ORIGINAL SCIENTIFIC ARTICLE

Opening the "Black Box": The Genetic and Biochemical Basis of Eye Evolution

Todd H. Oakley · M. Sabrina Pankey

Abstract Eyes provide a rich narrative for understanding evolution, having attracted the attention of preeminent scientists and communicators alike. Until recently, this narrative has focused primarily on the evolution of eye structure and far less on biochemistry or genetics. Although eye biochemistry was once likened to an unknown "black box;" the flood of discoveries in biochemistry is now allowing an increasingly detailed understanding of the processes involved in vision. As a result, evolutionary comparative ("tree-thinking") analyses that use these data currently allow a new and still unfolding narrative, both richer in detail and more comprehensive in scope. Rather than toppling evolutionary theory by finding irreducibly complex molecular machines, eye evolution provides detailed accounts of how natural processes tinker with existing genetic components, duplicating and recombining them, to yield complex, intricate, and highly functional eyes. Understanding the new biochemical narrative is critical for researchers and teachers alike, in order to answer anti-evolutionist claims, and to provide an up-to-date account of the state of knowledge on the subject of eye evolution.

Keywords Evolution · Phototransduction · Vision · Eyes · Phylogeny · Novelty · Complexity

The evolutionary history of eyes is one of the most intriguing and often-told stories in biology. It is a topic researched and discussed by members of the pantheon of evolutionists, including Darwin (1859), Salvini-Plawen and Mayr (1977), Gould (1994), and Dawkins (1996). The commonly told story is familiar and has not changed much since Darwin first proposed it: A light-sensitive nerve gradually changes over evolutionary time, adding complexity across the generations. To Darwin's sketch, Salvini-Plawen and Mayr (1977) added detail, and Nilsson and Pelger (1994) tested the temporal component. The ancient canon of gradual evolution is certainly valuable for demonstrating that some assumptions are met for the hypothesis that natural selection generates complexity, serving as a colorful and graphical example (Dawkins 1996). However, this account—if taken too far—can easily be criticized, as demonstrated, for example, by proponents of Intelligent Design (ID; Behe 1996) who point out that the Darwinian canon ignores complexity at the molecular level. The structure of this article is first to briefly present the "gradual morphological" account of the evolution of eye form and to describe how this account relates to understanding evolution by natural selection. Section II will point out two primary limitations of this "gradual morphological" model, including one highlighted by ID proponents. Third, the paper will present molecular and biochemical details describing how phototransduction (the cascade of signaling events that generate a nervous impulse in response to light) and complex lenses evolved. These two case studies provide specific details about how eyes evolved. The processes elucidated, such as duplication and co-option (the use of existing components during evolution in new contexts) generalize to any complex trait.

The Gradual–Morphological Model of Eye Evolution

"…reason tells me, that if numerous gradations from a perfect and complex eye to one very imperfect and simple, each grade being useful to its possessor, can be

T. H. Oakley (✉) · M. S. Pankey
Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, CA 93106, USA
e-mail: oakley@lifesci.ucsb.edu

shown to exist; if further, the eye does vary ever so slightly, and the variations be inherited, which is certainly the case; and if any variation or modification in the organ be ever useful to an animal under changing conditions of life, then the difficulty of believing that a perfect and complex eye could be formed by natural selection, though insuperable by our imagination, can hardly be considered real."—Charles Darwin (1859)

Darwin Establishes Eye Evolution Model

The gradual–morphological model of eye evolution begins with Darwin, in a chapter of *The Origin of Species* entitled "Difficulties on Theory". He recognized the conceptual leap (certainly influenced by his wife Emma whose highly skeptical margin comment survives on a handwritten draft) to understanding how something as "perfect" as an eagle's eye evolved by natural selection. To make such a leap, Darwin argued that the complexity we see today evolved gradually in a long series of intermediate steps, each step a better adaptation than the previous. Darwin's model (quoted above) primarily explicates the now well-known requirements for natural selection: variation, heritability, and differential survival. He envisions a gradual and linear accumulation of complexity leading to the evolution of complex eyes, driven by natural selection acting on slight, heritable variations. Darwin also notes that the process of natural selection will lead to the particular pattern of numerous gradations from simple to complex eyes. Such a hypothesis predicts that—in general—species with complex eyes are descended from species with simpler and simpler eyes, the farther back in time the ancestry is traced.

Unfortunately, such a pattern may be very difficult to test because it requires knowledge of numerous ancestral species in a direct line from a living species with complex eyes. Eyes are not particularly common in the fossil record (compared to bones and teeth for example). Furthermore, if all lineages have a tendency toward increased eye complexity over time, which is called a universal driven trend (McShea 1996), such a history will be impossible to reconstruct by comparing living taxa (Oakley and Cunningham 2000), because if all descendents of a simple-eyed ancestor evolve complex eyes, we would confidently infer the ancestor to have had complex eyes too. Darwin realized this difficulty. He wrote "both the parent and all transitional varieties will generally have been exterminated by the very process of formation and perfection of the new form." Given that directional selection erases history, which is also not preserved in the fossil record, how can we begin to test the hypothesis of gradual increase from simple to complex eyes?

Darwin's approach to establishing a gradual increase in eye complexity was to tackle an important prerequisite first:

transitional forms between simple and complex eyes must be functional. We may envision eye evolution—as Dawkins did in *Climbing Mount Improbable*—as a slow and gradual climb up a metaphorical mountain with simple eyes at low altitude and complex eyes at the peaks. If different species lineages are proceeding along this climb at different paces, or if different "altitudes" (levels of eye complexity) serve different animals well for their particular mode of life (i.e., not all animals need highly complex eyes), then we should observe currently living species to be at all stages of complexity from simple to complex—they all will have progressed up Mt. Improbable, but to many different altitudes. Darwin first illustrated this concept by a brief review. Namely, various living species possess eyes of differing complexity. As such, one prerequisite for natural selection driving complexity is met: numerous grades of eye exist that are functional and useful to those animals possessing them.

Addition of Detail

With the advantage of over 100 additional years of accumulated knowledge on eye morphology, Salvini-Plawen and Mayr (1977) added exquisite detail to Darwin's approach by extensive review of eye and photoreceptor cell morphology in all animals. In particular, they illustrated specific, single-lineage evolutionary transitions from simple to complex eyes. The authors constructed "morphological sequences of differentiation," which consist of collections of eyes of differing and graduated complexity from closely related living taxa. Unlike Darwin, who collected examples from more distantly related animals, Salvini-Plawen and Mayr provided examples from closely related groups, such as within gastropods, and within bivalves. Similarly, a graduated series of events has been recently summarized for the evolution of vertebrate eyes (Lamb et al. 2007). Here, many molecular and developmental characteristics were used, but otherwise, the vertebrate series is conceptually similar to the "sequences of differentiation" illustrated for mollusks by Salvini-Plawen and Mayr (1977).

Nilsson and Pelger Test Time Dimension

In addition to these comparative studies of actual eyes, Nilsson and Pelger (1994) supported another prerequisite of the gradualist model: there has been enough time to evolve complex eyes. They began with a conceptual model based on actual eyes from nature. This model involves gradually increasing complexity, evolving in a linear series from simple to complex eyes. Their great contribution was to quantify each grade of this conceptual model as a percent change in morphological shape. By making conservative assumptions about the rate of morphological change and

population sizes, they concluded that eyes can evolve from simple photoreceptive spots to complex lens-eyes in only about 400 thousand generations. Although their model could have suggested billions of years were required for this transition, thereby casting doubt on a gradual model of eye evolution, this did not happen. In fact, just the opposite was true—they found that eyes could evolve rapidly in geologic time scales. Nilsson and Pelger's work therefore represents one of the few scientific tests of the gradualist model of eye evolution. Nevertheless, there exists an important criticism of the work. Hansen (2003) argued that Nilsson and Pelger neglected pleiotropy (multiple functions of a single gene) in their calculations by assuming that any change in morphology of one feature is free from effects on other features. Hansen (2003) suggested gene duplication as a way of reducing constraints imposed by pleiotropic interactions. As we shall see in a later section, duplication of genes has indeed played an important role in eye evolution.

Summary and Strengths

To summarize key events in the history of the gradual model of eye evolution, Darwin proposed the model and pointed out—by brief review—the fact that numerous functional eyes exist of varying complexity in living organisms. The absence of functional intermediates would have been a blow to the gradual model. Salvini-Plawen and Mayr extended Darwin's proposal by extensive review, proposing multiple specific candidates for linear series of eye evolution, including within gastropod mollusks. Later, Nilsson and Pelger tested the time component of the gradual model and found that complex lens eyes can indeed evolve quickly in geologic terms. Nilsson and Pelger's experiments assume that morphological functions can be optimized separately without tradeoffs (Hansen 2003) and assume a particular pathway of morphological evolution. The requirement of too much time also would have been a blow to the gradual model. Finally, these gradual-morphological models have been used extensively by Richard Dawkins in popular books arguing for the efficacy of natural selection. The models provide powerful and visual ways to imagine how eye evolution might have proceeded and are therefore valuable as tools to communicate how natural selection can produce amazingly complex structures, even with a random component of mutation (Dawkins 1996).

Limitations of the Gradual-Morphological Model

"Unfortunately, [equating microevolution with all of evolution] often results in an overemphasis on changes

in allele frequencies and an underemphasis on (or no consideration of) the origin of the different alleles and their properties. Both are important in evolution."—John Endler (1986)

"Scientific illustrations are not frills or summaries; they are foci for modes of thought."—Stephen Jay Gould (1991)

Despite its multiple strengths, the gradual-morphological model of eye evolution has at least two shortcomings. First, the model usually takes as a starting point variation in morphology caused by anonymous, unknown mutations, and therefore does not inquire as to what molecular changes might have been responsible for the origins of variation. A second shortcoming is that the gradual-morphological model leads people to view eye evolution as a single, linear path from simple to complex eye. In this simplified view, eye complexity is effectively reduced to a single parameter, instead of being viewed as an integrated set of many components. Eyes and their integrated sets of components (mainly proteins encoded by genes) do not have linear evolutionary histories; instead, their histories are branching histories, requiring a phylogenetic or "tree thinking" perspective (O'Hara 1997). Examining eye evolution with a "tree thinking" perspective illustrates how components of currently integrated structures have differing histories, assembled from other systems over evolutionary time. This section discusses in more detail these two drawbacks of the gradual-morphological model of eye evolution.

Origins of Variation in Eye Evolution

Missing from the gradual-morphological model is any specific detail about how eye variation might have originated, which is rooted in the paucity of knowledge on the molecular basis of vision available to Darwin, Mayr, and their contemporaries. Of course, evolutionists since the modern synthesis have known that the source of heritable variation in structures like eyes is mutation of the genes involved in "making" those eyes. However, the purely morphological models completely lack any connection between genes and form. Such a connection is required to be able to identify how a specific mutation gives rise to a specific change in phenotype. That connection was not elucidated to the level of specific nucleotide changes in DNA until recently. Instead, variation was assumed to be abundant, and its cause was conceived as anonymous mutations that change eye form in an unspecified way. Today, as a result of numerous ongoing studies in biochemistry and genetics, we have increasingly detailed information about the genetic components involved in phototransduction (conversion of a light signal into a nervous impulse, see Appendix 1, 2), photoreception

(sensing light), vision (formation of images from light), and eye development. In many cases, scientists have elucidated specific genes and often specific nucleotide changes that are involved in different visual systems. When such mutations arise in a population of organisms, they serve as the origin of variation upon which natural selection can act.

Ultraviolet sensitivity of birds provides an exceptionally clear example, where a specific amino acid change is known to modulate the functionality of an opsin protein. Opsins (see Appendix 1) are proteins expressed in photoreceptive cells. An opsin gains sensitivity to light by binding to a light-reactive chemical called a chromophore. Amino acid changes in opsins modulate the wavelength (color) sensitivity of the protein/chromophore complex. Among several bird species, Yokoyama et al (2000) found a correlation between wavelength sensitivity and which amino acid is present at a particular site in the opsin protein. Three birds with UV vision have cysteine amino acids in position 84 (C84) of one of their opsin genes. Two other birds and a frog, which have violet (and not UV) sensitivity, possess a serine at position 84 (S84) of one of their opsin genes. Such a correlation suggests a causal relationship between the amino acid at position 84 of opsin, and the color sensitivity of that opsin. Increasingly, biologists are able to test explicitly hypotheses based on correlation, that a given molecular change is the cause of a phenotypic change: Yokoyama et al (2000) were able to do just this. They experimentally altered C84 to S84 in the opsin protein of a zebra finch. This altered protein was expressed in cell culture, and the wavelength sensitivity changed from UV to blue. The reciprocal experiment was also conducted. Changing C84 in both pigeon and chicken opsins altered the proteins' sensitivities from violet to UV in cell culture. These experiments provide extremely strong evidence that position 84 of bird SWS (short wave sensitive) opsin would change color sensitivity of the organism, if the amino acid could be altered directly in an animal, perhaps by transgenics.

The evolution of opsins sensitive to different wavelengths (colors) is not the only example where specific molecular changes are linked to changes in protein or organismal phenotype (e.g. Kozmik et al. 2008; Menon et al. 2001; Salcedo et al. 2003; Yokoyama 1997). In addition, several studies have investigated the molecular basis of the multiple steps in phototransduction, from opsin activation to nervous impulse. These steps involve interactions between proteins, and the specific amino acids that mediate such interactions have been elucidated. For example, different opsins activate different G-proteins, which are mediated by specifically known amino acids in opsin, as demonstrated by altering those amino acids experimentally (Franke et al. 1990; Marin et al. 2000). In turn, different G-proteins activate either phospholipase C (PLC) or phospho-

diesterase (PDE), and these interactions are mediated by specific amino acid motifs, again demonstrated by biochemical experiments (Artemyev et al. 1992; Venkatakrishnan and Exton 1996).

In addition to biochemical phenotypes, the molecular basis of at least one morphological phenotype has also been demonstrated. Open rhabdomes, where photoreceptor cells that are spread out within each facet of the eye, as opposed to tightly packed, are present in the compound eye of flies. The open rhabdome is associated with expression of the gene *eyes shut*, also called *spam* (Husain et al. 2006; Zelhof et al. 2006). This correlation was tested by mis-expression of the *spam* gene in an eye with tightly packed receptors. Turning on expression of *spam* in an eye with a normally closed rhabdome spread the photoreceptor cells apart, effectively causing an open rhabdome eye by mis-expression of this one gene. These results are consistent with *spam* expression as having causal role in the evolution of open rhabdoms from closed rhabdoms (Zelhof et al. 2006).

In a final example, biologists have demonstrated molecular components that are involved in light reactive behavior of the nematode *Caenorhabditis elegans* (Ward et al. 2008). Worms that have a mutated gene encoding the ion channel CNG lack a particular light response. CNG is an ion channel involved in phototransduction in other animals (Appendix 2). Therefore, even though *C. elegans* lacks any pigmented eye, the molecular basis of its photic response shares characteristics with other animals.

These biochemical and molecular experiments are exceedingly important for understanding the evolution of eyes and vision because they are beginning to provide an understanding of the relationships between changes in genes and changes in phenotype. Nevertheless, determining the molecular basis of eyes and vision alone cannot tell us about evolution. In addition, we need to take a comparative approach. For example, if two closely related species share a trait, such as C84 in opsin, we would often infer that the common ancestor of those species also had the trait. Such inferences rely on phylogenetics, a field that has developed highly sophisticated techniques for modeling evolution and for inferring the probabilities of past events based on common ancestry (Cunningham et al. 1998; Pagel 1999). Although many people conceive of phylogenetics as applying to species relationships and to relationships among members of gene families, phylogenetic thinking can also be applied to other levels of biological organization (Arendt 2003; Geeta 2003; Oakley 2003; Oakley et al. 2007; Serb and Oakley 2005). Each component of any animal eye has an evolutionary history that can be reconstructed by comparative techniques. Now that the field of biology has elucidated characteristics of eye components, comparative analyses can be conducted on the eye components themselves. This allows, and forces, an

extension of the gradual-morphological models, which often failed to consider the possibility of separate histories of eye components. This is the second shortcoming of gradual-morphological models: they have not taken advantage of "tree-thinking" approaches.

Tree-Thinking and Multidimensional Time in Eye Evolution

Students of biology at all levels, but especially those less experienced, commonly have a strong tendency to view evolution as a linear series of events. Furthermore, their perceived series often proceeds from simple to complex and often equates highest perceived complexity with most human-like. This way of thinking has conceptual antecedents at least to Aristotle's "Great Chain of Being" and to other prominent biologists like Linnaeus, Lamark, and even Haeckel (Dayrat 2003). The biases are further reenforced by commonplace graphics portraying evolution itself as a parade of primates, from the knuckle-walker (bringing up the rear) to the modern human (leading the way), representing an "Iconography of Expectation" of increasing complexity (Gould 1989). Such tendencies may be mired in human bias, by a deep-seated need to view humans as special, as a pinnacle of evolutionary progress (Gould 1989). Although human vanity certainly seems logical, an additional, perhaps even more fundamental impediment to tree-thinking may be that it requires conceptions of time that are largely outside of human experience. Humans experience, imagine, understand, and communicate time as a linear phenomenon. We understand time unfolding as a process in one dimension. Phylogenetic "time" is different because it branches, leading to multiple parallel trajectories of evolutionary history. It takes practice to learn "tree-thinking," and it is challenging for anyone to communicate precisely about phylogenies when our every day language does not have to deal with similar phenomena (see also Crisp and Cook 2005; Gregory 2008; O'Hara 1997).

The gradual-morphological model of eye evolution falls into exactly the same trap as the graphical progression of primate through proto-humans to human: these iconographies reenforce the notion of evolution as a linear, progressive, and goal-oriented series of biological entities of increased complexity. In the case of eye evolution, Salvini–Plawen and Mayr's figure of gastropod eyes provides an outstanding example of the linear model of evolution, especially since the figure has been copied and elaborated. The progressive series of gastropod eyes begins with a light-sensitive patch (usually with no mention of its origin), followed by eyes with an increasingly deep cup. Next, a simple lens arises, usually with no mention of how the variation originates, beyond the notion that possessing a lens is a continuous extension of morphological variation.

Finally, at the end of the morphological sequence, an eye with a fully formed complex lens is illustrated (Fig. 1a).

It is important to think clearly about what such models can and cannot tell us. Darwin defended his model very well, writing:

> "In looking for the gradations by which an organ in any species has been perfected, we ought to look exclusively to its lineal ancestors; but this is scarcely ever possible, and we are forced in each case to look to species of the same group, that is to the collateral descendants from the same original parent-form, in order to see what gradations are possible, and for the chance of some gradations having been transmitted from the earlier stages of descent, in an unaltered or little altered condition."

In other words, Darwin was using living taxa as surrogates for unknown lineal ancestors. Darwin realized he was only testing a necessary requirement for natural selection to produce complex traits: intermediate forms of the trait must be functional and useful. Salvini-Plawen and Mayr were testing the same idea, using more closely related organisms. However, versions of Salvini-Plawen and Mayr's gastropod eye sequence, copied later by other authors, have shoehorned additional data into a hardened gradual-linear theory. A common example of one of these
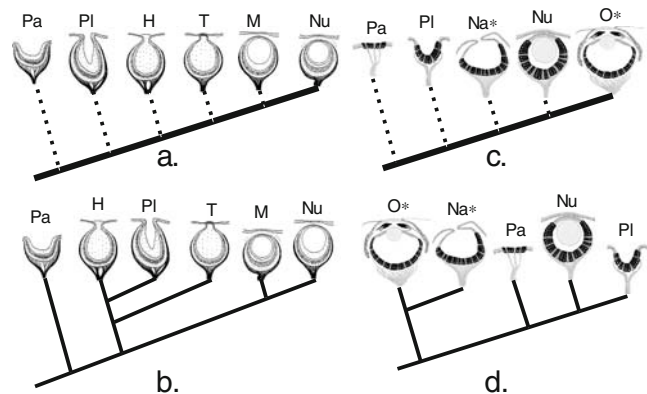

Fig. 1 Morphological series of eyes. **a** Salvini-Plawen and Mayr (1977) illustrated six eyes of varying complexity, from different living species of gastropods. They made the implicit assumption that the eyes of living species approximate the ancestral states of a pectinate (i.e., comb-like, where one descendent of each node does not branch any more) tree. This is illustrated by placing *dashed lines* leading to living species. **b** An actual estimate of gastropod phylogeny (based on molecular data and analyses of Ponder and Lindberg (1997) indicates that the tree is not pectinate. Therefore, the actual history of gastropod eyes is more complicated than a direct march from simple to complex. **c** Two cephalopod eyes (*asterisks*) were added to the gastropod eyes by Strickberger (1990). **d** An estimate of mollusk phylogeny again shows how the actual history is more complicated than a direction march to the complex eye. One interpretation is that the lens of the eyes originated separately in gastropods and cephalopods

extended mollusk eye series originated in Figure 3–1 (page 34) of Strickberger's (1990) textbook *Evolution*. The figure uses four of the gastropods illustrated by Salvini-Plawen and Mayr but adds two additional eyes, both from cephalopods: a nautilus and a squid (Fig. 1b). The nautilus and squid fit nicely into a gradual-linear series, intercalated between gastropods. Nautilus serves as a prime example of a pinhole eye, falling morphologically just between the pit eye of *Pleurotomaria* and the lens eye of *Nucella*. The squid eye, often heralded as a highly complex eye, especially because of its uncanny—but convergent—similarity to vertebrate eyes, now takes the pinnacle position of the linear series. Apparently, these eyes were added to enhance the representation of intermediate stages of eye complexity. Alone, this is not a problem, as long as people remember that the goal of such collections of organs is to show that eyes of intermediate complexity are functional and useful.

However, gradual-linear series of eyes are often elevated to direct models of how evolution actually proceeded. Yet without explicit statistical phylogenetic analyses, these collections of eyes should not be taken as models of how evolution actually did happen in the groups being illustrated. Nevertheless, in some cases, the linear series are presented as actual accounts of evolution, even though they are only drawings of eyes from currently living species, unaccompanied by any phylogenetic analysis. Although the caption in Strickberger (2000) is much more balanced, the caption of Strickberger (1990) illustrates the point, calling the figure "Some stages in the evolution of eyes as found in mollusks…". Ridley (2004) similarly, and somewhat more forcefully states "Stages in the evolution of the eye…". The figure is again reproduced in Encyclopedia Britannica (Ayala 2008) and in Ayala (2007), with a caption stating "Steps in the evolution of eye complexity in living mollusks. The simplest eye is found in limpets (far left), consisting of only a few pigmented cells, slightly modified from typical epithelial (skin) cells…..The octopus eye (far right) is quite complex, with components similar to those of the human eye such as cornea, iris, refractive lens, and retina."

There are at least two problems with viewing eyes of living species as an evolutionary model of how evolution actually proceeded. First, it promotes the fallacy of progress in evolution. The caption above is written as if the first mollusk eye is the same as a modern limpet's, and that evolution progressed linearly to the human-like complex octopus eye. Second, a gradual-linear model ignores complexities like convergence. In particular, adding cephalopod eyes to a group of gastropod eyes ignores the phylogenetic relationships of the animals. When placed on a phylogenetic tree of the animals, it becomes clear that the complex, lens eyes of gastropods and cephalopods probably originated separately (Fig. 1d).

Origins of Eye Components

"[the gradual-morphological model of eye evolution] merely adds complex systems to complex systems and calls that an explanation. This can be compared to answering the question 'How is a stereo system made?' with the words 'By plugging a set of speakers into an amplifier, and adding a CD player, radio receiver, and tape deck.'"—Michael Behe (1996) p. 38–39

"Although much is known about mutation, it is still largely a "black box" relative to evolution. Novel biochemical functions seem to be rare in evolution, and the basis for their origin is virtually unknown."—John Endler and Tracy McLellan (1988)

"…a satisfactory explanation of a biological phenomenon—such as sight…—must include its molecular explanation".—Michael Behe (1996)

In *Darwin's Black Box: The Biochemical Challenge to Evolution*, Michael Behe raises some important scientific questions about eye evolution, but then answers these questions with unscientific (namely, untestable) claims that visual biochemistry implies intelligent design. In 1996, he pointed out a "Black Box;" a then rather unexplored area of scientific inquiry. Behe's general claim was that the evolution of some multicomponent molecular processes had not yet been explained in detail. The evolutionary path leading to the multistep biochemistry of visual processes was one example. Although biochemists had elucidated many of the components of phototransduction (the process of responding to light with a nerve impulse) in flies and vertebrates, no one had yet explained in much detail how phototransduction evolved. Behe had rightly pointed out that contemporary communicators of evolution still focused on the morphological structures of eyes (Dawkins 1996), as Darwin had done, which may have little bearing on the evolution of the biochemistry.

Unfortunately, instead of pointing to the molecular evolution of multicomponent systems as a rich area for new scientific research and synthesis, Behe chose to commit scientific suicide by incorrectly claiming that the only way for multistep biochemistry to arise is by intelligent design. This and related arguments are often called "God in the gaps" because supernatural explanations are invoked to explain observations that science has not yet explained. As we shall see in this section, through the examination of detailed case studies, the gap in understanding of the molecular evolution of eye components is all but closed, highlighting the bankruptcy of the argument that design is required to explain the origins of biological features. Instead, the natural processes of mutation (espe-

cially mutations that duplicate genes) and selection have left clear traces as causal agents of the evolution of eyes.

The Evolutionary Origin of a Light-Sensitive Nerve

In *The Origin of Species*, Darwin hypothesized that the first step in the evolution of eyes involved the gain of photosensitivity in a nerve, writing, "…I may remark that several facts make me suspect that any sensitive nerve may be rendered sensitive to light…" Here, Darwin was making the assumption that variation leading to light sensitivity of previously non-light-sensitive nerves was abundant and that natural selection could then act on that variation. But how did those specific variations originate? How does a nerve become light sensitive? In his notebook, Darwin suggested that it might be impossible to understand how a nerve gained light sensitivity. He wrote "to show how the first eye is formed—how one nerve becomes sensitive to light,…is impossible." Because the mechanisms of heredity and the molecular mechanisms of photoreception were unknown, no specific hypothesis could even be posed in Darwin's time about the genesis of light perception. Things have changed.

Today, through an understanding of the distinct evolutionary histories of the components of vision, and more specifically of phototransduction (see Appendix 1, 2 for details on animal phototransduction), we can now pose a specific historical hypothesis for their origin: phototransduction originated within animals by modifying an existing signaling pathway. More specifically, at some time before the divergence of jellyfish and humans, but likely after the common ancestor of sponges and humans, the first light-sensitive animal opsin protein originated. This protein did not originate from nothing, nor was it newly breathed into an ancient animal genome by a designer. Instead, opsins arose by mutation of an existing receptor to render it light-sensitive. This historical hypothesis makes numerous predictions, and available data are consistent with the hypothesis. It also makes predictions that have not yet been tested, indicating promising areas for future research.

If the hypothesis that phototransduction arose within animals is valid, then some components of phototransduction should exist within animals (e.g., sponges, cnidarians, bilaterians), but should not exist (or should possess a different function) outside of animals (e.g., choanoflagellates, fungi). Such is the case for opsin, which is present as the primary photopigment gene in most animals. Recent research shows that various cnidarians, including a hydra, a sea anemone, a hydrozoan, and a box jellyfish, possess opsins (Kozmik et al. 2008; Plachetzki et al. 2007; Suga et al. 2008). At the same time, opsins are absent from sponges and non-animals (Fig. 2). In science, demonstrating the absence of something like a gene is difficult because a skeptic can always invent a reason why the target was accidentally missed. In the case of the opsins in question, their existence is very unlikely because scientists have determined the entire genome sequence of the sponge *Amphimedon queenslandica*, the choanoflagellate *Monosiga*, and numerous fungi. There is no evidence of opsin in any of those complete genome sequences. Some proteins of sponges and non-animal species closely related to animals are in fact rather similar to opsins, but in every case, the non-animal receptors lack characteristics that specifically define opsins. Therefore, although opsins might have been present at the origin of animals and lost in sponges, their absence instead strongly suggests that they originated within animals, before the common ancestor of humans, insects, and cnidarians, all of which possess opsins.

With current knowledge that opsin is the basis of light sensitivity, Darwin's question of how a nerve becomes light sensitive can be rephrased as, "how did animal opsins originate?" Proteins rarely originate from nowhere, and opsins are no exception. Opsins form a subfamily within a larger family of proteins called G-protein-coupled receptors (GPCRs), also sometimes called serpentine proteins because they snake back and forth across cell membranes. Since serpentine proteins are present in all animals and their close relatives—including sponges, *Monsiga*, and fungi—we know that this broad class of proteins long predates animals. In yeast (a fungus), these receptors are sensitive to pheromones, and they even direct a signal through proteins homologous to non-opsin phototransduction proteins. As such, a signaling pathway exists outside animals, which is very similar to phototransduction, except that the receptor protein detects pheromones, not light. Receptors outside
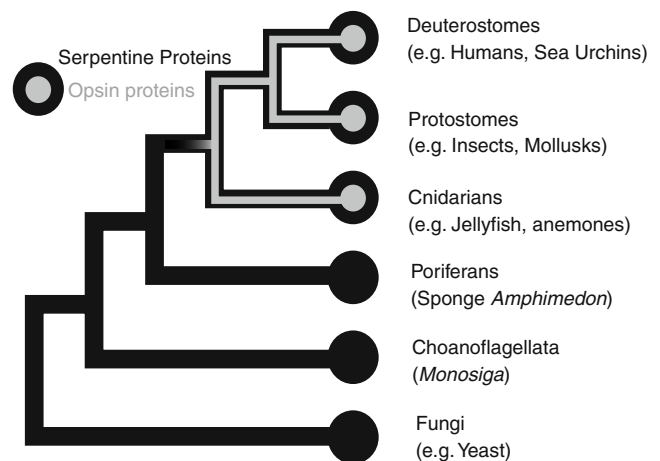

Fig. 2 Opsin originated by mutation of a "serpentine protein" [also called G-protein-coupled receptor (GPCR)]. Protostomes, deuterostomes, and cnidarians possess opsins, but they are absent from porifera and non-animals. Yet, these organisms possess non-opsin GPCR proteins. This indicates that opsin originated by a GPCR gaining light sensitivity, before the common ancestor of Cnidarians, protostomes (including flies), and deuterostomes (including humans)

animals share some characteristics with opsin, like snaking through a cell membrane seven times. It is one of these serpentine proteins that served as the progenitor of the first opsin protein, as evidenced by the similarity of opsins and other serpentine proteins.

Darwin's question can be refined further, to "how did a serpentine protein gain the ability to respond to light?" Since opsin's light-sensitivity is mediated by its ability to bind a light-reactive chemical, called a chromophore, the question can be even further refined to ask how a GPCR must be modified to bind a chromophore. In the case of opsin, we know that a particular amino acid—a lysine in the seventh membrane-spanning region—binds to the light-reactive chemical. Presumably then, a mutation changing an amino acid in the 7th trans-membrane region of a light-insensitive GPCR was involved in the acquisition of light sensitivity in animals. This fateful mutation, when coupled with numerous other mutations, was responsible for the origins of eyes and vision in animals. Although science has not yet tracked down every single mutation involved in the evolution of vision, the origin of opsins clearly illustrates, in richer detail than Darwin might have imagined, the natural processes that gradually allow the evolution of complex features.

The Evolutionary Origin of a Complex Lens

Lenses are a hallmark of complex camera type eyes of vertebrates and cephalopod mollusks. Critical molecular components of lenses are called crystallins, a collection of diverse proteins that are highly expressed in animal lenses. Crystallin proteins often have been co-opted during evolution when enzymes used for other purposes have become expressed in eyes (reviewed in Piatigorsky 2007)

Lenses like those of a squid eye that exist and function in water have high demands for bending light compared to lenses that function in air. This demand is rooted in the fact that cells are composed mainly of water. As such, aquatic lenses cannot take advantage of the transition of light entering watery cells of the eye from the external air, which bends the light. Instead, aquatic lenses must be very powerful. But the more powerful a lens, the more it must be curved, and the more curved a lens, the more aberration results in the image for a lens of a given size. Luckily for fish and squid, there is a solution to these demands, called the graded refractive index lens. These lenses can be compared to an onion, containing a central core and concentric layers surrounding that core. The core bends light very significantly (i.e., it has a high refractive index), while each layer outside the core bends light less and less, with the outer layer having the lowest refractive index. The rings of the "onion" thus form a graded series from high refraction in the middle to low refraction on the outside.

These types of lenses achieve high power with little aberration. How might these rather complicated and precise lenses have evolved? A graded lens was modeled by Nilsson and Pelger (1994) as part of their gradual series from photoreceptor spot to camera-type eye. Dawkins described the models this way:

> "The results were swift and decisive. A trajectory of steadily mounting acuity led unhesitatingly from the flat beginning through a shallow indentation to a steadily deepening cup, as the shape of the model eye deformed itself on the computer screen. The transparent layer thickened to fill the cup and smoothly bulged its outer surface in a curve. And then, almost like a conjuring trick, a portion of this transparent filling condensed into a local, spherical subregion of higher refractive index. Not uniformly higher, but a gradient of refractive index such that the spherical region functioned as an excellent graded-index lens."– Richard Dawkins (1995)

"Conjuring trick," indeed: How in fact does a "portion of transparent filling" condense into a local region with higher refractive index? What, for example, are the genetic changes? Do such morphological changes occur gradually, as envisioned by Darwin, Nilsson, and Dawkins? And do genetic changes show a similar pattern? Or have changes occurred in discrete, quantum steps? Results from recent comparative studies in one species are largely consistent with gradual change at the morphological level. Yet at the same time, those gradual changes were mediated by duplications at the level of the genes involved (Sweeney et al. 2007).

The squid-graded refractive index lens is dominated by S-crystallins, but not just one gene, rather about 25 related genes are expressed only in the lens at high concentration. These genes are in fact about 80% similar to each other in amino acid sequence and probably form a monophyletic group that is closely related to a liver-expressed enzyme. From this simple result, we can already see that a comparative approach adds a new dimension to the understanding of eye evolution. Numerous closely related genes indicate that those genes originated by duplication events, mutations that increased the number of crystalline genes. In addition, the crystallin genes probably had a different function, outside of the lens, before being recruited to lens function, as evidenced by a more distantly related gene, with expression in the liver (a more extensive phylogenetic analysis of the gene family could be used to more formally establish ancestral function).

The duplicated squid crystallin genes are the components of the lens that may allow its differing refractive index between center and periphery. Alterations (mutations) of the duplicated crystallin genes probably change the refractive index of the lens. The graded index lens is established by a

gradient of protein concentration, with higher protein concentrations leading to higher refractive power. In turn, protein concentration can be altered by changing the charge of the proteins: higher electrostatic charge of proteins causes them to repel each other more, resulting in a less densely packed group of proteins and lower refractive index. In the case of squid lenses, crystallins expressed closer to the center of the lens have on average lower electrostatic charges compared to proteins expressed at the periphery of the lens. The average phenotype of the genes tends to change in a graded fashion from center to edge of the lens, leading to a change in refractive index throughout the lens.

These data provide a more detailed picture of how a complex lens might have evolved. Dawkins', "conjuring trick," at least for this squid lineage, may actually have been a co-option event, a mutation causing the first crystallin protein to be expressed adjacent to the retina. Co-option of existing components provides a different perspective compared to traditional gradual models that imply anonymous variation leading to the origin of novel traits like a lens. Additionally, hints about the origins of variation within the lenses of cephalopods are recorded. Here, a combination of factors was required. Gene duplication mutations provided raw material. Other mutations must also have been involved, namely, those that direct expression of the crystallins to particular regions of the lens and those that altered the charge of the proteins, leading to different packing potential and ultimately different refractive indices. We can view these changes as perfectly in line with the gradual-morphological model of eye evolution: a lens forms, and mutations gradually and incrementally increase the complexity of the lens. At the same time, using a comparative approach to investigate the molecular components of the lens leads to a richer and more detailed understanding of the evolutionary pathway. Although the specifics of this case study probably do not apply generally to all lenses (for example, there is no evidence for numerous gene duplications of crystallins in some other species), we expect that generally similar mechanisms of co-option and duplication were important. Certainly, the evolution of complex lenses is an area for scientific study that does not require supernatural explanations like conjuring tricks or intelligent design.

Remaining "Black Boxes" and Summary

We have argued that analyzing molecular data with comparative approaches provides a powerful extension to more traditional models that examined only the morphological form of traits. Major advantages include the elucidation of possible mutational mechanisms involved in the generation of novelty. The origin of variation does not have to be an anonymous black box. In addition, a comparative approach allows a more nuanced understanding of evolutionary trajectories. Evolution usually does not proceed in a direct line from simple to complex. Rather, convergences and reversals are common. Evolution has no foresight.

Despite the ability to extend previous models, the endeavor of comparing molecular components of animal visual systems is still rather new. As such, there are many details and questions that science has not yet worked out. At the same time, the approaches and results outlined here will almost certainly apply to as yet unstudied aspects of evolution. Natural mechanisms of duplication, co-option, and divergence of components, when coupled with natural selection, are amazingly creative and powerful. There is no reason to resort to outmoded ways of dealing with these unknowns. The tendency to invoke the supernatural to explain things we do not yet understand is as old as humankind itself. However, history has taught us over and over that science can address many unknowns; it can open many "black boxes." Therefore, there is no reason and no value to invoking supernatural explanations for topics that science has not yet addressed or elucidated. The aim of this article is to provide an entrée into some of the details and nuances of eye evolution because it is time that everybody understands: we have come a long, long way since 1859.

Appendix 1. A technical overview of phototransduction

Described below is a scientific overview of animal phototransduction cascades—the molecular basis of vision. This overview is meant to introduce the major steps involved in phototransduction and provide references for additional information.

This pathway represents one of the best understood G-protein signaling pathways in biology. Light photons are captured by the proteins called opsins (Fig. 3). The signal is then transmitted intracellularly by G-proteins. Finally, G-proteins and their targets amplify this signal to elicit an electric current throughout the photoreceptor (Fig. 4).

Opsin: a model G protein-coupled receptor ('GPCR')

Responsible for initiating many cellular responses, GPCRs are membrane-bound proteins responsive to extracellular molecules such as hormones, odorants, or neurotransmitters. The GPCRs in phototransduction are known as opsins. While opsins have undergone considerable divergence in
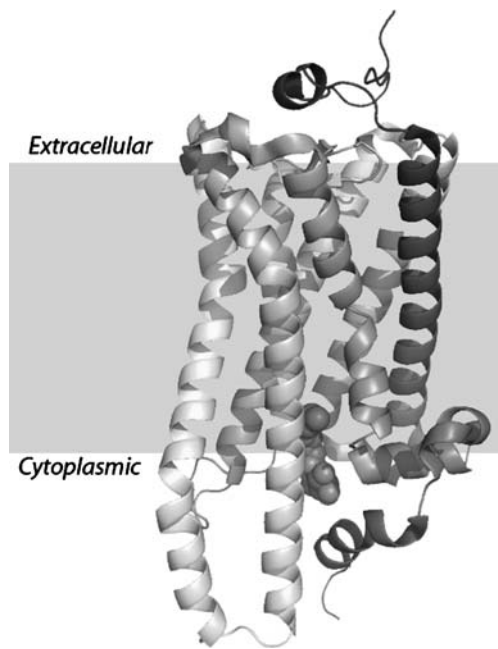
Fig. 3 Key features of rhodopsin. Light-sensitive retina (spacefill model) is bound within the opsin's membrane-spanning helices. G-protein binding occurs within the leftmost cytoplasmic loop. Structure revealed by Murakami and Kouyama (2008)

amino acid sequence in the 600 million years since they originated in animals (Plachetzki et al. 2007), many key structural aspects remain conserved.

Opsins are comprised of seven helices that span the cell membrane in photoreceptors (Palczewski et al. 2000). Bound within these helices is opsin's chromatophore, a chemical that undergoes a conformational change upon contact with photons in a specific range of wavelengths

(Terakita 2005). The chromophore of opsin is a vitamin A derivative called retinal. The energy from photons modifies a double bond and causes retinal to slightly lengthen in size. This induces opsin to expose intracellular binding regions recognized by G-proteins. Following photo-activation in some animal eyes, nearby proteins must act to restore opsin and its chromophore to the original state before new photons can initiate a response.

G-protein: heterotrimeric signaling molecules

G-proteins represent an ancient (older than animals) family of proteins that have diversified modestly relative to GPCRs with which they interact (Milligan and Kostenis 2006). While nearly all light-sensing animals rely on these G-proteins for phototransduction (as well as a host of other GPCR-mediated responses), different opsins will preferentially bind different classes of the $G\alpha$ subunit.

The majority of photoreceptors known presently use a $G\alpha$ protein belonging to either of two evolutionarily distinct classes: "$G\alpha$-q" and "$G\alpha$-t". As discussed in Box 2, photoreceptors that employ different opsins consequently will bind different targets downstream and induce contrasting cellular responses. The G-proteins responsible for relaying the light signal consist of three subunits (α, β, γ). The α subunit of this heterotrimeric protein binds opsin only after opsin's chromophore has accepted a photon and induced a conformational change. Upon binding, $G\alpha$ hydrolyzes a bound GDP for GTP and dissociates from G β-γ. At this point, $G\alpha$-GTP can begin to activate specific targets such as phosphodiesterase (PDE) or phospholipase C (PLC).
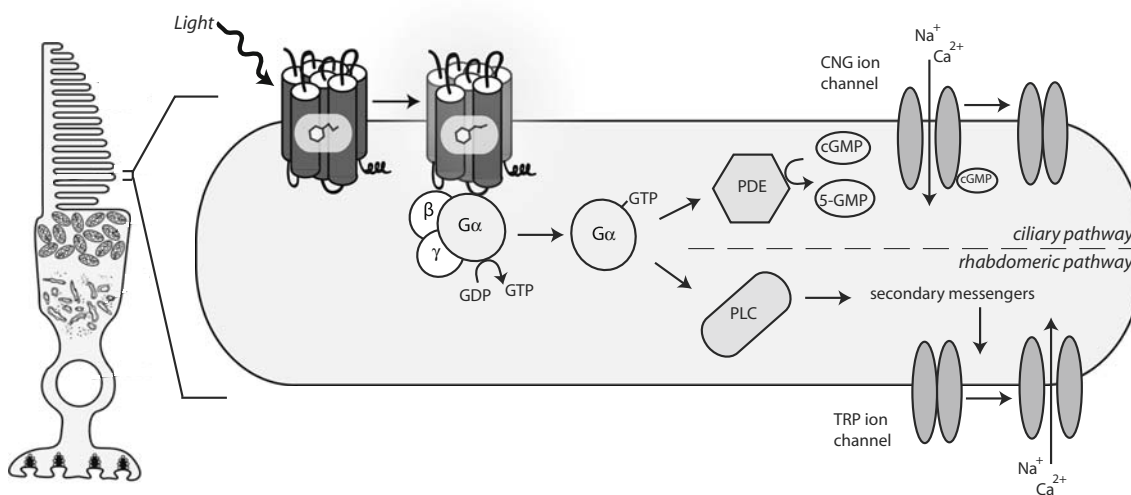

Fig. 4 Major steps of the phototransduction cascade occurring in an animal photoreceptor. Both ciliary and rhabdomeric pathways begin with light-activated opsin binding a G-protein. Following G-protein activation, phototransduction progresses through a either a PDE pathway culminating in the closure of ion channels or a PLC pathway leading to the opening of ion channels. Vertebrate rod cell reproduced from Cote (2006)

Downstream targets: G-protein targets and secondary messengers

In cells expressing Gα-q, PLC responds to activation by Gα-q-GTP by catalyzing the production of an enzyme (IP3) that causes the release of calcium from the endoplasmic reticulum. The subsequent opening of transient receptor potential (TRP) ion channels effectively lowers the cell's membrane potential (depolarization), inducing an electric current through the cell (Hardie 2001).

In cells bearing opsins that target Gα-t, PDE becomes activated by Gα-t-GTP to hydrolyze cellular cGMP into 5′ GMP. These cells rely on cyclic nucleotide-gated (CNG) ion channels, which only remain open in the presence of cGMP. Following photo-excitation and subsequent decrease in cellular cGMP, these channels close and raise the cell membrane potential (hyperpolarization), producing an electrical current (Yau and Baylor 1989).

Appendix 2. Phototransduction origin and evolution

While opsins have diversified tremendously since their origin in early Metazoans, the G protein pathway through which they signal light has undergone fewer, though substantial, changes. As a highly coordinated protein network, any change to one phototransduction component often necessitates changes in partnering proteins. This co-evolution of components has resulted in several homologous phototransduction pathways.

The present diversity in phototransduction pathways has arisen largely due to repeated opsin gene duplications and "co-option" events, where an opsin recruits new suited intracellular signaling components. Researchers today believe that all bilaterian animals use at least two major phototransduction pathways (highlighted in the white boxes below). Phototransduction pathways (See Appendix 1) are conventionally classed as either "ciliary" or "rhabdomeric," in reference to the cellular morphology of the photo-receptors in which they operate. Early work on the photoreceptor evolution recognized that vertebrate retinal cells bear an elaborately folded cilium, while the majority of photoreceptors comprising an invertebrate's eye rely on microvillous rhabdoms. Subsequent molecular evidence has largely supported this distinction (Eakin 1963, 1972; Fuortes and O'Bryan 1972; Yau and Baylor 1989). "Ciliary" photoreceptors bear a class of opsins distinct from the opsins of rhabdomeric cells (e.g., Arendt et al. 2004; Provencio et al. 1998). Likewise, intracellular signaling molecules such as Ga subunits (Ga-q or Ga-t), G-protein effectors (PLC or PDE), ion channels (TRP or CNG), and membrane potential changes (decrease or increase) are observed, respectively, in rhabdomeric and ciliary photoreceptors. (Gomez and Nasi 1995; Graham et al. 2008)

The photoreceptors responsible for the eye's primary vision are typically either entirely rhabdomeric or ciliary. However, improved genomic and transcriptomic screens of animal taxa have revealed that many animals have nonvisual photoreceptors that express the genes for additional photo-
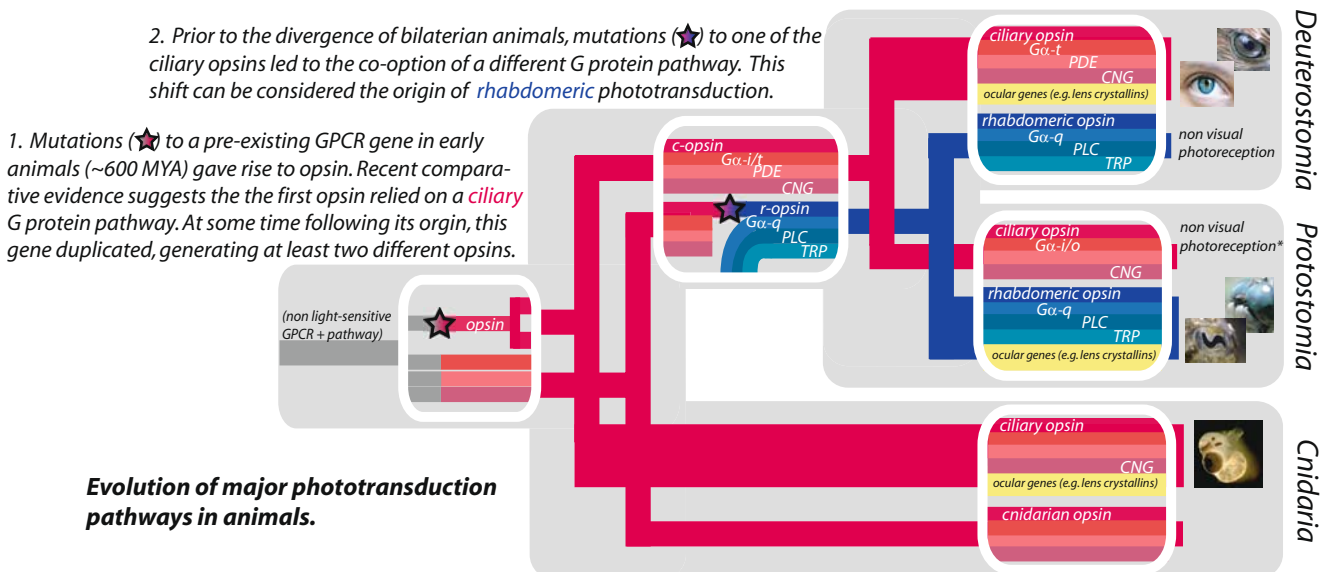

Fig. 5 Evolution of major phototransduction pathways in animals. 1 Mutations (Illustrated with *star graphic*) to a pre-existing GPCR gene in early animals (~600 MYA) gave rise to opsin. Recent comparative evidence suggests the first opsin relied on a ciliary G protein pathway (*red branches*). At some time following its origin, this gene duplicated, generating at least two different opsins. 2 Prior to the divergence of bilaterian animals, mutations (illustrated with *star graphic*) to one of the ciliary opsins led to the co-option of a different G protein pathway. This shift can be considered the origin of rhabdomeric (*blue branches*) phototransduction.

transduction pathways. For instance, while the rods and cones of vertebrate eyes rely on a canonical ciliary pathway (i.e., ciliary opsins, Ga-t, PDE, CNG ion channels), adjacent cells known as retinal ganglion cells (RGCs) express a suite of phototransduction genes formerly considered to belong only to the rhabdomeric cells of invertebrates (Isoldi et al. 2005; Panda et al. 2005). Figure 5 represents our current understanding of how different phototransduction pathways have emerged in animals.

Phylogenetic analyses of the opsin used in RGCs ("melanopsin") indicate that this protein is more closely related to the opsins used in the eyes of protostomes (insects, worms, mollusks, etc) than to the opsins of the nearby rods and cones. Indeed, a closer examination into both the specific signaling proteins and how the cell's membrane potential changes has supported the hypothesis that RGCs share recent ancestry with rhabdomeric photoreceptors.

The presence of multiple evolutionarily distinct photo-transduction pathways is observed outside of the retina as well. In some nonmammalian chordates, melanopsin and its associated rhabdomeric components are implicated in photosensitive dermal cells (Provencio et al. 1998; Koyanagi et al. 2005).

Research into the various visual and nonvisual photo-receptors of invertebrates has revealed that many of these divergent pathways are shared between vertebrates and invertebrates, attesting to the deep evolutionary history of these pathways. While the primary visual cells of many invertebrates use rhabdomeric phototransduction, recent work has uncovered ciliary components in nonvisual photoreceptors of certain insects (Velarde et al. 2005), polychaete annelids (Arendt et al. 2004), nematodes (Ward et al. 2008), and lancelets (Koyanagi et al. 2005). Interestingly, cnidarians use exclusively ciliary-like components for both visual and nonvisual light perception (Plachetzki et al. 2007; Suga et al. 2008; Kozmik et al. 2008).

While the number of locations of nonvisual photo-receptors varies by taxa (many cephalized animals tend to possess such cells in their brains or ganglia), their role remains to be firmly established. Work in the nematode *C. elegans* demonstrates that certain ciliary phototransduction components are employed in phototaxis. At present, RGCs (rhabdomeric) and pineal organs (ciliary) are believed to mediate circadian rhythm in vertebrates, while the ganglion-based photoreceptors (ciliary) in insects and annelids may carry out that function.

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