Evolutionary Constraint on Visual and Nonvisual Mammalian Opsins

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Abstract Animals have evolved light-sensitive G protein–coupled receptors, known as opsins, to detect coherent and ambient light for visual and nonvisual functions. These opsins have evolved to satisfy the particular lighting niches of the organisms that express them. While many unique patterns of evolution have been identified in mammals for rod and cone opsins, far less is known about the atypical mammalian opsins. Using genomic data from over 400 mammalian species from 22 orders, unique patterns of evolution for each mammalian opsins were identified, including photoisomerases, RGR-opsin (RGR) and peropsin (RRH), as well as atypical opsins, encephalopsin (OPN3), melanopsin (OPN4), and neuropsin (OPN5). The results demonstrate that OPN5 and rhodopsin show extreme conservation across all mammalian lineages. The cone opsins, SWS1 and LWS, and the nonvisual opsins, OPN3 and RRH, demonstrate a moderate degree of sequence conservation relative to other opsins, with some instances of lineage-specific gene loss. Finally, the photoisomerase, RGR, and the best-studied atypical opsin, OPN4, have high sequence diversity within mammals. These conservation patterns are maintained in human populations. Importantly, all mammalian opsins retain key amino acid residues important for conjugation to retinal-based chromophores, permitting light sensitivity. These patterns of evolution are discussed along with known functions of each atypical opsin, such as in circadian or metabolic physiology, to provide insight into the observed patterns of evolutionary constraint.

Keywords encephalopsin, melanopsin, neuropsin, RGR-opsin, peropsin, atypical

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Life has evolved in the presence of consistent rhythms of light and dark. This light has been used for adaptive advantage by allowing an organism to interact with its external world, through focusing and detection of coherent, image-forming light, as well as to synchronize to daily and seasonal rhythms via the detection of ambient, scattered light. While several mechanisms of light detection are known, all animals use a single family of retinal-based G protein–coupled receptors (GPCRs), known as opsins, for light detection. For their light-detecting function, energy from an incident photon isomerizes a retinal-based chromophore, resulting in a structural change within the opsin protein that initiates a G protein signaling event. This protein family evolved prior to the evolution of animal visual systems, suggesting that the first functions for opsins were nonvisual, such as detecting time of day information based on light intensity.

Research over the past two decades has strongly indicated that several of these opsin classes play specific and crucial roles in circadian entrainment in mammals. While other classes of photopigments, such as cryptochromes, function as circadian photoreceptors in insects (Emery et al., 1998; Stanewsky et al., 1998), in vertebrates all known circadian photopigments are opsin-based. In particular, recent work has suggested that the nonvisual opsins OPN4 (melanopsin), OPN5 (neuropsin), and OPN3 (encephalopsin) each have unique functions for circadian rhythmicity in the mammal. Hence, an analysis of the evolution of these photoreceptive proteins may provide insights into the evolution of circadian rhythmicity in animals.

Opsins can be broadly classified as visual opsins, atypical opsins (i.e., nonvisual), or photoisomerases. This functional classification largely mirrors the evolutionary divergence of these opsins and has been analyzed based on the exon-intron structure of each opsin gene (Bellingham et al., 2003). The visual opsins and encephalopsins are referred to as C-type or ciliary opsins (regardless of the expression in cilia). In addition, a second class of opsins with a more distant divergence is the R-type or rhabdomeric opsins (regardless of the association with rhabdomeres), which include the atypical opsin OPN4 and invertebrate visual opsins. Cnidopsins are another class of opsins that are not found in vertebrate lineages (Plachetzki et al., 2007). Finally, Group 4 (RGR/G_o) opsins share a common evolutionary lineage and consist of photoisomerases and the atypical opsin, OPN5. While each of these 4 classes have distinct genomic organization, they all evolved from duplication events from a single melatonin receptor-like GPCR in stem eumetazoans prior to the Cambrian explosion and subsequently diversified prior to the vertebrate evolution (Feuda et al., 2012; Fleming et al., 2020). Thus, all vertebrates contain a similar repertoire of opsin genes. Despite the vast array of opsins found in the animal kingdom, the most recent common ancestor to all mammals likely had just 10 (Figure 1a) (Gerkema et al., 2013). Loss of opsin genes in stem mammals is often attributed to a "nocturnal bottleneck" during the Mesozoic era, affecting both visual and nonvisual opsins (Gerkema et al., 2013). Which opsins persist is dependent on the function of each opsin and the lighting environment in which the species inhabits. Ecological niches that promote diversification of the light-detecting mechanisms commonly include marine or subterranean habitats or strictly nocturnal lifestyles. The following analysis describes the mutational rate, relative site-by-site variation, and unique deviations in opsin evolution, as well as a brief summary of the currently known functions for each opsin that has contributed to the observed pattern of evolution.

MATERIALS AND METHODS

Opsin Sequence Acquisition

Protein sequences used in this study were acquired from multiple publicly available resources, namely, NCBI (Sayers et al., 2009), UCSC Genome Browser (Haeussler et al., 2019), UniProt (UniProt Consortium, 2019), Ensembl (Cunningham et al., 2019), Bat1KT (Jebb et al., 2020; Teeling et al., 2018), and the Ruminant Genome Database (L. Chen et al., 2019), as well as select publications (Meredith et al., 2013; Newman and Robinson, 2006; Sadier et al., 2018). Accession numbers and assemblies used for each species' opsin sequence can be found in supplemental data 1. Fragmented and incomplete sequences were not used in analyses. Splice variants were excluded from the analysis.

Substitutions/Site and Evolutionary Rate

Amino acid sequences were aligned using the MUSCLE algorithm. Substitutions/site was calculated in MEGA-X (Kumar et al., 2018) using the Poisson correction model (Zuckerkandl and Pauling, 1965). The shape parameter for the discrete gamma distribution was first estimated under the Jones-Taylor-Thornton (JTT) model (Jones et al., 1992). Species list was entered into TimeTree (Kumar et al., 2017) to generate a phylogenetic tree and determine estimates of divergence between species (Boc et al., 2012). Evolutionary rate was determined by pairwise

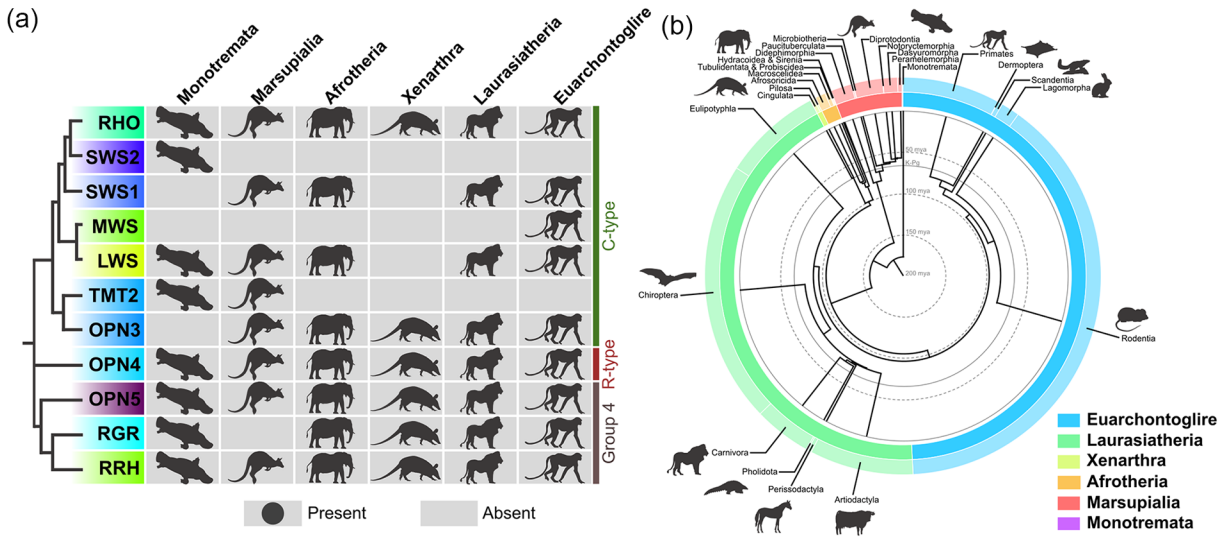

Figure 1. Opsin expression in major branches of the mammalian lineage. (a) Opsin expression in each infraclass/superorder of mammals is indicated by a symbol with the maximum absorption and phylogeny of each opsin. (b) Radial phylogenetic tree of all 27 mammalian orders proportioned by the number of species within each order. Abbreviations: RHO = rhodopsin; SWS2 = short-wavelength-sensitive 2; SWS1 = short-wavelength-sensitive 1; MWS = medium-wavelength-sensitive; LWS = long-wavelength-sensitive; TMT2 = teleost multiple tissue opsin 2; OPN3 = encephalopsin; OPN4 = melanopsin; OPN5 = neuropsin; RGR = RGR-opsin; RRH = peropsin.

substitutions/site divided by evolutionary distance (i.e., twice the time since species divergence).

Similarity Identity Matrices

Similarity and identity matrices were constructed using MatGat (Campanella et al., 2003) using the BLOSUM62 substitution matrix and opsin protein sequences.

Site-by-Site Relative Mutational Rate

The relative mutational rate for each amino acid position was determined under the JTT model using a discrete gamma distribution with 8 categories, allowing some sites to stay invariant (Jones et al., 1992; Kumar et al., 2018). Rates were scaled such that the average evolutionary rate for each protein was 1. The color scale was set such that the minimum rate for each protein was gray, the average rate (1) was yellow, and the maximum rate corresponded to the maximum absorption of each opsin. Only sites that were present in the ancestral amino acid sequence were used for analysis to prevent skewing by lineage-specific insertions.

Ancestral Sequence Reconstruction

Stem mammalian ancestral sequences were reconstructed using MUSCLE-aligned sequences and phylogenetic trees generated from each species using TimeTree (Kumar et al., 2017) within Mega-X (Kumar et al., 2018) based on maximum likelihood using the JTT model (Jones et al., 1992). Available opsin sequences from *Gallus, Alligator mississippiensis*, and *Anolis carolinensis* were used as outgroup sequences.

PER2::LUCIFERASE Rhythms

All animal experiments were carried out according to Institutional Animal Care and Use Committee guidelines at University of Washington, Seattle, WA. Wild-type; $Per2^{Luciferase/Luciferase}$ or $Opn3^{LacZ/LacZ}$; $Per2^{Luciferase/Luciferase}$ mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. The dorsal interscapular fat pads and inguinal white adipose tissue were dissected into cold Hank's Balanced Salt Solution (Gibco). Interscapular white fat was dissected away from brown fat under a dissecting microscope. Small (~1 mm^3) pieces of adipose tissue were placed on cell culture inserts (Millipore, PICMORG50) and cultured in sealed dishes in Dulbecco's Modified Eagle Medium (Cellgro) containing B27 serum-free supplement (Life Technologies), 0.1 mM D-Luciferin potassium salt (Biosynth), 352.5 µg/mL sodium bicarbonate, 10 mM HEPES, and 25 units/mL penicillin and 25 µg streptomycin (Life Technologies). Bioluminescence was monitored at 10-min intervals for 2.5 days using a Lumicycle 32 photometer device (Actimetrics, www.actimetics.com) contained within a 36 °C incubator.

RESULTS AND DISCUSSION

Visual Opsins

Rhodopsin (RHO) is the dim light–sensitive opsin expressed in rod cells in the mammalian retina that is necessary for scotopic vision (λ_{max} ~500 nm) (Imai et al., 2007). RHO is well-conserved in all mammals assessed. This sequence conservation includes animals living in virtually lightless environments, such as subterrestrial mammals, or limited-light environments, such as nocturnal and marine mammals (Figures 1a and 2a). RHO is even well-conserved in species of mole rats, which often possess a rudimentary eye that lacks a lens and is covered in fur and skin (Emerling and Springer, 2014a; Hendriks et al., 1987). Despite these limitations, mole rats are still able to detect breaches in tunnels and possess negative phototaxis. This ability demonstrates that even if conventional image-forming vision has been lost, there is continued selective pressure for opsin-mediated photodetection (Cooper et al., 1993; Kott et al., 2010). In addition, blind mole rats can entrain to a light-dark cycle, a nonvisual function of the retina that can be mediated via RHO, cone opsins, or OPN4, which remain present and functional in this fossorial mammal (David-Gray et al., 1998, 1999; Esquiva et al., 2016; Hannibal et al., 2002; Janssen et al., 2000). The RHO sequences that have diverged the most from the predicted ancestral sequence belong to the squirrel family of rodents (Sciuridae) and tree shrews (order Scandentia) (Figure 3a and Suppl. Fig. S2A), both of which, intriguingly, possess cone-dominant retinas (Kryger et al., 1998; Müller and Peichl, 1989).

Unlike RHO, the cone opsins are remarkably diverse within the mammalian class (Figure 3b and 3c). There are 4 different mammalian cone opsins: short-wavelength-sensitive 1 (SWS1; λ_{max} ~430 nm), short-wavelength-sensitive 2 (SWS2; λ_{max} ~440 nm), medium-wavelength-sensitive (MWS; λ_{max} ~530 nm), and long-wavelength-sensitive (LWS1; λ_{max} ~560 nm) (Merbs and Nathans, 1992; Wakefield et al., 2008). Most mammals possess 2 cone opsins, SWS1 and MWS/LWS, for dichromatic vision. There was likely redundant function between the two SWS opsins in early mammals, as monotremes (i.e., egg-laying mammals) retained SWS2 and lost SWS1, whereas all other mammals reciprocally retained SWS1 and lost SWS2 (Figure 1a) (Wakefield et al., 2008). Notably, trichromacy is only found within primates and some marsupials within the mammalian class (Arrese et al., 2002, 2005; Hunt et al., 1998). In primates, trichromacy arose independently in old world monkeys, apes, and humans (parvorder Catarhhini) and in new world howler monkeys (genus *Alouatta*) through a duplication event of LWS and sequence divergence in the former and unequal crossover of polymorphic alleles in the latter. By contrast, in trichromatic marsupials, there is no genetic evidence of an additional

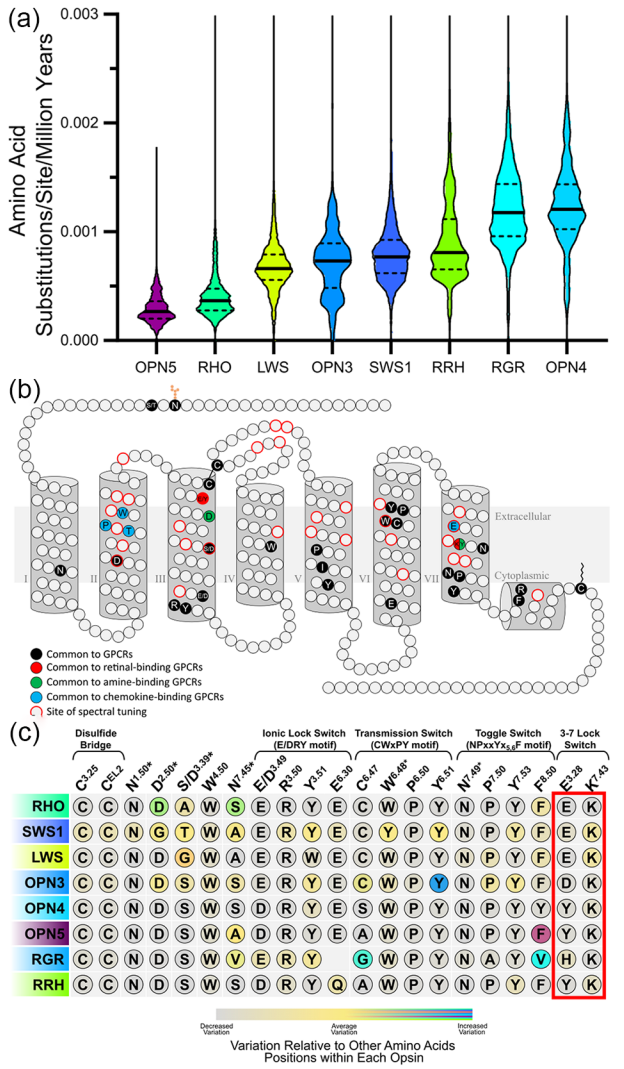

Figure 2. Summary of sequence analysis of mammalian opsin variants. (a) Pairwise mutational rates between all mammalian sequences for each opsin. Solid black line indicates median and dashed lines indicate interquartile range. Rate was determined by dividing amino acid substitutions/site by twice the time since divergence for each pair of species. (b) Representative GPCR structure with highly conserved residues indicated as well as cites of spectral tuning indicated by a red border. Whereas each transmembrane sequence occurs in the same spot between GPCRs, the NxS/T sequence for N-glycosylation can occur anywhere along the cytosolic N-terminus strand. (c) Average relative mutational rate for highly conserved GPCR and photoreceptive (red box) amino acid residues for each opsin. Ballesteros-Weinstein annotation was used to identity amino acid position. Asterisk indicates function in Na^+-binding pocket. For relative mutational rate for all positions within an opsin, see supplemental data 3. Abbreviations: OPN5 = neuropsin; RHO = rhodopsin; LWS = long-wavelength-sensitive; OPN3 = encephalopsin; SWS1 = short-wavelength-sensitive 1; RRH = peropsin; RGR = RGR-opsin; OPN4 = melanopsin; GPCR = G protein–coupled receptor.

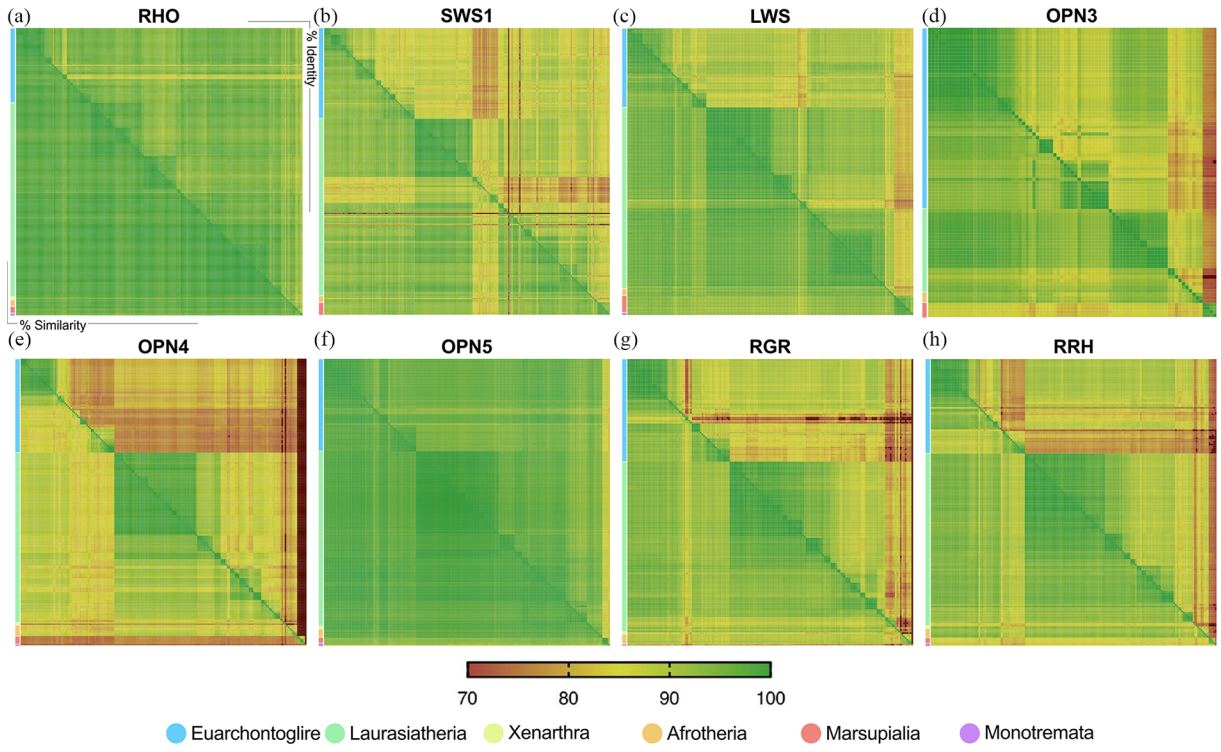

Figure 3. Similarity-identity heatmap for each mammalian opsin (a-h) Similarity scores (%) in lower left and identity scores in upper right. Color-coded branch of mammals to the left of each heatmap for reference. For high-resolution heatmaps labeled with each species name, see supplemental data 2. Dark red indicates that the value falls beyond the limits of the scale. Abbreviations: RHO = rhodopsin; SWS1 = short-wavelength-sensitive 1; LWS = long-wavelength-sensitive; OPN3 = encephalopsin; OPN4 = melanopsin; RGR = RGR-opsin; RRH = peropsin.

opsin gene. Due to the limited number of species expressing SWS2 and MWS opsins, they were not included in further analyses. The remaining 2 cone opsins, SWS1 and LWS, have evolved with a moderate degree of sequence conservation relative to other mammalian opsins (Figure 2b and 2c). Each has been lost in specific lineages, always occurring in animals with restricted light availability, such as marine, nocturnal, or subterrestrial mammals. For example, SWS1 has been lost in all cetaceans (toothed and baleen whales) (Fasick and Robinson, 2016; Meredith et al., 2013; Peichl et al., 2001), all pinnipeds (seals) (Levenson et al., 2006; Peichl et al., 2001; Peichl and Moutairou, 1998), all xenarthrans (armadillos, sloths, and anteaters) (Emerling and Springer, 2014b), and night monkeys (family Aotidae) (Jacobs et al., 1993). Similarly, LWS function has been lost in many whale species (most baleen whales, some toothed whales) (Meredith et al., 2013), all xenarthrans (Emerling and Springer, 2014b), naked mole rats (Kim et al., 2011), and star-nosed and golden moles (Emerling and Springer, 2014a).

Numerous amino acid residues have been identified in RHO, SWS1, and LWS that shift the spectral sensitivity of the opsin to shorter or longer wavelengths (Bowmaker, 2008; Lin et al., 2017; Musilova et al., 2019; Nathans, 1990; Yokoyama, 2008) (Figure 2c). While these mutations have been best studied in RHO homologues, they are broadly conserved across species. The absorption spectrum of retinal within an opsin is altered by these amino acid residues, altering the energetics of retinal isomerization. Notable residues for cone opsin spectral tuning include five which red-shift MWS/LWS (permitting trichromacy when both MWS and LWS are present) and $F^{2.53}$ (in Ballesteros-Weinstein nomenclature, corresponding to Phe-86 in bovine RHO) in SWS1, which shifts the λ_{max} of SWS1 into the UV spectrum (Cowing et al., 2002; Hunt et al., 2007; Yokoyama, 2000; Yokoyama and Radlwimmer, 1998). The predicted mammalian ancestral sequence for SWS1 contains this substitution, indicating that stem mammals had visual perception into the UV range, although it is a locus of increased variability (Suppl. Fig. S3B). While these loci are the best predictors for visual opsins, they may still be informative in spectral tuning of the remaining opsins, although such predictions should be determined experimentally.

In addition to the variation in amino acid residues necessary for spectral tuning, increased sequence variation in the amino acid residues necessary for light sensitivity, such as the seventh transmembrane

lysine residue, is seen in SWS1 and LWS. This observation is due to the inclusion of organisms that have lost the opsin protein's photosensitive functions and therefore have reduced constraint on these key amino acid residues but have retained an identifiable genomic sequence (Figure 2c). These patterns of evolution indicate the variable need for color vision in distinctly illuminated environments. In addition, environmental lighting does not only impact the visual function of rod and cone opsins but also influences nonvisual functions of these opsins. Within the retina, visual opsins in rods and cones can signal to intrinsically photosensitive retinal ganglion cells to mediate nonvisual responses such as circadian entrainment and the pupillary light response, independent of OPN4, although in the absence of OPN4, these responses are not sustained as well (Altimus et al., 2008; Güler et al., 2008; Lucas et al., 2003; Panda et al., 2002, 2003). Visual opsins are also found in extraretinal tissues, potentially suggesting additional nonvisual roles. Overall, when evolutionary pressure favors cone opsin function, they are conserved with moderate sequence constraint relative to other mammalian opsins; however, their necessity understandably varies with the environmental light of the animal. While these results largely recapitulate previous findings on mammalian opsin evolution, they serve as an important point of reference for the subsequent analysis into the mammalian evolution of nonvisual opsin evolution.

Encephalopsins

Encephalopsins are a unique, yet poorly understood class of opsins. While several encephalopsins are present in teleost and saurospid genomes, most mammals have only retained the blue light–sensitive OPN3, although monotremes and marsupials have retained teleost multiple tissue opsin 2 (TMT2, λ_{max} ~470 nm) (Kato et al., 2016; Sakai et al., 2015). Their expression is intriguingly not limited to the eye, as they are found throughout the body in a variety of cell types (Blackshaw and Snyder, 1999; Halford et al., 2001). Among the mammalian opsins, OPN3 stands out as the only opsin that has not yet been expressed as a functional photopigment in vitro. While zebrafish OPN3 has been shown to function as a blue light photopigment with λ_{max} of 465 nm and murine OPN3 has been shown to bind the retinaldehyde pigment, reconstitution of functional photopigment has remained elusive (Ozdeslik et al., 2019; Sugihara et al., 2016). Whether this is due to technical issues with heterologous expression or whether OPN3 has limited photoreceptive function is currently unclear (Olinski et al., 2020).

Outside of mammals, encephalopsins appear to function to synchronize peripheral clock gene rhythmicity to the external light-dark cycle (Cavallari et al., 2011; Moutsaki et al., 2003; Poletini et al., 2015). In mammals, in tissues such as the retina and skin, OPN3 appears neither necessary nor sufficient for local light entrainment (Buhr et al., 2015, 2019). The most apparent role to date for OPN3 in circadian function is in setting the amplitude of core clock gene oscillation. Retinal clock gene amplitude is diminished in the absence of OPN3 (Buhr et al., 2015). This phenomenon appears to occur in other OPN3-expressing tissues, as loss of *Opn3* dampens clock gene amplitude in interscapular and inguinal adipocytes (Figure 4a and 4b). These data suggest that OPN3 has dedicated functions within the circadian clock but leave open the question of whether it can serve as a photopigment for entrainment or phase shifting.

Lipolysis, or the mobilization of lipids from adipocytes, occurs with a circadian pattern that is controlled, in part, by clock genes within adipocytes. Mice with specific conditional loss of the clock gene *Bmal1* in adipocytes have disrupted lipolysis, increased weight gain in response to high-fat diet, increased adipocyte size, and a reduced metabolic rate (Paschos et al., 2012; Shostak et al., 2013). Loss of *Opn3* from adipocytes also results in impaired lipolysis, increased weight gain in response to high-fat diet, increased adipocyte size, and a reduced metabolic rate (Nayak et al., 2020; Sato et al., 2020). Interestingly, OPN3 is downregulated in adipocytes in response to high-fat diet (Choi et al., 2015). The findings that OPN3 facilitates clock gene rhythmicity in adipocytes help to reconcile these metabolic phenotypes. However, OPN3 and light were also demonstrated to interdependently modulate adaptive thermogenesis and glucose uptake in adipocytes, suggesting that OPN3 also functions in the photoreceptive pathway. Additional photoreceptive functions suggested for OPN3 include photorelaxation of smooth muscle within the uterus and airway (Barreto Ortiz et al., 2018; Wu et al., 2020; Yim et al., 2019, 2020). A non-light-dependent role for OPN3 has also been proposed in epidermal melanocytes, where it heterodimerizes with melanocortin 1 receptor to regulate pigmentation (Ozdeslik et al., 2019). A splice variant of OPN3, which excludes the second exon and subsequently truncates the protein within the helix, is unlikely to function as a light-sensitive protein or even as a GPCR, but may still function as a negative modulator of other proteins (Haltaufderhyde et al., 2015). Taken together, these diverse functions are expected to produce different patterns of evolution than seen with visual opsins.

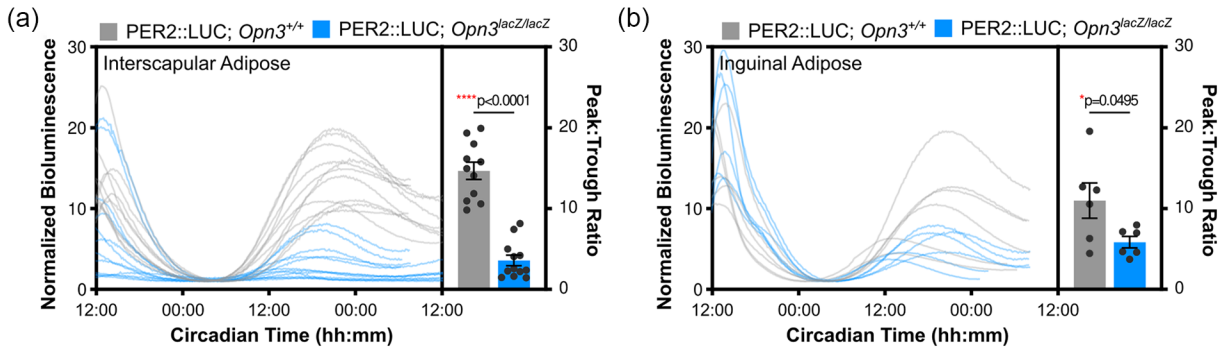

Figure 4. ***Opn3*** **facilitates PER2::LUCIFERASE amplitude ex vivo. (a) PERIOD2::LUCIFERASE rhythms from interscapular and (b) inguinal adipocytes with normalized peak:trough ratios.** ***P*** **values from Student's** ***t*** **test.**

In Euarchontoglires (e.g., primates and rodents), OPN3 is well-conserved with little sequence divergence (Figures 2a and 3d). However, in the laurasiatherian superorder, OPN3 remains well-conserved in carnivora, but has been lost in the Vespertilionidae family of bats, camelids, pigs, and ruminants (Figure 3d and Suppl. Fig. S2D). In xenarthrans, no complete sequences were identified for OPN3, but between sloths and armadillos all regions of the sequence could be identified, indicating either poor sequence coverage of OPN3 or, less likely, a recent loss in both branches of xenarthrans. Finally, OPN3 could not be found in the platypus genome, suggesting additional loss in monotremes. Altogether, these results suggest that the lighting environment may play some role in OPN3 sequence constraint (e.g., bats and early monotremes, but not subterrestrial rodents). It is possible, however, that other factors such as food and water availability or metabolic rate may place additional demands on sequence conservation (e.g., even-toed ungulates). An important caveat to genomic analysis of OPN3 is its high GC content in the first exon, leading to poor sequence coverage (Wang et al., 2011). In addition, domesticated animals (goat, cattle) serve as reference genomes for other artiodactyla species. Potential selection against the metabolic effects of OPN3 during domestication, similar to the absence of the thermogenic gene UCP1 from adipocytes in pigs (Berg et al., 2006), may bias the analysis of nondomesticated OPN3 artiodactyla sequences due to poor alignment for the reference genomes.

Melanopsin

OPN4 is a blue light–sensitive (λ_{max} ~480 nm) atypical opsin expressed in intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina that mediates a range of nonvisual functions during development through adulthood. These functions include negative phototaxis, retinal vascular development in neonates, circadian photoentrainment of the suprachiasmatic nucleus, and the pupillary light response (Do, 2019; Hattar et al., 2002, 2003; Johnson et al., 2010; Lucas et al., 2003; Panda et al., 2002; Provencio et al., 1998; Rao et al., 2013; Ruby et al., 2002). Our analysis demonstrates that OPN4 shows the most sequence diversity within mammals relative to other opsins (Figure 2a). A previous study has demonstrated that OPN4 sequence variation is greater between 2 Euarchontoglire species than between 2 marsupials that diverged at similar evolutionary times (Pires et al., 2007). Our results demonstrate that the source of this variation is due to increased mutation rates in rodents, rather than increased mutations in primates or increased genetic constraint in marsupials (Suppl. Fig. S2E). Across all mammals, most of the observed diversity arises from mutations in the N- and C-termini of the protein (Suppl. Fig. S3E). These changes commonly alter the number of phosphorylatable serine and threonine residues on the C-terminus, which is known to alter the deactivation kinetics of OPN4 (Blasic et al., 2012a, 2012b; Blasic et al., 2014; Somasundaram et al., 2017). Proximal C-terminal serine and threonine residues appear more likely to regulate protein function. Reduced serine and threonine residue sites have been suggested to preserve rod function in animals occupying low-light environments, such as armadillos (Fasick and Robinson, 2016), by modulating the kinetics of the pupillary light response (Fasick and Robinson, 2016; Somasundaram et al., 2017). Our mammalian class-wide analysis of the C-terminus of OPN4 is consistent with this, although notably whales were not observed to have a decrease in phosphorylatable residues, whereas mouse-like rodents were found to have an increase (Figure 5). When separating these sites of phosphorylation into previously described clusters (P-I through P-IV) (Valdez-Lopez et al., 2020), there is an elevation of phosphorylatable sites in rodents in P-II, the cluster with the most influence on

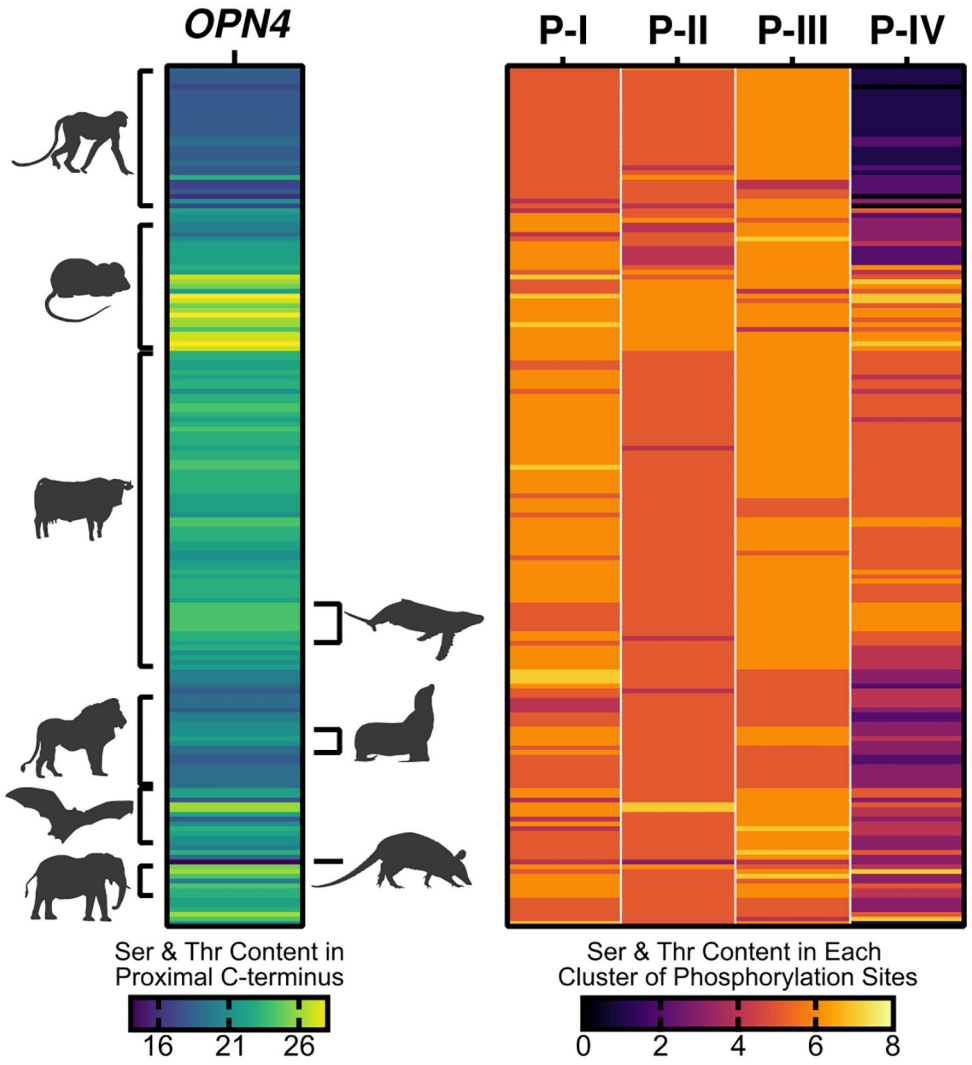

Figure 5. Phosphorylatable sites on the proximal C-terminus of OPN4. Total serine and threonine residue content on the C-terminal tail between the eighth helix and alternative splice sites. Species are ordered by evolutionary relationship, with symbols indicating primates, rodentia, artiodactyla, carnivora, chiroptera, and afrotheria (left) or cetacea, pinnepedia, or xenarthra (right). On the right, the sum of serine and threonine residues within each cluster of phosphorylatable sites (P-I through P-IV). Abbreviation: OPN4 = melanopsin.

OPN4 deactivation (Figure 5). The cluster with the most diversity across mammals is P-IV (Figure 5), which has minimal influence on OPN4 deactivation (Valdez-Lopez et al., 2020). Interestingly, we did not find any mammalian lineages with a loss of OPN4, indicating its importance for all mammalian life independent of the lighting environment.

Increased sequence variation in OPN4 does not include contribution from various isoforms of OPN4. OPN4 has 2 isoforms, a short (OPN4S) and a long form (OPN4L) differing only in the distal C-terminus (Hughes et al., 2012; Pires et al., 2009). OPN4S is the predominant isoform and is found in the M1 subtype of ipRGCs, whereas OPN4L is found in both M1 and non-M1 ipRGCs (Pires et al., 2009). Both isoforms have similar spectral tuning and response amplitudes in vitro; however, differences in the C-terminus may alter post-translational regulation of the protein in vivo (Pires et al., 2009). The functions of these isoforms have reflected their relative expression and subtype specificity, in which OPN4S exclusively functions in pupillary light response and OPN4L exclusively functions in negative masking, whereas both OPN4S and OPN4L function in phase shifting and sleep induction in mice (Jagannath et al., 2015). The developmental roles of OPN4 are likely limited to OPN4S, as it is the first of the two isoforms to be expressed in the retina (Hughes et al., 2012).

Unlike the previously discussed opsins, OPN4 (and other rhabdomeric opsins such as arthropod opsins) can undergo a process known as photoreversal. During photoreversal, the all-*trans*-retinal remains bound to the opsin and a subsequent photon isomerizes retinal back to a *cis*-configuration, eliminating the

need for extrinsic regeneration of the chromophore, as is present in the visual cycle. This characteristic likely involves numerous amino acid residues stabilizing all-*trans*-retinal within the opsin and is not unique to rhabdomeric opsins, as non-eutherian OPN5 (discussed in the next section) and lamprey parapinopsin (a ciliary opsin) also possess photoreversal (Koyanagi et al., 2004; Yamashita et al., 2010). Distinct from other mammalian opsins, OPN4 is tristable, forming stable complexes with 11-*cis*-, all-*trans*-, and 7-*cis*-retinal, and this property of OPN4 allows for sustained signaling and broad absorption which is beneficial for its nonvisual functions (Emanuel and Do, 2015). This stability is consistent with a view that retinal remains covalently bound to melanopsin, preventing bleaching observed in other pigments (Sexton et al., 2012).

Neuropsin

OPN5 is a violet light–sensitive (λ_{max} ~380 nm) opsin expressed in the retina, cornea, skin, brain, and testes (Kojima et al., 2011; Tarttelin et al., 2003; Yamashita et al., 2010). While OPN5 in birds and amphibians is bistable, binding all-*trans*-retinal with a second absorption peak in blue wavelengths, mammalian OPN5 does not bind to all-*trans*-retinal and lacks this second absorption peak (Yamashita et al., 2010). A single mutation is responsible for this shift at location 4.57 (corresponding to A168 in bovine RHO) with an alanine residue conferring bistability and a threonine residue, present in most mammals, lacking bistability (Yamashita et al., 2014). Interestingly, monotremes and marsupials have $A^{4.57}$, suggesting that monostable OPN5 evolved in eutherian mammals and was not present in stem mammals (Suppl. Fig. S3F). Only recently have functions been identified for mammalian OPN5. OPN5 functions to regulate perinatal vascular development and entrains local retinal, corneal, and skin clock gene rhythmicity independent of the suprachiasmatic nucleus; it also plays a minor role in behavioral entrainment/phase shifting (Buhr et al., 2015, 2019; Díaz et al., 2020; Nguyen et al., 2019; Ota et al., 2018). In the mouse, OPN5 functions in the medial preoptic nucleus of the hypothalamus to regulate energy metabolism and thermogenesis (Zhang et al., 2020). Importantly, all of these known functions of OPN5 are dependent on light. Consistent with this finding, ex vivo phase shifting of the skin and cornea has an action spectrum that matches the in vivo absorption spectrum of OPN5 (Buhr et al., 2019; Díaz et al., 2020).

In birds, OPN5 is expressed in tanycytes that line the third ventricle, where it detects seasonal changes in light to regulate circannual physiology and behavior independent of the retina (Nakane et al., 2010). However, in the murine hypothalamus, OPN5 is not expressed in tanycytes (unpublished data). Interestingly, numerous studies have converged on a mammalian *Opn5*$^+$*/Lepr*$^+$*/Ptger3*$^+$*/Bdnf*$^+$*/Adcyap1*$^+$*/Qrfp*$^+$ preoptic population as a potent regulator of energy metabolism and thermogenesis that also functions in regulating entry to torpor (Hrvatin et al., 2020; Moffitt et al., 2018; Takahashi et al., 2020; Tan et al., 2016; Yu et al., 2016; Zhang et al., 2020). The functions of these marker genes provide insight into the role of this OPN5-expressing cell type, as leptin receptor (*Lepr*) signaling conveys information about the energy status of an organism, prostaglandin receptor EP3 signaling mediates pyrexia, and *Qrfp* regulates activity and food intake. It is conceivable that this neuronal population serves as a central hub, integrating temperature, seasonal lighting, and food availability to regulate seasonal changes in metabolism in mammals.

Despite the seemingly disjoint functions of OPN5, it is the most conserved mammalian opsin (Figure 2a). We were unable to identify any mammalian species that had lost OPN5 or that had greatly deviated from the ancestral sequence (Figure 3f and Suppl. Fig. S2F). Most mutations occur in the N- or C-termini of the protein (Suppl. Fig. S3F), while all residues necessary for GPCR or photoreceptive function remained intact (Figure 2c). Most of the residues associated with spectral tuning in the visual opsins show little variation, suggesting that there is little deviation from its known violet absorption (Suppl. Fig. S3F). In addition, there are several potential isoforms of OPN5; however, no functions or tissue-specific expression of these isoforms has been reported. It is likely that extraretinal functions of OPN5 may mediate this extreme sequence conservation in mammals, as mice lacking hypothalamic OPN5 have profound metabolic dysregulation.

Photoisomerases

Photoisomerases bind all-*trans* retinal and convert it to 11-*cis*-retinal upon light stimulation, recycling chromophore to maintain the visual cycle in parallel with retinal isomerase (Chen et al., 2001; Morshedian et al., 2019; Radu et al., 2008). RGR and RRH are both expressed within the retinal pigmented epithelium, while RGR is additionally expressed in Müller glia within the retina (Morshedian et al., 2019; Shen et al., 1994; Sun et al., 1997). It should be noted that RGR expression in Müller glia in diurnal animals, such as the human and cattle, is far greater than in nocturnal animals, such as the mouse (Zhang et al., 2019). This observation is consistent with an important role of RGR as a photoisomerase in the cone visual cycle, which occurs in photopic light conditions during the

day (Palczewski and Kiser, 2020; Ward et al., 2020; Zhang et al., 2019).

RGR is poorly conserved relative to other opsins (Figure 2a) and notably is absent in marsupial genomes (Figure 1a). In addition, decreased genetic constraint is seen in hystricomorphs, the rodent suborder containing guinea pigs, mole rats, chinchillas, and degus (Figure 3g and Suppl. Fig. S2G). RGR likely does not function as a signaling GPCR due to accumulated mutations in the ionic lock switch, a domain necessary for receptor activation and coupling to G proteins upon activation (Figure 2c) (Vogel et al., 2008). For example, Euarchontoglires, laurasiatherians, and xenarthrans all have accumulated mutations in the highly conserved E/DRY motif within the third helix, while all mammals lack the highly conserved glutamate ($E^{6.30}$) located within the sixth helix that serves as a counterion to the switch in the inactive state (Palczewski et al., 2000; Vogel et al., 2008). However, other features, such as those necessary for GPCR structure and retinal binding, remain conserved in mammals, supporting RGR as a photoreceptive but nonsignaling GPCR. A splice variant, in which 4 amino acids in the first extracellular loop are inserted, greatly reduces photoisomerization, while a second splice variant, in which 38 amino acids are excluded from a region spanning the sixth and seventh transmembrane helices, abolishes the ability of RGR to regenerate 11-cis retinal (Zhang et al., 2019). It is currently unclear what the consequences are from the loss of RGR in marsupials or mutations in hystricomorphs, but in mice, loss of RGR results in cones losing sensitivity during light exposure and reduced electroretinogram b-wave amplitude following constant light exposure (Chen et al., 2001; Morshedian et al., 2019). It is conceivable that diurnal animals with high visual acuity would be under increased genetic constraint relative to nocturnal mammals or animals that do not rely heavily on the visual system without additional adaptations to regenerate chromophore for the cone visual cycle.

Peropsin is moderately conserved (Figure 2a) with no identifiable loss in mammalian lineages. Increased sequence variation is seen in 2 groups: mouse-like rodents (i.e., myomorpha) and the order eulipotyphla (e.g., shews, hedgehogs) (Figure 3h and Suppl. Fig. S2H). Unlike RGR, RRH has retained the DRY sequence within the ionic lock switch as well as other GPCR features necessary for structure, G protein signaling, and retinal binding (Figure 2c). While no functions for RRH have been established in mammals beyond photoisomerization and retinal transport, it is interesting that arachnid RRH is in an active, potentially signaling state in the dark and is suppressed by light (Cook et al., 2017; Nagata et al., 2018). Not only do these results suggest that RRH may have G protein signaling functions, but that the functions of RRH are conserved in all mammals, regardless of their lighting environment although decreased sequence constraint in subterrestrial/nocturnal mammals demonstrate a spectrum of biological selection.

Genetic Constraint in the Human Population

While evolutionary analysis provides insight into genetic constraint on homozygous variants across time, they provide little evidence for ongoing constraint in human populations. Fortunately, available genomic data on allelic variants from more than 140,000 individuals from the Genome Aggregation Database (gnomAD) allow us to understand continued selective pressure on hemizygous opsin sequence variants as determined by the ratio of observed-to-predicted variants for each gene (Karczewski et al., 2020). Of note, MWS and LWS cannot be analyzed with this method due to their location on the X chromosome (Vollrath et al., 1988). No opsins demonstrate selection against synonymous variants (i.e., changes in nucleotides that do not change amino acid sequence), indicating that the sequences are not located in hypomutated regions of the genome (Siepel et al., 2005) and that any constraint is not due to the primary DNA sequence, as might be the case for noncoding regulatory RNA elements (e.g., microRNA [miRNA], long noncoding RNAs [lncRNA]) (Morris and Mattick, 2014) (Figure 6a). However, OPN3 and OPN5 demonstrate reduced variation to missense variants (i.e., mutations that result in a change in amino acid sequence) (Figure 6b). Finally, SWS1, OPN3, and OPN5 all demonstrate selection against nonsense variants (i.e., premature stop codons, frameshift mutations, mutations at splice junctions) (Figure 6c). Thus, there is continued selective pressure against hemizygous variants for the two atypical opsins, OPN3 and OPN5, and the visual opsin, SWS1, in the modern human population.

It is important to consider that although not constrained against hemizygous variants at the population level, the remaining opsins RHO, OPN4, RGR, and RRH are likely still under selective pressure as variants of RHO and RGR are known to cause ocular diseases, such as retinitis pigmentosa (Dryja, McGee, Hahn, et al., 1990; Dryja, McGee, Reichel, et al., 1990; Li et al., 2016; Morimura et al., 1999), and OPN4 polymorphisms have been associated with seasonal affective disorder (Roecklein et al., 2009). Mutations in SWS1, MWS, LWS, and the locus control region for MWS/LWS are known to cause a variety of types of color blindness (Neitz and Neitz, 2011). Consistent with its role in photorelaxation of airway smooth muscles, OPN3 has been identified as a risk locus for

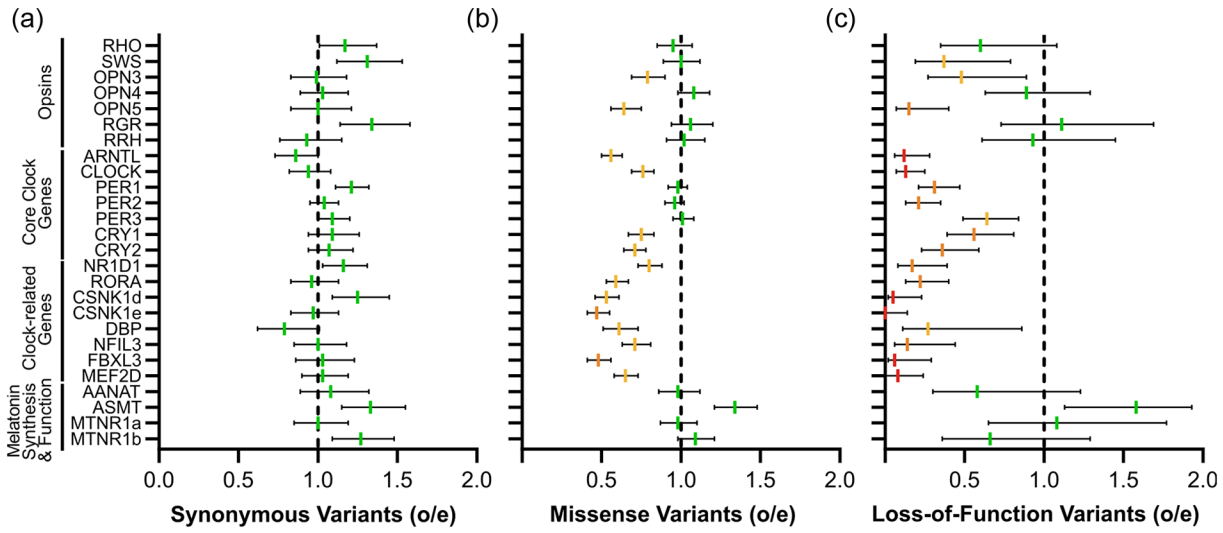

Figure 6. Genetic constraint in human populations. Genetic variation in opsins and clock-related genes from 141,456 humans accessed from the Genome Aggregation Database (Karczewski et al., 2020). Ratio of observed-to-expected genetic variants for (a) synonymous, (b) missense, and (c) loss-of-function variants. Green indicates that the confidence interval of observed-to-expected variants does not exceed the null hypothesis. Yellow, orange, and red indicate increasing constraint against variants. Abbreviations: RHO = rhodopsin; SWS = short-wavelength-sensitive; OPN3 = encephalopsin; OPN4 = melanopsin; OPN5 = neuropsin; RGR = RGR-opsin; RRH = peropsin.

asthma susceptibility (White et al., 2008). To date, no human diseases have been associated with OPN5.

Nonphotic Stimulation of Opsins

Conjugation to a chromophore, such as 11-*cis*-retinal, is necessary for animal opsins to detect and respond to light, and this conjugation is dependent on a crucial lysine residue within the seventh helix ($K^{7.43}$) and a counterion in the third helix (commonly $E^{3.28}$ or $Y^{3.28}$) (Yan et al., 2003). An important caveat to the above analysis is that any stimulus that results in isomerization of retinal is sufficient for opsin G protein signaling (Kandori et al., 2001; Kukura et al., 2005). While light is the primary source of this isomerization, thermal (Pérez-Cerezales et al., 2015; Roy et al., 2020; Shen et al., 2011; Sokabe et al., 2016; Yau et al., 1979) and mechanical (Senthilan et al., 2012; Zanini et al., 2018) forces may also isomerize opsin-conjugated retinal, although the role of the chromophore in these functions may be restricted to opsin trafficking rather than direct detection of stimuli (Leung and Montell, 2017). Current data on OPN5 suggest that it functions only via light-mediated stimulation in the retina, skin, and hypothalamus (Buhr et al., 2015, 2019; Nguyen et al., 2019). Recent studies in smooth muscle and adipocytes demonstrate that OPN3 may also function in a light-dependent manner (Nayak et al., 2020; Sato et al., 2020; Wu et al., 2020; Yim et al., 2019, 2020); however, it has also been proposed that OPN3 has light-independent functions in skin, mediated via conjugation to other GPCRs such as the melanocortin 1 receptor (Ozdeslik et al., 2019). Unlike RHO (Jastrzebska et al., 2015; Ploier et al., 2016; Zhang et al., 2016), OPN3 does not appear to homodimerize (Felce et al., 2017). Whether OPN3 heterodimerization is limited to melanocortin 1 receptor or whether it can conjugate to other GPCRs remains to be determined. It is also currently unclear whether GPCR dimerization plays a role in light-mediated functions of OPN3.

Given that opsins can respond to stimuli other than light and can serve functions other than G protein signaling, such as vitamin A transport, it is important to outline criteria that should be met when attributing a photosensitive function to an opsin. (1) a light-dependent biological response should have a wavelength-dependent action spectrum. This action spectrum could be narrow if involving a single opsin or broad if multiple opsins are involved in the response. To reduce thermal isomerization of retinal, this spectrum should be measured in a temperature-controlled manner since light sources (such as LEDs) can have different thermal efficiencies. (2) An opsin with an absorption spectrum corresponding to the biological response should be expressed within the biological system. Finally, (3) functional loss of the opsin should alter or abolish the action spectrum of the biological response. If the response is mediated by a single opsin, the response should be lost entirely; however if multiple opsins are functioning together, the wavelengths and kinetics that the opsin functions

within will be disrupted. For example, the pupillary light response can be mediated via rod or cone input to ipRGCs, such that in the absence of a single opsin, the biological effect would remain intact; however, the kinetics of this response or wavelengths at which it occurs differ. One of the most sensitive ways to determine this effect would be to selectively mutate the seventh transmembrane lysine, leaving the remainder of the opsin intact. Currently, chromophore conjugation to this lysine residue is necessary for all known light-mediated opsin functions. In addition, selective mutations to the E/DRY sequence would be insightful to determine light-dependent signaling events from other potential opsin functions. Currently available gene-editing techniques make this feasible and could also be incorporated into cell type–specific and temporally controlled methods. Together, these criteria would indicate that a given light-sensitive response is opsin-dependent.

CONCLUSION

Loss of visual opsins follows predictable patterns based on limited-light environments of nocturnal, subterrestrial, or marine mammals. While this factor has shaped the evolution of nonvisual opsins, such as loss of OPN3 in vespertilionid bats, phosphorylation sites of OPN4, or reduced constraint in RGR and RRH sequences in rodents, other factors are likely in play, which is unsurprising given the diverse functions of these atypical opsins. OPN3 and, to a greater extent, OPN5 are well-conserved across mammals, and this conservation continues in modern humans, whereas OPN4 appears to be the most diverse of the mammalian opsins. Better understanding of the functions of these opsins in the variety of tissues that express them will provide us with new insight into the physiology and behavior of organisms that have reduced constraint of these nonvisual opsins.

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NOTE

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