INVITED REVIEW

The origins of colour vision in vertebrates

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The capacity for colour vision is mediated by the comparison of the signal intensities from photoreceptors of two or more types that differ in spectral sensitivity. Morphological, physiological and molecular analyses of the retina in an agnathan (jawless) fish, the lamprey *Geotria australis*, may hold important clues to the origins of colour vision in vertebrates. Lampreys are extant representatives of an ancient group of vertebrates, the origins of which are thought to date back to at least the early Cambrian, approximately 540 million years ago. *G. australis* possesses five photoreceptor types, each with cone-like ultrastructural features and different spectral sensitivities. Recent molecular genetic studies have also revealed that five visual pigment (opsin) genes are expressed in the retina, each of which is orthologous to the major classes of vertebrate opsin genes. These findings reveal that multiple opsin genes originated very early in vertebrate evolution, prior to the separation of the jawed and jawless vertebrate lineages, thereby providing the genetic basis for colour vision in all vertebrates.

The detection of all aspects of the visual scene including prey, predator or mate is dramatically improved by the added dimension of chromatic detail and the large variety of identified visual pigments in both invertebrates and vertebrates indicates colour vision offers a substantial selective advantage. In vertebrates, molecular phylogenetic analyses have established that the vertebrate visual pigments evolved along five distinct lines and that these lineages were in place before the teleost fish diverged from other vertebrates, about 400 million years ago (MYA).[1] The five vertebrate visual pigment gene lineages diverged from a single common ancestor that had evolved prior to the divergence of the vertebrates and invertebrates, about 700 MYA.[2]

Therefore, colour vision is inferred to have evolved in the earliest vertebrates between 400 and 700 MYA. Our understanding of the origins of colour vision or colour discrimination beyond this period are still a matter of contention due to the paucity of relevant species examined and the necessity to identify the full complement of photoreceptor types present within the one species. Generally, it is agreed that the ancestral vertebrate visual system was based on relatively unspecialised, cone-like photoreceptors, functioning under photopic conditions and that at least two spectrally distinct classes of photoreceptor (each expressing a different opsin gene) were present at a very early stage. Two further major lines of new evidence suggest that colour

vision evolved more than 300 million years earlier. This is based on the presence of four cone types with different coloured oil droplets in the Australian lungfish, *Neoceratodus forsteri*[3] and at least three types of cone-like photoreceptors in the southern hemisphere lamprey, *Geotria australis*,[4] although little work has been done in the remaining 'primitive' groups; the Chondrichthyes (sharks and rays) and the non-Actinopterygia (early ray-finned fishes). This further supports the hypothesis that colour vision may have appeared very early in vertebrate evolution. Establishing the morphological and spectral characteristics of the photoreceptor types and the molecular evolution of the visual pigments in representatives of all of the 'primitive'
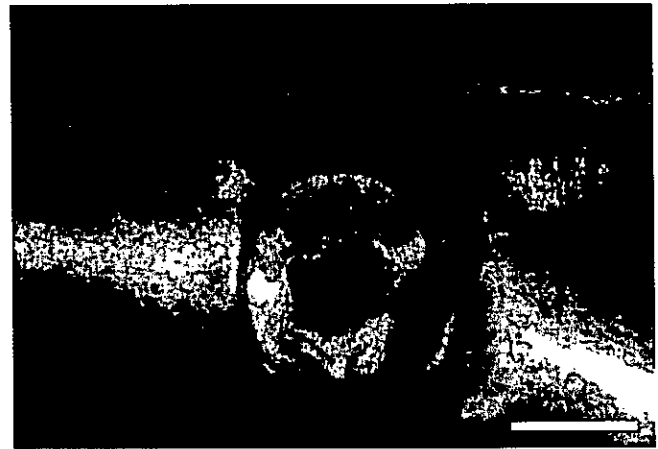
A   **B**

Figure 1a. Dorso-lateral view of the southern hemisphere lamprey, *Geotria australis*, a representative of the earliest extant vertebrates, which date back over 540 million years. Scale bar: 30 mm.

Figure 1b. Close-up of the head and eye of *G. australis*. The pupil is partially obscured by a dorsal irideal flap. Scale bar: 1 mm.

A   **B**

Figure 2a. Transmission electron micrograph of the retina of *G. australis* cut in transverse section showing the complement of photoreceptor types (numbered 1 to 5) based on size and the presence or absence of filtering pigments, localised within the endoplasmic reticulum located in the myoid region. Scale bar: 2 μm.

Figure 2b. High power of the plasma membrane and where it invaginates the discs (arrowheads) of the outer segment of the photoreceptor depicted by 5 in Figure 2a. This cone-like feature was found to be present in all five morphologically-characterised receptor types. cp, calycal process. Scale bar: 0.2 μm.

vertebrate groups would be a major advance in our understanding of the evolution of colour vision. Recent analysis of the first vertebrates, the lampreys or agnathans, has revealed that the distinction between the rods and cones early in vertebrate evolution is not clear-cut and that this group may hold vital clues to the origins of colour vision.

THE DUPLEX RETINA AND THE CHARACTERISATION OF RODS AND CONES

Historically, it has been established that the vertebrate retina, including that of humans, contained two independent visual systems. Schultz[5] proposed, albeit with some scepticism at that time, that two morphologically distinct photoreceptor types, namely rods and cones, mediated scotopic and photopic vision, respectively. Over the course of the past 500 million years of vertebrate evolution, retinal photoreceptors have undergone radical changes in response to environmental and other driving forces and some have even transformed from one type to another according to changes in ecological activity, that is, according to the
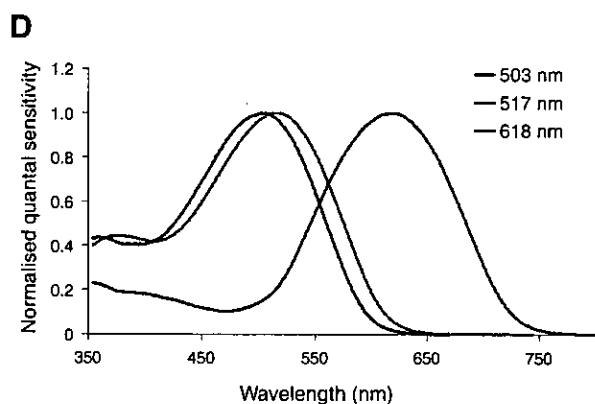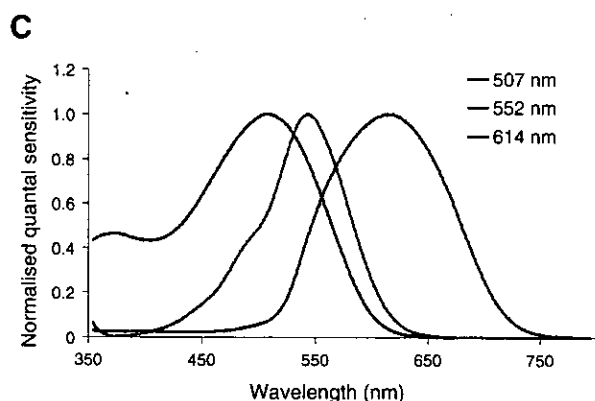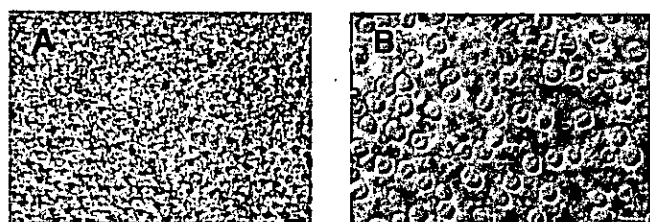
Figures 3a and 3b. Wholemount views of the retina in downstream (A) and upstream (B) *Geotria australis* showing the packing, the sizes and the coloured filters (orange/yellow) of the different photoreceptor types. Note the increase in receptor size in upstream individuals and the presence of a colourless ellipsosome, an intracellular structure that may act to focus light onto the outer segment. Scale bar: 10 µm.

Figures 3c and 3d. Summary of the calculated photoreceptor quantal spectral sensitivity curves for three of the five types of photoreceptors identified in downstream (C) and upstream (D) migrants of *Geotria australis*. The peak sensitivities are provided in the legends. The difference in the spectral sensitivities between the two adult migration phases is due to the inclusion of a yellow/orange short wavelength absorbing pigment located in the myoid regions of each of the three photoreceptor types, however, this spectral filter has a particularly large effect on the medium wavelength sensitive receptor (green line). Quantal spectral sensitivity was calculated by modelling the absorptance of the visual pigment in the outer segment and the spectral transmittance of this photostable pigment. Adapted from Collin and associates.[31]
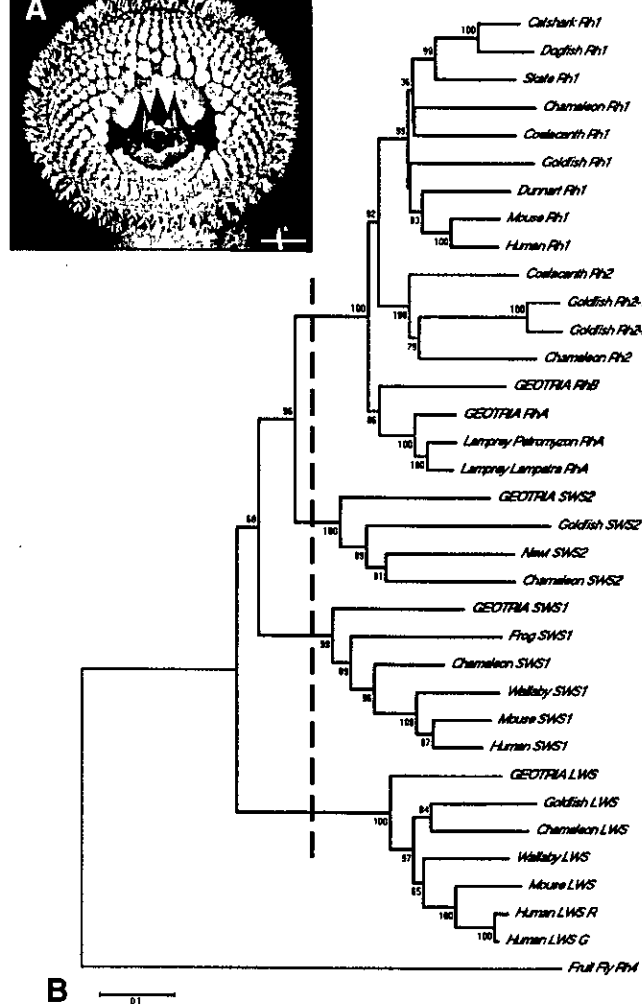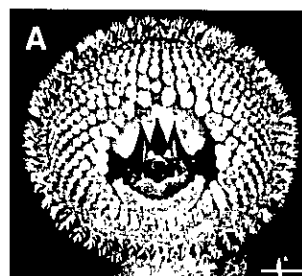
Figure 4a. The suctorial (jawless) mouth of the upstream *Geotria australis*, which is used to digest blood and flesh from its hosts. Scale bar: 10 mm.

Figure 4b. Phylogenetic tree of codon-matched nucleotide sequences showing the relationships between the opsin genes of *G. australis*, the northern hemisphere lampreys, *Lampetra japonica* and *Petromyzon marinus*, representative jawed vertebrates and an invertebrate outgroup. See text for explanations of the different opsin groups. The dashed line indicates the predicted genetic complement of opsins present in the most recent common ancestor of the jawed and jawless vertebrates, *ca.* 540 million years ago. The number at each branch point reflects its robustness (max. 100). The scale bar is calibrated in nucleotide substitutions per site. Reproduced with permission from Collin and co-workers.[35]

transmutation theory of Walls.[6] In fact, in many primitive species of vertebrate, the distinction between rods and cones is not at all clear.

The morphological characterisation of vertebrate photoreceptors still remains an important method of differentiating between rods and cones in many species. The shape of the outer segment (cylindrical in rods and tapered in cones) has traditionally been used to characterise receptors but this has given equivocal results in many species, due to the similarity of each receptor type in different regions of the retina. Other morphological features are less ambiguous and underlie important electrochemical signalling during the phototransduction cascade, for example, the arrangement of the plasma membrane surrounding the outer segment.[7] Some of the morphological criteria remain ambiguous.

In humans, the photoreceptor mosaic performs the first neural coding of colour information by sampling the retinal image with arrays of three different cones with overlapping spectral sensitivities.[8] The maximum sensitivities (λ_{max}) fall into the short, medium and long wavelength regions of the visible spectrum (400 to 750 nm). Other mammalian species such as rodents[9,10] and many species of birds[11,12] and fishes[13,14] possess receptors, which extend the spectral range into the ultraviolet region. The λ_{max} of a photoreceptor remains an important measure of spectral sensitivity and can be useful for discriminating between rods and cones, although in some species the peak sensitivities can be identical.[15]

Molecular examination of photoreceptor visual pigments is an important and more definitive method of characterising rods and cones (especially when combined with *in situ* hybridisation) and in assessing the potential for colour vision in the early vertebrates. Visual pigments in vertebrates comprise a protein, opsin, to which a chromophore, the aldehyde of vitamin A, is attached. Vitamin A occurs in two forms, A_1 and A_2, either of which can combine with an opsin protein to form a visual pigment. Packed into the membranes of the discs within the outer segments, these

transmembrane proteins comprise a polypeptide chain of approximately 350 amino acids, which form seven transmembrane alpha helices linked by extra membrane loops. Mammalian photoreceptors possess only Vitamin A_1-based opsin visual pigments, while both Vitamin A_1- and A_2-based visual pigments are found in fish, amphibians and reptiles.[15] Changes in the photoreceptor spectral sensitivity are mediated by different ratios of the two chromophores and/or variations in the genes coding the opsin protein.[16,17] Vertebrate retinal opsins are classified into five evolutionarily distinct groups:

1. Rod photoreceptor *(Rh1)*
2. Rh1-like *(Rh2)*
3. Short wavelength sensitive *(SWS1)*
4. SWS1-like *(SWS2)*
5. Long *(LWS)* or medium *(MWS)* wavelength sensitive *(LWS/MWS)* opsin clusters.[18]

The rhodopsin *Rh1* pigments are usually expressed in rods and the remainder in cones. Gene duplication, followed by nucleotide substitution, provides the basis of functional differentiation of the various groups of opsins.

CHARACTERISATION OF RODS AND CONES IN THE FIRST VERTEBRATES

Lampreys and hagfishes are the sole survivors of the very early agnathan (jawless) stage in vertebrate evolution.[19] Recent studies[20,21] have shown that lampreys, or their very close relatives, had already evolved by the lower Cambrian period (*ca* 540 million years ago). The present day hagfishes possess poorly-developed eyes, often without a lens or vitreous body, and an undifferentiated retina. On morphological criteria, only a single rod-like photoreceptor with a peak absorbance around 500 nm has been identified,[22,23] although Vigh-Teichmann and colleagues[24] found two unidentified classes of outer segments that could be distinguished on ultrastructural and immunocytochemical criteria. No other data exist on the photoreceptor types for this primitive group of agnathans with degenerate eyes, which are different from the lampreys in most respects and generally are considered to have a com-

mon ancestry but to have evolved independently.[19]

A number of northern hemisphere (or holarctic) lampreys have been examined and possess two morphologically different photoreceptor types—a short (putative rod) and a long (putative cone) receptor—although the classification of the receptors has been a subject of contention for almost 150 years (reviewed in Crescitelli[25] and Collin, Potter and Braekevelt[4]). At least in terms of the classical criterion used to differentiate rods from cones (the continuity of the outer segment discs with the extracellular matrix), both morphologically-identified receptor types are cones.[26-28] The functional divergence of these two receptor types has been examined by other criteria, including microspectrophotometry,[29,30] visual pigment anion sensitivity[30] and electrophysiology[29] but uncertainty still exists regarding the identity of the two receptor types. Electroretinographic (ERG) responses recorded in the river lamprey, *Lampetra fluviatilis* by Govardovskii and Lychakov[29] show typical cone responses for both receptor types, including the short receptors (putative rods) that do not saturate at high illuminances and contribute to both photopic and scotopic vision.

Certainly, the results of recent studies of a species of southern hemisphere lamprey, *Geotria australis* leave little doubt that at least the potential for colour vision may have arisen very early in vertebrate evolution and that ancestral photoreception was based on a cone-like photoreceptor. In adult (both downstream and upstream migrants) retina of *G. australis* (Figure 1), five types of photoreceptor have been identified morphologically,[4,31] all of which possess cone-like features (Figure 2). Thus far, microspectrophotometric analysis has confirmed only the peak absorbances of three of these receptor types, with λ_{max} values of 506/500 nm, 515/515 nm and 610/616 nm for the downstream and upstream phases, respectively[31] (Figure 3). It was not until the visual pigments of each of these receptor types was examined at the molecular level that the potential for colour vision in these ancient vertebrates was fully realised.

THE EVOLUTION OF VISUAL PIGMENTS IN VERTEBRATES

It has been postulated that all five classes of visual pigment co-existed in the common ancestor of the vertebrates, as the divergence of all five opsin gene lineages was thought to predate the different vertebrate lineages.[32-34] This conclusion was based on sequence comparisons of the opsin genes from only a few freshwater teleost species and did not take into account the full genomic set of opsin gene sequences of any of the extant descendants of earlier vertebrate groups, such as the sharks and rays or the jawless vertebrates.

Recent molecular genetic studies have revealed five visual pigment (opsin) genes, all of which are expressed in the retina in the southern hemisphere lamprey *Geotria australis*.[35] A codon-matched nucleotide sequence alignment of the opsin genes from all of the major phylogenetic groups of vertebrates provided the basis for estimating the evolutionary distances between the gnathostome and *G. australis* opsin cDNAs and the resulting phylogenetic tree shows that the *G. australis LWS*, *SWS1* and *SWS2* opsin genes are orthologous to the *LWS*, *SWS1* and *SWS2* opsin genes of jawed vertebrates (gnathostomes), with the branching order within each gene group largely following the accepted order of appearance of the vertebrate species[35] (Figure 4). The two unique opsin genes in *G. australis*, *RhA* and *RhB*, were most closely related to the gnathostome *Rh1* and *Rh2* genes, however, both *RhA* and *RhB* are equally distantly related to the gnathostome *Rh1* gene lineage as they are to the *Rh2* gene lineage (Figure 4). This suggests that the most recent common ancestor of the jawed and jawless vertebrates probably possessed just a single ancestral *Rh* opsin gene, as well as *LWS*, *SWS1* and *SWS2* opsin genes. Some time later, after the separation of the agnathan and gnathostome groups, two independent gene duplication events occurred, one in the Agnatha to give *RhA* and *RhB* opsins, and the other in the gnathostomes to produce the *Rh1* (rod) and *Rh2* (green cone) opsin genes in the jawed vertebrates.

Therefore, in contrast to other investigators who have speculated that the ancestors of all vertebrates must have possessed all five of the major types of retinal photoreceptor opsin genes for example, *LWS*, *SWS1*, *SWS2*, *Rh1* and *Rh2*, Collin and co-workers[35] provided the first evidence to demonstrate unequivocally that the molecular events giving rise to four of the five phylogenetic classes of vertebrate visual pigment opsin genes had already taken place by the early Cambrian period and prior to the evolution of the first jawed vertebrates. Uniquely, they also suggest that the duplication of the ancestral *Rh* opsin gene, to give rise to the true rod (*Rh1*) and the green cone (*Rh2*) opsin genes of the jawed vertebrates, occurred specifically within the gnathostome lineage and independently of the jawless vertebrate lineage. It follows that the agnathan lineage probably does not possess a true rod photoreceptor, retaining cone-like features in all photoreceptors.[35,36]

THE PUTATIVE ORIGINS OF COLOUR VISION

Palaeontological evidence from the Silurian and Devonian periods shows that the lateral eyes of the ancestral vertebrates possessed well-developed image-forming eyes, which were rotated within their orbits by six extraocular muscles as in modern vertebrates.[37] These heavily armoured ostracoderms were benthic, presumably feeding by extracting food in or on the substrate. Depending on the light environment encountered by these early vertebrate ancestors, two theories have been proposed on the evolution of multiple photoreceptor types:

1. Those fish that possessed two visual pigments, one matching the background and the other offset from the background illumination, could detect targets that were spectrally different from the background.[38,39]

2. The evolution of at least two spectral classes of photoreceptors would eliminate the considerable noise associated with the significant flicker produced by light passing through the surface ripples in shallow water, thereby enabling

the earlier detection of predators.[40]

Both of these theories assume that vision in the ancestral vertebrates was cone-based (photopic), with colour vision evolving possibly later as a biproduct, following the development of colour opponency via horizontal cells. Despite the absence of any behavioural evidence of colour vision in either the ancestral or early vertebrates, it is highly likely that colour vision dates back at least 540 million years with the ancestral vertebrates possessing four major types of vertebrate cone opsins, each presumably sensitive to different parts of the light spectrum. Although the selection pressures underlying the origins of colour vision are still unknown, the early vertebrates lived in a shallow water environment, where a wide light spectrum could be exploited by the evolution of multiple visual pigments and utilised in observing a range of complex environments and animals, some of which were inherently colourful.[40,41]

Linked to the evolution of the four cone opsin genes in the ancestral vertebrates is the evolution of opsin gene expression control mechanisms that, until recently, have been assumed to direct stable and unchanging expression of only one opsin gene in each morphologically distinct type of photoreceptor.[42] On the contrary, there is an accumulating weight of evidence that suggests that not only can two or more opsin genes be co-expressed in the same photoreceptor[43,44] but that opsin gene expression is plastic and changes in response to a diverse array of cues including hormonal, developmental and environmental light stimuli.[45-48] The presence of four opsin genes in the ancestral vertebrates raises the question of the ancestral state of opsin gene regulation, that is, whether it is static or plastic. Our own investigations of *Geotria australis* have found striking differences in the morphological and spectral characteristics of the retinal photoreceptors present in the two major stages of the anadromous, adult lifecycle.[31] These findings suggest that temporal plasticity in opsin gene expression is the primitive condition for vertebrates and may be a common phenomenon throughout vertebrate evolution.

Therefore, the 'fanciful' and much-criticised theory of Walls[6] that the ancestral photoreceptor type was based on a cone and that rods evolved as a transmutation with a view to increasing sensitivity with the development of rhodopsin was exceptionally prescient, especially given that his findings were based on light microscopical analysis of the retina of *Geotria australis* prior to 1942.[6]

FUTURE DIRECTIONS AND EVOLUTIONARY IMPLICATIONS

In addition to the importance of the ancestral vertebrates possessing four different (putatively cone) visual pigment genes and the implications for the origins of colour vision, the next hurdle to negotiate is when dim light or rod-based (scotopic) vision evolved. As the ultimate definition of the vertebrate rod photoreceptor is provided by the expression of the true rod *Rh1* opsin gene, identification of the timing of the origin of this opsin gene will identify the time frame of the first possible appearance of the true rod photoreceptor in vertebrates. Based on the findings of Collin and co-workers,[35] photopic (bright light, cone-based) colour vision originated first in the ancestral vertebrates, while full scotopic (dim light, rod-based) vision developed later, being dependent on the evolution of the true *Rh1* opsin gene, probably only within the jawed vertebrate lineage.

In summary, further study is required to establish the selective forces driving the evolution of photoreception and increase our understanding of the phylogenetic basis of the duplicity theory. Multidisciplinary approaches will continue to identify the evolutionary constraints placed on the shape, photodynamics, spectral sensitivity and molecular structure of photoreceptors in vertebrates.

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