The Evolution and Function of Melanopsin in Craniates

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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

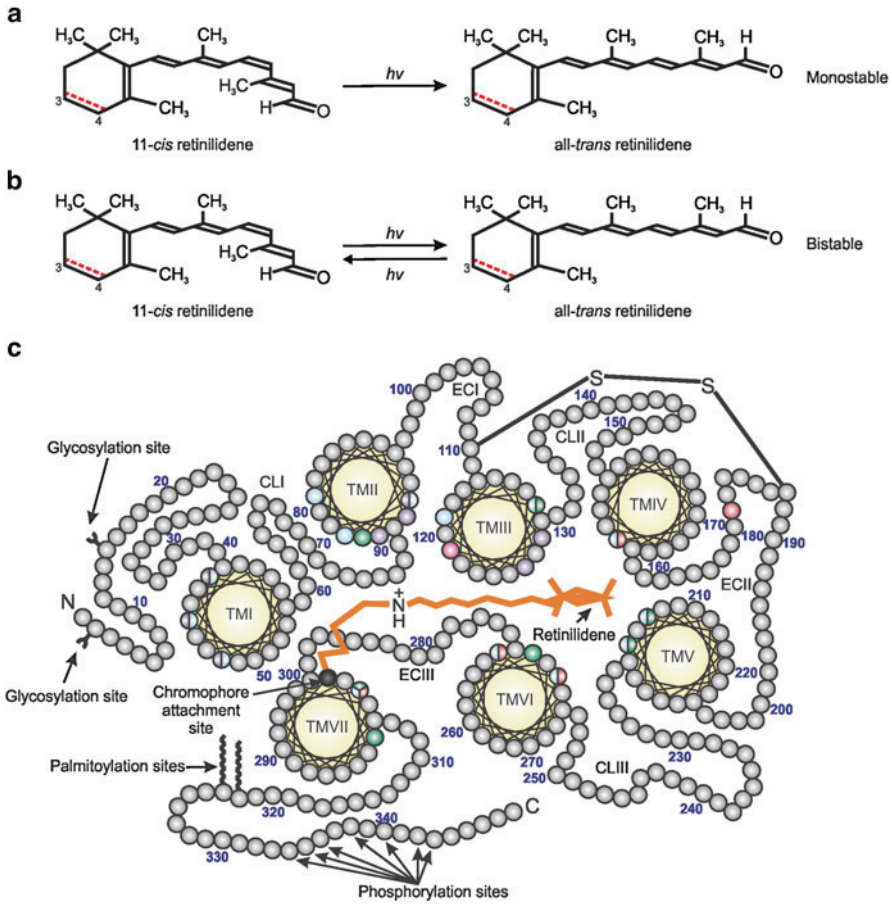

Fig. 2.1 A diagram of the structure of a typical photopigment (e.g. rod opsin). (**a**, **b**) The initial step in phototransduction consists of photon (*hv*) absorption by 11-*cis* retinal, which photoconverts to all-*trans* retinal. Vertebrate photopigments are broadly divided into rhodopsins that 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

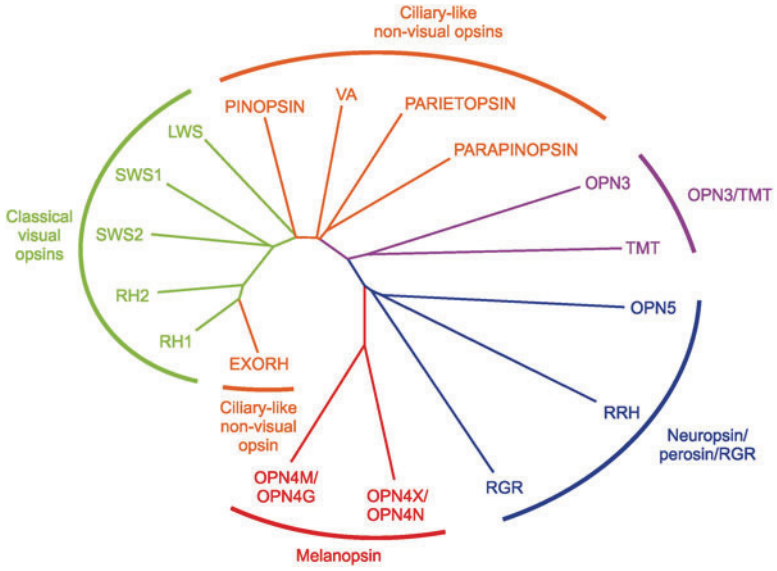

Fig. 2.2 A phylogenetic tree showing the evolution of known opsin-based pigments in the craniates and their classification into five main classes. Mammal-like/gnathostome melanopsin, OPN4M/OPN4G; *Xenopus*-like/non-therapsid melanopsin, OPN4X/OPN4N; retinal G protein-coupled receptor, RGR; retinal pigment 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

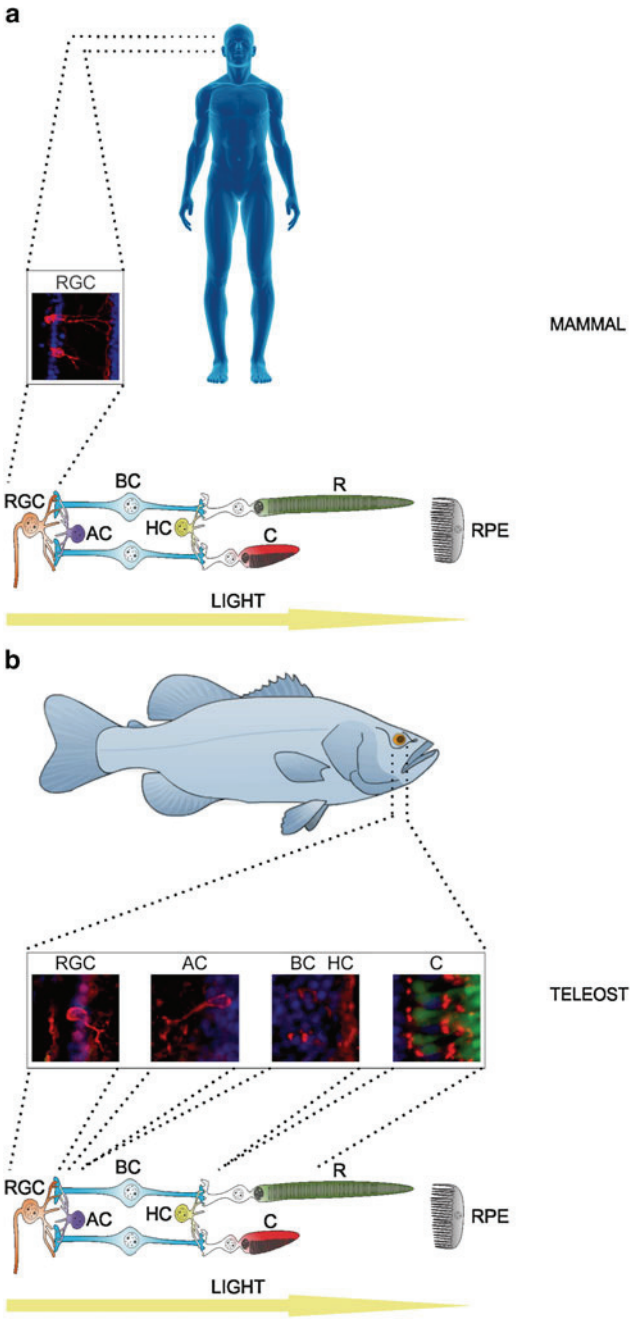

Fig. 2.3 A schematic showing immunocytochemically detected melanopsin expression (coloured *fluorescent red*) in the retina of (**a**) a typical mammal (e.g. human, *Homo sapiens*) compared to (**b**) a representative teleost (e.g. zebrafish, *Danio rerio*). In humans, the OPN4M protein is

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)

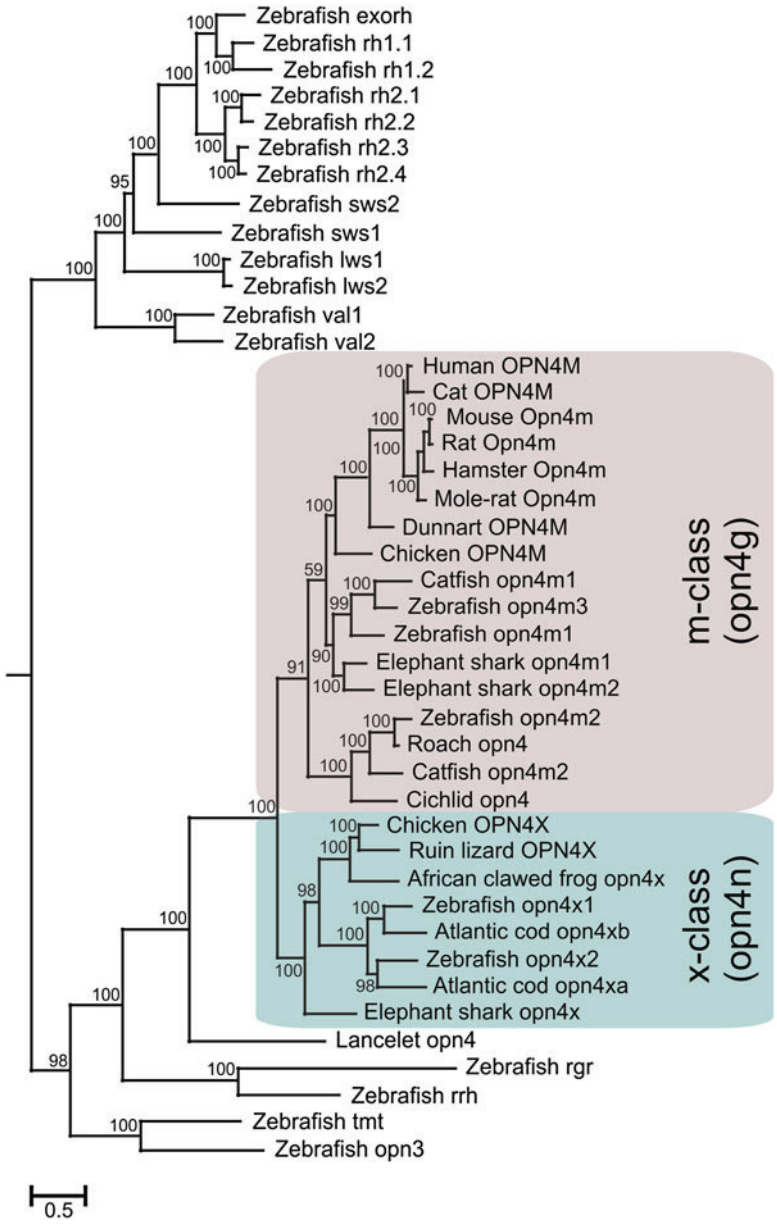

Fig. 2.4 The evolution of craniate melanopsin photopigments. Phylogenetic analyses based on a codon-matched nucleotide alignment of various chordate melanopsin cDNA sequences compared to published visual and non-visual photosensory pigments of the zebrafish (*Danio rerio*), showing the two main *opn4* 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

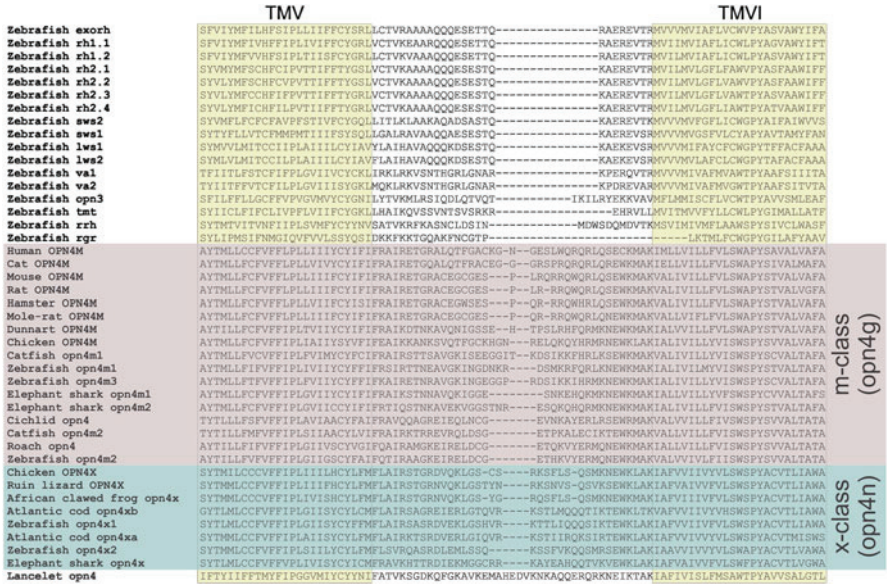

Fig. 2.5 Amino acid alignment of the third cytoplasmic loop (CLIII) of the chordate pigments shown in the phylogenetic tree of Fig. 2.4. Gaps were inserted to maintain a high degree of identity and are indicated by *dashes* (–), whereas *boxed yellow shading* denotes two transmembrane (TM) domains (i.e. 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

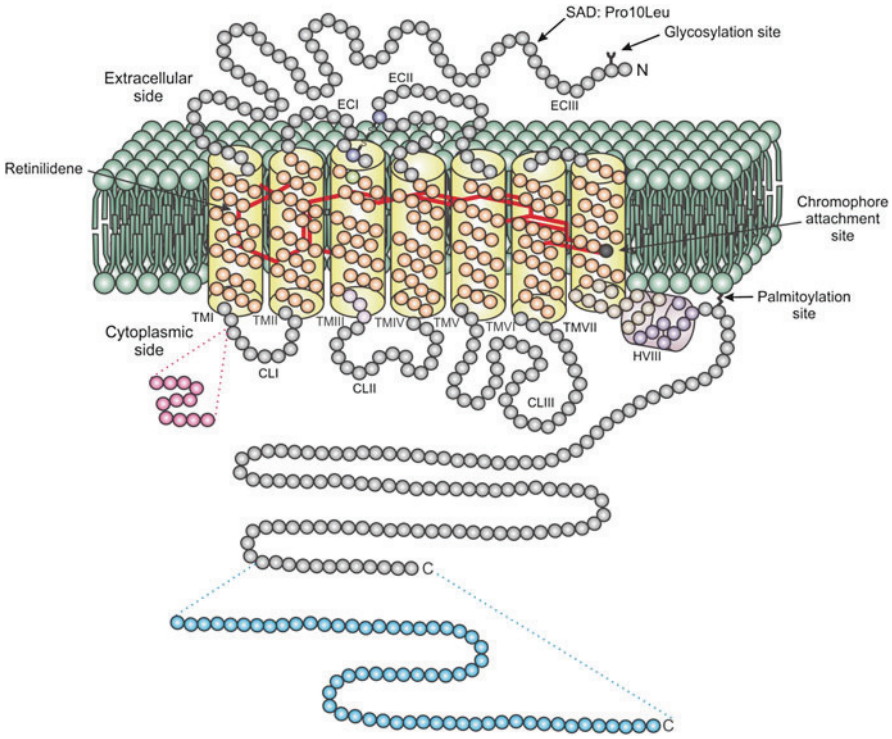

Fig. 2.6 A side-view schematic representation of the human melanopsin pigment showing the presence of seven transmembrane (TM) domains (TMI-VII) (*yellow*), three extracellular loops (ECI-III), three cytoplasmic loops (CLI-III), an amino-terminus (N), a carboxyl-terminus (C), a putative eighth cytoplasmic 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

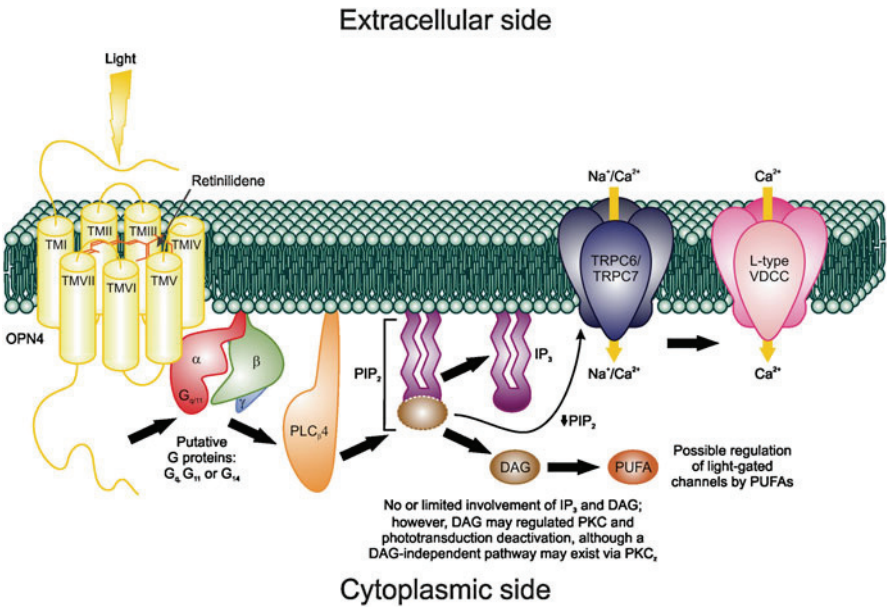

Fig. 2.7 A diagram showing the current hypothesis of how the melanopsin (OPN4) phototransduction cascade generates a depolarising current, thus converting photons into electrical signals. Initially, photostimulation of the retinal chromophore (*orange*) within the melanopsin pigment (*yellow*) results 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 PLCβ4$^{-/-}$ 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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