008), mantis shrimp (Porter et al. 2009), and butterflies (Wakakuwa et al. 2010). Through DNA mutations at key sites in these newly duplicated opsin genes, visual pigments can acquire broadened sensitivities including both UV (Yuan et al. 2010) and red sensitivity in butterflies (Frentiu et al. 2007).

In addition to visual pigment tuning, other factors can influence the sensitivity of retinal photoreceptors. These factors involve filters, which control the wavelengths of light that actually reach the light-sensitive receptors. Filtering can occur in the cornea and lens, which often absorb short (UV) wavelengths, because of substances deposited in the lens (Thorpe et al. 1993). Some organisms also have colored oil droplets, which are deposited within the photoreceptors but located ahead of the visual pigments such that the light first passes through the oil droplets. Birds (Hart 2001), reptiles (Loew et al. 2002), and even insects (Zaccardi et al. 2006; Arikawa et al. 2009) have colored oil droplets. These pigments are thought to narrow the spectral range of visual pigment absorption and so enhance color discrimination (Govardovskii 1983). In butterflies, two different filtering pigments can even be used in combination with the same visual pigment to produce rhabdoms with different spectral sensitivity and so enable color discrimination where none would otherwise be possible (Zaccardi et al. 2006).

In characterizing visual systems and testing for visual sensitivity differences between species, it is ideal to explore all four mechanisms of visual pigment tuning, as well as filtering pigments. Sequencing opsin genes and quantifying opsin expression through quantitative PCR are now relatively common techniques (Kashiyama et al. 2009; Carleton 2011; Hofmann et al. 2012). When number and diversity of opsin genes increases, retinal transcriptomes or even whole-genome sequences can provide insights (Raible et al. 2006; Plachetzki et al. 2007; Tong et al. 2009; Porter et al. 2013). Chromophore differences and filtering pigment effects are most easily quantified by measuring λ_{max} in vivo using microspectrophotometry (MSP) (Loew and Lythgoe 1978; Bowmaker 1984; Hart 2001). It is also possible to quantify which retinal chromophore is present using high-pressure liquid chromatography (Provencio et al. 1992; Loew et al. 2002) and then reconstitute the visual pigment by expressing the opsin protein in cell lines and combining them with the appropriate chromophore (Oprian 1993).

8.3 Models for How Vision Might Drive Speciation

Visual communication requires a visual signal to be generated, sent through the environment, and detected by a receiver (Bradbury and Vehrencamp 2011). With the exception of bioluminescence, visual signals are generated when a signaler is illuminated by sunlight (or moonlight or starlight), and that light is reflected. The signal is then transmitted through the environment, and detected by the receiver's visual system. Selection on signalers and receivers tends to maximize the fidelity and efficiency

of signal detection. Because the environment can shape both the illuminant spectra and the transmission of the signal, and because the visual system evolves to maximize signal detection in a given environment, the environment can have a strong effect in shaping visual communication. The environmental effects have been examined most often in aquatic environments, where large spectral shifts result as water quality and pathlength vary. The effects in the terrestrial environment are more subtle.

There are several key ideas as to how the environment shapes visual communication and the optimal location of visual pigment absorption (Munz and McFarland 1977; Lythgoe 1979; Loew 1995). If an organism has just one visual pigment, its peak absorption typically matches that of the background illumination. This is called the sensitivity hypothesis and argues that matched pigments maximize sensitivity so the organism can operate over a range of light levels. Further, a matched pigment has been shown to be useful for detecting dark objects against the bright background space light. If the organism has a second visual pigment, the peak absorbance of this pigment is best offset from that of the background light, so that it can detect contrasting signals. A contrasting pigment has been shown to help organisms detect bright objects at wavelengths shifted away from that of the background light spectrum (Munz and McFarland 1977; Levine and MacNichol 1982).

If the environmental light spectrum varies between locations or over evolutionary time, this may cause selective pressure on the visual system to adjust and track the peak illuminant spectrum. The classic example is the blue shift in the oceanic light spectrum that occurs with depth as a result of light absorption by clear water. The visual pigments in organisms living at great depths blue shift so that the rod pigment remains matched to the downwelling light spectrum. This has been observed in marine and freshwater systems (Munz and McFarland 1977; Hunt et al. 1996; Sugawara et al. 2005; Yokoyama 2008). Cone pigments also blue shift with depth, as demonstrated for the cottoid fishes of Lake Baikal (Bowmaker et al. 1994; Bowmaker and Hunt 2006). Cones also spectrally shift to adapt to the light spectrum transmitted through water in different habitats. In the Lutjanid fishes found off the coast of Australia, individuals sampled from the edge of the reef have blue-shifted visual pigments that match the blue-shifted light spectrum of the clear ocean waters, while fish sampled close to the coast have red-shifted visual pigments that better match the transmission of light through the chlorophyll-stained waters (Lythgoe et al. 1994). Differential expression of opsins also helps organisms adapt to the light spectrum. In the killifish *Lucania goodie*, individuals from clear springs express more shorter wavelength opsins and those from tea-stained waters express more longer wavelength opsins (Fuller et al. 2003, 2004).

8.3.1 Models for the Role of Vision in Speciation

The idea that the environment might shape visual sensitivities and visual signals, and contribute to signaling divergence has been formalized in the sensory drive hypothesis (Endler 1992). While there are a number of other models that seek to

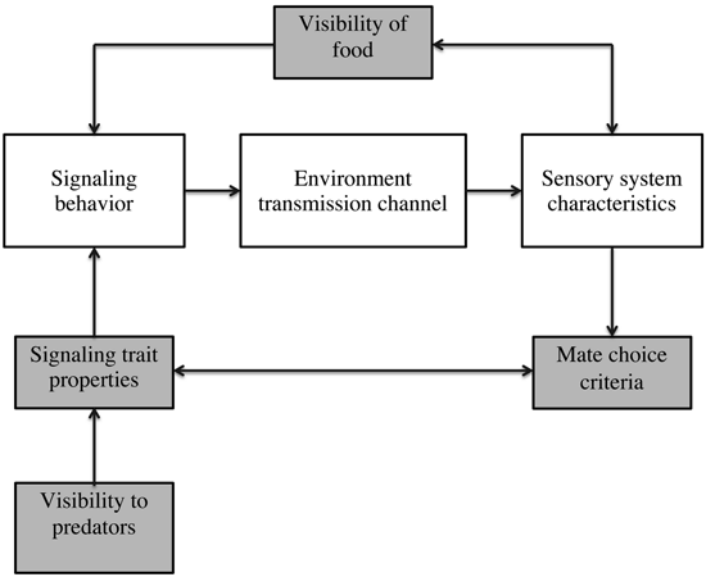

Fig. 8.2 The sensory drive process involves the interaction of a number of factors. Here, we slightly rotate Endler's original picture of sensory drive to place the environment, and it's spectral transmission properties at the center of the model. The environment then shapes both sensory capabilities and signaling characteristics. These are also modified by the success of foraging, the ability to attract mates, and the success at avoiding predation (modified from Endler 1992)

explain how inherent sensory biases or preferences affect communication and speciation, the sensory drive hypothesis is the model that most explicitly incorporates the environment's role in shaping sensory systems and thus contributing to divergence and potentially also speciation (Endler and Basolo 1998).

As originally formulated, the sensory drive hypothesis suggests that signalers and receivers are linked by their need to transfer information (Fig. 8.2). Signals and their detection will therefore evolve together, with their evolution being directed by the environment (Endler 1992). More specifically, the model proposes that habitat will determine the wavelengths that are maximally transmitted, the visual system will adapt to maximize signal detection, and the signals will then follow to maximally stimulate the visual system (Endler 1993; Boughman 2002).

The role of sensory drive in speciation arises in variable environments. If a population is originally in a uniform environment, and part of the population moves to a new environment, that environment might select for different visual sensitivities, leading to visual system evolution. As a result, signals might then change to better stimulate the new visual sensitivities and lead to a shift in visual communication. This could then contribute to behavioral isolation of these two incipient species. An alternative scenario is that the initial environment is not uniform, but instead is graded, such that the illuminant spectrum changes with water depth or canopy cover. These different microhabitats might select for different visual sensitivities.

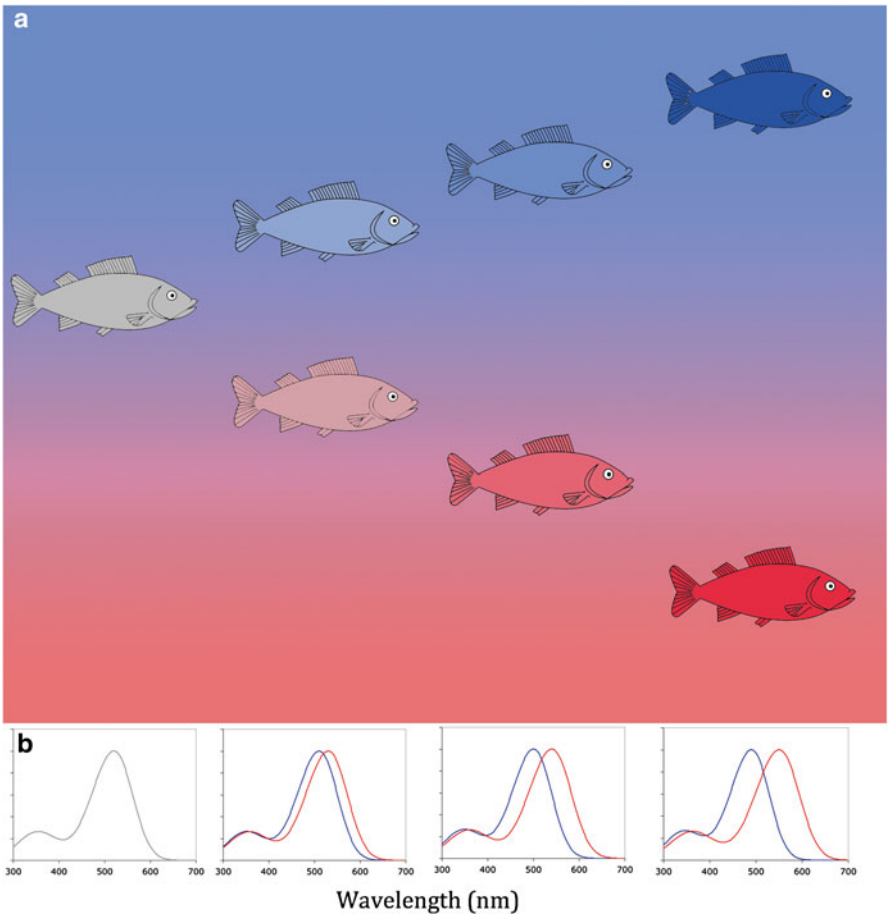

Fig. 8.3 One scenario for how sensory drive might work over time is shown for a heterogeneous environment. (**a**) A population might diverge in response to a habitat gradient, here shown as depth, with divergence increasing with time. (**b**) Selection first acts to alter the sensitivity of visual pigment absorption spectra over time. Here, the shallower individuals become more short wavelength (*blue*)-sensitive while the deeper individuals become longer wavelength (*red*)-sensitive. These visual sensitivities then select for male signaling which best stimulates those sensitivities, causing male colors to shift through time. At the end of such a process, the shallow and deep individuals have different visual sensitivities and visual signals, which then contribute to behavioral isolation and result in two new species

As a result, the divergent habitats would lead to divergence in vision, followed by signal divergence, and could contribute to the population splitting and then speciating (Fig. 8.3). Some would therefore consider visual sensitivity to be a "magic" trait where divergent selection for ecological adaption, simultaneously leads to non-random mating (Gavrilets 2004; Servedio et al. 2011).

The process by which a given population might diverge in a heterogeneous environment by sympatric speciation was modeled by Kawata et al. (2007). They considered female preferences for male traits and made three assumptions: (1) spectral sensitivity adapts to the ambient light regime; (2) female sensitivity depends only on the absorption of visual pigments; and (3) females prefer males with nuptial colors that best stimulate her visual pigments. Visual pigment sensitivity and male color reflectance were controlled by a number of mutable, genetic factors and the light environment varied with depth. Their simulations suggest that sympatric speciation could occur over a broad set of conditions through sensory drive. For a population that started with one set of visual sensitivities and one primary male signaling color (Fig. 8.4a), it produced two populations, which differed in male color (Fig. 8.4b) and female sensitivity (Fig. 8.4c) that were both associated with water depth. They further found that if variation in the light spectrum with depth (the light gradient) was too steep or too shallow, speciation would not occur (Fig. 8.5). This depended on the dispersal distance (Fig. 8.5d). Presumably, if the light gradient is too shallow, animals can inhabit any depth (dispersal distances are large) and there is no reduction in gene flow (Fig. 8.5a). If the light gradient is too steep, individuals cannot adapt to such large changes in light spectrum so that both sensitivity types are essentially constrained to the same location. These small dispersal distances enable too much gene flow for speciation to occur (Fig. 8.5c). Intermediate gradients therefore enable individuals to spread out, reducing gene flow among parts of the population so they can diverge.

8.4 Visual Pigment Variation and Speciation

The role of visual pigment variation in speciation has not been tested for many taxa. There are several requirements for visual photopigments to affect speciation. First, some evolutionary force must alter visual pigment sensitivities. Second, these sensitivity differences must affect mating success (either ecologically through habitat preference or behaviorally through male territoriality or female mate choice). Third, these differences must lead to divergence perhaps in mating signals or preferred habitat, such that after speciation, the two populations remain reproductively isolated.

Typically, the first line of evidence that speciation might be driven by visual pigment evolution is for visual pigment sensitivities to differ between sister species. Based on this expectation, there are three possibilities for comparisons of visual pigment diversity within closely related taxa: (1) Visual pigment absorbance does not vary between closely related species. In that case, vision has played no role in speciation. (2) Visual pigment absorbance might vary between sister taxa, but it is not yet clear whether the variation is involved in speciation. (3) Visual pigments vary between sister taxa, with visual pigment differences linked to variation in light environment and to differences in visual signals, supporting the idea that visual pigments have indeed led to the formation of new species. We will discuss each of these cases in turn.

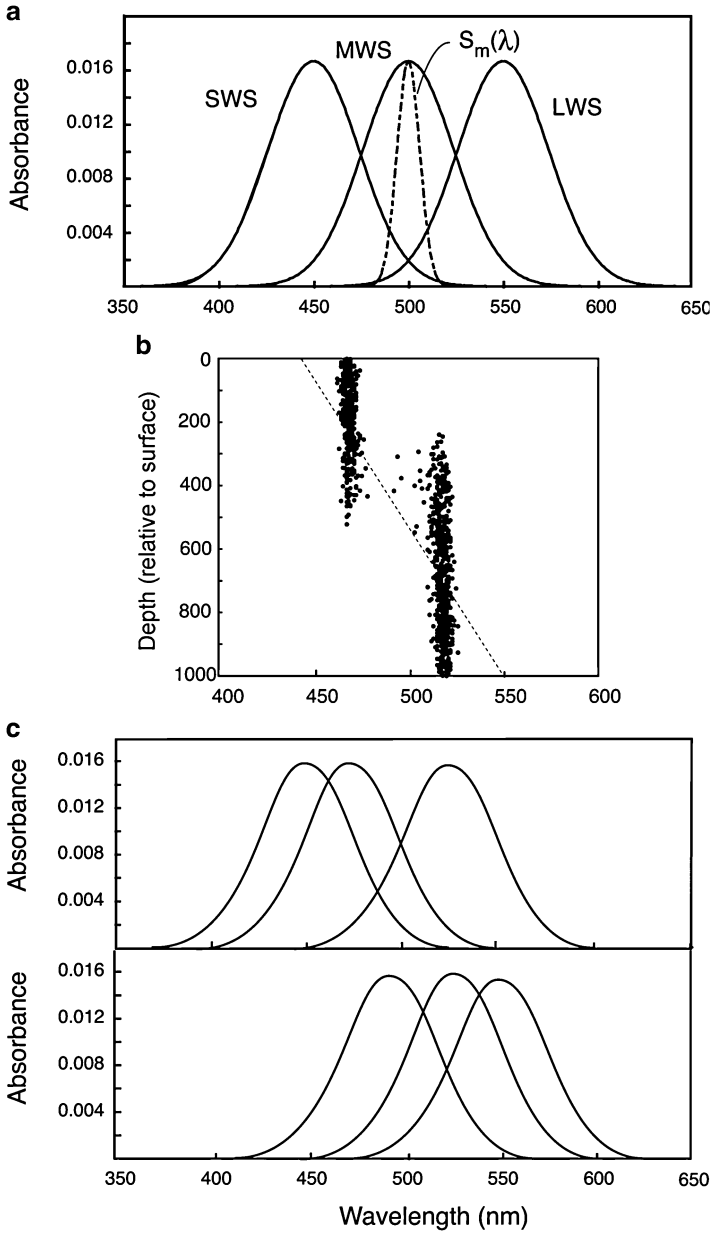

Fig. 8.4 Modeling results of sexual selection in a diverse environment where light spectrum shifts to longer wavelength with depth, based on figures 1 and 5 from Kawata et al. 2007, with permission from BMC Evol Biol. (**a**) The visual sensitivities of the short (SWS), medium (MWS) and long (LWS) wavelength-sensitive visual pigments and the reflectance spectrum of the male color signal (S_m) for the initial population prior to any evolution in vision or male color. (**b**) The final bimodal distribution of male colors peaking between 470 and 520 nm and their variation with depth after the evolutionary simulation of sensory drive. (**c**) The shift in visual pigment sensitivities for individuals that are reproductively isolated. These would correspond to individuals that are shallow (*top*) and or deeper (*bottom*)

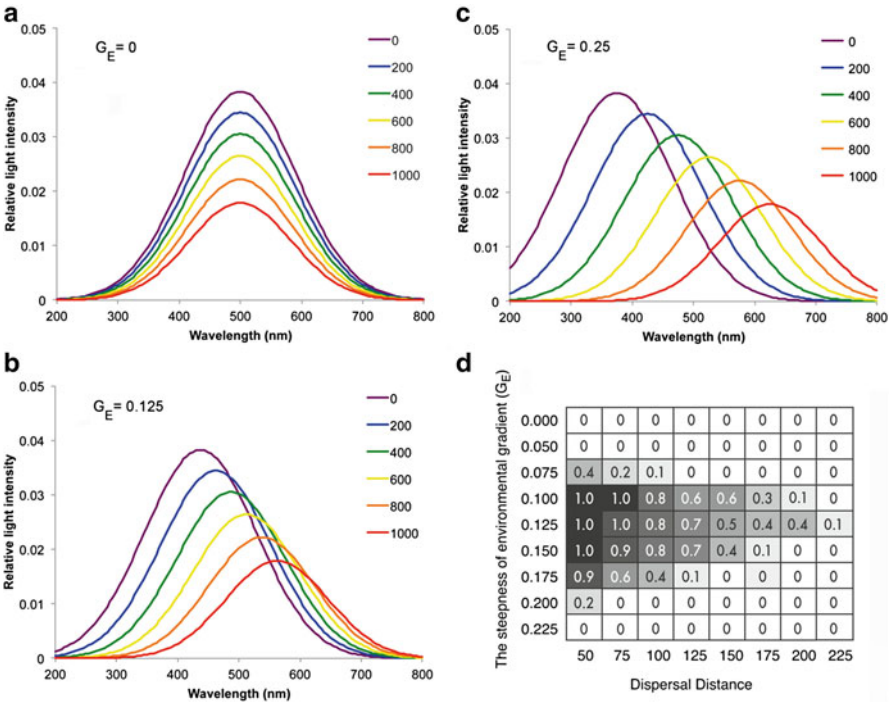

Fig. 8.5 The probability of speciation and how it depends on the light gradient and dispersal distance, based on the simulations of Kawata et al. (2007). Kawata et al. use the factor G_E to indicate how quickly the light spectrum shifts to longer wavelength with depth. Here, the corresponding spectra (which are normal distributions) are calculated for (**a**) $G_E=0$, (**b**) $G_E=0.125$, and (**c**) $G_E=0.25$. Spectra depend on the depth, y, which vary from 0 to 1,000 as shown in the legend. We use Kawata's equation to calculate the peak of the light spectrum using $500+G_E\times(y-500)$. (**d**) This panel (figure 2a from Kawata et al. 2007) shows the probability of speciation (from 0 to 1) for different values of G_E and different dispersal distances. Speciation does not occur for light gradients that are too shallow, $G_E<0.05$, or too steep, $G_E>0.2$

8.4.1 Examples Where Visual Pigment Evolution Is Unlikely to Drive Speciation

Proving that vision and in particular evolution of visual pigments is directly involved in driving speciation is a difficult task. Therefore, we begin by identifying some systems where visual pigment evolution is clearly not driving species divergence. In these cases, visual sensitivities have been found to be nearly identical across a group of species. These examples come primarily from terrestrial vertebrates. The terrestrial photic environment can differ somewhat with location, e.g. deep in the forest versus out in an open meadow. However, there is generally much less spectral variation in the terrestrial than in the aquatic environment, because terrestrial lighting is dominated by sunlight transmitted through air. The largest spectral differences are associated with time of day rather than habitat (Munz and McFarland 1977; Loew

and McFarland 1990). As a result of the lack of variation in the light environment across habitats, it may be that there is relatively little selection to shift visual sensitivities among terrestrial animals.

One group whose visual sensitivities have been compared across species is the Caribbean anoles. Anoles are a classic example of an adaptive radiation, and have convergently evolved similar ecomorphs on several Caribbean islands (Losos et al. 1998). These lizards occupy different habitats with different lighting conditions from grass, to tree trunks, to tree crowns. Anoles communicate with pushup displays to show their colorful dewlaps, and dewlap color differs between species. Therefore, one might expect that anoles differ in their visual sensitivities. However, a study of 17 species selected from different lighting environments and with different dewlap colors showed remarkable similarities in their visual pigment sensitivities (Loew et al. 2002). All 17 species had four visual pigments, one UV (λ_{max} 365 nm), one short (λ_{max} 455 nm), one medium (λ_{max} 495 nm), and one long (λ_{max} 564 nm) wavelength-sensitive. Visual pigment peak absorbances differed by only a few nanometers between species from Central America to Cuba to Jamaica and Puerto Rico. The chromophore used was also consistent across all the Caribbean species, being A1. There were some differences in oil droplet cutoff wavelengths, which might impact color discrimination. But the visual pigments of these anoles inhabiting different terrestrial habitats and using distinct dewlap colors were very similar.

One study did find some visual communication differences between two species of sympatric anoles: *Anolis cristatellus* and *A. cooki* (Leal and Fleishman 2002); the two species occupy similar habitats but display against different backgrounds. *A. cristatellus* has a highly UV-reflective dewlap and displayed against UV poor backgrounds, while *A. cooki*'s dewlap had low UV reflectance and displayed against a high UV background. Visual differences determined by electroretinograms identified some subtle sensitivity differences, though no differences were observed at the short end of the spectrum. Therefore, this study suggests that differences in visual communication can be achieved by variation in display location, and does not always require variable visual pigment sensitivities.

The bowerbirds of Australia are another adaptive radiation that has been tested for visual sensitivity differences. Male bowerbirds build complex displays or bowers to attract females. These bowers are often decorated with colorful objects. Males also have colorful patches or body colors that are used to attract females for mating (Frith and Frith 2004). The visual systems of 12 bowerbird species, as well as a catbird outgroup, were examined by characterizing visual pigments, ocular media, and oil droplet spectra for six of the species and sequencing the rod and cone opsin genes for all of them (Coyle et al. 2012). Little variation was found in either the visual pigment peak absorbances, or the transmission cutoffs for lenses and oil droplets, and the opsin gene sequences were essentially identical across species. This suggests that differences in visual sensitivity have not played a role in male bowerbird displays or bowerbird speciation.

8.4.2 Examples Where Visual System Evolution Could Potentially Drive Speciation

There are a number of systems where variation in visual pigment absorbances has been found (Levine and MacNichol 1982; Briscoe and Chittka 2001; Hart and Hunt 2007). However, few of these studies have examined variation at the level of sister taxa. If sister taxa have been compared and found to differ, such differences have often not been tested to determine whether they play any role in speciation. In spite of these limitations, we note here a number of genera which show variation in visual sensitivity and which require further testing to assess the role of vision in directing speciation.

Several studies have compared visual pigments of closely related invertebrate species. A review of 47 different hymenopterans found significant variation within families, including evidence for 10–20 nm shifts in λ_{max} between species in the same genus including *Melapona* (stingless bees) and *Bombus* (bumblebees) (Peitsch et al. 1992). Variation also occurs within other insect genera that include fireflies, back swimmers, and even crickets (Briscoe and Chittka 2001). Studies of diversity between different species of crabs have shown that their visual pigment λ_{max} values can vary by over 42 nm, with variations up to 20 nm within a given genus (Cronin and Forward 1988; Jordao et al. 2007). MSP comparisons between six species of mantis shrimp also found that the midband visual pigments can vary by 10–25 nm between species from the same genus from different habitats, with the peripheral visual pigment varying by up to 60 nm (Cronin et al. 1996). However, a similar study in crayfish found very little visual pigment variation between species (Crandall and Cronin 1997).

Some of the greatest λ_{max} variation in invertebrates occurs in butterflies (Bernard and Remington 1991; Briscoe and Chittka 2001; Stavenga and Arikawa 2006). Some butterflies have the typical insect complement of three visual pigments: ultraviolet, blue and long wavelength, with considerable variation within genera. For example, the long class varies by 10 nm in *Heliconius* (Yuan et al. 2010) and more than 30 nm in *Limenitis* (Frentiu et al. 2007). In addition, there have been numerous lineage-specific gene duplications followed by opsin sequence divergence, which have considerably expanded visual pigment diversity. This includes duplication of the UV (Briscoe et al. 2010), blue (Sison-Mangus et al. 2008; Awata et al. 2009), and long wavelength (Briscoe 2000, 2001; Frentiu et al. 2007; Awata et al. 2010) opsin genes. These can contribute to substantial shifts (>30 nm) in sensitivity between closely related species (Briscoe 2008).

Other mechanisms for spectral tuning among closely related species have also been examined. A study of three stomatopod species living at different depths found no evidence for differences in visual pigments within species (Cronin et al. 2002). However, they found significant variation in filtering pigments, with deeper individuals having blue-shifted absorption (Cronin et al. 2001; Cronin and Caldwell 2002). Butterflies also use different screening pigments to alter the sensitivity of photoreceptors and can obtain different sensitivities with one visual pigment and

two filters that behaviorally enables color discrimination (Wakakuwa et al. 2004; Zaccardi et al. 2006). Opsin expression can also vary between species, such as found in comparisons of three species of *Drosophila* where eye size was studied (Posnien et al. 2012); the authors suggest that in the more crepuscular *D. mauritiania*, an increase in the number of dorsal ommatidia, along with an increase in UV opsin expression, may provide enhanced sensitivity to lower light levels. Variation in butterflies extends to differences in male/female expression of pigments in particular photoreceptors as well as co-expression of different pigments in the same receptor (Sison-Mangus et al. 2006; Awata et al. 2010; Ogawa et al. 2012). However, in spite of all this diversity, only a few invertebrate studies have explored whether visual pigment variation could play a role in speciation (see below).

Teleost fishes show some of the greatest visual pigment diversity. Several early surveys identified significant variation across species, though these did not typically include closely related species (Loew and Lythgoe 1978; Levine and MacNichol 1979). A number of more recent studies have however focused on single genera. A large survey of coral reef fish found variation in the genera *Chromis* and *Acanthurus* (Losey et al. 2003). A study of 12 species of snapper (Order Perciformes, Family *Lutjanidae*) showed that the peak sensitivities of visual pigments blue shift as fish habitat changes from estuarine to open ocean (Lythgoe et al. 1994). Based on a recent phylogeny by Miller and Cribb (2007), this work suggests that sister taxa have quite different visual pigment sensitivities which could contribute to speciation (Fig. 8.6). Other fishes show significant differences as well. Two populations of

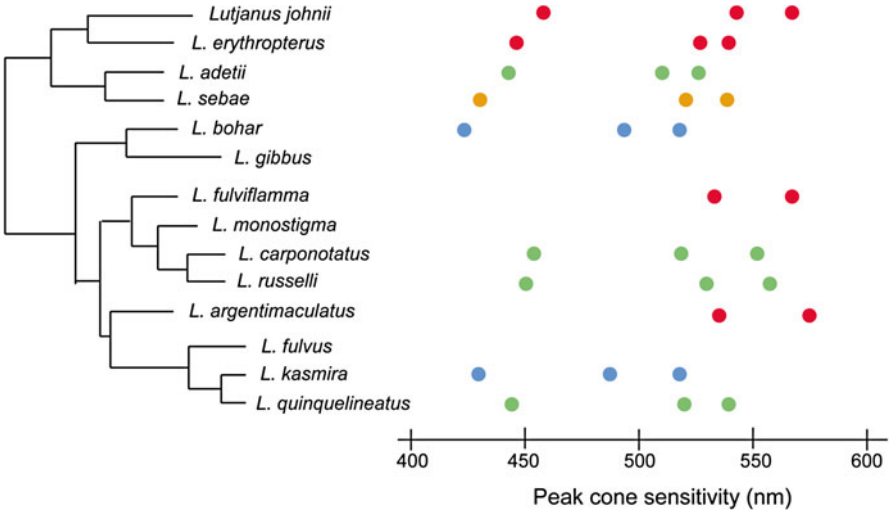

Fig. 8.6 Visual pigment sensitivities vary between closely related species of Australian snappers from the genus *Lutjanus*. Phylogenetic relationships are taken from Miller and Cribb (2007). The visual pigment peak sensitivities determined by microspectrophotometry are from Lythgoe et al. (1994). Visual pigment sensitivities are colored by the habitat from which Lythgoe et al. sampled individuals: estuaries (*red*), near shore reef (*yellow*), midshelf reef (*green*) and outer reef (*blue*)

the freshwater killifish *Lucania goodei*, which inhabit clear or tannin-stained waters, differ in opsin gene expression. Fish from clearer spring water express shorter wavelength pigments than those in tannin-stained waters (Fuller et al. 2004, 2005). This could lead to divergence and ultimately speciation. A study of 17 species from the cottoid fish radiation in Lake Baikal showed differences in visual pigment sensitivity, with fish visual pigments blue shifting with habitat depth (Bowmaker et al. 1994). These differences are likely the result of both differences in opsin sequence and gene expression. Visual pigments in cichlid fishes have also been measured, with closely related species showing both small and large differences in λ_{max} (van der Meer 1995; Carleton et al. 2005; Jordan et al. 2006). The largest shifts are the result of differences in gene expression, though subtle differences in opsin sequence also contribute (Hofmann et al. 2009; O'Quin et al. 2010).

Some variation of visual pigments has been documented in birds (Hart and Hunt 2007; Odeen and Hastad 2013). Most of this variation involves altering the shortest wavelength visual pigment to be either UV- or violet-sensitive by changes in just a few key amino acids (Wilkie et al. 2000; Yokoyama and Shi 2000; Carvalho et al. 2007; Hunt et al. 2009). In addition, the role of oil droplets in shaping peak cone sensitivities has been documented (Hart 2001). Most of these studies have examined individual species rather than a range of species within a genus. Therefore, it is not yet known whether such variation plays a significant role in speciation.

The variation observed in mammals is similar to that in birds. Much of it involves the loss of the SWS1 or LWS gene (Jacobs 2013). Loss of SWS1 has occurred in both nocturnal mammals (David-Gray et al. 2002; Tan et al. 2005; Zhao et al. 2009) and diving marine mammals (Levenson and Dizon 2003; Newman and Robinson 2005), with the additional loss of the LWS gene in some deep diving marine mammals (Meredith et al. 2013). While there can be variation in the sensitivity of the SWS1 gene (Carvalho et al. 2012) and LWS gene, as well as variation in whether primates have one or two distinct LWS genes (Hunt et al. 2009; Jacobs 2009; Davies et al. 2012), it is unclear if this plays a role in mate choice and speciation. However, it has been suggested that trichromacy in primates evolved so that they could discriminate emotional and social states (e.g. blushing), which might be important for primate mating (Changizi et al. 2006).

8.4.3 Examples Where Visual Divergence Is Likely to Drive Speciation

There are a few examples where the role of visual pigments or visual sensitivities in speciation has been explored. Here, we will discuss several cases where closely related species have been shown to differ in visual sensitivities. Differences are linked to the light environment, and these differences have led to divergence in visual signaling. Although often not proven, these examples provide the best evidence that evolution of visual pigments, or at least visual sensitivities, can drive speciation.

Evolution of visual pigments and mating signals has been demonstrated in invertebrates, particularly in bioluminescent organisms. In fireflies, species in the same genus can differ in both their visual pigment peak absorbance and their bioluminescent signal by tens of nanometers (Lall et al. 1980, 2009). They can also differ in screening pigments, which further specialize their visual sensitivities to match mating signals (Lall et al. 1980, 1988; Cronin et al. 2000). Correspondence between bioluminescent signals and visual sensitivities has also been observed in click beetles (Lall et al. 2010). The mating signals are tuned to time of day, with nocturnal species using green flashes and visual sensitivities while twilight species tend to use more yellow flashes. Yellow twilight signals are thought to provide better offset from the sunlight reflecting off green foliage. The correspondence between male sexual signals (flashes) and female visual sensitivities offer evidence that evolution of visual pigments or at least visual communication has contributed to reproductive isolation, and perhaps promoted speciation. Further evidence might be gained by looking for evidence of positive selection in either the opsins or luciferase genes between sister taxa.

Butterflies have brilliant mating colors that are sometimes aposematic (i.e. present as a warning coloration). They also have distinct mating preferences based on these colors. In one study, the mating preference of white *Heliconius cydno* and yellow *H. pachinus*, as well as hybrids, were used to QTL map male color preference and relate it to the locus controlling yellow wing color (Kronforst et al. 2006). Interestingly, both preference and wing color map to the same location, which is the location of *wingless*. This suggests that *wingless* could be a "magic" locus controlling both female signal and male preference (Gavrilets 2004; Servedio et al. 2011). This is not however the location of the butterfly visual pigment genes and thus mate choice is not likely due to visual pigment evolution. The question then remains what is the mechanism that controls preference. The authors suggest that either there is an inversion which contains both a preference gene (whatever that is) and a color gene (which could be *wingless*), or perhaps the same gene controls both. If it is the same gene, it might cause both pigmentation in the wing and screening pigments in the retina. Ommochrome pigments have been found in both locations and their occurrence in different ommatidia does contribute to long wavelength discrimination in *Heliconius erato* (Zaccardi et al. 2006). However, it remains unclear whether long wavelength discrimination is necessary to distinguish white and yellow wing colors or how this would influence mate preference.

Another butterfly study has made a link between butterfly visual sensitivities and wing colors (Briscoe et al. 2010; Bybee et al. 2012). These authors found a duplicated UV-sensitive opsin gene that is shared across the genus *Heliconius*. The gene duplicate shows signs of positive selection and the selected sites map to the retinal binding pocket. Epi-microspectrophotometry reveals that these genes encode pigments with peak sensitivities at 355 and 398 nm, a difference which could considerably enhance color discrimination in this region. They correlate the UV gene duplication with the evolution of yellow wing pigment patterns within this genus, which contribute significant UV reflectivity. They make the case that added color discrimination in the UV range is important because *Heliconius* must distinguish congeners from mimetic

species. Such differences may have contributed to mate choice early in the evolution of the *Heliconius* group and therefore may have played a role in speciation as this group of butterflies split off from other closely related groups. Other butterfly groups, such as the Pieridae, also have opsin gene duplications suggesting they should be explored for opsin differences within the family and for possible correlations with coloration (Wakakuwa et al. 2010). It seems that butterflies are likely to provide evidence for speciation as a result of evolution of visual sensitivity at the very least, and perhaps evolution of visual pigments, after further study.

Fishes have the most variation in visual pigment sensitivities. Therefore, it is not surprising that our best evidence for visual pigment evolution driving vertebrate speciation is in fishes. One of the first studies to demonstrate sensory drive and its role in speciation was carried out on stickleback fishes (Boughman 2001). This study showed that sticklebacks inhabit different light environments that transmit more or less red light. Further, females in blue-shifted environments are more red-sensitive and those in red-shifted environments have less red sensitivity based on the optomotor response. Finally, male sticklebacks in blue-shifted waters have larger red patches for signaling and those in red-shifted waters have smaller red areas (Fig. 8.7). Though these correlations with the light spectrum are somewhat unexpected, they do show that males use more red signaling in the environments where females are more red-sensitive. And preference tests showed females preferred redder males when they were more red-sensitive. These important differences in visual communication were demonstrated to contribute to reproductive isolation. The only thing missing from this study is a link to the evolution of visual pigments. The visual pigment opsin genes in stickleback populations occupying different light environments have been sequenced by Flamarique et al. (2013) and, although they find very little change in the LWS opsin gene sequence, opsin gene expression does differ, with fish in red-shifted waters utilizing more red/red double cones and those in clearer waters having red/green double cones. Therefore, the differences in red sensitivity measured by Boughman (2001) could be due to increased LWS opsin expression, rather than divergence in opsin sequence. This agrees with recent studies in cichlid fishes, where increased LWS gene expression was correlated with increased optomotor sensitivity (Smith et al. 2012). Further work on the stickleback system showed that LWS gene expression differences were not plastically controlled by the light environment, but rather were genetically determined (Flamarique et al. 2013). Taken together, these data support the idea that sensory drive is altering visual sensitivities and visual signals and thereby contributing to stickleback speciation.

Another well-studied fish group is a set of nine species of surf perch (Embiotocidae) that live in the kelp forests off the California coast. Using MSP, Cummings and Partridge (2001) examined their dichromatic visual systems and found variation in visual pigment absorbances with shifts of up to 20–30 nm in both short and long wavelength cones. These differences were shown to improve color detection for some species and luminance detection in others (Cummings 2004). Modeling of surf perch colors within the context of visual sensitivities suggested that color pattern divergence evolved to maximize color detection in those species with color optimized visual systems, and for maximal luminance in those species

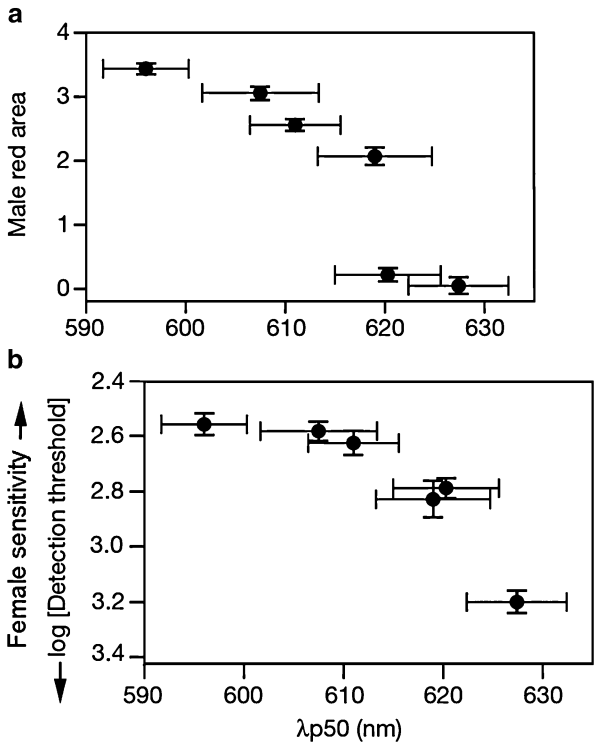

Fig. 8.7 Male red color and female red sensitivity are correlated with water color and thereby correlated with each other (modified from Boughman 2001, figure 1). Water color at nesting sites is characterized by the λp50 value, the wavelength that divides the radiant light spectrum in half (half of all photons are at shorter wavelengths and half are at longer wavelengths). Male red area was scored visually using a qualitative scale for intensity and area. Female sensitivity was measured using optomotor response to moving stripes under illumination at 640 nm. Here, the plot for log detection threshold has been inverted from the original to show increasing female sensitivity on the *y* axis. Both male red area and female sensitivity to red light decrease in habitats where the water color is more red-shifted

whose visual systems were specialized for luminance detection. Therefore, sensory biases set the direction of conspecific male signal evolution (Cummings 2007). Such differences could be important for maintaining reproductive isolation (and therefore possibly generating divergence) among this largely sympatric group of species. Studies to examine the molecular basis of differences in visual pigment sensitivity could provide additional support for opsin gene selection and therefore the role of sensory drive in the evolution of these species.

Lake Victoria cichlids provide probably the strongest case that we have for divergence of visual pigments and color signals leading to speciation (Seehausen et al. 2008; Miyagi et al. 2012). Although the Victorian cichlids are thought to have evolved quite recently, they show significant variation in, and selection on, their LWS opsin gene (Terai et al. 2002; Spady et al. 2005). Long wavelength sensitivity

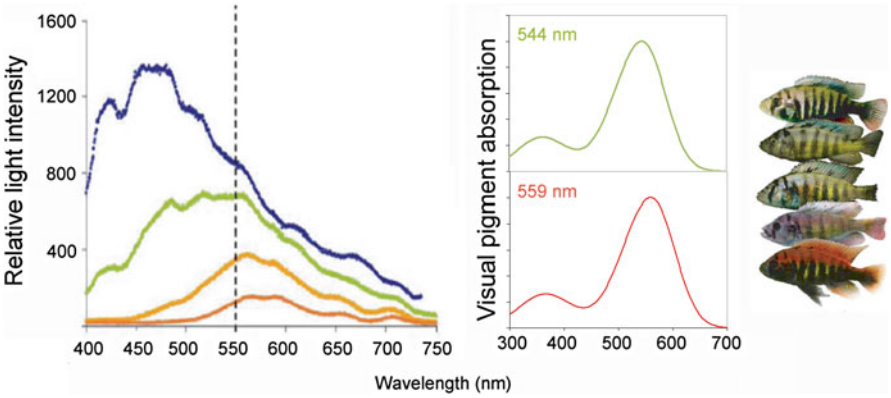

Fig. 8.8 The waters of Lake Victoria are relatively more transmissive to longer wavelength light than that of other lakes. The spectra show downwelling light at the surface (*blue*), 0.5, 1, and 2 m depth. As a result, cichlids that live deeper have evolved a longer wavelength LWS opsin gene which produces a longer wavelength visual pigment ($\lambda_{max} = 559$ nm) while cichlids that live shallower have a LWS opsin gene produces a shorter pigment ($\lambda_{max} = 554$ nm). As a result, females prefer redder males at depth and bluer males in the shallows. Therefore, the gradient in spectral environment leads to differentially sensitive visual pigments, and evolution of male color patterns (shown at *right*), resulting in speciation (modified from Seehausen et al. 2008, figure 1)

varies between species (Maan et al. 2006) and this occurs through variation in both LWS sequence (Terai et al. 2002, 2006) and gene expression (Carleton et al. 2005). Because Lake Victoria is relatively eutrophic, the light spectrum shifts to longer wavelengths with depth. As a result, fish that live deeper tend to have longer wavelength LWS opsin genes (Fig. 8.8). This difference has been selected and there is significant sequence divergence of a 5 kb region around the LWS opsin locus, including the promoter, between taxa from different habitats (Terai et al. 2006; Seehausen et al. 2008). This provides further evidence that visual sensitivity has adapted to water depth.

In addition to vision varying with depth, fish color also varies with deeper species having more red coloration than shallower species (Fig. 8.8). This has been observed in a number of species from both rock dwelling and sand dwelling habitats in Lake Victoria (Seehausen et al. 2008; Miyagi et al. 2012). This suggests that male signaling has evolved to better stimulate the visual sensitivities at different depths and be better transmitted through the waters. Calculations suggest that red male colors are 10 % brighter to the longer wavelength pigment relative to the shorter wavelength pigment, which might make males more attractive and could have implications for male mating success (Carleton et al. 2005). As a result of the light gradient that occurs in these lakes, the visual system has diverged and led to a divergence of male colors, which then contribute to behavioral isolation between these incipient species (Seehausen and van Alphen 1998). This system seems to be a clear example of visual pigment evolution driving speciation. The one weak link in the argument is the fact that when females are behaviorally tested in the lab for their

preference for male colors, there is no correlation between female preference and their LWS opsin gene sequence. However, it may be that fish require the murkier waters found in the wild to show such preferences (Seehausen et al. 2008). Experiments to confirm a link between female LWS opsin sequence and female mate preference under more natural lighting would be interesting and would help bolster the role of visual pigment evolution in driving cichlid speciation.

8.5 The Need for More Studies

The sensory drive mechanism, first proposed in 1992, stimulated evolutionary biologists to consider whether sensory systems could contribute to speciation (Endler 1992). The concept that habitat differences would contribute to the evolution of sensory systems and then to divergence in signaling seemed a logical argument for how new species might arise. However, in the past 20 years, the data to support this mechanism have been slow in coming. We can make several observations based on the studies that have been done.

The first is that visual system diversity seems markedly reduced in the terrestrial environment. Whether this is because not enough systems have yet been examined or whether the air does not modify the light spectrum sufficiently to provide a divergent selective force as in aquatic habitats is still to be determined. Several key adaptive radiations, including anoles (Loew et al. 2002) and bowerbirds (Coyle et al. 2012), where we might expect visual sensitivities to contribute to speciation, have not supported the sensory drive hypothesis. However, the visual diversity found in insects, and in particular butterflies, would seem to counter the argument that not much happens in terrestrial environments. Perhaps it is something specific to terrestrial vertebrates, which reduces visual pigment diversity. More terrestrial studies are needed, with an emphasis on studies of closely related species, to examine the extent of visual sensitivity variation, and then to determine the molecular mechanism underlying that variation, and its role in behavioral isolation. This is needed in vertebrates, which have not yet yielded systems with significant variation, and invertebrates, which seem poised to offer strong evidence for the role of visual pigment evolution in speciation.

If we accept that terrestrial light environments generally show less variation, we might consider the variation in the light spectrum with time of day (Munz and McFarland 1977). One change, which might drive the evolution of visual pigments, would be for organisms to alter the time of day when they are breeding. Fireflies seem to be one group of organisms that have taken advantage of these temporal differences (Lall et al. 2009) and it would seem useful to explore other closely related species that have undergone diurnal to crepuscular transitions. Such changes could contribute to both ecological isolation due to temporal shifts, and behavioral isolation through visual pigment sensitivity shifts.

Aquatic systems seem to have larger potential variation in visual pigment sensitivities with more variation between closely related species. While there are several

tantalizing examples in fishes that suggest visual sensitivities can drive speciation, further work is needed to fill in the pieces of the puzzle for several systems. Therefore, with a few possible exceptions, we are still lacking definitive evidence that visual pigment evolution has contributed to speciation.

In addition to considering the contribution of visual pigment evolution, it is worth expanding this question to consider any visual sensitivity differences that arise in the photoreceptors that might cause speciation. Such studies would help support the powerful sensory drive model for how new species might come about. This includes examining filtering pigments and chromophore shifts and how they might contribute. Ideally, future studies should provide all the following evidence to support the role of diverging visual sensitivities in speciation. Such studies should:

1. Compare visual sensitivities among closely related taxa from a variety of animal groups with different habitats and ecologies to identify sister taxa that differ.
2. Determine the molecular basis for any differences in visual sensitivities including opsin sequence changes, differential gene expression, chromophore shifts, gene duplications, and filtering pigments.
3. Link visual sensitivity differences to mating signals such as color or luminance signals.
4. Identify any causative environmental factors, such as light spectrum or light intensity, that might contribute to visual sensitivity and signal divergence.
5. Test for evidence of selection between taxa by examining the region around loci controlling visual sensitivity (which could be the opsins) and visual signaling.
6. Test whether differences in visual sensitivity and signaling between sister taxa contribute to behavioral isolation and therefore could have led to speciation.

These individual pieces of evidence would then build a convincing argument that evolution of visual sensitivity and perhaps of visual pigments has led to the generation of new species, in fulfillment of the sensory drive theory.

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