Takahisa Furukawa
James B. Hurley
Satoru Kawamura *Editors*

Vertebrate Photoreceptors

Functional Molecular Bases

Springer

Vertebrate Photoreceptors

Takahisa Furukawa • James B. Hurley
Satoru Kawamura
Editors

Vertebrate Photoreceptors

Functional Molecular Bases

Springer

Editors
Takahisa Furukawa
Institute for Protein Research
Osaka University
Suita, Osaka, Japan

Satoru Kawamura
Graduate School of Frontier
Biosciences
Osaka University
Suita, Osaka, Japan

James B. Hurley
Department of Biochemistry
University of Washington
Seattle, WA, USA

ISBN 978-4-431-54879-9 ISBN 978-4-431-54880-5 (eBook)
DOI 10.1007/978-4-431-54880-5
Springer Tokyo Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014931983

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Our vision entirely relies on photoreceptors in our retina. The main role of these cells is to transduce the capture of a photon into an electrical signal that is processed in the retina and sent to the brain to induce the sensation of vision. In the retina, cells are layered to process the visual information step by step. This layered structure of the cells is advantageous for the study of photoreceptors: they all are in the outermost layer of the retina. Thanks to this layered structure, we can identify a photoreceptor easily and reliably and measure its light response, and we can collect photoreceptors in a quantity large enough and pure enough for biochemical studies. Furthermore, photoreceptors are polarized and each part is compartmented: the outer segment, the inner segment, the cilia connecting the outer segment and the inner segment, the ellipsoid, the myoid, the nucleus, and the synaptic terminal. Each part can be recognized easily under a light microscope, which makes it straightforward to study the specialized role of each compartment. Thanks to all of these advantages, photoreceptors have been studied from various perspectives including protein chemistry, biochemistry, electrophysiology, cell biology, and developmental biology utilizing a variety of simple and sophisticated experimental methods. Mutant animals with defects in genes that are expressed only in photoreceptors allow investigators to circumvent lethality issues. Molecular genetics tools such as photoreceptor-specific promoter sequences are available to express mutant proteins specifically in photoreceptor neurons. Therefore, investigators have been able to exploit powerful molecular genetics tools to manipulate the expression or deletion of a normal or mutant protein to be able to perform in vivo biochemical and physiological studies on the molecular mechanisms underlying the function and the structure of vertebrate photoreceptors. Because photoreceptors are critical for our vision, gene mutations that cause photoreceptor dysfunction and/or degeneration lead to visual impairment or blindness in humans. Subjugation of photoreceptor diseases such as retinitis pigmentosa and macular dystrophy cannot be achieved without a correct understanding of photoreceptor development, photoreceptor metabolism, survival and death of photoreceptor neurons, and function. In addition, stem cell and iPSC technology have provided a foundation for realizing therapies

based on regeneration and tissue design for currently incurable photoreceptor and retinal degeneration diseases.

This book is meant to provide a series of comprehensive views on various important aspects of vertebrate photoreceptors. The chapters are selected from fields of studies that have contributed a broad understanding of the birth, development, structure, function, and death of photoreceptor neurons. The underlying common word in all of the chapters that is used to describe these mechanisms is "molecule". Only with this word can we understand how these highly specific neurons function and survive. We hope that this theme will be evident throughout each of the chapters in the book. It is challenging for even the foremost researchers to cover all aspects of the subject. We hope that understanding photoreceptors from several different points of view that share a molecular perspective will provide readers with a useful interdisciplinary perspective.

Photoreceptors are the most deeply studied sensory receptor cells, but readers will find that many important questions remain. We still do not know how photoreceptors, visual pigments, and their signaling pathways evolved, how they were generated and how they are maintained. This book will make clear what is known and what is not known. As has always been the case, pioneering studies in photoreceptors will surely guide the studies of sensory cells in general. In addition, because the retina is a part of the central nervous system, developmental aspects of photoreceptors will provide insights into developmental mechanisms of the brain. Photoreceptors, how they work, how they develop, and how they stay alive are topics of intrinsic general interest.

We hope that this book will help readers to develop an integrated understanding of vertebrate photoreceptors as a whole. In this sense, the book will be a guide for graduate students in studies of photoreceptors and other sensory cells. For the same reason, we hope that this book will help to stimulate multidisciplinary collaboration among researchers with different types of expertise and that such studies may open up new and important avenues of research in this field.

Osaka, Japan — Takahisa Furukawa
WA, USA — James B. Hurley
Osaka, Japan — Satoru Kawamura

Contents

Chapter 1
Evolution and Diversity of Visual Pigments in Connection with Their Functional Differences

Keita Sato and Yoshinori Shichida

Abstract Living organisms have generated and optimized their photoreceptor cells to acquire information from the outer environment. Among the photoreceptor cells, rod and cone photoreceptor cells (rods and cones) present in vertebrate retinas have evolved to mediate vision. Rods are responsible for dim light vision whereas cones act for bright light and color vision. Corresponding to their difference in physiological role, both cells contain functional proteins constituting signal transduction cascades with different properties. Visual pigment is the upstream protein that absorbs light and triggers the subsequent signal transduction cascade. It has diversified into five groups: the L (LWS/MWS), S (SWS1), M1 (SWS2), M2 (RH2), and rhodopsin (RH1) groups. The visual pigments contained in the first four groups are collectively called cone pigments, and they have similar molecular properties, although they have a wide range of absorption maxima (from 359 to 584 nm) that work for color discrimination. Cone pigments exhibit molecular properties distinct from rod pigment rhodopsins, such as their thermal stability and kinetics of retinal incorporation and release, which contribute to the difference in cellular response between cones and rods. Because rod and cone pigments have a common ancestor, they must have acquired diversified molecular properties during the course of molecular evolution. In this chapter, the molecular basis of the evolution and diversification of vertebrate visual pigments as related to their physiological functions is described.

Keywords Diversity • Evolution • Vision • Visual pigments

K. Sato • Y. Shichida (✉)
Department of Biophysics, Graduate School of Science,
Kyoto University, Kyoto 606-8502, Japan
e-mail: shichida@rh.biophys.kyoto-u.ac.jp

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_1, © Springer Japan 2014

1.1 Introduction

The surface of the Earth is supplied with an abundance of light from the sun. For living organisms, light is an essential source of energy for metabolism and a major information carrier from outer environments. Vision is a primary light reception system in animals. Animals have eyes, specialized organs for light reception in vision. In vertebrates, the retina, a multilayered neural tissue in the eye, contains photoreceptor cells and neural cells that perform subsequent signal processing and eventually send a signal to the central nervous system. Vertebrate photoreceptor cells are specialized neural cells for photoreception and are classified into two types, rods and cones. The rods and cones differ in morphology and respond to light differently (Fig. 1.1). Both have a segmented structure: outer segment, inner segment, nucleus, and synapse. The terms "rod" and "cone" are derived from the morphology of their outer segments: namely, they have rod- and cone-shaped outer segments, respectively. Rods exhibit higher light sensitivity than cones and cones exhibit faster response and greater adaptation than rods. These differences in cellular properties between rods and cones correspond to their physiological roles for dim light vision and bright light and color vision, respectively. The outer segments of rods and cones have highly stacked membrane structures. In rods, the membranous structure of outer segments consists of stacked inner membranes, called disk membranes. In contrast, the stacked membranes are continuous with the cell membrane in cones. In both cells, these stacked membrane structures contain the components of the photo-signal transduction system, including visual pigments and downstream enzymes. Rods and cones have their respective subtypes of molecular components, which contribute to the differences in cellular responses and discrimination of physiological roles.

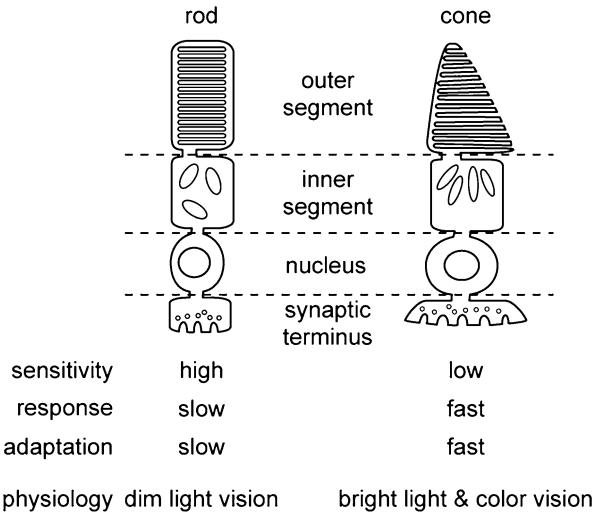

Fig. 1.1 Physiological differences between rod and cone photoreceptors

1.2 General Remarks on Vertebrate Visual Pigments

Visual pigments are membrane proteins located in the stacked membrane structures in the outer segments of photoreceptor cells. They are members of the protein family opsin, a type of serpentine G protein-coupled receptor (GPCR). Opsin is generally composed of a protein moiety having seven transmembrane α-helices and its ligand chromophore retinal, the aldehyde form of vitamin A. Retinal is covalently bonded to an amino substituent of a highly conserved lysine residue in transmembrane helix 7 of opsin through a Schiff base (imine) linkage. In visible light-absorbing opsins, the Schiff base is protonated, which results in delocalization of the pi electron in the conjugated double-bond system of the retinal Schiff base, enabling the absorption of visible light. The positively charged, protonated Schiff base is stabilized by a negatively charged glutamic acid at position 113 in the case of vertebrate visual pigments (Zhukovsky and Oprian 1989; Sakmar et al. 1989; Nathans 1990). This negatively charged glutamic acid is called the counterion. Almost all known opsins utilize all-*trans* and 11-*cis* forms of retinal as their chromophores. 11-*cis*-retinal functions as an inverse agonist, whereas all-*trans*-retinal works as an agonist to induce the formation of an active state of opsin, which results in activation of its cognate G protein. Thus, the visual pigment contains 11-*cis*-retinal in its resting state to suppress the G-protein activity and converts to the active state upon isomerization of the 11-*cis*-retinal into the all-*trans* form by absorption of a photon. Vertebrate photoreceptor cells have a specific G-protein subtype called transducin that is a close relative of the G_i subtype of G proteins (Stryer et al. 1981). Visual pigment promotes a GDP–GTP exchange reaction on the α-subunit of transducin in a light-dependent manner. Similarly to other GPCRs that bind a diffusible ligand, opsins are also phosphorylated and bind to arrestin in a signal shutdown process (Bennett and Sitaramayya 1988; Palczewski et al. 1992).

1.3 Subgroups of Vertebrate Visual Pigments

Based on the identities of their amino acid sequences, visual pigments are classified into five distinct groups: rhodopsin (Rh1), M2 (Rh2), M1 (SWS2), S (SWS1), and L (LWS/MWS) groups (Okano et al. 1992; Yokoyama 1994; Yoshizawa 1994; Shichida and Imai 1998; Ebrey and Koutalos 2001). Each group is also characterized by the absorption maxima of its pigments: rhodopsin, 478–516 nm; M2, 467–509 nm; M1, 418–474 nm; S, 359–436 nm; and L, 508–584 nm. The wide range of absorption maxima of the cone pigments enables color discrimination, and the different features of these pigments are adaptive to the different habitats of the respective animals. Almost all known vertebrates have rhodopsin and cone pigment genes. The phylogenetic tree of vertebrate visual pigments strongly suggests that the full set of visual pigments was completed in the common ancestor of vertebrates (Fig. 1.2). However, some genes were later multiplied, and some were lost.

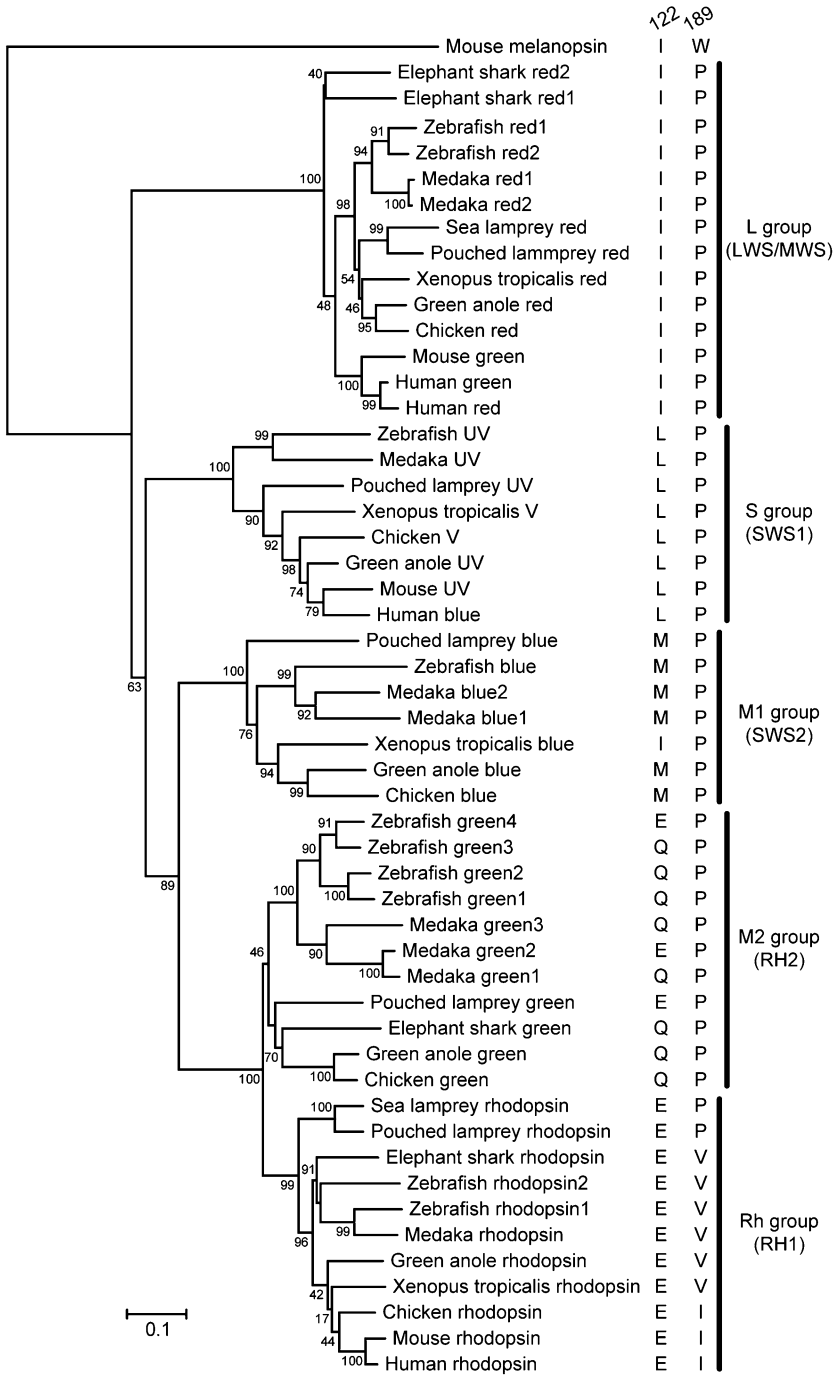

Fig. 1.2 Phylogenetic tree of vertebrate visual pigments constructed by the neighbor-joining method. Amino acids at positions 122 and 189 (bovine rhodopsin numbering) and group names are shown

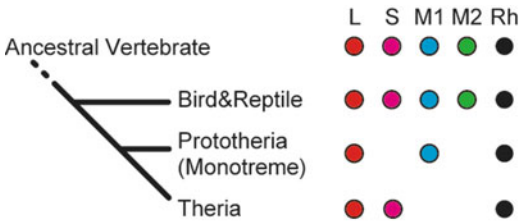

Fig. 1.3 Schematic view of phylogeny of vertebrate visual pigments found in birds, reptiles, and mammals

Compared to nonmammalian vertebrates, mammals have lost the M1 and M2 groups of visual pigments, probably because of the nocturnal habitat of the common ancestor of mammals. As a result, most mammals have only three types of visual pigments: the rhodopsin, S, and L groups. There are two exceptions. The platypus has M1 instead of S (Wakefield et al. 2008) (Fig. 1.3). Additionally, some of the New World monkeys and most Old World monkeys, including macaques and humans, have duplicated L group genes that can discriminate light in green to red regions (Surridge et al. 2003); that is, those primates are trichromatic, whereas other mammals are dichromatic. Birds have four types of cone pigments, which means that they have tetrachromatic vision. Fishes have various numbers of rod pigment and cone pigments as a result of gene duplication (Hofmann and Carleton 2009).

Additionally, fishes, amphibians, and reptiles use 3,4-dehydroretinal as the chromophore according to their living conditions, which generally results in a red shift of the absorption maxima of visual pigments, because 3,4-dehydroretinal has an extended conjugated double-bond system compared to normal retinal. It was previously thought that use of 3,4-dehydroretinal as a chromophore of visual pigment could be one of the evolutionary adaptations of animals living in turbid water environments where long-wavelength light tends to be transmitted without scattering. In fact, tadpoles use 3,4-dehydroretinal, whereas frogs use retinal (Liebman and Entine 1968). Also, eels in rivers use 3,4-dehydroretinal, and they change to using retinal when they go to sea to lay eggs (Schwanzara 1967). However, several experimental facts have been reported that are not accounted for by the simple adaptive evolution just described. For example, it has been reported that 3,4-dehydroretinal-based visual pigment increases in winter in salmon and bullfrog (Makino et al. 1983; Temple et al. 2006). The developmental stage in amphibians also affects the retinal/3,4-dehydroretinal ratio in the retina (Crescitelli 1973; Azuma et al. 1988). Additionally, there is a report indicating that breeding at higher temperature and with higher light intensity increases the composition of 3,4-dehydroretinal in bullfrog retina (Tsin and Beatty 1980). Through control of the retinal/3,4-dehydroretinal ratio, these species tune their absolute and wavelength sensitivities.

1.3.1 Rhodopsin

Rhodopsin (Rh1 group) is a photoreceptive protein in rod photoreceptor cells in most cases [with some exceptions, for example, green anoles, which have pure cone retina and rhodopsin-expressing cone photoreceptor cells (McDevitt et al. 1993)].

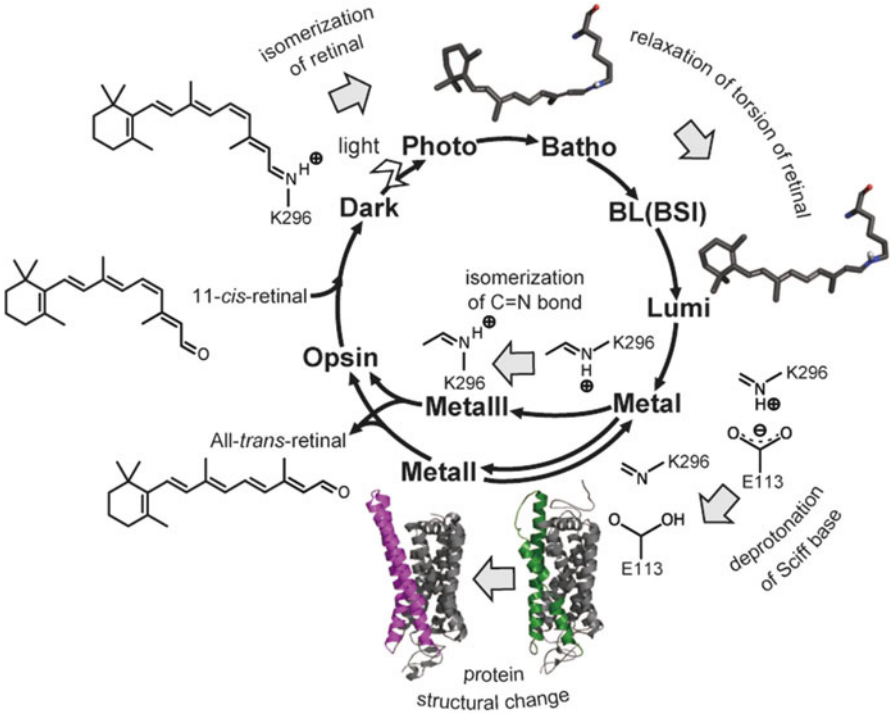

Fig. 1.4 Schematic view of photobleaching process of rhodopsin

The absorption maximum of rhodopsin is located at about 500 nm, and this value does not vary much except in some aquatic species, which have blue-shifted absorption spectra (Hunt et al. 1996; Fasick and Robinson 1998; Fasick and Robinson 2000). This blue shift is basically the result of replacements at positions 83, 122, or 292 (Yokoyama 2000) to adapt their dim light vision to the light environment in the deep sea.

Because the bovine eyeball has been easily available for experimentation and the visual pigment contained in the retina is almost exclusively rhodopsin, bovine rhodopsin has been deeply investigated for many years using various biochemical and spectroscopic techniques. Rhodopsin has 11-*cis*-retinal as a chromophore in the dark state and undergoes a series of light-induced reactions, called the photobleaching process, upon absorption of light (Hubbard and Wald 1952; Yoshizawa and Shichida 1982; Shichida and Imai 1998; Shichida 1999) (Fig. 1.4). Light excites a pi electron of the conjugated double-bond system of 11-*cis*-retinal chromophore to its Franc–Condon state. With relaxation of the pi electron from the excited state to the ground state, the C11=C12 bond in the conjugated double-bond system undergoes *cis–trans* isomerization (Kandori et al. 2001). The time constant of this reaction is approximately 200 fs, and quantum yield is 0.65 (Schoenlein et al. 1991; Kakitani et al. 1992; Kim et al. 2001). This ultrafast and highly efficient isomerization is one of the

molecular bases of the high sensitivity of light perception in vision. Because *cis–trans* isomerization causes an extension of the length of the chromophore, the chromophore should be in a highly twisted conformation in the restricted chromophore-binding site in the primary intermediates, photo and batho (Mizukami et al. 1993; Han and Smith 1995; Nakamichi and Okada 2006), and this causes an elevation of potential energy. In fact, calorimetric studies showed that about 60 % of the photon energy is stored in the batho intermediate as an increase in enthalpy, mainly because of the distortion of the chromophore (Cooper 1979; Schick et al. 1987). Then, by using the potential energy, the twisted retinal chromophore induces stepwise conformational changes of the protein moiety, resulting in formation of several intermediate states, lumi, BL (BSI), and meta-I, and finally forming a state that activates transducin. The active state of visual pigment is called meta-II. In both rod and cone pigments, meta-II has an absorption maximum in the ultraviolet region, indicating that the Schiff base chromophore loses the proton upon formation of meta-II (Matthews et al. 1963; Doukas et al. 1978; Shichida et al. 1993). The proton on the Schiff base in rhodopsin mediates the interaction between the chromophore and a counterion; thus, its transfer to the counterion results in the loss of interaction, inducing a flexible conformation near the Schiff base (Robinson et al. 1992; Jäger et al. 1994). Therefore, the proton transfer is one of the causes of large conformational changes that occur in the process of formation of meta-II that promotes GDP–GTP exchange of the G protein transducin (Longstaff et al. 1986). The transition from meta-I to meta-II is a reversible reaction. Thus, meta-I and meta-II are in equilibrium. This equilibrium mixture decays into the meta-III intermediate through thermal isomerization of the C15=N bond (Vogel et al. 2003). Meta-III retains all-*trans*-retinal and interacts with transducin; however, the activation efficiency is greatly decreased compared to that of meta-II (Zimmermann et al. 2004). This result shows that all-*trans* 15-anti and all-*trans* 15-syn retinal Schiff bases act as full agonists and weak partial agonists of rhodopsin, respectively. Concurrently with formation of meta-III, meta-II dissociates into the free retinal and protein moiety opsin. These two reactions proceed in parallel, and the ratio of the two reactions depends on the pigment and the conditions. Finally, meta-III also dissociates into retinal and opsin. The physiological role of the meta-III intermediate is still unknown. In previous studies, meta-III has been speculated to function as partial storage of a visual pigment to modulate the photosensitivity of the photoreceptor in light-adapted conditions (Heck et al. 2003; Bartl and Vogel 2007). Our experiments using knock-in mice in which rhodopsin was replaced by the E122Q mutant demonstrated that the decay of meta-III dominates the recovery of the rod after moderate photobleaching (Imai et al. 2007).

1.3.2 Cone Pigments

As already described, cone pigments are classified into four groups: M2 (Rh2), M1 (SWS2), S (SWS1), and L (LWS/MWS) groups. Cone pigments are normally expressed in stacked membranes of cone outer segments. However, there are a

Table 1.1 Identities and similarities between amino acid sequences of human rhodopsin (Rh) and chicken visual pigments

	Hu Rh	Ch Rh	Ch M2	Ch M1	Ch S	Ch L
Human Rh	—	0.858	0.690	0.490	0.449	0.389
Chicken Rh	0.937	—	0.724	0.486	0.451	0.397
Chicken M2	0.837	0.851	—	0.517	0.487	0.418
Chicken M1	0.693	0.702	0.710	—	0.479	0.385
Chicken S	0.641	0.651	0.659	0.665	—	0.402
Chicken L	0.565	0.570	0.585	0.586	0.576	—

The amino acid sequences of human rhodopsin and chicken visual pigments were aligned using mafft, and then identity and similarity values for each pair were calculated. The components in the upper triangle show the identities between the two amino acid sequences, and the components in the lower triangle show the similarities calculated for the two amino acid sequences based on the BLOSUM62 similarity matrix (Katoh and Standley 2013)

few exceptions as have been described for rhodopsins. One is the case of the Tokay gecko, in which the pigments belonging to the groups of cone visual pigments are embedded into rod-like photoreceptor cells (Kojima et al. 1992). Another example is the case of amphibians that have two types of rods, red rods and green rods. Red rods are canonical rods that express rhodopsin in their outer segments, whereas green rods have M1 (SWS2) group cone pigments as their photoreceptive proteins (Röhlich and Szél 2000; Ma et al. 2001).

1.3.2.1 M2 (RH2)

Based on phylogenetic analysis, the M2 and rhodopsin groups appear to have emerged from a common ancestor (Okano et al. 1992). Because of the high similarity of their sequences and absorption properties, the M2 group is also called the rhodopsin-like RH2 group (Yokoyama 1994). In fact, the M2 and rhodopsin groups show approximately 70 % sequence identity. This pair is more highly similar than any other pair of two groups (Table 1.1). However, they have distinct molecular natures from each other. Comparative studies on rhodopsin and M2 group cone pigments have been performed mainly by using chicken rhodopsin and green-sensitive cone pigment (chicken green). Spectroscopic studies showed that rhodopsin and chicken green exhibit very similar photobleaching processes (Shichida et al. 1994; Imai et al. 1995). However, the kinetics of the late thermal reaction differ between them (Shichida et al. 1994; Imai et al. 1995; Kuwayama et al. 2005; Sato et al. 2012). Especially, the decay rate of meta-II of chicken green is 100-fold faster than that of chicken rhodopsin (see following).

1.3.2.2 M1 (SWS2)

The absorption maxima of M1 group visual pigments are distributed between those of the S and M2 groups. M1 visual pigments have absorption maxima at 418–474 nm (Takahashi and Ebrey 2003). In mammals, monotremes have M1

group instead of S group pigments for detection of shorter-wavelength light (Fig. 1.3). This finding suggests that the common ancestor of prototheria and theria had a full set of the five groups of visual pigments and that gene losses had occurred independently in prototherian and therian lineages (Deeb 2010). The photobleaching process of chicken blue cone pigment, which belongs to the M1 group, has been investigated in detail by low-temperature spectroscopy (Imai et al. 1997b). The absorption maxima of batho, BL, and lumi intermediates of chicken blue are shorter than those of the respective intermediates of chicken green and rhodopsin. However, the maxima of the meta intermediates of these visual pigments are similar, which shows that the retinal chromophore changes its position in the protein moiety so as to change the interacting amino acids in the transition from lumi to meta-I. Additionally, the absorption spectrum of the batho intermediate of chicken blue displays vibrational fine structure. Vibrational fine structure of the absorbance spectrum of the batho intermediate is also observed in S group pigments, but not in rhodopsin, M2, or L group pigments, which suggests that the retinal conformation of the batho intermediate of M1 and S group pigments is more restricted than that of rhodopsin, M2, and L group pigments (Yoshizawa and Wald 1963; Yoshizawa and Wald 1967; Imai et al. 1995; Vought et al. 1999).

1.3.2.3 S (SWS1)

S group pigments have absorption maxima in the shortest wavelength region among visual pigments. Their absorbance ranges from violet to ultraviolet, achieved by control of the protonation–deprotonation of the retinal Schiff base. In general, protonated and deprotonated retinal Schiff bases have absorbances in the visible and ultraviolet (UV) regions, respectively. The conversion between violet and ultraviolet during the molecular evolution of S group cone pigments occurred independently in multiple strains. In avian species, protonation of the retinal Schiff base in S group visual pigments is mainly controlled by the amino acid residues at positions 86 and 90 (Carvalho et al. 2007). The most probable ancestral avian S pigment has S86 and S90, and it should be a violet-sensitive pigment; that is, its retinal Schiff base should be protonated. In separate lineages, the amino acid at 90 was replaced by cysteine, and/or the amino acid at 86 was replaced by phenylalanine, alanine, methionine, cysteine, or isoleucine. It has been shown that F86 or C90 is sufficient for deprotonation of the Schiff base but A86 or C86 is not sufficient (Carvalho et al. 2007; Hunt et al. 2009). Most mammals have a violet-sensitive S pigment. Some rodent and marsupial species, such as mouse and dunnart, have UV-sensitive pigments. In mammals, control of protonation–deprotonation of the Schiff base is mainly done by substitution at position 86. All the UV pigments in mammals have F86, and Y86 is a common replacement seen in violet pigments of nonprimate mammals (Fasick et al. 2002; Hunt et al. 2009). In primates, detailed studies have shown that the seven sites synergistically affect the absorption maxima of S group pigments (Shi et al. 2001; Carvalho et al. 2012).

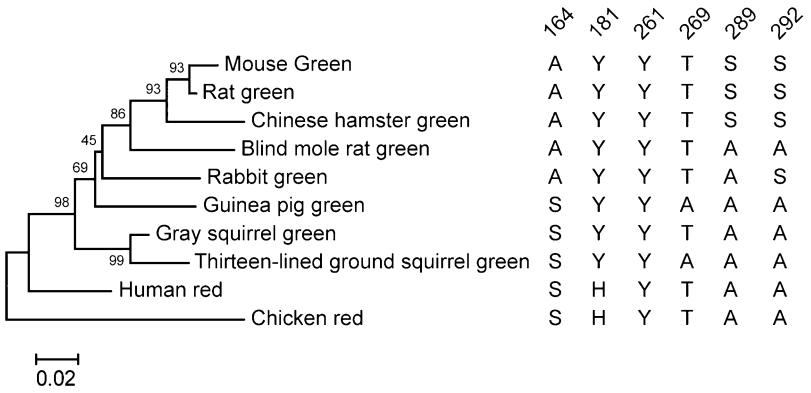

Fig. 1.5 Functionally important amino acid replacements in L group pigment. Amino acids responsible for regulation of absorption wavelength and anion binding shown on *right*

1.3.2.4 L (LWS/MWS)

The absorption maximum of a visual pigment is generally determined by the physicochemical properties of amino acid residues surrounding the retinal chromophore. However, L group cone pigments can bind to chloride within the protein moiety and shift the absorption maximum to a longer wavelength (Crescitelli 1977; Fager and Fager 1979; Shichida et al. 1990). Although a carboxylic residue at 181 is one of the highly conserved residues in the opsin family, most L group pigments have a histidine residue at this position, resulting in formation of a chloride-binding site in their protein moieties. An early study demonstrated that histidine at 181 together with lysine at 197 constituted the chloride-binding site (Wang et al. 1993). However, later studies showed that alanines at 289 and 292 are also constituents of the chloride-binding site (Davies et al. 2012b; Yamashita et al. 2013). In L pigments of some animals, such as guinea pig and blind mole rat, the amino acid residue at 181 is replaced by tyrosine, resulting in a decrease of the chloride-dependent spectral red shift (Fig. 1.5). On the other hand, L pigments of mouse, rat, and hamster have serines at position 289 and 292 instead of alanines, in addition to tyrosine at position 181. These replacements cause almost no shift of absorption maximum upon binding of chloride. L pigments of mouse, rat, and hamster are the most strongly blue-shifted pigments among L group pigments.

Furthermore, it has been shown that L pigment exhibits a thermal recovery reaction from the photo-activated intermediate state to the original dark state (Imamoto et al. 1994). The physiological function of the thermal recovery is not yet clear. The visual pigments in other groups do not show such a molecular nature. Thus, this could be related to the specific functions of L pigments.

1.3.3 Difference in Molecular Properties Between Rhodopsin and Cone Pigments

The prominent differences between rhodopsin and cone pigments are the decay rate of the active state meta-II and its turnover rate of G-protein activation, the kinetics of the incorporation of 11-*cis*-retinal, the rate of spontaneous activation, and thermal stability. As compared to cone pigments, rhodopsin has a 100-fold slower time constant for decay of the active state but has more than twofold larger amplification efficiency for G-protein activation (Shichida et al. 1994; Imai et al. 1995; Tachibanaki et al. 2012; Imamoto et al. 2013). The difference in decay time of meta-II between rhodopsin and cone pigment might not be related to the rate of photoresponse because the off-kinetics of photoreceptor cells largely depends on the rates of visual pigment phosphorylation by G protein-coupled receptor kinase (GRK), arrestin binding to the phosphorylated visual pigment, guanylyl-cyclase (GC) activation, and guanylate cyclase-activating protein (GCAP) binding to the GC. Instead, it is the difference in amplification efficiency that causes the difference in photoresponse between rods and cones. In addition, the large difference between rhodopsin and cone pigments in the release of all-*trans*-retinal from the protein moiety causes the difference in recovery from the light-adapted state between rods and cones (Imai et al. 2007).

Visual pigments are regenerated by binding to 11-*cis*-retinal. The kinetics of regeneration are also markedly different between rhodopsin and cone pigments. The regeneration of rhodopsin is much slower than that of cone pigment, which might correspond to the slower adaptation of rods for outside light environments. In contrast, rhodopsin has a much lower rate of spontaneous activation than cone pigments (Donner 1992; Sakurai et al. 2007; Fu et al. 2008), which accounts for the low noise and low threshold of rods. These distinct molecular properties are closely related to the physiological roles of rods and cones, namely, dim and bright light vision, respectively. Furthermore, compared to cone pigments, rhodopsin is thermally stable (Okano et al. 1989). Its stable nature largely contributes to the integrity of the outer segment of the rod photoreceptor cell itself. Rhodopsin knockout mice show loss of the outer segment of photoreceptor cells and gradually lose the photoreceptor layer of the retina (Lem et al. 1999). Additionally, many mutations in rhodopsin identified as causes of retinitis pigmentosa are related to thermal stability or appropriate folding of rhodopsin (Mendes et al. 2005). These findings show that rhodopsin also works as a structural protein to sustain the architecture of rods.

Based on mutational studies, it has been shown that two residues at positions 122 and 189 are involved in the large difference in the kinetics of retinal release and incorporation (Imai et al. 2005). Glutamic acid at position 122 (in the bovine rhodopsin numbering system) is highly conserved in vertebrate rhodopsins (Fig. 1.2). On the other hand, the residue at 122 is a neutral amino acid such as

isoleucine or glutamine in most cone pigments, although some of the M2 group pigments in fishes have glutamic acid at 122. The residue at 189 is nearly completely conserved as proline in cone pigments, but rhodopsins, except for those of lamprey and hagfish, contain leucine at this position. Comparative mutational studies on chicken green-sensitive cone pigment (chicken green) and chicken rhodopsin have shown that the replacements of the residues of rhodopsin at position 122 and 189 with the corresponding residues of chicken green caused acceleration of the meta-II decay and 11-*cis*-retinal incorporation, and the opposite replacements caused reverse effects in chicken green (Imai et al. 1997a; Kuwayama et al. 2002). Thus, these two residues are the determinants of the molecular properties of rhodopsin and cone pigments.

1.4 Nonvisual Opsins Closely Related to Vertebrate Visual Pigments

In the phylogenetic tree covering all the known opsins, many nonvisual opsins constitute sister groups with that of vertebrate visual pigments (Fig. 1.6). They are classified into five groups named the pinopsin, VA/VAL-opsin, parietopsin, parapinopsin, and opn3 groups. These opsins diverged from a common ancestor, and thus it is meaningful to compare the molecular nature of these nonvisual or nonvertebrate opsins to obtain insight into the evolution of vertebrate visual pigments.

1.4.1 Pinopsin

It is well known that pineal glands in nonmammalian vertebrates are photosensitive (Deguchi 1981). Pinopsin is the opsin that was first isolated from chicken pineal gland (Okano et al. 1994). It is also the nonvisual opsin that was first isolated and characterized from vertebrates. Pinopsin bound to 11-*cis*-retinal maximally absorbs blue light at about 470 nm, and it can activate transducin light dependently at a level comparable to vertebrate rhodopsins (Nakamura et al. 1999). Pinopsin is the closest relative of the visual pigments. Pinopsin is found in bird, reptile, and amphibian cDNA or genome resources, but not in teleost fishes. It is possible that exo-rhodopsin (see following) takes the place of pinopsin in the teleost fish lineage. Pinopsin and other nonvisual opsins described in the following section have an extracellular loop 2 that is shorter by two amino acids compared to vertebrate visual pigments. In pinopsin, this affects the kinetics of the formation and decay of the active state (Nakamura et al. 2001).

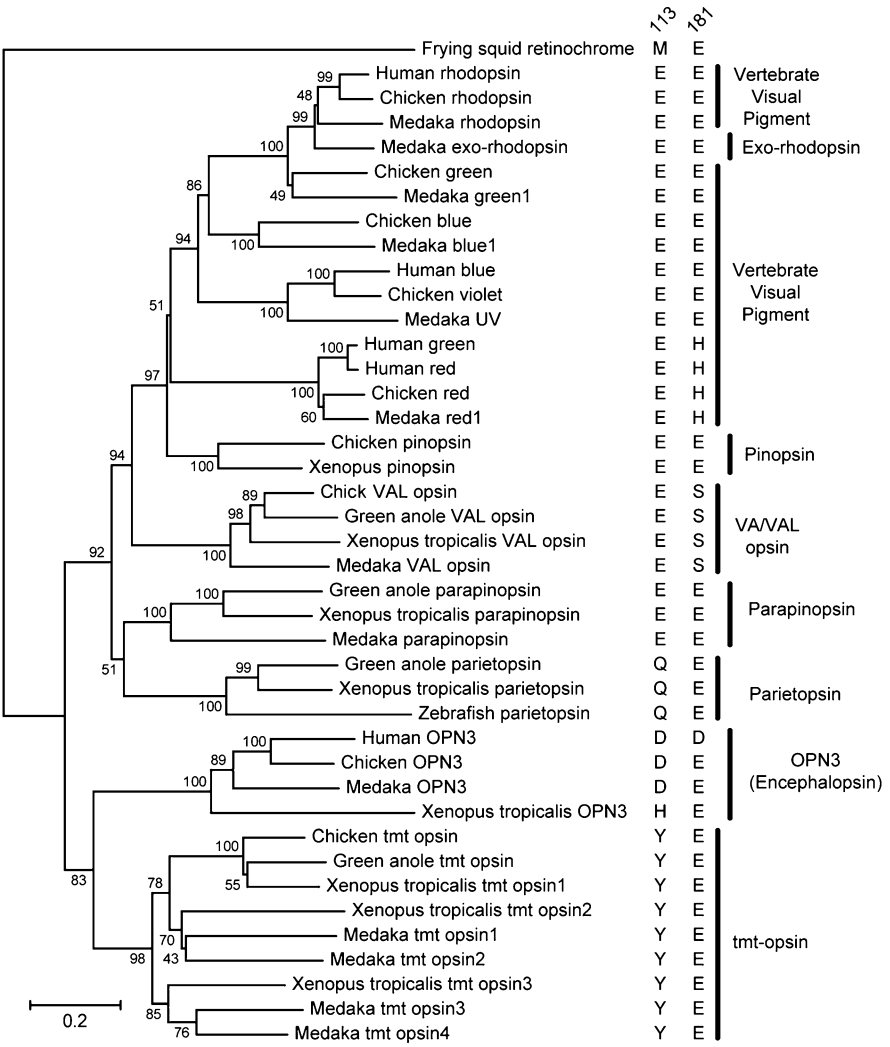

Fig. 1.6 Phylogenetic tree of opsins homologous to vertebrate visual pigments constructed by the neighbor-joining method. Amino acids at positions 113 and 181 (bovine rhodopsin numbering) and group names are shown

1.4.2 VA/VAL-Opsin

VA opsin was named after "vertebrate ancient opsin" because it branched phylogenetically from the ancestral position of vertebrate visual pigments and pinopsins (Soni and Foster 1997). Additionally, VA-long (VAL) opsin was found in zebrafish as a variant of VA opsin with a long C-terminal tail (Kojima et al. 2000). Some studies have shown that the absorption maximum of VA/VAL

opsin is at 480–500 nm in the 11-*cis*-retinal bound form (Kojima et al. 2000; Kojima et al. 2008; Davies et al. 2012a). Additionally, it has been shown that VAL opsin can activate G_i and G_t in a light-dependent manner (Sato et al. 2011). Interestingly, VA/VAL opsin has serine at site 181, where other opsins have a highly conserved carboxylic residue. This point simply suggests that glutamate at 113 is a counterion of the protonated Schiff base of VA/VAL opsin, and S181 would have a special role in the functionality of VA/VAL opsin, as does H181 of vertebrate L group cone pigment.

1.4.3 Parietopsin

Some animal species, including lizards and frogs, have a third eye or parietal eye on the top of their heads. Photoreceptor cells in the parietal eye have two different cell responses: blue light hyperpolarization and green light depolarization (Su et al. 2006). These photoreceptor cells have two different pigments, pinopsin and parietopsin. From recombinant expression experiments, pinopsin and parietopsin have absorption maxima in blue and green light regions, respectively (Su et al. 2006). Thus, pinopsin and parietopsin would mediate blue and green light responses, respectively, in the single photoreceptor cell of the parietal eye. Pinopsin and parietopsin are thought to activate gustducin and G_o subtypes of G protein, respectively, in the parietal photoreceptor cells, although this has not yet been biochemically confirmed. The counterion of parietopsin is the glutamic acid at position 181, which is different from that of vertebrate visual pigment. However, the photobleaching process of parietopsin is rather similar to that of vertebrate rhodopsin, and parietopsin does not show the photoreversible reaction between the 11-*cis*-retinal bound and all-*trans*-retinal bound forms often seen in opsins whose counterion is at 181 (Sakai et al. 2012).

1.4.4 Parapinopsin

Parapinopsin was first isolated from the pineal organ of catfish (Blackshaw and Snyder 1997). Thereafter, parapinopsin of the Japanese lamprey was investigated in detail by using the recombinant protein (Koyanagi et al. 2004). The dark state of parapinopsin has its absorption maximum in the UV wavelength region. Upon absorption of light, parapinopsin converts to an active state having the absorption maximum in the visible wavelength region. Re-irradiation of the active state converts it back to the original parapinopsin. That is, the 11-*cis*-retinal bound dark state and all-*trans*-retinal bound active state are both stable at physiological temperature and are interconvertible by absorption of light. This property is called “bistable nature”. Additionally, it has been clearly shown that the counterion of the Schiff base in the active state is glutamic acid at 181 (Terakita et al. 2004).

1.4.5 Opn3 and tmt-Opsin

Opn3 was identified in 1999 as one of the nonvisual opsins that mammals possess (Blackshaw and Snyder 1999). The tissue distribution of Opn3 has been shown to be certain areas in the brain and testis. However, because of the difficulty of recombinant expression, its molecular properties have not been characterized and its function is still unknown. Teleost multiple tissue (tmt)-opsin is a close homologue of mammalian Opn3. It was first isolated from teleost and found in multiple tissues (Moutsaki et al. 2003). After that, it was shown that many nonmammalian vertebrates have opsins classified as tmt-opsins, and that some kinds of invertebrates have opn3 homologues. Very recently, it was shown that recombinant pufferfish tmt-opsin and mosquito Opn3 constitute photosensitive pigments with 11-*cis*-retinal and that they activate G_i and G_o subtypes of G protein in a light-dependent manner (Koyanagi et al. 2013).

1.4.6 Exo-Rhodopsin

Exo-rhodopsin was first found in the zebrafish pineal gland (Mano et al. 1999). Although it is clustered with rod-visual pigment rhodopsin in the phylogenetic tree, it is present in the pineal gland and not in retinal cells. Thus it was named extraocular rhodopsin, exo-rhodopsin. Exo-rhodopsin is only found in bony fishes, and thus it was derived from gene duplication of rhodopsin in the ancestor of bony fishes.

1.5 Evolution of Vertebrate Visual Pigments

Several lines of evidence have suggested that vertebrate visual pigments evolved and diversified in a stepwise manner. One of the important aspects of their evolutionary process was the counterion displacement. About 10 years ago, we tried to identify the counterion, which is the molecular basis for visible light absorption in opsin, in various opsins, because glutamic acid at position 113, which was identified as a counterion in vertebrate visual pigments, is not conserved in other opsins. We thereby found that glutamic acid at position 181 acts as the counterion in these opsins (Terakita et al. 2000; Terakita et al. 2004). It is notable that almost all the pigments in the opsin family, including vertebrate visual opsins, have a glutamic or aspartic acid at position 181, so that glutamic acid at position 181 may have been an ancestral counterion in vertebrate visual opsin. Thus, we speculate that counterion displacement from 181 to 113 occurred during the molecular evolution of vertebrate visual opsins. One of the advantages of the counterion displacement would be to increase the G-protein activation efficiency, that is, vertebrate rhodopsin exhibits

G-protein activation efficiency about 50-fold higher than that of invertebrate opsins (Terakita et al. 2004). Therefore, ancestral and invertebrate opsins contain a counterion at position 181, but vertebrate opsin acquired a new counterion at position 113 during the course of molecular evolution, resulting in the acquisition of highly efficient G-protein activation (Shichida and Matsuyama 2009).

In the vertebrate opsin clade in the phylogenetic tree, parietopsin has a counterion at position181. VA/VAL opsin is likely to have a counterion at position 113, because it has a conserved serine at position 181. The dark state of parapinopsin has the absorption maximum in the UV region, but the photoactivated state has a visible absorption spectrum, and it was shown that the glutamate at 181 is the counterion (Terakita et al. 2004). On the other hand, the glutamic acid at position 113 is completely conserved in vertebrate visual pigments, and this residue acts as a counterion of the chromophore Schiff base (Sakmar et al. 1989; Nathans 1990; Tsutsui et al. 2008). Consequently, the counterion position of vertebrate visual pigment was displaced to position 113 from 181 at least before diversification of the five groups of visual pigment during the course of evolution. In addition to the high efficiency of G-protein activation, several studies on vertebrate visual pigments have shown that the counterion has multiple functions in visual pigments such as suppression of constitutive activity, spectral tuning, and facilitation of photoisomerization, even in UV-sensitive visual pigments of which the Schiff base is not protonated (Tsutsui and Shichida 2010).

The G-protein activation efficiency is significantly lower in parapinopsin and VAL opsin than in vertebrate rhodopsin (Terakita et al. 2004; Sato et al. 2011). Thus, there is some relationship between the efficiency of G-protein activation and whether the active state has a deprotonated or protonated Schiff base. That is, the G protein-activating state of vertebrate visual pigments is meta-II, which has UV absorbance and a deprotonated Schiff base, whereas those of parapinopsin and VAL opsin have visible absorbance and a protonated Schiff base. In vertebrate rhodopsin, the efficient suppression and activation of G protein would be caused by the acquisition of the opsin lock. That is, the counterion at position 113 is involved in helix 3, so that a salt bridge formed between the counterion and the protonated Schiff base chromophore in rhodopsin and between the counterion and lysine residue in opsin works as a lock between helices 3 and 7, resulting in increased stability of opsin (Robinson et al. 1992). Absorption of a photon then causes proton transfer from the protonated Schiff base to the counterion, resulting in unlocking of the salt bridge and induction of opsin conformational change that activates G protein with high efficiency (Longstaff et al. 1986; Jäger et al. 1994; Knierim et al. 2007; Mahalingam et al. 2008). In fact, it was demonstrated that parapinopsin undergoes a relatively small helix movement compared to vertebrate rhodopsin in the formation of the active state (Tsukamoto et al. 2009). These data suggest that vertebrate visual pigments have acquired the ability to form a highly active meta-II intermediate during the evolutionary process to achieve a high signal-to-noise ratio in vision. Such a high efficacy of G-protein activation has not been necessary for the functions of parapinopsin or VAL opsin. It is expected that key amino acid replacement(s) will be discovered in future studies.

It was experimentally confirmed that not only vertebrate visual pigment but also VAL opsin and pinopsin are able to induce GDP–GTP exchange in transducin, the visual G protein (Nakamura et al. 1999; Sato et al. 2011). On the other hand, squid rhodopsin and chicken opn5m, opsins phylogenetically distant from vertebrate visual pigments, cannot activate transducin (Terakita et al. 2002; Yamashita et al. 2010). These observations imply that, with the emergence of transducin, vertebrate visual pigments obtained specificity for G-protein activation in the ancestral state before the branching of VA/VAL opsin and other opsins.

1.6 Concluding Remarks

Vertebrate visual pigments would have evolved from a common ancestor along with some kinds of vertebrate nonvisual opsins and have diversified for sensing wide ranges of wavelength and intensity of light. The respective visual pigments in animals underwent replacements of amino acids to achieve appropriate color palette and kinetic properties, and the animals adapted their ability to sense light according to the light environment of their habitats as a result of spontaneous mutation and natural selection. A number of studies have demonstrated that specific amino acid replacements occur in specific phylogenetic lines and have shown how they affect the molecular properties. Moreover, they have shown relationships between the molecular properties of visual pigments and electrophysiological responses at the cellular level and between amino acid substitutions and distributions of animal species. Additionally, analysis of nonvisual pigments would give us insight into the ancestral state of vertebrate visual pigments.Vision and visual pigments would be a good model for understanding evolution and diversification at molecular and physiological levels in accordance with the environment surrounding individual species. Further understanding of diversified vertebrate visual pigments will inform us about the evolutionary history behind the nuanced system of photosensing by living organisms.

References

Azuma M, Seki T, Fujishita S (1988) Formation of visual pigment chromophores during the development of *Xenopus laevis*. Vision Res 28:959–964. doi:10.1016/0042-6989(88)90072-7

Bartl FJ, Vogel R (2007) Structural and functional properties of metarhodopsin III: recent spectroscopic studies on deactivation pathways of rhodopsin. Phys Chem Chem Phys 9:1648–1658. doi:10.1039/b616365c

Bennett N, Sitaramayya A (1988) Inactivation of photoexcited rhodopsin in retinal rods: the roles of rhodopsin kinase and 48-kDa protein (arrestin). Biochemistry 27:1710–1715. doi:10.1021/bi00405a049

Blackshaw S, Snyder SH (1997) Parapinopsin, a novel catfish opsin localized to the parapineal organ, defines a new gene family. J Neurosci 17:8083–8092

Blackshaw S, Snyder SH (1999) Encephalopsin: a novel mammalian extraretinal opsin discretely localized in the brain. J Neurosci 19:3681–3690
Carvalho LS, Cowing JA, Wilkie SE et al (2007) The molecular evolution of avian ultraviolet- and violet-sensitive visual pigments. Mol Biol Evol 24:1843–1852. doi:10.1093/molbev/msm109
Carvalho LS, Davies WL, Robinson PR, Hunt DM (2012) Spectral tuning and evolution of primate short-wavelength-sensitive visual pigments. Proc Biol Sci 279:387–393. doi:10.1098/rspb.2011.0782
Cooper A (1979) Energy uptake in the first step of visual excitation. Nature (Lond) 282:531–533. doi:10.1038/282531a0
Crescitelli F (1973) The visual pigment system of *Xenopus laevis*: tadpoles and adults. Vision Res 13:855–865. doi:10.1016/0042-6989(73)90048-5
Crescitelli F (1977) Ionochromic behavior of gecko visual pigments. Science 195:187–188
Davies WIL, Turton M, Peirson SN et al (2012a) Vertebrate ancient opsin photopigment spectra and the avian photoperiodic response. Biol Lett 8:291–294. doi:10.1098/rsbl.2011.0864
Davies WIL, Wilkie SE, Cowing JA et al (2012b) Anion sensitivity and spectral tuning of middle- and long-wavelength-sensitive (MWS/LWS) visual pigments. Cell Mol Life Sci 69:2455–2464. doi:10.1007/s00018-012-0934-4
Deeb SS (2010) Visual pigments and colour vision in marsupials and monotremes. In: Deakin JE, Waters PD, Graves JAM (eds) Marsupial genetics and genomics. Springer, The Netherlands, pp 403–414
Deguchi T (1981) Rhodopsin-like photosensitivity of isolated chicken pineal gland. Nature (Lond) 290:706–707. doi:10.1038/290706a0
Donner K (1992) Noise and the absolute thresholds of cone and rod vision. Vision Res 32:853–866. doi:10.1016/0042-6989(92)90028-H
Doukas AG, Aton B, Callender RH, Ebrey TG (1978) Resonance Raman studies of bovine metarhodopsin I and metarhodopsin II. Biochemistry 17:2430–5
Ebrey T, Koutalos Y (2001) Vertebrate photoreceptors. Prog Retin Eye Res 20:49–94. doi:10.1016/S1350-9462(00)00014-8
Fager LY, Fager RS (1979) Halide control of color of the chicken cone pigment iodopsin. Exp Eye Res 29:401–408
Fasick JI, Robinson PR (1998) Mechanism of spectral tuning in the dolphin visual pigments. Biochemistry 37:433–438. doi:10.1021/bi972500j
Fasick JI, Robinson PR (2000) Spectral-tuning mechanisms of marine mammal rhodopsins and correlations with foraging depth. Vis Neurosci 17:781–788
Fasick JI, Applebury ML, Oprian DD (2002) Spectral tuning in the mammalian short-wavelength sensitive cone pigments. Biochemistry 41:6860–6865. doi:10.1021/bi0200413
Fu Y, Kefalov V, Luo D-G et al (2008) Quantal noise from human red cone pigment. Nat Neurosci 11:565–571. doi:10.1038/nn.2110
Han M, Smith SO (1995) NMR constraints on the location of the retinal chromophore in rhodopsin and bathorhodopsin. Biochemistry 34:1425–1432. doi:10.1021/bi00004a037
Heck M, Schädel SA, Maretzki D et al (2003) Signaling states of rhodopsin. Formation of the storage form, metarhodopsin III, from active metarhodopsin II. J Biol Chem 278:3162–3169. doi:10.1074/jbc.M209675200
Hofmann CM, Carleton KL (2009) Gene duplication and differential gene expression play an important role in the diversification of visual pigments in fish. Integr Comp Biol 49:630–643. doi:10.1093/icb/icp079
Hubbard R, Wald G (1952) Cis-trans isomers of vitamin A and retinene in the rhodopsin system. J Gen Physiol 36:269–315. doi:10.1085/jgp.36.2.269
Hunt DM, Fitzgibbon J, Slobodyanyuk SJ, Bowmakers JK (1996) Spectral tuning and molecular evolution of rod visual pigments in the species flock of cottoid fish in Lake Baikal. Vision Res 36:1217–1224. doi:10.1016/0042-6989(95)00228-6
Hunt DM, Carvalho LS, Cowing JA, Davies WL (2009) Evolution and spectral tuning of visual pigments in birds and mammals. Philos Trans R Soc Lond B Biol Sci 364:2941–2955. doi:10.1098/rstb.2009.0044

Imai H, Imamoto Y, Yoshizawa T, Shichida Y (1995) Difference in molecular properties between chicken green and rhodopsin as related to the functional difference between cone and rod photoreceptor cells. Biochemistry 34:10525–10531

Imai H, Kojima D, Oura T et al (1997a) Single amino acid residue as a functional determinant of rod and cone visual pigments. Proc Natl Acad Sci USA 94:2322–2326

Imai H, Terakita A, Tachibanaki S et al (1997b) Photochemical and biochemical properties of chicken blue-sensitive cone visual pigment. Biochemistry 36:12773–12779. doi:10.1021/bi970809x

Imai H, Kuwayama S, Onishi A et al (2005) Molecular properties of rod and cone visual pigments from purified chicken cone pigments to mouse rhodopsin in situ. Photochem Photobiol Sci 4:667–674. doi:10.1039/b416731g

Imai H, Kefalov V, Sakurai K et al (2007) Molecular properties of rhodopsin and rod function. J Biol Chem 282:6677–6684. doi:10.1074/jbc.M610086200

Imamoto Y, Imai H, Yoshizawa T, Shichida Y (1994) Thermal recovery of iodopsin from its meta I-intermediate. FEBS Lett 354:165–168

Imamoto Y, Seki I, Yamashita T, Shichida Y (2013) Efficiencies of activation of transducin by cone and rod visual pigments. Biochemistry 52:3010–3018

Jäger F, Fahmy K, Sakmar TP, Siebert F (1994) Identification of glutamic acid 113 as the Schiff base proton acceptor in the metarhodopsin II photointermediate of rhodopsin. Biochemistry 33:10878–82

Kakitani H, Hatano Y, Shichida Y, Imamoto Y, Tokunaga F, Kakitani H (1992) Excited state dynamics of retinal proteins as studied by Fourier transform of optical absorption spectrum I. Development of analytical method. Photochem Photobiol 56:977–987

Kandori H, Shichida Y, Yoshizawa T (2001) Photoisomerization in rhodopsin. Biochemistry (Mosc) 66:1197–1209

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30:772–780. doi:10.1093/molbev/mst010

Kim JE, Tauber MJ, Mathies RA (2001) Wavelength dependent *cis-trans* isomerization in vision. Biochemistry 40:13774–13778

Knierim B, Hofmann KP, Ernst OP, Hubbell WL (2007) Sequence of late molecular events in the activation of rhodopsin. Proc Natl Acad Sci USA 104:20290–20295. doi:10.1073/pnas.0710393104

Kojima D, Okano T, Fukada Y et al (1992) Cone visual pigments are present in gecko rod cells. Proc Natl Acad Sci USA 89:6841–6845

Kojima D, Mano H, Fukada Y (2000) Vertebrate ancient-long opsin: a green-sensitive photoreceptive molecule present in zebrafish deep brain and retinal horizontal cells. J Neurosci 20:2845–2851

Kojima D, Torii M, Fukada Y, Dowling JE (2008) Differential expression of duplicated VAL-opsin genes in the developing zebrafish. J Neurochem 104:1364–1371. doi:10.1111/j.1471-4159.2007.05093.x

Koyanagi M, Kawano E, Kinugawa Y et al (2004) Bistable UV pigment in the lamprey pineal. Proc Natl Acad Sci USA 101:6687–6691. doi:10.1073/pnas.0400819101

Koyanagi M, Takada E, Nagata T et al (2013) Homologs of vertebrate Opn3 potentially serve as a light sensor in nonphotoreceptive tissue. Proc Natl Acad Sci USA. doi:10.1073/pnas.1219416110

Kuwayama S, Imai H, Hirano T et al (2002) Conserved proline residue at position 189 in cone visual pigments as a determinant of molecular properties different from rhodopsins. Biochemistry 41:15245–15252

Kuwayama S, Imai H, Morizumi T, Shichida Y (2005) Amino acid residues responsible for the meta-III decay rates in rod and cone visual pigments. Biochemistry 44:2208–2215

Lem J, Krasnoperova NV, Calvert PD et al (1999) Morphological, physiological, and biochemical changes in rhodopsin knockout mice. Proc Natl Acad Sci USA 96:736–741. doi:10.1073/pnas.96.2.736

Liebman PA, Entine G (1968) Visual pigments of frog and tadpole (*Rana pipiens*). Vision Res 8:761–IN7. doi:10.1016/0042-6989(68)90128-4

Longstaff C, Calhoon RD, Rando RR (1986) Deprotonation of the Schiff base of rhodopsin is obligate in the activation of the G protein. Proc Natl Acad Sci USA 83:4209–4213

Ma J, Znoiko S, Othersen KL et al (2001) A visual pigment expressed in both rod and cone photoreceptors. Neuron 32:451–461. doi:10.1016/S0896-6273(01)00482-2

Mahalingam M, Martínez-Mayorga K, Brown MF, Vogel R (2008) Two protonation switches control rhodopsin activation in membranes. Proc Natl Acad Sci USA 105:17795–17800. doi:10.1073/pnas.0804541105

Makino M, Nagai K, Suzuki T (1983) Seasonal variation of the vitamin A2-based visual pigment in the retina of adult bullfrog, *Rana catesbeiana*. Vision Res 23:199–204. doi:10.1016/0042-6989(83)90143-8

Mano H, Kojima D, Fukada Y (1999) Exo-rhodopsin: a novel rhodopsin expressed in the zebrafish pineal gland. Mol Brain Res 73:110–118. doi:10.1016/S0169-328X(99)00242-9

Matthews RG, Hubbard R, Brown PK, Wald G (1963) Tautomeric forms of metarhodopsin. J Gen Physiol 47:215–40

McDevitt DS, Brahma SK, Jeanny J-C, Hicks D (1993) Presence and foveal enrichment of rod opsin in the "all cone" retina of the American chameleon. Anat Rec 237:299–307. doi:10.1002/ar.1092370302

Mendes HF, van der Spuy J, Chapple JP, Cheetham ME (2005) Mechanisms of cell death in rhodopsin retinitis pigmentosa: implications for therapy. Trends Mol Med 11:177–185. doi:10.1016/j.molmed.2005.02.007

Mizukami T, Kandori H, Shichida Y et al (1993) Photoisomerization mechanism of the rhodopsin chromophore: picosecond photolysis of pigment containing 11-cis-locked-8-membered-ring-retinal. Proc Natl Acad Sci USA 90:4072–4076

Moutsaki P, Whitmore D, Bellingham J et al (2003) Teleost multiple tissue (tmt) opsin: a candidate photopigment regulating the peripheral clocks of zebrafish? Mol Brain Res 112:135–145. doi:10.1016/S0169-328X(03)00059-7

Nakamichi H, Okada T (2006) Crystallographic analysis of primary visual photochemistry. Angew Chem Int Ed Engl 45:4270–3. doi:10.1002/anie.200600595

Nakamura A, Kojima D, Imai H et al (1999) Chimeric nature of pinopsin between rod and cone visual pigments. Biochemistry 38:14738–14745

Nakamura A, Kojima D, Okano T et al (2001) Regulatory mechanism for the stability of the meta II intermediate of pinopsin. J Biochem 129:329–334

Nathans J (1990) Determinants of visual pigment absorbance: identification of the retinylidene Schiff's base counterion in bovine rhodopsin. Biochemistry 29:9746–52

Okano T, Fukada Y, Artamonov ID, Yoshizawa T (1989) Purification of cone visual pigments from chicken retina. Biochemistry 28:8848–8856. doi:10.1021/bi00448a025

Okano T, Kojima D, Fukada Y et al (1992) Primary structures of chicken cone visual pigments: vertebrate rhodopsins have evolved out of cone visual pigments. Proc Natl Acad Sci USA 89:5932–5936

Okano T, Yoshizawa T, Fukada Y (1994) Pinopsin is a chicken pineal photoreceptive molecule. Nature (Lond) 372:94–97. doi:10.1038/372094a0

Palczewski K, Rispoli G, Detwiler PB (1992) The influence of arrestin (48K protein) and rhodopsin kinase on visual transduction. Neuron 8:117–126. doi:10.1016/0896-6273(92)90113-R

Robinson PR, Cohen GB, Zhukovsky EA, Oprian DD (1992) Constitutively active mutants of rhodopsin. Neuron 9:719–25

Röhlich P, Szél A (2000) Photoreceptor cells in the *Xenopus* retina. Microsc Res Tech 50:327–37. doi:10.1002/1097-0029(20000901)50:5<327::AID-JEMT2>3.0.CO;2-P

Sakai K, Imamoto Y, Su C-Y et al (2012) Photochemical nature of parietopsin. Biochemistry 51:1933–1941. doi:10.1021/bi2018283

Sakmar TP, Franke RR, Khorana HG (1989) Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. Proc Natl Acad Sci USA 86:8309–13

Sakurai K, Onishi A, Imai H et al (2007) Physiological properties of rod photoreceptor cells in green-sensitive cone pigment knock-in mice. J Gen Physiol 130:21–40. doi:10.1085/jgp.200609729

Sato K, Yamashita T, Ohuchi H, Shichida Y (2011) Vertebrate ancient-long opsin has molecular properties intermediate between those of vertebrate and invertebrate visual pigments. Biochemistry 50:10484–10490. doi:10.1021/bi201212z

Sato K, Yamashita T, Imamoto Y, Shichida Y (2012) Comparative studies on the late bleaching processes of four kinds of cone visual pigments and rod visual pigment. Biochemistry 51:4300–4308. doi:10.1021/bi3000885

Schick GA, Cooper TM, Holloway RA et al (1987) Energy storage in the primary photochemical events of rhodopsin and isorhodopsin. Biochemistry 26:2556–2562. doi:10.1021/bi00383a022

Schoenlein RW, Peteanu LA, Mathies RA, Shank CV (1991) The first step in vision: femtosecond isomerization of rhodopsin. Science 254:412–415

Schwanzara SA (1967) The visual pigments of freshwater fishes. Vision Res 7:121–148. doi:10.1016/0042-6989(67)90079-X

Shi Y, Radlwimmer FB, Yokoyama S (2001) Molecular genetics and the evolution of ultraviolet vision in vertebrates. Proc Natl Acad Sci USA 98:11731–11736. doi:10.1073/pnas.201257398

Shichida Y (1999) Visual pigment: photochemistry and molecular evolution. In: Toyoda J, Murakami M, Kaneko A, Saito T (eds) The retinal basis of vision. Elsevier, Amsterdam, pp 23–37

Shichida Y, Imai H (1998) Visual pigment: G-protein-coupled receptor for light signal. J Cell Mol Life Sci 54:1299–1315

Shichida Y, Matsuyama T (2009) Evolution of opsins and phototransduction. Philos Trans R Soc Lond B Biol Sci 364:2881–2895. doi:10.1098/rstb.2009.0051

Shichida Y, Kato T, Sasayama S et al (1990) Effects of chloride on chicken iodopsin and the chromophore transfer reactions from iodopsin. Biochemistry 29:5843–5848

Shichida Y, Okada T, Kandori H et al (1993) Nanosecond laser photolysis of iodopsin, chicken red-sensitive cone visual pigment. Biochemistry 32:10832–10838

Shichida Y, Imai H, Imamoto Y et al (1994) Is chicken green-sensitive cone visual pigment a rhodopsin-like pigment? A comparative study of the molecular properties between chicken green and rhodopsin. Biochemistry 33:9040–9044

Soni BG, Foster RG (1997) A novel and ancient vertebrate opsin. FEBS Lett 406:279–283

Stryer L, Hurley JB, Fung BK-K (1981) Transducin: an amplifier protein in vision. Trends Biochem Sci 6:245–247. doi:10.1016/0968-0004(81)90089-X

Su C-Y, Luo D-G, Terakita A et al (2006) Parietal-eye phototransduction components and their potential evolutionary implications. Science 311:1617–1621. doi:10.1126/science.1123802

Surridge AK, Osorio D, Mundy NI (2003) Evolution and selection of trichromatic vision in primates. Trends Ecol Evol 18:198–205. doi:10.1016/S0169-5347(03)00012-0

Tachibanaki S, Yonetsu S, Fukaya S et al (2012) Low activation and fast inactivation of transducin in carp cones. J Biol Chem 287:41186–41194

Takahashi Y, Ebrey TG (2003) Molecular basis of spectral tuning in the newt short wavelength sensitive visual pigment. Biochemistry 42:6025–6034. doi:10.1021/bi020629+

Temple SE, Plate EM, Ramsden S et al (2006) Seasonal cycle in vitamin A1/A2-based visual pigment composition during the life history of coho salmon (*Oncorhynchus kisutch*). J Comp Physiol A 192:301–313. doi:10.1007/s00359-005-0068-3

Terakita A, Yamashita T, Shichida Y (2000) Highly conserved glutamic acid in the extracellular IV–V loop in rhodopsins acts as the counterion in retinochrome, a member of the rhodopsin family. Proc Natl Acad Sci USA 97:14263–14267. doi:10.1073/pnas.260349597

Terakita A, Yamashita T, Nimbari N et al (2002) Functional interaction between bovine rhodopsin and G protein transducin. J Biol Chem 277:40–46. doi:10.1074/jbc.M104960200

Terakita A, Koyanagi M, Tsukamoto H et al (2004) Counterion displacement in the molecular evolution of the rhodopsin family. Nat Struct Mol Biol 11:284–9. doi:10.1038/nsmb731

Tsin ATC, Beatty DD (1980) Visual pigments and vitamins A in the adult bullfrog. Exp Eye Res 30:143–153. doi:10.1016/0014-4835(80)90108-6

Tsukamoto H, Farrens DL, Koyanagi M, Terakita A (2009) The magnitude of the light-induced conformational change in different rhodopsins correlates with their ability to activate G proteins. J Biol Chem 284:20676–20683. doi:10.1074/jbc.M109.016212

Tsutsui K, Shichida Y (2010) Multiple functions of Schiff base counterion in rhodopsins. Photochem Photobiol Sci 9:1426. doi:10.1039/c0pp00134a

Tsutsui K, Imai H, Shichida Y (2008) E113 is required for the efficient photoisomerization of the unprotonated chromophore in a UV-absorbing visual pigment. Biochemistry 47:10829–10833. doi:10.1021/bi801377v

Vogel R, Siebert F, Mathias G et al (2003) Deactivation of rhodopsin in the transition from the signaling state meta II to meta III involves a thermal isomerization of the retinal chromophore CN double bond. Biochemistry 42:9863–9874. doi:10.1021/bi034684+

Vought BW, Dukkipatti A, Max M et al (1999) Photochemistry of the primary event in short-wavelength visual opsins at low temperature. Biochemistry 38:11287–11297. doi:10.1021/bi990968b

Wakefield MJ, Anderson M, Chang E et al (2008) Cone visual pigments of monotremes: filling the phylogenetic gap. Vis Neurosci 25:257–264. doi:10.1017/S0952523808080255

Wang Z, Asenjo AB, Oprian DD (1993) Identification of the Cl^--binding site in the human red and green color vision pigments. Biochemistry 32:2125–2130

Yamashita T, Ohuchi H, Tomonari S et al (2010) Opn5 is a UV-sensitive bistable pigment that couples with G_i subtype of G protein. Proc Natl Acad Sci USA 107:22084–22089. doi:10.1073/pnas.1012498107

Yamashita T, Nakamura S, Tsutsui K et al (2013) Chloride-dependent spectral tuning mechanism of L-group cone visual pigments. Biochemistry. doi:10.1021/bi3016058

Yokoyama S (1994) Gene duplications and evolution of the short wavelength-sensitive visual pigments in vertebrates. Mol Biol Evol 11:32–39

Yokoyama S (2000) Molecular evolution of vertebrate visual pigments. Prog Retin Eye Res 19:385–419

Yoshizawa T (1994) Molecular basis for color vision. Biophys Chem 50:17–24

Yoshizawa T, Shichida Y (1982) Low-temperature spectrophotometry of intermediates of rhodopsin. Methods Enzymol 81:333–354

Yoshizawa T, Wald G (1963) Pre-lumirhodopsin and the bleaching of visual pigments. Nature (Lond) 197:1279–1286

Yoshizawa T, Wald G (1967) Photochemistry of iodopsin. Nature (Lond) 214:566–571. doi:10.1038/214566a0

Zimmermann K, Ritter E, Bartl FJ et al (2004) Interaction with transducin depletes metarhodopsin III: a regulated retinal storage in visual signal transduction? J Biol Chem 279:48112–48119. doi:10.1074/jbc.M406856200

Zhukovsky EA, Oprian DD (1989) Effect of carboxylic acid side chains on the absorption maximum of visual pigments. Science 246:928–930

Chapter 2
Phototransduction in Rods and Cones

Satoru Kawamura and Shuji Tachibanaki

Abstract Light is detected by visual pigment, and this detection signal is converted to an electrical signal in the photoreceptor cell. In vertebrates, there are two classes of photoreceptors, rods and cones, and they respond to light by membrane hyperpolarization. The mechanism that produces a hyperpolarizing light response, the phototransduction cascade consisting of a series of enzymatic reactions, is now well understood in rods. Rods and cones function under different light conditions. Rods are highly light sensitive, so that they are functional in very dim light. Cones are less light sensitive and they are functional in daylight. Thanks to these two classes of cells, our vision covers a light intensity range more than 10^8 fold from the darkness under starlight to dazzling brightness at high noon on a tropical island. In addition to the difference in light sensitivity, the time required for recovery of a hyperpolarizing response to a flash of light is much shorter in cones than in rods. This higher time resolution in cones makes it possible to detect an object moving quickly in daylight. In cones, the phototransduction cascade is similar to that in rods. The differences in light sensitivity and time resolution between rods and cones could, therefore, stem from differences in the efficiencies of reactions or the lifetime of an active species in the cascade. This chapter describes the similarities and the differences in the phototransduction cascade between rods and cones studied biochemically in carp (*Cyprinus carpio*).

Keywords Cones • Light sensitivity • Rods • Time resolution

S. Kawamura (✉) • S. Tachibanaki
Graduate School of Frontier Biosciences, Osaka University, Yamada-oka 1-3,
Suita, Osaka 565-0871, Japan
e-mail: kawamura@fbs.osaka-u.ac.jp

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_2,

2.1 Introduction

Studies of the mechanism that generates light responses in vertebrate photoreceptors were initiated in the early 1960s by measuring the light-stimulated membrane voltage changes with an intracellular glass microelectrode in a carp retina. The finding was that photoreceptors responded to light by a membrane hyperpolarization (Tomita 1965). This was a surprising finding because studies in invertebrate photoreceptors had shown that they respond to light with a membrane depolarization. Shortly after the finding of hyperpolarizing light responses in vertebrate photoreceptors, it was found that the hyperpolarization is accompanied by an increase in membrane resistance (Toyoda et al. 1969). From this measurement, it became evident that vertebrate photoreceptors respond to light by closing a cation channel.

The molecule responsible for detecting light is visual pigment, which was first found in the 1850s to 1870s (reviewed by Hubbard 1977). The pigment in rods is called rhodopsin and is present only in the outer segment (OS) portion of a rod (Fig. 2.1a). This pigment bleaches when it absorbs light. Wald and colleagues found that rhodopsin (and afterward visual pigment in cones also) is composed of the chromophore, 11-*cis* retinal, and the protein, opsin (reviewed by Dowling 2000). The actual molecule that absorbs light is 11-*cis* retinal. They also found that 11-*cis* retinal is isomerized to all-*trans* retinal by light and that this isomerization is the first step of detection of light in the photoreceptors.

The mechanism of closure of a cation channel in the plasma membrane of a rod OS was extensively studied mainly biochemically by using membrane preparations of purified rod OS. As a consequence, an enzyme hydrolyzing cGMP, cGMP phosphodiesterase (PDE), was found to be activated in the light (Miki et al. 1973), which suggested a light-induced decrease in the cytoplasmic cGMP concentration in a rod OS. PDE was then found to be activated by a trimeric GTP-binding protein, transducin (Tr), that is activated by bleached rhodopsin (Fung and Stryer 1980). Although most of the key players in the phototransduction cascade were found, the link between hydrolysis of cGMP and the closure of the cation channel only became evident when Fesenko's group found a cGMP-gated cation channel in the plasma membrane of a rod OS in 1985 (Fesenko et al. 1985). With this finding, the key players acting in the initial rising phase of a light response (actually, in the initial hyperpolarizing phase) were fully connected. Details of the phototransduction cascade in rods are described in Sect. 2.3.

After a light stimulus, rod membrane hyperpolarization must be returned to the dark resting level. For this, all the activated players must be inactivated and the cGMP that had been hydrolyzed must be replenished. In addition to the players acting on the rising phase of a response, players acting in the recovery phase, and therefore acting in the inactivation of activated players, are known: these are described in Sect. 2.4.

The investigations that led to the elucidation of the phototransduction cascade were conducted initially in rods because purified rod OS can be obtained rather easily in an amount sufficient for biochemical study. Thanks to this advantage, the rod photoreceptor is one of the best understood cells in the study of signal

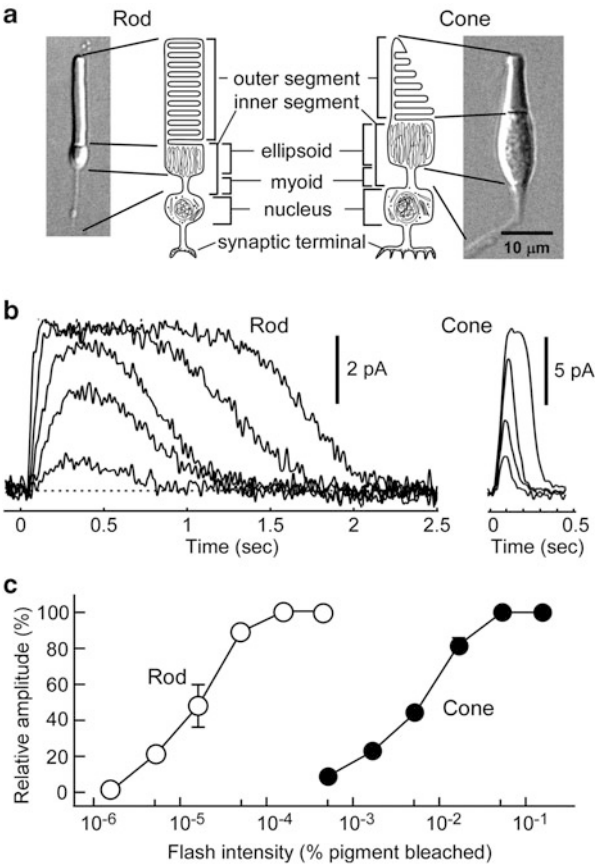

Fig. 2.1 Carp rods and cones, and their responses to flashes of light. (**a**) A mechanically dissociated carp rod (*left*) and a red-sensitive cone (*right*), and their schematic drawings. (**b**) A light response family of a rod (*left*) and that of a red-sensitive cone (*right*). Outer segment membrane currents were recorded with a suction electrode by giving light flashes of various intensities, and the measured current responses are superimposed. Light intensities used in measurement in rods varied from 5.1×10^{-6} % bleach to 5.1×10^{-4} % bleach (corresponding to 8–800 molecules of visual pigment activated per rod; R*/rod), and those in cones varied from 1.6×10^{-3} % bleach to 0.16 % bleach (2,100–210,000 R*/cone). (**c**) Light intensity–response amplitude relations of the responses recorded from a rod (*open circles*) and a red-sensitive cone (*filled circles*) shown in (**b**). (Reproduced with permission from Fig. 1 in Kawamura and Tachibanaki 2008)

transduction both qualitatively and quantitatively. In most vertebrate retinas, there are two types of photoreceptors, rods and cones. It has been known that similar players (cone orthologues) are expressed in cones. Probably a cone orthologue acts slightly differently from a rod orthologue, so such differences may contribute to the differences in light sensitivity, for example, between rods and cones. In this chapter, we first explain the phototransduction cascade in rods, and then the cascade in cones is explained based mainly on our own studies that show the similarities and the differences in the cascade between rods and cones.

2.2 General Features of Rod and Cone Light Responses

Figure 2.1a shows a carp rod (left) and a cone (right) with their schematic drawings, and Fig. 2.1b shows superimposed membrane current responses (shutdown and reflowing of an inward current; see following) to light flashes with ~10-ms duration of various intensities recorded in a carp rod (left) and a carp red cone (right) with a suction electrode (see Sect. 2.3.1.4 for how the response can be measured). The relationship between the relative amplitude of a response and the flash intensity is plotted in Fig. 2.1c. In subsequent paragraphs, we use the term light response to indicate an electrical response evoked by a light stimulus.

From Fig. 2.1b,c, rod and cone light responses are characterized as follows:

1. In both rods and cones, light responses are evoked in a graded manner. The amplitude of a response is a function of flash intensity.
2. The time course is rather slow in rod responses and much more transient in cone responses. This time course difference can be rephrased as the difference in time resolution. Cones respond to the onset and the offset of a light stimulus relatively well, but rods do not, so that time resolution is better in cones than in rods. For this reason, flicker fusion frequency is much higher in daylight where cones operate and it is low in the dark where rods operate.
3. As seen in Fig. 2.1c, cones require much more light to operate than rods. In the case of carp, red cones require about 300 times more light than do rods to evoke a half-maximal response.

The rod–cone differences shown in (2) and (3) are generally observed in any animal species, but quantitatively, the rod–cone differences vary among species.

2.3 Phototransduction Cascade in Rods

The phototransduction cascade is now well understood in rods (Fig. 2.2) (Pugh and Lamb 2000; Kawamura and Tachibanaki 2008; Luo et al. 2008). In short, light is detected in rods by a light-absorbing receptor molecule, rhodopsin. [Light-absorbing receptor molecules are generally called visual pigment, and rhodopsin is a special name for visual pigment in rods. In cones, the pigments are named differently. For this reason, when abbreviated, we call visual pigment, including rhodopsin, as "R" (receptor molecule) in this chapter.] Rhodopsin, when it has not yet absorbed light, contains 11-*cis* retinal in the dark in the inside of a rhodopsin molecule. With the absorption of light, 11-*cis* retinal is isomerized to all-*trans* retinal, and rhodopsin is activated. Activated rhodopsin (R*) activates a heterotrimeric GTP-binding protein, transducin (Tr). Activated Tr (Tr*) activates a hydrolyzing enzyme of cGMP, cGMP phosphodiesterase (PDE). In the plasma membrane of a rod OS, a cGMP-gated cation channel is present. In the dark, cytoplasmic cGMP concentration is high and cGMP binds to the channel to open it to allow an inward current flow. On absorption

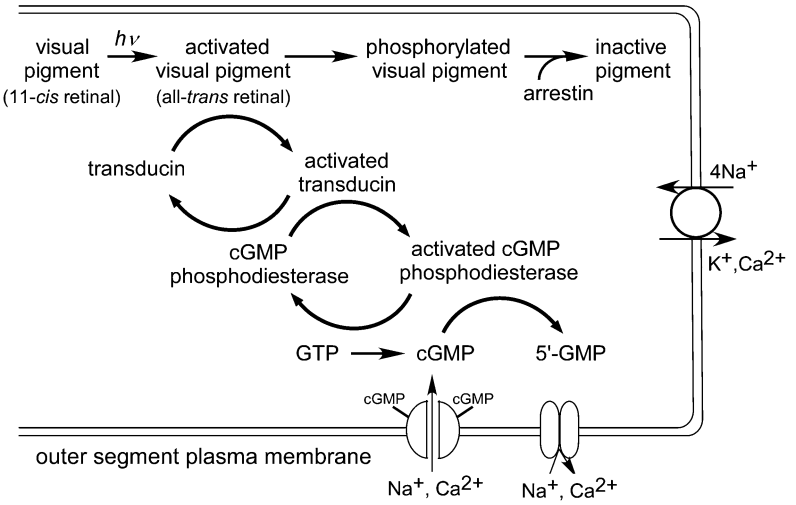

Fig. 2.2 Simplified phototransduction cascade. In the outer segment of a rod and a cone, a cGMP hydrolytic pathway to evoke a light response is present. See details in text. (Reproduced from Fig. 4 in Kawamura and Tachibanaki 2012)

of light by rhodopsin, cGMP is hydrolyzed by activated PDE (PDE*). As a result, cGMP dissociates from the channel to close it. Because of the cessation of the inward current, a rod is hyperpolarized.

Upon termination of the light stimulus, the inward current is restored, and the rods depolarize back to their dark state. To recover to the dark level, all of the activated species, R*, Tr*, and PDE*, must be inactivated and, in addition, cGMP concentration should be restored to its dark level.

In the following paragraphs, the mechanisms that create the active species, R*, Tr*, and PDE*, and the mechanisms that inactivate these molecules as well as the mechanisms that restore cGMP concentration, are summarized. The light response of a rod or cone is a consequence of all these reactions.

2.3.1 Mechanism That Creates the Active Species, R, Tr*, and PDE*, in the Rising Phase of a Light Response*

2.3.1.1 Activation of Rhodopsin

Rhodopsin is a membrane-bound protein with seven α-helices spanning across the membrane. As is known for this family of proteins, it couples with a heterotrimeric GTP-binding protein, which in photoreceptors is known specifically as transducin. Rhodopsin is made up of a chromophore, 11-*cis* retinal, and the protein moiety, opsin. Rhodopsin absorbs green light maximally at around 500 nm in its 11-*cis*

retinal-bound form. For this reason, when rhodopsin is seen in the dark by giving a brief light flash, it is colored in red. Light isomerizes 11-*cis* retinal to all-*trans* retinal in rhodopsin, which is the first step of light detection in rods. As a result of the isomerization of the chromophore, opsin changes its conformation gradually, and finally bleaches to the state where chromophore does not absorb visible light. During this bleaching process, metastable products, called bleaching intermediates, are formed. Among these, a product formed within milliseconds after absorption of light, metarhodopsin II, is believed to be the molecular species physiologically relevant to R*. In metarhodopsin II, the original arrangement of the transmembrane 7α-helices is reorganized, and the helix 6 is rotated and tilted to expose the interacting site of opsin with Tr (Choe et al. 2011; Standfuss et al. 2011).

2.3.1.2 Activation of Transducin (Tr)

R* activates transducin (Tr). Tr is a heterotrimeric GTP-binding protein composed of α-, β-, and γ-subunits ($Tr_{\alpha\beta\gamma}$). The α-subunit (Tr_{α}) is the functional unit of this protein and contains the binding cleft for GTP (and GDP) and also the interaction sites to R* and PDE. The β- and the γ-subunit form a membrane-associated complex ($Tr_{\beta\gamma}$) and this complex is required to form a trimeric complex ($Tr_{\alpha\beta\gamma}$). At the resting state, Tr_{α} retains GDP in its binding cleft. On activation of R, R* binds to the C-terminal of the GDP-bound form of Tr_{α} in the $Tr_{\alpha\beta\gamma}$ complex and probably opens the nucleotide-binding cleft in Tr_{α} (Noel et al. 1993). R* catalyzes the exchange of bound GDP for GTP in Tr. The GTP-bound form of Tr_{α} (Tr_{α}-GTP) is the active form, Tr*. Once GDP is replaced by GTP, Tr_{α}-GTP dissociates from both R* and $Tr_{\beta\gamma}$ and activates PDE. Tr_{α} has GTPase activity intrinsically, so that bound GTP is hydrolyzed to GDP after some period of time (see Sect. 2.3.2.2). The GDP-bound form of Tr_{α} again interacts with R* for replacement of GDP by GTP. This activation cycle of Tr_{α} continues as long as R* is present, so that a single R* can activate many Tr molecules. This Tr_{α} activation step is one of the reactions that amplify the photon capture signal in rods. In the GTP-bound form of Tr_{α}, a conformational change takes place on the surface of Tr_{α}, so that the effector molecule, the γ-subunit of PDE, can interact with Tr_{α}-GTP.

2.3.1.3 Activation of cGMP Phosphodiesterase (PDE)

PDE is a heterotetrameric complex composed of one α-, one β-, and two γ-subunits ($PDE_{\alpha\beta2\gamma}$). The α- (PDE_{α}) and the β- (PDE_{β}) subunits are the catalytic subunits responsible for hydrolysis of cGMP, and the γ-subunit (PDE_{γ}) is the inhibitory subunit. Each of the PDE_{γ} binds to either PDE_{α} or PDE_{β}. PDE with two γ-subunits bound is the most inactive form of PDE. At the resting state, a C-terminal region of PDE_{γ} binds to PDE_{α} or PDE_{β} to inhibit their catalytic activities. Tr_{α}-GTP binds to this PDE_{γ} C-terminal region and sequesters it into a binding pocket in Tr_{α}-GTP to release the PDE_{γ} inhibitory constraint on PDE_{α} or PDE_{β} (Slep et al. 2001).

For simplicity, we do not refer to each subunit in the following sections, and indicate the inactive form of PDE as PDE and the active form as PDE*. We refer to the GTP-bound form of T_α, Tr_α-GTP, as Tr*.

Tr* activates PDE by relieving the inhibitory constraint on PDE_α or PDE_β. PDE* then hydrolyzes cGMP, an intracellular second messenger in the rod OS. Rod PDE* is a highly effective at hydrolyzing cGMP. Each of the catalytic subunits can hydrolyze 1,000 cGMP molecules per second (Leskov et al. 2000).

2.3.1.4 Closure of the cGMP-Gated Cation Channel

In the plasma membrane of a rod OS, a cGMP-gated cation channel is present (Fesenko et al. 1985). This is the first channel to be identified among the class of cyclic nucleotide-gated channels. The channel is a hetero-tetramer composed of three CNGA1 subunits and one CNGB1 subunit, each of which binds one cGMP molecule (Zhong et al. 2002).

In the dark, the cytoplasmic cGMP concentration ($[cGMP]_{cyt}$) is less than 10 μM, and the Michaelis constant (K_m) of the channel to cGMP is around 50 μM. Taking the cooperative binding of cGMP to the channel into consideration, it has been estimated that the fraction of the channel opened is only 1 % or so in the dark (Yau and Nakatani 1985). In the light, cGMP is hydrolyzed by PDE*. With this decrease in $[cGMP]_{cyt}$, cGMP bound to the channel dissociates from the channel to result in a closure of the channel. Because the inward current flowing into the rod OS in the dark is blocked, a rod is hyperpolarized in the light. The upward deflection of light responses shown in Fig. 2.1b results from cessation of the inward current, and this current was measured with an electrode into which the OS portion of a rod was tightly sucked (suction electrode).

The cGMP-gated channel allows permeation of cations including Ca^{2+}. In the dark where the channel is open, Ca^{2+} flows into the rod OS. In the light where the channel is closed, the flow of Ca^{2+} into a rod OS is blocked. In the plasma membrane of a rod OS, an Na^+/K^+, Ca^{2+} exchanger is present to extrude Ca^{2+} that enters in the dark (Yau and Nakatani 1984). Because this exchanger operates even in the light, the Ca^{2+} concentration in a rod OS decreases to about 10 nM under intense light (Gray-Keller and Detwiler 1994). This Ca^{2+} decrease is the key event for the photoreceptor light adaptation (see Chap. 4).

2.3.2 Mechanism of the Recovery of a Light Response

The foregoing reactions explain the mechanism of the rising phase of a light response (in reality, it is a fall of the inward current maintained in the dark). All the activated species, R*, Tr*, and PDE*, must be inactivated when the light stimulus ceases.

2.3.2.1 Inactivation of R*

Inactivation of R* is attained by two steps of reactions: first, by phosphorylation of R* with a visual pigment kinase, G protein-coupled receptor kinase 1 (GRK1), and second, by binding of arrestin (Arr) to phosphorylated R*.

GRK1 specifically phosphorylates R* to terminate the activation of Tr by R*. GRK1 is inactive in its resting state, and on interaction with R*, GRK1 becomes activated to phosphorylate R*. In bovine rhodopsin, there are seven serine and threonine residues in the C-terminal region, and these are potential phosphorylation sites. In biochemical studies, more than five phosphates are reported to be incorporated into this region, but under physiological situations, fewer than four phosphates seem to be incorporated at the maximum (Kennedy et al. 2001).

Phosphorylation of R* greatly reduces the ability of R* to activate Tr. However, its reduction is not perfect, and Arr binds to the phosphorylated R* to almost completely shut down the activity of R*. After stimulation of rhodopsin by light, all-*trans* retinal dissociates from opsin, and chromophore-free opsin is formed. The chromophore-free form of opsin activates Tr, although the activity is quite low (Cornwall and Fain 1994). However, after a huge amount of rhodopsin has been bleached, enough of this form of opsin is present to result in significant activation of Tr. The details of whether phosphates and/or Arr have been removed at this stage are not known. In any event, it is believed that Tr activation is completely terminated at the time when all the bleached pigment has been fully regenerated by binding of 11-*cis* retinal to opsin.

The activity of GRK1 is regulated by a Ca^{2+}-binding protein, S-modulin (Kawamura and Murakami 1991) or recoverin (Dizhoor et al. 1991). As already shown (Sect. 2.3.1.4), Ca^{2+} permeates the cGMP-gated channel. As a consequence of the closure of the channel in the light, cytoplasmic Ca^{2+} concentration decreases during light response. At an early phase of the rise of a light response, Ca^{2+} concentration is close to that in the dark and rather high in a rod OS. Under this condition, S-modulin/recoverin inhibits the GRK1 activity to extend the lifetime of R* to prolong Tr* activation (Kawamura 1993). When Ca^{2+} concentration decreases at a later phase of a light response, GRK1 is disinhibited to phosphorylate R* effectively to inactivate R*.

2.3.2.2 Inactivation of Tr*

Tr* (actually Tr_{α}*-GTP) hydrolyzes its bound GTP with an intrinsic GTPase activity, so that Tr* is inactivated by itself; that is why the inactive form of Tr contains bound GDP. The lifetime of Tr*, therefore, is determined by how long it takes to hydrolyze GTP. In the heterotrimeric G-protein system, a protein that facilitates GTP hydrolysis is known to be present. Generally it is called a GTPase-accelerating protein (GAP), and in the case of rods, it is RGS9-1 (He et al. 1998). This protein forms a complex with a protein called Gβ5, and the

complex is anchored to the membranes through another protein, R9AP. Although RGS9-1 facilitates the hydrolysis of GTP in Tr*, inactivation of Tr*, that is, the hydrolysis of GTP, is the rate-limiting step to terminate a light response in the phototransduction cascade in rods (Krispel et al. 2006).

2.3.2.3 Inactivation of PDE*

PDE is activated by the GTP-bound form of Tr, Tr*, that releases the inhibition by PDE_γ directed to the catalytic subunit, either PDE_α or PDE_β. With hydrolysis of GTP in Tr*, the C-terminal of PDE_γ sequestered in a binding pocket in Tr_α* (Slep et al. 2001) is released from the pocket, and thereby binds to the catalytic subunit to inhibit the cGMP-hydrolyzing activity of PDE_α or PDE_β. Inactivation of PDE seems to be instantaneous after hydrolysis of GTP in Tr_α*, because the time courses of GTP hydrolysis and PDE inactivation matched well (Angleson and Wensel 1993).

2.3.2.4 Restoration of cGMP Concentration

The rise of a light response is caused by hydrolysis of cGMP. For the recovery of a response, cGMP concentration must be restored to its dark level. The enzyme responsible for the synthesis of cGMP is guanylate cyclase (GC). This protein is membrane bound and its activity is regulated by a Ca^{2+}-binding protein, GCAP (guanylate cyclase-activating protein) (Palczewski et al. 1994). As shown already, Ca^{2+} concentration in a rod OS decreases during a light response. GCAP detects this decreasing Ca^{2+} concentration and activates GC to accelerate cGMP synthesis and, therefore, facilitates the recovery of a response to the dark level.

2.3.3 Other Reactions Necessary to Recover to the Dark State

The foregoing recovery is attained by terminating the activation reaction: R* is inactivated by phosphorylation and Arr binding; Tr* is inactivated by GTP hydrolysis; PDE* is inactivated by binding of PDE_γ to $PDE_{\alpha\beta(\gamma)}$ after hydrolysis of GTP in Tr*; cGMP concentration, once lowered in the light, returns to the dark level with its synthesis by GC. The Ca^{2+} concentration in a rod OS once lowered in the light goes back to the dark level by reopening of the cGMP-gated channel during the recovery of a response. However, as for rhodopsin, there are at least two reactions that should occur for a rod to recover fully to the dark state.

When a rod is stimulated briefly with a weak light flash, a light response is generated and recovered, as just shown. In this case, rhodopsin usage is negligible: in a case of a rod, a half-saturating amplitude of a light response is evoked with bleach of less than 100 molecules of rhodopsin among more than 10^7 rhodopsin molecules in a rod OS. However, when a rod is stimulated with a strong light for a

long time, bleached pigment accumulates. It is noteworthy that bleached pigment does not regenerate by itself, and for this reason, rhodopsin runs out under continuous bright light unless it is regenerated by some mechanism. For regeneration, opsin should bind to 11-*cis* retinal. In rods, all-*trans* retinal does not convert to 11-*cis* retinal. Instead, all-*trans* retinal is reduced to its alcohol, all-*trans* retinol, which cannot be used for regeneration of rhodopsin with this form. Therefore, in the cells other than photoreceptors, mechanisms converting all-*trans* retinol to 11-*cis* retinal or 11-*cis* retinol are present, and these retinoids are supplied to rods (and cones) to regenerate visual pigment. These mechanisms are called the visual cycle or retinoid cycle. One mechanism that supplies 11-*cis* retinal to both rods and cones is present in the pigment epithelium. The other mechanism is present in Müller cells and supplies 11-*cis* retinol, not 11-*cis* retinal, to cones specifically. This topic is discussed in more detail in Chap. 3.

The other necessary reaction is the dephosphorylation of rhodopsin. The phosphates incorporated into R* should be removed by the phosphatase activity for rhodopsin to recover to the dark state. Currently, dephosphorylation of rhodopsin or opsin is not understood well. It is certain that the phosphates are removed, but it is not known at what stage it takes place, before dissociation of all-*trans* retinal from opsin or after that, before binding to 11-*cis* retinal or after that, or independently of these reactions.

2.4 Differences in the Activation Steps in the Phototransduction Cascade between Carp Rods and Cones

As already described (Sect. 2.2), rods and cones function under different light conditions and show different response characteristics. From the findings that cone orthologues of the rod players in the cascade are present in cones, the phototransduction cascade in cones is believed to be similar to that in rods. Therefore, the differences in the rod and cone light responses are probably the result of the differences in the biochemical characteristics of the reactions in the cascade and/or expression levels of the proteins in the cascade. We planned to study these issues biochemically many years ago, and for this purpose, we first needed to obtain purified cones.

In our past experience, we knew that in carp, mechanically dissociated cones sediment more easily than rods when they are kept in a test tube. Based on this finding, we tried to purify carp cones by density gradient centrifugation. Fortunately, by using a stepwise Percoll density gradient, we could purify carp rods at the 45/60 % (wt/vol) interface and cones at the 75/90 % (wt/vol) interface (Tachibanaki et al. 2001) (Fig. 2.3). The cone fraction contained both single and double cones with three kinds of visual pigments. The ratio of the three kinds of pigments was 3 (red) to ~1 (green) to ~1 (blue), which was determined spectrophotometrically.

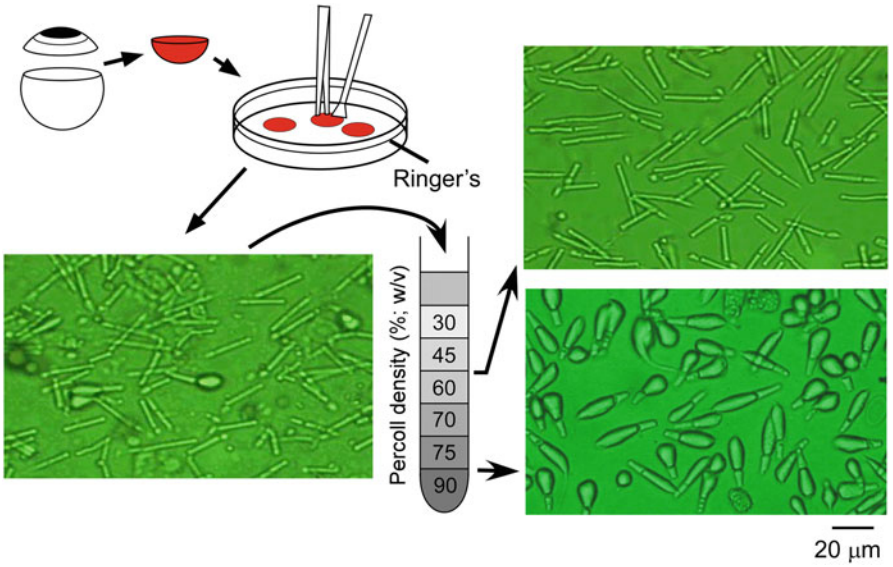

Fig. 2.3 Purification of carp rods and cones. Mechanically dissociated carp rods and cones were layered at the top of a stepwise Percoll density gradient. After centrifugation, rods are found at the 45/60 % interface and cones are found at the 70/90 % interface. [Modified from Fig. 1 in Tachibanaki et al. (2001). Copyright (2001) National Academy of Sciences USA]

Carp retina contains UV-sensitive cones, but their content is low and could not be measured with our spectrophotometry. Contamination of cones in a purified rod fraction and that of rods in purified cone fraction were both less than 1 %. Using these purified preparations of carp rods and cones, we examined similarities and differences in the phototransduction cascade between rods and cones. To do this, we quantified the number of molecules in the phototransduction cascade to estimate their concentrations and compared the activity of each of the reactions in the cascade (Table 2.1).

2.4.1 Differences in Visual Pigment

1. Content of visual pigment
   Expression levels of visual pigments in the outer segments (OS) are determined in many animal species by microspectrophotometric measurements (Harosi 1975). From measured absorption intensities of a pigment in the OS and the OS dimensions, it is known that the pigment density is ~3 mM in all animal species in both rods and cones. In this estimation, the structure of the OS, that is, stacks of disks in a rod OS or a lamellar structure of the plasma membranes in a cone OS (Fig. 2.1a), is not taken into consideration, and only the outer

Table 2.1 Concentrations and activities of phototransduction proteins in carp rods and cones[a]

Photoreceptor	Molecule		Concentration in outer segment	Activity
Rod	Visual pigment		3 mM[b]	143 transducin-activated/bleached pigment/s[c]
	Transducin		0.28 mM[c]	1 PDE molecule activated/transducin[e]
	RGS9		3.3 μM[c]	3.2 GTP hydrolyzed/Tr*·RGS9-s (0.045 GTP hydrolyzed/Tr*-s)[c]
	cGMP phosphodiesterase		11 μM (in frog)[d]	15 cGMP hydrolyzed/pigment-present/s[e]
	Visual pigment kinase (GRK1)		12 μM[f]	1.5 phosphorylations/GRK1/s[f]
	S-modulin/recoverin		53 μM[g] (as a cytoplasmic concentration)	Maximum inhibition to 40 % of GRK1 activity[g]
	Guanylate cyclase	GC-R1	3.9 μM[h]	0.79 cGMP formed/GC-R1/s[h]
		GC-R2	0.3 μM[h]	
	GCAP	GCAP1	0.8 μM[h]	11.8 cGMP formed/[GC-R1·GCAP1]/s[h]
		GCAP2	1.8 μM[h]	6.8 cGMP formed/[GC-R1·GCAP2]/s[h]
Cone	Visual pigment		3 mM[b]	30 transducin-activated/bleached pigment/s[c]
	Transducin		0.28 mM[e]	1 PDE molecule activated/transducin[e]
	RGS9		75 μM[c]	
	cGMP phosphodiesterase		(Similar to that in carp rods)[e]	15 cGMP hydrolyzed/pigment-present/s[e]
	Visual pigment kinase (GRK7)		120 μM[f]	14 phosphorylations/GRK7/s[f]
	S-modulin/recoverin (Visinin)		1.2 mM[g] (as a cytoplasmic concentration)	Maximum inhibition to 20 % of GRK7 activity[g]
	Guanylate cyclase	GC-C	72 μM[h]	1.7 cGMP formed/GC-C/s[h]
	GCAP	GCAP3	33 μM[h]	5.7 cGMP formed/[GC-C·GCAP3]/s[h]

[a]Modified from Table 1 in Kawamura and Tachibanaki (2012); [b]Harosi (1975); [c]Tachibanaki et al. (2012); [d]Dumke et al. (1994); [e]Koshitani et al. (2014); [f]Tachibanaki et al. (2005); [g]Arinobu et al. (2010); [h]Takemoto et al. (2009)

dimensions of the OS are considered. It is believed that half of the rod OS volume is occupied by disk membranes and that the cytoplasm volume in a rod OS is approximately half of the OS total volume estimated from the outer dimensions of a rod OS. Similarly, in a cone OS, the cytoplasmic volume is believed to be about half of the volume estimated from the outer dimensions of a cone OS.

2. Activation of visual pigment
   The light sensitivity of a pigment was compared between rod and cone visual pigments (Okano et al. 1992). Using chicken rhodopsin and chicken cone pigment, it was shown that the light sensitivity of chicken red cone pigment is very similar to that of chicken and bovine rhodopsin. Because the pigment concentration is similar among photoreceptors in general, as already stated, the probability of quantum catch by a photoreceptor is approximately proportional to the OS volume.

2.4.2 Differences in Transducin

1. Content of transducin
   Quantification of transducin (Tr) can be made by quantifying the amount of GTP bound to Tr, because the binding stoichiometry of GTP to Tr is 1:1. However, because GTP is hydrolyzed by an intrinsic GTPase activity of Tr, it is difficult to quantify the bound GTP. To solve this problem, a nonhydrolyzable analogue of GTP, GTPγS, is frequently used, and its light-dependent binding to rod or cone membranes is quantified. For the quantification, the isotope-labeled form of GTPγS, ^{35}[S]GTPγS, is usually used. This GTP analogue binds to transducin firmly, so that it can be quantified with a filter-binding assay in which GTPγS-bound form of transducin is trapped on a filter membrane. Because the binding is not covalent, transducin should not be denatured; otherwise, GTPγS is liberated from Tr.

   In carp rod membranes, the content of transducin was approximately about 1/10 of rhodopsin (0.094 molecules of transducin per rhodopsin present; 0.094 Tr/R) (Tachibanaki et al. 2012), similar to other animal species. Quantification of transducin in cone membranes was slightly difficult because a significant portion of GTPγS bound to cone membranes in the dark: in rod membranes, the binding in the dark is negligible. However, our recent quantification showed that the content of cone transducin is similar to that of rod transducin (Koshitani et al., 2014).
2. Activation of transducin
   Binding time course of GTPγS was measured in carp rod membranes (Tachibanaki et al. 2012), and the rate of GTPγS-binding was 143 transducin molecules activated per R* per second (143 Tr*/R*-s) (Fig. 2.4). For the measurement of GTPγS-binding in cone membranes, we utilized a rapid quench apparatus to terminate the reaction in a subsecond range, because GTPγS

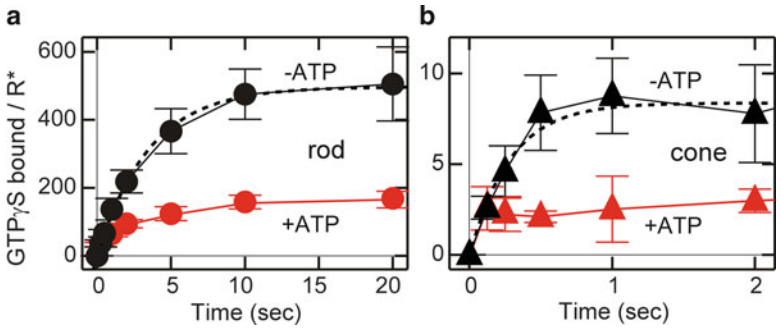

Fig. 2.4 Time course of transducin activation in rod and cone membranes. (**a**) Light-dependent GTPγS binding in rod membranes in absence (*filled black circles*) and presence (*filled red circles*) of ATP. *Vertical axis* shows number of GTPγS molecules bound per visual pigment bleached. (**b**) Light-dependent GTPγS binding in cone membranes in absence (*filled black triangles*) and presence (*filled red triangles*) of ATP. [This research was originally published in *Journal of Biological Chemistry* (Tachibanaki et al. 2012). © The American Society for Biochemistry and Molecular Biology]

binding, including other reactions in the phototransduction cascade in cone membranes also (see below), ceases very quickly after a light stimulus. In our measurements with cone membranes, the rate of transducin activation was 30 Tr*/R*-s. Thus, transducin activation in cone membranes is about fivefold less efficient than in rods, which probably reduces the light sensitivity of a cone by a factor of 5 compared to a rod. In the presence of visual pigment phosphorylation, Tr activation in cones seems to be completed in our earliest time point (125 ms; Fig. 2.4b).

2.4.3 Differences in cGMP Phosphodiesterase (PDE)

1. Content of PDE
   Rod PDE is composed of two heterologous catalytic subunits (PDE_{α} and PDE_{β}) and two identical inhibitory subunits (PDE_{γ}). In contrast, cone PDE comprises two identical catalytic subunits (PDE_{α}) and two identical inhibitory subunits (PDE_{γ}).

   The content of PDE in rods has been estimated by amino acid analysis and reported to be 1/270 of that of rhodopsin (Dumke et al. 1994). Bovine cone PDE has been purified and its specific activity found to be similar to that of bovine rod PDE (Gillespie and Beavo 1988). In our carp cone membranes, full PDE activity normalized by the visual pigment present was very similar between rod and cone membranes, which suggested that the content of PDE is comparable in carp rods and cones (Koshitani et al. 2014).
2. Activation of PDE
   Activation of PDE is attained by removal of the inhibitory constraint of PDE_{γ} on $PDE_{\alpha\beta}$ in rods, and a similar mechanism seems to be present in cones. Binding of

Tr_{α}-GTP to PDE_{γ} is 1:1 in rods. In our recent study, we compared the effectiveness of PDE activation by Tr* between carp rods and cones by measuring PDE activities in the presence of known concentration of stably active form of Tr_{α}, Tr_{α}-GTPγS. The dependence of PDE activation by Tr_{α}-GTPγS was very similar between rods and cones: PDE was activated by a similar dose of Tr_{α}-GTPγS in both rod and cone membranes (Koshitani et al., 2014). Because the expression level of PDE is similar between rods and cones, as just mentioned, the similarity of PDE activation by Tr_{α}-GTPγS indicates that the number of PDE molecules activated by a unit number of Tr_{α}-GTPγS is similar between rods and cones. In other words, PDE activation by Tr* is very similar between carp rods and cones.

2.4.4 *Differences in cGMP-Gated Cation Channel*

1. Activation of the channel by cGMP
   The rod cGMP-gated channel is composed of three CNGA1 subunits and one CNGB1 subunit (Zhong et al. 2002), whereas the cone channel is composed of two CNGA3 subunits and two CNGB3 subunits (Peng et al. 2004). The K_m value and the Hill coefficient of the cGMP binding to the channel are similar between rods and cones (Yau and Nakatani 1984; Picones and Korenbrot 1994).
2. Density of the channel
   Although the surface membrane area is estimated to be tenfold larger in catfish cones than in salamander rods, the total number of the cGMP-gated channel was calculated to be comparable between these two photoreceptors (Haynes and Yau 1990). This finding is consistent with the observation that the current flowing into the OS in the dark is similar between these two cells. The result indicates that the density of the channel is lower in cones.

2.4.5 *Summary of the Differences in the Activation Steps in the Phototransduction Cascade Between Rods and Cones*

As summarized, in carp, the contents of Tr and PDE expressed in the unit of per visual pigment present are similar between rods and cones. Visual pigment concentrations are generally similar between rods and cones, which indicates that Tr and PDE concentrations are also similar between rods and cones in carp.

The only difference in the reactions in the rising phase of a light response was the step of Tr activation by R* in carp: activation of transducin was fivefold less efficient in cones than in rods. In previous studies made in other laboratories using other animal species, rod pigment was expressed in cones or cone pigment was expressed in rods in *Xenopus* (Kefalov et al. 2003). In this study, identical light responses were recorded as those recorded from the wild type, which indicates that

the amplification at the step from R* to Tr is very similar no matter whether the pigment or transducin is a rod type or a cone type. However, in mice, fourfold less efficient signal amplification was observed in a rod expressing a mouse cone pigment (Sakurai et al. 2007). When rod transducin was expressed in cones or cone transducin was expressed in rods in mice (Deng et al. 2009), light responses obtained in these animals were comparable with those found in the wild type. However, in other study in mice, twofold lower signal amplification was observed when cone transducin was expressed in rods (Chen et al. 2010). In wild-type mice, the amplification seems to be several times lower in cones than in rods (Nikonov et al. 2006). Although the responsible reactions were not measured in these studies, it can be generally concluded that the signal amplification is only several times lower in cones than in rods at most in both carp and mice.

2.5 Differences in the Inactivation Reactions in the Cascade Between Carp Rods and Cones

2.5.1 Differences in Inactivation of Activated Visual Pigment

Inactivation of activated visual pigment (R*) is attained first by phosphorylation on R* by a visual pigment kinase (GRK) and second by binding of Arr. In many animal species, rod and cone types of GRK and Arr are expressed differentially in rods and cones, but this rule is not simply applied to all animals (Shimauchi-Matsukawa et al. 2005). For example, in mice, a rod type of GRK (GRK1) is expressed in both rods and cones, whereas in humans, the rod-type GRK1 is expressed in rods but both GRK1 and a cone-type GRK (GRK7) are expressed in cones (Weiss et al. 2001). In carp, GRK1 in rods and GRK7 in cones are expressed (Table 2.1).

In purified carp rods and cones, we measured a time course of R* phosphorylation (Fig. 2.5) (Tachibanaki et al. 2005). R* phosphorylation was enormously faster in cones than in rods. In the measurement shown, the rate of phosphorylation was approximately five phosphates incorporated into a single molecule of R* per second (5 Pi/R*-s) in cones and approximately 0.1 Pi/R*-s in rods: the rate was 50 times faster in cones than in rods. The steady level of the phosphorylation per R* was close to 3 in both rods and cones. The rate in cones (5 Pi/R*-s), therefore, suggests that each molecule of R* in cones is fully phosphorylated in less than 1 s. How many phosphates need to be incorporated into opsin for termination of a light response? We compared the time courses of pigment phosphorylation in cone membranes and a light response in a cone cell. During this study, we knew that the rate of phosphorylation is a function of light intensity, probably because the expression level of GRK is much lower than that of R even in cones (see following; Table 2.1). For this reason, when one wants to compare the phosphorylation and light response directly, it is necessary to use a similar intensity of light in the two types of measurements.

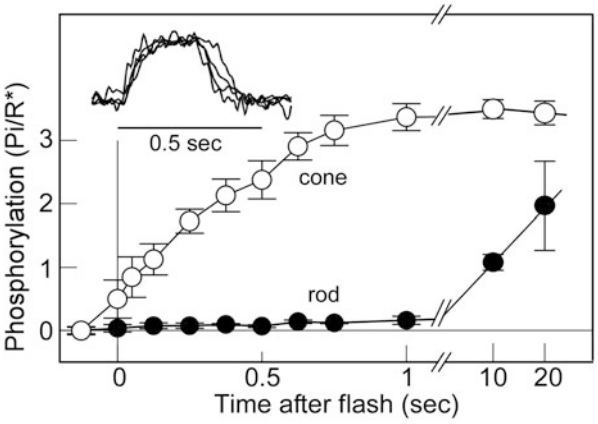

Fig. 2.5 Time course of visual pigment phosphorylation in rod and cone membranes. Visual pigment phosphorylation time course was measured in rod (*filled circles*) and cone (*open circles*) membranes. A light flash was given at time 0, and the amount of phosphate incorporated into a bleached visual pigment molecule (*Pi*/*R**) was measured at the time indicated. *Inset* is a collection of four cone membrane current responses recorded from four different cells by giving a light flash of which intensity was similar to that used for the measurement of the pigment phosphorylation in cone membranes. [Modified from Fig. 1 in Tachibanaki et al. (2005). Copyright (2005) National Academy of Sciences USA]

The R* phosphorylation time course was compared with a light response evoked by a light flash of similar intensity used for the measurement of the phosphorylation time course (Fig. 2.5, inset). It is seen that when two phosphates were incorporated the response almost recovered to the dark level. This result indicates that R* inactivation is complete when two phosphates as an average were incorporated into R*. This kind of comparison was only possible in cones, because light response measurements and biochemical phosphorylation measurements can be made at a similar light intensity in cones but not in rods. Because rods are highly light sensitive in evoking a light response, the light intensity needed for the phosphorylation measurement is too high to record a light response stably for more than 30 s.

This faster R* phosphorylation in cones is the result of much higher GRK activity in cones (Table 2.1): the expression level determined in a unit of per visual pigment present is 10 times higher in cone GRK7 than rod GRK1. Furthermore, the phosphorylation activity of a single molecule of GRK7 is 10 times higher in GRK7 than in GRK1. As a whole, GRK activity in cones is 100 times higher in cones than in rods in carp. It should be mentioned that this difference is based on the unit area of rod and cone membranes because the difference is expressed in a unit of per visual pigment present of which concentration is very similar between rods and cones.

How many Tr molecules are activated per R* during its lifetime in cones? In the presence of ATP, we measured the Tr activation and found that Tr* activation completed within 125 ms after a light flash in cone membranes (Fig. 2.4b) (Tachibanaki et al. 2012). By this time point, R* inactivation seems to be completed. Because the rate of transducin activation was found to be 30 Tr*/R*-s in cone membranes (Sect. 2.4.2), it can be calculated that fewer than 4 Tr* molecules (30×0.125) are formed during the lifetime of R* in cone membranes in the presence of R* phosphorylation.

As shown in Sect. 2.3.2.1, GRK is regulated by S-modulin/recoverin in a Ca^{2+}-dependent manner (Kawamura 1993). S-modulin/recoverin in rods and its cone orthologue, visinin, were interchangeable (Arinobu et al. 2010). However, the expression level of visinin was about 20 times higher than S-modulin/recoverin (Table 2.1), and as a result of this higher expression level of visinin, the effect of these Ca^{2+}-binding proteins is expected to be larger in cones than in rods.

2.5.2 *Differences in Inactivation of Tr**

Transducin is activated by binding of GTP, and activated transducin (Tr*) is inactivated by hydrolysis of bound GTP with its intrinsic GTPase activity. The rate of GTP hydrolysis is therefore the rate of inactivation of Tr. The rate was measured in carp rod and cone membranes (Tachibanaki et al. 2012), and it was found to be 0.082 GTP molecules hydrolyzed per Tr* per second (0.082 GTP/Tr*-s) in rods. The rate was 2.1 GTP/Tr*-s in cones in our previous estimation (Tachibanaki et al. 2012), but based on our recent quantification of Tr in cones (Koshitani et al., 2014), we believe that the rate could be close to 1 GTP/Tr*-s in cones. Even after this correction, the rate of Tr* inactivation is more than 10 times (1/0.082) higher in cones than in rods in carp retina.

For inactivation of a trimeric GTP-binding protein including transducin, a GAP protein that activates hydrolysis of GTP is known to be present. The higher GTP hydrolysis in cones shown above is probably the result of a higher expression level of RGS9-1: it is more than 20 times higher in cones than in rods in carp (Tachibanaki et al. 2012).

2.5.3 *Differences in Inactivation of PDE*

Inactivation of PDE in cones was measured in purified cone membranes using a pH assay method (Tachibanaki et al. 2001). PDE inactivation was generally faster in cones. However, the rate of inactivation was a function of the flash intensity used: the higher the intensity, the lower the rate. For this reason, it was hard to describe the difference quantitatively. In the measurement of PDE activity with the pH assay method, it is necessary to use light stimulus of the intensity much higher than that

used for eliciting light responses, and in this case, a significant amount of R* and/or Tr* still remains activated even after the time close to their "lifetimes," which may be why quantitative measurements of PDE inactivation are difficult. In rods, the time courses of GTP hydrolysis (Tr* inactivation) and PDE inactivation are similar (Angleson and Wensel 1993). If we can assume that the same situation can be applied in cones, PDE inactivation could be more than tenfold faster in cones than in rods as the rate of Tr* inactivation is higher by more than ten times in cones (see above).

2.5.4 *Differences in Restoration of cGMP Concentration*

In carp rods and cones, different sets of guanylate cyclase (GC) and GCAPs are expressed: in rods, GC-R1 and GC-R2, and GCAP1 and GCAP2 are expressed, whereas GC-C and GCAP3 are expressed in cones (Takemoto et al. 2009) (Table 2.1). The expression levels of GCAPs were about half that of GCs in both rods and cones. When GC activities were measured in washed rod and cone membranes, the activity was 36 times higher in cone membranes than in rod membranes. Because the activity expressed in the unit of per visual pigment present was insensitive to Ca^{2+}, GCAPs seemed to have been washed out during preparation of membranes in this study. Thus, this result showed that the basal GC activity in a unit volume is 36 times higher in cones than in rods.

GC was activated by GCAPs at low Ca^{2+} concentrations. Based on the GC activities measured in the presence of GCAPs, the expression levels of GC(s) and GCAP(s), and the K_m of each pair of GC and GCAP, we estimated GC activities in intact cells in the dark and in the light, and in rods and cones (Fig. 2.6a). As shown already (Sect. 2.3.1.4), under bright light, Ca^{2+} concentrations decrease to about 10 nM in rods. At low Ca^{2+} concentrations in our estimation, the activity was 22 μM cGMP synthesized/second (cGMP/s) in rods and 250 μM cGMP/s in cones. This higher activity in cone membranes results from the approximately 20-fold higher expression level of GC-C and GCAP3 in cones than that of GC-Rs and GCAP1 plus GCAP2 in rods (Table 2.1).

The foregoing estimation was based on our biochemical measurement of GC activity in the presence of physiological concentrations of GCAPs at low and high Ca^{2+} concentrations. Similar cGMP synthetic activities at low Ca^{2+} concentrations were obtained in our electrophysiological estimation of the GC activity in pseudo in situ conditions: truncated rod and cone outer segments were perfused with GTP, and the concentration of synthesized cGMP from GTP was estimated by measuring the current flowing through the cGMP-gated channel (Fig. 2.6b) (Takemoto et al. 2009). The GC activities obtained in these measurements were comparable with those estimated biochemically in the presence of GCAPs: 29 μM cGMP/s in rods and 140–300 μM cGMP/s in cones at low Ca^{2+} concentrations.

GC activities could be estimated from light responses shown in Fig. 2.1b. Assuming that only 1 % of the cGMP-gated channel is open in the dark (Yau and

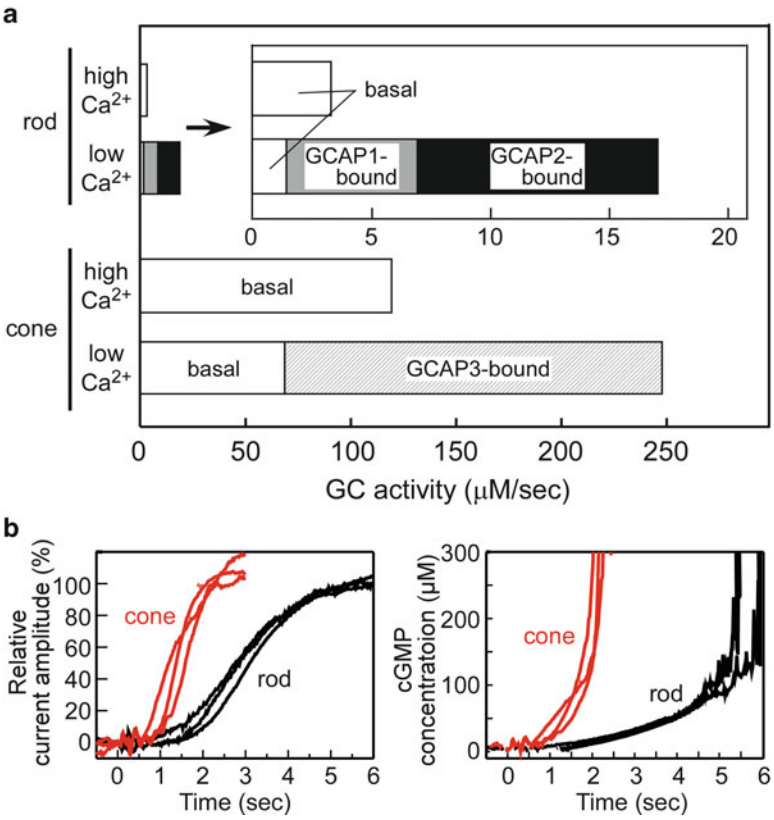

Fig. 2.6 Guanylate cyclase (*GC*) activity in carp rods and cones. (**a**) In situ GC activity was estimated from the expression levels of GCs and guanylate cyclase-activating protein (*GCAP*), the determined specific activity of GCAP-free and GCAP-bound form of a single GC molecule, and determined Michaelis constant in each pair of GC and GCAP in rods and cones. Results in rods are shown as an *inset* with an expanded activity scale. (**b**) *Left*: Time courses of generation of currents induced by perfusion of 1 mM GTP in three independent truncated outer segment (OS) preparations of a frog rod (*black traces*) and a carp red cone (*red traces*). *Right*: Calculated time courses of rise of mean intracellular cGMP concentrations in preparations shown on the *left*. [Modified from Fig. 4 in Takemoto et al. (2009). Copyright (2009) National Academy of Sciences USA]

Nakatani 1985), and the K_m value and the Hill coefficient of the channel being 30 μM and 2.5 in rods and 60 μM and 2.3 in cones (see Takemoto et al. 2009), we determined the GC activities to be 5.5 μM cGMP/s in rods and 90 μM cGMP/s in cones from the light responses shown in Fig. 2.1b. These activities estimated from light responses are broadly in agreement with those obtained biochemically or electrophysiologically in truncated preparations as described.

2.5.5 *Summary of the Differences in the Inactivation Reactions in the Cascade Between Rods and Cones in Carp*

In general, inactivation of the activated players in the cascade such as R*, Tr*, and PDE* is much faster in cones than in rods. Although the effect of Arr has not yet been studied, R* inactivation is 100 times faster, and Tr* inactivation (and therefore, PDE* inactivation, too) is more than 10 times faster in cones. All these reactions tend to shorten the time to terminate the hydrolysis of cGMP. In addition, replenishment of cGMP is more than 10 times faster in cones. As a consequence of these much more effective reactions, a cone light response peaks much earlier than a rod response. This earlier time-to-peak makes the peak amplitude of a light response small and thereby brings about low light sensitivity. This small amplitude together with lower amplification of the photon capture signal is the major determinant of the lower light sensitivity of cones compared to rods.

The more effective inactivation of the activated players and more effective replenishment of cGMP in cones than in rods are caused by the higher expression levels of the molecules (GRK7, RGS9-1, and GC-C/GCAP3) responsible for the recovery of a light response in cones.

Differences in light response recovery between rods and cones also have been studied in zebrafish and mice. When GRK1 in mouse cones is knocked out (Lyubarsky et al. 2000) or GRK7 in zebrafish cones is knocked down (Rinner et al. 2005), dark adaptation is slowed. The recovery phase of a light response recorded from single cones in $Nrl^{-/-}$/$GRK1^{-/-}$ mice was slowed (Nikonov et al. 2005). These studies show that visual pigment kinase is necessary for normal recovery of a light response in cones. When cone arrestin is knocked out, recovery of a light response is delayed (Nikonov et al. 2006). This effect is more significant at higher light intensity, but the effect is relatively small at low light intensity. Studies on the phototransduction cascade in cones are now intensively conducted mainly using genetically engineered mice. It would be required, however, that the phenotypically observed differences are confirmed at the molecular level.

References

Angleson JK, Wensel TG (1993) A GTPase-accelerating factor for transducin, distinct from its effector cGMP phosphodiesterase, in rod outer segment membranes. Neuron 11:939–949

Arinobu D, Tachibanaki S, Kawamura S (2010) Larger inhibition of visual pigment kinase in cones than in rods. J Neurochem 115:259–268

Chen CK, Woodruff ML, Chen FS et al (2010) Replacing the rod with cone transducin α subunit decreases sensitivity and accelerates response decay. J Physiol (Lond) 588:3231–3241

Choe HW, Kim YJ, Park JH et al (2011) Crystal structure of metarhodopsin II. Nature (Lond) 471:651–655

Cornwall MC, Fain GL (1994) Bleached pigment activates transduction in isolated rods of the salamander retina. J Physiol (Lond) 480:261–279
Deng W-T, Sakurai K, Liu J et al (2009) Functional interchangeability of rod and cone transducin α-subunits. Proc Natl Acad Sci USA 106:17681–17686
Dizhoor AM, Ray S, Kumar S et al (1991) Recoverin: a calcium sensitive activator of retinal rod guanylate cyclase. Science 251:915–918
Dowling JE (2000) George Wald in Biographical Memoirs, vol. 78 (2000), pp 298–317. http://books.nap.edu/openbook.php?record_id=9977&page=298
Dumke CL, Arshavsky VY, Calvert PD et al (1994) Rod outer segment structure influences the apparent kinetic parameters of cyclic GMP phosphodiesterase. J Gen Physiol 103:1071–1098
Fesenko EE, Kolesnikov SS, Lyubarsky AL (1985) Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. Nature (Lond) 313:310–313
Fung BKK, Stryer L (1980) Photolyzed rhodopsin catalyzes the exchange of GTP for bound GDP in retinal rod outer segments. Proc Natl Acad Sci USA 77:2500–2504
Gillespie PG, Beavo JA (1988) Characterization of a bovine cone photoreceptor phosphodiesterase purified by cyclic GMP-sepharose chromatography. J Biol Chem 263:8133–8141
Gray-Keller MP, Detwiler PB (1994) The calcium feedback signal in the phototransduction cascade of vertebrate rods. Neuron 13:849–861
Harosi FI (1975) Absorption spectra and linear dichroism of some amphibian photoreceptors. J Gen Physiol 66:357–382
Haynes LW, Yau KW (1990) Single-channel measurement from the cyclic GMP-activated conductance of catfish retinal cones. J Physiol (Lond) 429:451–481
He W, Cowan CW, Wensel TG (1998) RGS9, a GTPase accelerator for phototransduction. Neuron 20:95–102
Hubbard R (1977) Preface to the English translations of Boll's on the anatomy and physiology of the retina and of Kühne's chemical processes in the retina. Vision Res 17:1247–1248
Kawamura S (1993) Rhodopsin phosphorylation as a mechanism of cyclic GMP phosphodiesterase regulation by S-modulin. Nature (Lond) 362:855–857
Kawamura S, Murakami M (1991) Calcium-dependent regulation of cyclic GMP phosphodiesterase by a protein from frog retinal rods. Nature (Lond) 349:420–423
Kawamura S, Tachibanaki S (2008) Rod and cone photoreceptors: molecular basis of the difference in their physiology. Comp Biochem Physiol A Mol Integr Physiol 150:369–377
Kawamura S, Tachibanaki S (2012) Explaining the functional differences of rods versus cones. WIREs Membr Transp Signal 1:675–683
Kefalov V, Fu Y, Marsh-Armstrong N et al (2003) Role of visual pigment properties in rod and cone phototransduction. Nature (Lond) 425:526–531
Kennedy MJ, Lee KA, Niemi GA et al (2001) Multiple phosphorylation of rhodopsin and the in vivo chemistry underlying rod photoreceptor dark adaptation. Neuron 31:87–101
Koshitani Y, Tachibanaki S, Kawamura S (2014) Quantitative aspects of cGMP phosphodiesterase activation in carp rods and cones. J Biol Chem 289:2651–2657
Krispel CM, Chen D, Melling N et al (2006) RGS expression rate-limits recovery of rod photoresponses. Neuron 51:409–416
Leskov IB, Klenchin VA, Handy JW et al (2000) The gain of rod phototransduction: reconciliation of biochemical and electrophysiological measurements. Neuron 27:525–537
Luo DG, Xue T, Yau KW (2008) How vision begins: an odyssey. Proc Natl Acad Sci USA 105:9855–9862
Lyubarsky AL, Chen C, Simon MI et al (2000) Mice lacking G-protein receptor kinase 1 have profoundly slowed recovery of cone-driven retinal responses. J Neurosci 20:2209–2217
Miki N, Keirns JJ, Marcus FR et al (1973) Regulation of cyclic nucleotide concentrations in photoreceptors: an ATP-dependent stimulation of cyclic nucleotide phosphodiesterase by light. Proc Natl Acad Sci USA 70:3820–3824
Nikonov SS, Daniele LL, Zhu X et al (2005) Photoreceptors of $Nrl^{-/-}$ mice coexpress functional S- and M-cone opsins having distinct inactivation mechanisms. J Gen Physiol 125:287–304

Nikonov SS, Kholodenko R, Lem J et al (2006) Physiological features of the S- and M-cone photoreceptors of wild-type mice from single-cell recordings. J Gen Physiol 127:359–374
Noel JP, Hamm HE, Sigler PB (1993) The 2.2 Å crystal structure of transducin-alpha complexed with GTP gamma S. Nature (Lond) 366:654–663
Okano T, Fukada Y, Shichida Y et al (1992) Photosensitivities of iodopsin and rhodopsins. Photochem Photobiol 56:995–1001
Palczewski K, Subbaraya I, Gorczyca WA et al (1994) Molecular cloning and characterization of retinal photoreceptor guanylyl cyclase-activating protein. Neuron 13:395–404
Peng C, Rich ED, Varnum MD (2004) Subunit configuration of heteromeric cone cyclic nucleotide-gated channels. Neuron 42:401–410
Picones A, Korenbrot JI (1994) Analysis of fluctuations in the cGMP-dependent currents of cone photoreceptor outer segments. Biophys J 66:360–365
Pugh EN, Lamb TD (2000) Phototransduction in vertebrate rods and cones: molecular mechanisms of amplification, recovery and light adaptation. Handbk Biol Phys 3:183–255
Rinner O, Makhankov YV, Biehlmaier O, Neuhauss SC (2005) Knockdown of cone-specific kinase GRK7 in larval zebrafish leads to impaired cone response recovery and delayed dark adaptation. Neuron 47:231–242
Sakurai K, Onishi A, Imai H et al (2007) Physiological properties of rod photoreceptor cells in green-sensitive cone pigment knock-in mice. J Gen Physiol 130:21–40
Shimauchi-Matsukawa Y, Aman Y, Tachibanaki S et al (2005) Isolation and characterization of visual pigment kinase-related genes in carp retina: Polyphyly in GRK1 subtypes, GRK1A and 1B. Mol Vis 11:1220–1228
Slep KC, Kercher MA, He W et al (2001) Structural determinants for regulation of phosphodiesterase by a G protein at 2.0 Å. Nature (Lond) 409:1071–1077
Standfuss J, Edwards PC, D'Antona A et al (2011) The structural basis of agonist-induced activation in constitutively active rhodopsin. Nature (Lond) 471:656–660
Tachibanaki S, Tsushima S, Kawamura S (2001) Low amplification and fast visual pigment phosphorylation as mechanisms characterizing cone photoresponses. Proc Natl Acad Sci USA 98:14044–14049
Tachibanaki S, Arinobu D, Shimauchi-Matsukawa Y et al (2005) Highly effective phosphorylation by G protein-coupled receptor kinase 7 of light-activated visual pigment in cones. Proc Natl Acad Sci USA 102:9329–9334
Tachibanaki S, Yonetsu S, Fukaya S et al (2012) Low activation and fast inactivation of transducin in carp cones. J Biol Chem 287:41186–41194
Takemoto N, Tachibanaki S, Kawamura S (2009) High cGMP synthetic activity in carp cones. Proc Natl Acad Sci USA 106:11788–11793
Tomita T (1965) Electrophysiological study of the mechanisms subserving color coding in the fish retina. Cold Spring Harb Symp Quant Biol 30:559–566
Toyoda J, Nosaki H, Tomita T (1969) Light-induced resistance changes in single photoreceptors of *Necturus* and gekko. Vision Res 9:453–463
Weiss ER, Ducceschi MH, Horner TJ, Li A et al (2001) Species-specific differences in expression of G-protein-coupled receptor kinase (GRK) 7 and GRK1 in mammalian cone photoreceptor cells: implications for cone cell phototransduction. J Neurosci 21:9175–9184
Yau KW, Nakatani K (1984) Electrogenic Na–Ca exchange in retinal rod outer segment. Nature (Lond) 311:661–663
Yau K-W, Nakatani K (1985) Light-suppressible, cyclic GMP-sensitive conductance in the plasma membrane of a truncated rod outer segment. Nature (Lond) 317:252–255
Zhong H, Molday LL, Molday RS et al (2002) The heteromeric cyclic nucleotide-gated channel adopts a 3A:1B stoichiometry. Nature (Lond) 420:193–198

Chapter 3
Regeneration of 11-*cis*-Retinal in Visual Systems with Monostable and Bistable Visual Pigments

John C. Saari

Abstract In this chapter, the biochemistry of visual pigment regeneration in vertebrates is discussed and comparisons are made with regeneration in selected invertebrate systems. There are clear differences in the mechanisms of regeneration of 11-*cis*-retinoid between retinas that rely on rhabdomeric and ciliary visual pigments. Those pigments that employ c-opsins rely on a dark, enzymatically catalyzed isomerization process. Those that employ r-opsins rely on photoreversibility of a stable meta state of the visual pigment and an independent photoisomerase. Outside the vertebrate retina, no dark isomerase has been characterized except for isomerooxygenase (*NinaB*), which is involved in de novo generation of chromophore from carotenoids and xanthophylls in *Drosophila*. Similarities in reaction types and the use of chaperones in both ciliary and rhabdomeric visual systems are largely dictated by the chemical properties of the retinoid chromophore. The toxicity of retinal and its potential for oxidation and generation of other signaling molecules (retinoic acids in vertebrates) require that chaperones bind and sequester the retinoid and that dehydrogenases are available to reduce it to the less toxic retinol. The limited solubility of retinoids requires chaperones to bind and protect the retinoids during transit between cellular and extracellular compartments. Finally, the energy requirement for 11-*cis*-retinoid production from all-*trans*-precursors requires that the reaction take place at the oxidation level of retinol, where a cleavable, energy-yielding bond can be generated and coupled with isomerization or via photoisomerization where the energy of light can be harnessed.

Keywords Retinoid cycle • Rhodopsin cycle • Vision • Visual cycle • Vitamin A

J.C. Saari (✉)
Departments of Ophthalmology and Biochemistry, University of Washington School of Medicine, Box 357350, Seattle, WA 98195-7350, USA
e-mail: jsaari@u.washington.edu

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_3, © Springer Japan 2014

3.1 Introduction

All known photosensory systems are based on a complex of a chromophore and a protein. A prevalent chromophore in all three domains of life is a *cis*-retinal, almost always 11-*cis*-retinal or its derivatives 11-*cis*-3-dehydroretinal (11-*cis*-retinal 2), 4-hydroxy-11-*cis*-retinal, or 3-hydroxy-11-*cis*-retinal. Many different proteins, generally called opsins, are employed to generate sensory pigments with a wide variety of spectral properties. Absorption of a photon by the chromophore of these systems results in an isomerization in which the *cis*-double bond at position 11 is converted to a *trans*-double bond. This 11-*cis*- to all-*trans*-retinal photoisomerization is a key event because the change in shape of the chromophore is transmitted to the opsin, causing it to change its conformation and to interact with a G protein downstream in a signal transduction pathway. In addition, *cis*/*trans* isomerization allows the receptor to be active for a defined period of time before inactivation by one of several mechanisms. However, this *cis*/*trans* isomerization also necessitates a mechanism to regenerate the original chromophore isomeric conformation, a "resetting mechanism."

There appeared to be a clear difference in the mechanism of regeneration of 11-*cis*-isomers in vertebrates and invertebrates based on the stability and spectral characteristics of the signaling state, generally called a meta state. The complex of chromophore and protein in the meta state of *bistable* sensory pigments was stable and capable of absorbing another photon, thus regenerating the original state. Illumination of such pigments generated a photoequilibration consisting of *cis* forms of the chromophore (nonsignaling, dark forms) and *trans* forms (active signaling meta-forms). Generally, these bistable pigments were found in rhadomeric, invertebrate photosensory systems and employed an opsin of the rhabdomeric lineage (an r-opsin) (Terakita 2005; Yau and Hardie 2009). In contrast, the meta state of *monostable* sensory pigments did not absorb visible light and was not stable, allowing dissociation of the chromophore and protein complex. Regeneration of monostable pigments required an enzymatic cycle of reactions, known initially as a visual cycle (Wald 1968) and now often called a retinoid or regeneration cycle. This cycle was capable of functioning in the dark because of an enzymatically catalyzed all-*trans*- to 11-*cis*-retinoid reaction. Monostable visual pigments were generally found in photosensory systems derived from a modified cilium and their opsins fell within group of ciliary or c-opsins (Terakita 2005; Yau and Hardie 2009). Thus, vertebrates regenerated their visual pigments via a series of enzymatic reactions occurring in adjacent cells whereas invertebrates regenerated their visual pigments via a cell-autonomous, light-driven "back-isomerization."

However, it was clear that photoregeneration would cease at night and would not be useful for organisms with rhabdomeric photoreceptors in environments where there were too few photons to allow for efficient absorption, for instance, deep-sea creatures. In addition, photoregeneration could not account for recycling of photolyzed chromophore released from internalized and degraded opsin, for de novo synthesis of 11-*cis*-retinal from precursors such as vitamin A, xanthophylls, and carotenoids, and for turnover of rhodopsin. Thus, it was apparent that rhabdomeric

visual systems required an additional form of 11-*cis*-retinal generation and several enzymatic transformations of vitamin A and derivatives. These processes were first examined in squid retina, which was the first rhabdomeric system that was explored in molecular detail. Production of 11-*cis*-retinal from all-*trans*-retinal was found to depend on a protein called retinochrome and, surprisingly, the reaction was light dependent (Hara and Hara 1965). Subsequently, photoisomerases were reported in several other rhabdomeric visual systems (*Drosophila*, honey bee, blowfly) and may be a feature of all visual systems based on bistable pigments. Recently, the enzymes and proteins responsible for a regeneration cycle in *Drosophila* have been explored at the molecular level and collectively termed a visual cycle (Wang et al. 2010).

At present, we have molecular information about chromophore regeneration cycles of relatively few organisms, but some patterns are beginning to emerge. The vertebrate *rod regeneration cycle* remains the best characterized with its chemistry well defined, its enzymology nearly settled, and our understanding of its roles in retinal diseases well advanced (Thompson and Gal 2003; Travis et al. 2006). Details regarding a vertebrate *cone regeneration cycle* have emerged within the past decade, but several aspects remain poorly characterized or controversial. As already mentioned, a *molluscan (squid) regeneration cycle* was described decades ago employing a "reverse rhodopsin" (photoisomerase) to regenerate 11-*cis*-retinal. The features of an *insect regeneration cycle* have been outlined with more clarity in recent years. Finally, intrinsically photosensitive retinal ganglion cells (ipRGCs) represent a special case of an r-opsin-based signaling system, melanopsin, in a vertebrate retina. There are many other invertebrate systems where electrophysiology and morphology suggest interesting transformations of the chromophore, but the molecular details remain elusive (Hillman et al. 1983).

In this chapter, I review the basic chemistry, enzymology, and chaperones of the regeneration cycles for which we have some molecular information. A comparison of vertebrate visual systems with monostable ciliary opsins and invertebrate visual systems with bistable r-opsins reveals several parallel themes. Some of these are likely to be consequences of the use of the same chromophore, which is potentially toxic and whose chemistry at physiological temperatures is limited. Others likely reflect a common ancestral origin for components.

3.2 Vertebrate Rod Regeneration Cycle

We now have a detailed understanding of the chemical reactions, chaperone proteins, enzymology, and roles in retinal disease of the vertebrate rod regeneration cycle.

3.2.1 Description of the Cycle

Changes in the colors of the retina in response to illumination featured prominently in discovery and elucidation of the vertebrate rod regeneration cycle. For instance, Kühne (1879) and Boll (1877) noted that frog retina was reddish when prepared in

the dark and turned first yellow and then colorless in the light. The term used for this loss of color, bleaching, became synonymous with photoisomerization when the molecular basis of the phenomenon was determined (Wald 1968). These early physiologists also noted that the original red color was restored in the dark if the retina was placed in apposition to the retinal pigment epithelium (RPE), a single layer of cells at the back of the retina between the choroidal blood supply and photoreceptor cell outer segments. Later Wald demonstrated that the original change in color was related to the visual process and that the system could be regenerated in the dark by enzymatic processes that occurred, in part, in RPE cells (Wald 1935). Studies by Dowling in the rat provided the first indication of the enzymatic changes that occurred during the dark regeneration cycle (Dowling 1960), but the critical dark isomerase activity was not detected until the mid-1980s (Bernstein and Rando 1986; Bernstein et al. 1987) and the enzyme responsible was not identified until the mid-2000s (Jin et al. 2005; Moiseyev et al. 2005; Redmond et al. 2005). The vertebrate regeneration cycle is now known to consist of enzymes that catalyze transformations of the retinoids, binding proteins that solubilize and protect the retinoids, and undefined processes associated with transport of the retinoids, particularly 11-*cis*-retinal, across lipid bilayers and aqueous compartments.

A brief description of the rod visual cycle follows (Fig. 3.1). In subsequent sections, well-established facets of the cycle are mentioned in passing and some active research areas are discussed in more detail. The reader is referred to other reviews of the rod visual cycle that have recently appeared for more details and for evidence supporting the roles of components of the cycle (Tang et al. 2013; Lamb and Pugh 2004, 2006; Kiser et al. 2011; Parker and Crouch 2010; Kiser et al. 2014; Saari 2012; Travis et al. 2006). Following photoisomerization of the 11-*cis*-retinal in rhodopsin, all-*trans*-retinal is released from its binding pocket within opsin and reduced to all-*trans*-retinol by retinol dehydrogenase 8 (RDH8) and other dehydrogenases of the rod photoreceptor cells. A member of the ABC-transporter family, ABCA4, is present in rod outer segment membranes and can rescue any all-*trans*-retinal that mislocates to the inner leaflet of rod outer segment (ROS) membranes by "flipping" its orientation to the cytosolic leaflet (Illing et al. 1997; Beharry et al. 2004; Sun and Nathans 1997). All-*trans*-retinol leaves the ROS and traverses the interphotoreceptor matrix (IPM) chaperoned by interphotoreceptor matrix retinoid-binding protein (IRBP). Within the RPE, all-*trans*-retinol is bound by cellular retinol-binding protein type 1 (CRBP1) and esterified by lecithin:retinol acyltransferase (LRAT) to form all-*trans*-retinyl palmitate and other long-chain fatty acid esters. Isomerohydrolase or isomerase 1 (RPE65) hydrolyzes and isomerizes all-*trans*-retinyl palmitate to generate palmitate and 11-*cis*-retinol. Cellular retinaldehyde-binding protein (CRALBP; gene designation *RLBP1*) binds 11-*cis*-retinol and facilitates its oxidation to 11-*cis*-retinal, catalyzed by RDH5 and other *cis*-specific dehydrogenases. 11-*cis*-retinal binds tightly to CRALBP and is possibly released from the binding protein and from RPE by interaction with acidic phospholipids or other unidentified acidic compounds. 11-*cis*-retinal traverses the IPM, chaperoned by IRBP, enters the photoreceptor ROS, and associates with opsin to regenerate rhodopsin.

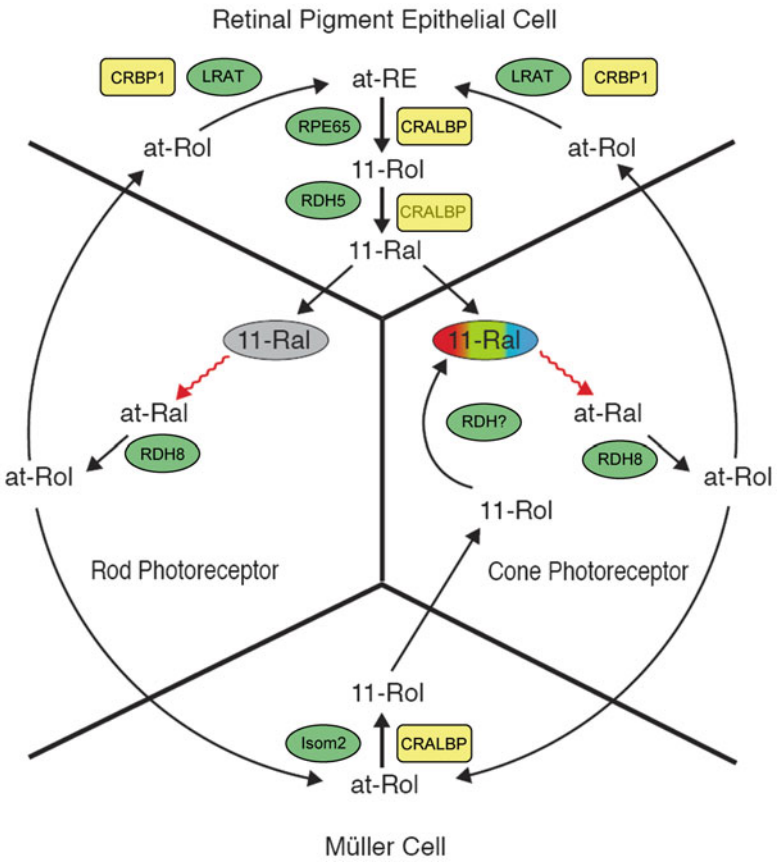

Fig. 3.1 Reactions of vertebrate rod and cone visual cycles. Reactions occurring in four cells are shown and are described in more detail in Sects. 3.2 and 3.3. *at-Ral* all-*trans*-retinal, *at-Rol* *all-trans-retinol*, *at-RE* all-*trans*-retinyl ester, *11-Ral* 11-*cis*-retinal, *11-Rol* 11-*cis*-retinol, *CRALBP* cellular retinal-binding protein, *CRBP1* cellular retinol-binding protein type 1, *Isomer2* *cis–trans* isomerase type 2, *LRAT* lecithin:retinol acyltransferase, *RDH8* retinol dehydrogenase type 8, *RDH?* uncharacterized retinol dehydrogenase, *RPE65* retinal pigment epithelium protein of 65 kDa, aka isomerohydrolase. Rhodopsin and cone visual pigments are depicted as *purple* and *tricolored ovals*, respectively. Photochemical isomerizations are shown as *red squiggly arrows*. (Modified from Saari 2012)

3.2.2 Active Research Areas

Our understanding of the rod visual cycle and its role in normal physiology and disease has seen remarkable progress since the advent of DNA cloning, sequencing, and the generation of transgenic mice. The following remarks provide background for subsequent discussion or point out areas that remain unresolved at present. Note that the topics are not inclusive and reflect the author's interests and background.

3.2.2.1 Dehydrogenases of the Vertebrate Rod Visual Cycle

There are several dehydrogenases catalyzing the oxidation/reduction reactions of the vertebrate rod visual cycle: RDH8 and RDH12 catalyzing reduction of all-*trans*-retinal and RDH5, RHD10, and RHD11 catalyzing the oxidation of 11-*cis*-retinol (reviewed in Kiser et al. 2011; Kiser et al. 2013; Parker and Crouch 2010). In contrast, there is only one gene for the enzyme encoding the quintessential reaction of the rod visual cycle, isomerization of all-*trans*-retinoid to 11-*cis*-retinoid. Although the reason for the redundancy of the dehydrogenases is not known, it is possible that this reflects the chemical reactivity of both all-*trans*- and 11-*cis*-retinals with the resultant need for strict control of their cellular concentrations. In addition, all-*trans*-retinal is a precursor for all-*trans*-retinoic acid, a potent signaling molecule that controls numerous biological processes (Tanoury et al. 2013). Thus, it is essential to control the amount of all-*trans*-retinal and restrict its use for vision in this context.

3.2.2.2 Dark Isomerization

It has been known since the nineteenth century that rhodopsin could regenerate in the dark if the neural retina was in apposition to the RPE (Kühne 1879; Boll 1877). However, nearly 100 years would elapse before this dark isomerization reaction was detected in extracts of RPE, and then nearly another 20 years before the molecular identity of the isomerase was known. Studies in frogs established that the isomerization had to take place at the oxidation level of retinol because all-*trans*-retinol labeled with tritium on C-15 retained its radioactivity during isomerization (Bernstein and Rando 1986). This observation was followed by a demonstration of isomerization of all-*trans*-retinol to 11-*cis*-retinol catalyzed by an enzyme in RPE extracts (Bernstein et al. 1987). Subsequent studies revealed that the substrate for the isomerization was all-*trans*-retinyl ester (Trehan et al. 1990; Moiseyev et al. 2003), which was generated from all-*trans*-retinol added to RPE extracts by the powerful LRAT activity of RPE (Saari and Bredberg 1988, 1989; Barry et al. 1989). Hydrolysis of the retinyl ester bond was postulated to provide the energy needed for generation of the hindered 11-*cis*-retinol (Deigner et al. 1989); thus, the enzyme was named an isomerohydrolase. Three reports in 2005 established that the enzyme responsible for the isomerohydrolase activity was RPE65, a major protein of the retinal pigment epithelium (Jin et al. 2005; Moiseyev et al. 2005; Redmond et al. 2005; Redmond 2009). Debate regarding whether the mechanism of the reaction involved Sn2 nucleophilic attack (Deigner et al. 1989) or carbocation formation (McBee et al. 2000) was eventually resolved in favor of the latter (Kiser et al. 2009; Kiser et al. 2013; Redmond et al. 2010; Poliakov et al. 2011). REP65 is a member of a family of non-heme iron-containing enzymes. One family member catalyzes symmetrical cleavage of β-carotene and other carotenoids and xanthophylls to all-*trans*-retinal (β-carotene monooxygenase 1, BCMO1), one catalyzes asymmetrical cleavage of β-carotene and lycopene (β-carotene dioxygenase 2, BCDO2), and another catalyzes the

simultaneous cleavage and isomerization of zeaxanthin and other xanthophylls to 3-OH-all-*trans*-retinal and 3-OH-11-*cis*-retinal (ninaB; see Sect. 3.5.2.1 following).

3.2.2.3 Release of 11-*cis*-Retinal from CRALBP

Various experimental approaches including in vitro enzymology (Saari and Bredberg 1982; Saari et al. 1994), analysis of the phenotype of knockout mice (Saari et al. 2001), and that of humans with *RLBP1* mutations (Burstedt et al. 2003), supported a role for CRALBP in rod physiology that included acceptance of 11-*cis*-retinol by apo-CRALBP from RPE65 and facilitation of its oxidation to 11-*cis*-retinal by RDH5. This result implied that retinoids "flow through" the binding pocket of CRALBP during their processing in RPE. For this to occur, 11-*cis*-retinal must be released from CRALBP and from the RPE cell and travel to the ROS. However, CRALBP binds 11-*cis*-retinal with high affinity (K_d ~ 10 nM), and attempts to find conditions in which it would release the retinoid were unsuccessful (Saari et al. 2009). A breakthrough occurred when CRALBP was reported to bind to a subset of anionic lipids (Saari et al. 2009). Based on a qualitative assay, CRALBP bound most avidly to phosphatidic acid (PA) and phosphatidylinositol-3,4,5-*tris*-phosphate (PIP3), with less avid binding noted for phosphatidylserine (PS). No binding occurred with phosphatidylcholine (PC) or phosphatidylethanolamine (PE). Binding to acidic lipid surfaces was all the more remarkable because the isoelectric point of CRALBP is acidic (pI ~ 5.1). Analysis of the electrostatic surface potential of a model of the CRALBP structure (no crystallographic structure was available at the time) revealed a positively charged recess, which was subsequently found in the structure of CRALBP when it became available from X-ray crystallographic studies (He et al. 2009). Finally, incubation of CRALBP 11-*cis*-retinal with small unilamellar vesicles containing acidic lipids perturbed the structure of CRALBP and allowed access to the bound retinoid. Notably, the efficacy of release by acidic lipids was in parallel with their ability to bind to CRALBP. One of the residues in this basic recess is mutated in humans with Bothnia dystrophy (R234W) (Burstedt et al. 2001). Analysis of the expressed mutant protein and its crystal structure revealed that the mutation interrupts a cascade of side-chain flips, resulting in impaired release of 11-*cis*-retinal (Golovleva et al. 2003; He et al. 2009). At present there are no physiological data to support the hypothesis that 11-*cis*-retinal is released from CRALBP by acidic lipids. Analysis of mice expressing CRALBP in which the amino acid residues of the basic recess have been mutated would provide valuable information.

3.2.2.4 Release of 11-*cis*-Retinal from RPE and Traverse of the IPM

The mechanism by which 11-*cis*-retinal is released from RPE cells remains unknown. Simple partition of the free retinoid in the RPE apical membrane seems unlikely given the readiness of retinal to form Schiff bases with amines

and the presence of phosphatidylethanolamine in membranes. Export via a member of the ABC-transporter family comes to mind, but there is no evidence to support this idea at present. It also seems unlikely that naked 11-*cis*-retinal could survive transit of the IPM, which contains an abundance of proteins, without some sort of chaperone. IRBP is present in this compartment (Bunt-Milam and Saari 1983) and is likely to bind the protein during its transit. However, the affinity of IRBP for 11-*cis*-retinal is relatively weak (Noy 2000) and the aldehyde group does not appear to be protected when bound.

3.2.2.5 Origin of the Vertebrate Visual Cycle

The development of methods for probing genomic sequences and the steady acquisition of genomic data from multiple organisms have fostered attempts to understand the origin of the visual cycle in vertebrates. Early studies (reviewed by Kusakabe et al. 2009) suggested that the last common ancestor of tunicates and vertebrates already employed components of the vertebrate visual cycle. In particular, molecular phylogeny and genomic analysis provided evidence for the presence of orthologues of RPE65, CRALBP, and BCMO in the genome of the ascidian *Ciona intestinalis*, considered to be a close living relative of vertebrates (Takimoto et al. 2007). More recently, others have challenged that notion based on closer analysis of genomic sequences (Albalat 2012; Poliakov et al. 2012) and analysis of enzymatic activities (Poliakov et al. 2012). Poliakov et al. considered the presence of both LRAT and RPE65 activities to be a fundamental hallmark for a vertebrate type of visual cycle because the retinyl ester synthetase generates substrate for the isomerase. A *Ciona* BCMO sequence, previously consider to be a RPE65 orthologue, lacked critical functional amino acid residues typical of RPE65, and cellular extracts had oxygenase cleavage activity but no RPE65 (isomerohydrolase) activity. They further demonstrated that *Ciona*, and *Branchiostoma belcheri* (amphioxus, a cephalochordate), lacked LRAT and RPE65 orthologues. The most primitive vertebrate eye that they examined with RPE65 and LRAT activity was that of the lamprey. In their view, the vertebrate visual cycle arose with the development of the primitive vertebrate eye.

3.3 Vertebrate Cone Regeneration Cycle

3.3.1 Description of the Cone Visual Cycle

Evidence supporting the regeneration of vertebrate cones, separated from RPE, was apparent in early physiological studies of cone function (Goldstein 1970). In the late 1980s and early 1990s, important mechanistic features of cone

regeneration were determined in several laboratories. In particular, it was noted that cones could regenerate their visual pigment from 11-*cis*-retinol as well as 11-*cis*-retinal whereas rods could only regenerate with 11-*cis*-retinal (Ala-Laurila et al. 2009; Jones et al. 1989). Independently, two retinoid-binding proteins, cellular retinol-binding protein type 1 (CRBP1) and CRALBP, were localized to RPE and unexpectedly to Müller cells in the neural retina (Bunt-Milam and Saari 1983; Bok et al. 1984; Saari et al. 1984; Saari et al. 1995), indicating that vitamin A metabolism was a feature of these principal glial cells of the retina. Cultured Müller cells from chicken retina generated 11-*cis*-retinol following incubation with all-*trans*-retinol (Das et al. 1992). Subsequently, several enzymatic activities were discovered in cone-enriched retinas, primarily chicken, and a plausible cycle of reactions was proposed, occurring partly in Müller cells and partly in cones (Mata et al. 2002) (Fig. 3.1). Recently, a combination of methods was used to demonstrate that mouse Müller cells were responsible for an early rapid phase of mouse cone visual pigment regeneration whereas 11-*cis*-retinal from RPE was responsible for a slower phase (Kefalov 2012; Wang and Kefalov 2011).

The cone visual cycle as currently proposed for mouse involves the participation primarily of cones and Müller cells (Mata et al. 2002; Kaylor et al. 2013), with contributions from metabolism in RPE and rods. All-*trans*-retinal, released from photolyzed cone visual pigments, is reduced within the cone cell by RDH8 to produce all-*trans*-retinol, which diffuses to the adjacent Müller cell, perhaps chaperoned by IRBP. Müller cells could also receive all-*trans*-retinol from rod photoreceptors, as mentioned earlier. Within Müller cells, all-*trans*-retinol is isomerized to 11-*cis*-retinol, catalyzed by isomerase 2 (see following discussion). CRALBP, long known to be present in Müller cells (Bunt-Milam and Saari 1983), is thought to provide preferential binding of 11-*cis*-retinol (Saari and Bredberg 1987), thus generating stereospecificity, and to drive the equilibrium catalyzed by an isomerase (Kaylor et al. 2013), similar to its role in RPE where it also facilitates oxidation of 11-*cis*-retinol to 11-*cis*-retinal (Winston and Rando 1998; Stecher et al. 1999; Saari et al. 1994; 2001). *Rlbp1*$^{-/-}$ (CRALBP-knockout) mice showed delayed cone resensitization, consistent with a role of CRALBP in cone visual pigment regeneration (Saari et al. 2001). 11-*cis*-retinol is then oxidized to 11-*cis*-retinal within cones by an unidentified dehydrogenase. Association with a cone opsin completes the cycle. It is noteworthy that the product of the reactions of the cone visual cycle released from Müller cells is 11-*cis*-retinol, which can be utilized by cones but not by rods (Ala-Laurila et al. 2009). This feature eliminates what would otherwise be an unfavorable competition for 11-*cis*-retinal because rods outnumber cones by 10 to 20 to 1 in most mammalian retinas. Although the rod signal saturates at a relatively low isomerization rate, the rhodopsin chromophore continues to isomerase as the illumination intensity rises, further increasing the demand for 11-*cis*-retinal.

3.3.2 Active Research Areas

Molecular characterization of the cone visual cycle is still in its infancy relative to the more mature understanding of the rod visual cycle. The molecular identity of the cone visual cycle isomerase is of particular interest. Several candidates have been proposed, and conflicting claims remain to be resolved.

3.3.2.1 Isomerases of the Cone Regeneration Cycle

The molecular nature of the Müller cell isomerase (variously called isomerase 2, isomerase I, and RPE65c) remains unsettled. Recently, three candidate enzymes have been proposed in three different species. To further complicate matters, evidence has been published that RPE65 is expressed in mouse cone inner segments (Tang et al. 2011). It is possible that some of the discrepancies in these reports relate to species differences or to cone-dominated versus rod-dominated retina specializations. It would be surprising if the cone visual cycle isomerase differed so dramatically among these three species given the propensity of nature to modify existing systems rather than invent new ones.

An RPE65 paralogue, RPE65c, which showed 80 % and 79 % identity at the deduced amino acid sequence level with zebrafish RPE-specific RPE65a and human RPE65, respectively, was cloned from zebrafish retina cDNA (Takahashi et al. 2011). The expressed protein had isomerohydrolase activity, which was dependent on iron, typical of the β-carotene monooxygenase (BCMO) family of enzymes to which RPE65 belongs. Immunocytochemistry revealed its expression in cells of the inner retina, presumably Müller cells, but not RPE, which expressed RPE65a.

A candidate for a Müller cell isomerase of mouse retina (termed isomerase 2), dihydroceramide Δ4-desaturase-1 (DES1), was described that acts directly on all-*trans*-retinol, converting it to 11-*cis*-retinol (Kaylor et al. 2013), a reaction postulated in the original formulation of a cone visual cycle (Mata et al. 2002). The enzyme is a member of a membrane desaturase family of enzymes and was previously thought to be solely involved in catalyzing the last step in the biosynthesis of ceramide. The enzyme was localized to Müller cells and immunoprecipitated with CRALBP. It is surprising that the isomerase for the rod visual cycle (RPE65, isomerase 1) and that proposed for the cone visual cycle (isomerase 2) would not be related. However, the high rate of turnover of cone visual pigment during daylight (Rodieck 1998) and the slow enzymatic turnover of RPE65 (Redmond et al. 2005) could have contributed to the different solutions to the problem of retinoid isomerization in rods and cones.

Expression of a cDNA cloned from a chicken cDNA library yielded a protein that was 90 % identical to human RPE65 at the amino acid sequence level and which showed isomerohydrolase activity (Moiseyev et al. 2008). The specific enzymatic activity of the chicken RPE65 was approximately 12-fold higher than that from rod-dominant species (mouse, human), which was suggested to be related

to the faster turnover of visual pigment in cones. Other studies of chicken retina corroborated an isomerase activity (isomerase II) with biochemical properties different from RPE65, although the activity appeared to be lower (Muniz et al. 2009).

3.4 Regeneration Cycle in Molluscs

3.4.1 Description of the Molluscan Visual Cycle

Studies of the squid (*Todarodes pacificus*) eye were the first to provide molecular information regarding components of a visual pigment regeneration cycle (reviewed by Hara and Hara 1991). In a landmark study, squid rhodopsin was shown to possess two of what are now the defining characteristics of bistable visual pigments, a stable metastate and photoreversibility, that is, the generation of 11-*cis*-retinal upon absorption of a second photon (Hubbard and St. George 1958). A crystal structure of squid rhodopsin provided some structural information regarding the bistable nature of the chromophore (Murakami and Kouyama 2008). In squid, the side chain of Tyr111 appears to replace a glutamate that is present in all c-rhodopsin structures examined. The ionization state of this tyrosine hydroxyl remains unchanged during photoactivation, perhaps allowing regeneration via absorption of a second photon (Murakami and Kouyama 2008; Schertler 2008). Squid rhodopsin can thus be considered the prototypic bistable visual pigment. Considering the bistable nature of the visual pigment, it was something of a surprise when a second protein was discovered that bound all-*trans*-retinal with high affinity and converted it to 11-*cis*-retinal upon absorption of a photon (Hara and Hara 1965), essentially a "reverse rhodopsin." This protein, called retinochrome, has 7-transmembrane helices, similar in topology to opsin. Although rhodopsin is found in the rhabdomeric microvilli of squid retina, retinochrome was found in inner segments and basal regions of the outer segments (Hara and Hara 1976, 1991; Katagiri et al. 2001). Given the minimal solubility of retinoids in aqueous solutions, an additional component was needed if the functions of the two photosensitive proteins were to be coupled. This need led the investigators to search for an additional, bridging protein, which was discovered and named retinal-binding protein (RALBP) (Terakita et al. 1989, Ozaki et al. 1994) RALBP was found in both inner and outer segments. Thus, regeneration of a seemingly simple bistable visual pigment was demonstrated to be highly complex with three different retinal-binding proteins and uncharacterized dehydrogenases involved (Hara and Hara 1991). The investigators termed the functional unit the rhodopsin-retinochrome system, which has been subsequently demonstrated in several other molluscans. A summary of the squid regeneration cycle follows (Fig. 3.2).

Absorption of a photon by squid rhodopsin converts 11-*cis*-retinal to all-*trans*-retinal in the microvilli of rhabdomers and generates metarhodopsin. This signaling state is stable and can be photoisomerized back to the dark, nonsignaling state by

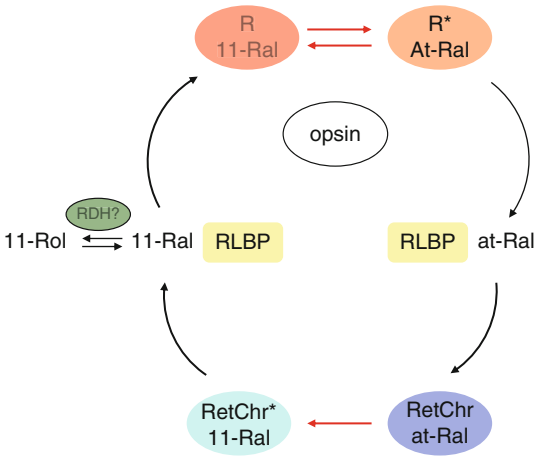

Fig. 3.2 Known reactions of a squid visual cycle. Reactions are discussed in more detail in Sect. 3.4. Abbreviations for retinoids as described in the legend to Fig. 3.1. *R* squid rhodopsin, *R** photolyzed squid rhodopsin, *RetChr* retinochrome, *RetChr** photolyzed squid retinochrome, *RLBP* retinal-binding protein. Photochemical isomerizations are shown in *red*

absorption of a second photon. Photoreversibility sets up a photoequilibrium, which determines the signaling output from the photoreceptor cells (Tsukamoto and Terakita 2010). Absorption of a photon by all-*trans*-retinal bound to retinochrome in the inner segments and basal regions of the outer segment converts it to 11-*cis*-retinal. Rhodopsin has a greater affinity for 11-*cis*-retinal whereas retinochrome has a greater affinity for all-*trans*-retinal. Thus, the products of the two photosystems are effectively coupled by an exchange mediated by RALBP, whose distribution bridges both inner and outer segments.

3.4.2 Active Research Areas

Although squid retina was initially the subject of a great deal of experimentation, major advances in understanding the rhodopsin-retinochrome system were confined to the determination of the structure of squid rhodopsin. It is unfortunate that the availability of sequence of the mouse genome and the relatively ease of genetic manipulation with this species may have discouraged further characterization of this systems.

3.4.2.1 Is There a Dark Isomerase?

What function does retinochrome play that is not already met by the photoreversibility of bistable squid rhodopsin? Its discoverers argue that retinochrome likely acts as a reservoir for 11-*cis*-retinal, which fills during daylight and contributes its chromophore to rhodopsin in the dark (Ozaki et al. 1983). Squid retina contains

about as much retinochrome as rhodopsin (Ozaki et al. 1983), and in keeping with a reservoir role for the protein, its amount increases markedly during light adaptation (Hara and Hara 1976). In addition, meta-retinochrome slowly regenerates (i.e., 11-*cis*-retinal thermally isomerizes to all-*trans*-retinal) in the dark, effectively readying the photoisomerase for daylight function. To date, no dark isomerases have been reported in squid retina.

3.5 Regeneration Cycle in Insects (*Drosophila*, Honey Bee, Blowfly)

3.5.1 Description of the Drosophila *Visual Cycle*

Although *Drosophila melanogaster* employs a bistable visual pigment and depends on photoregeneration of its visual pigment (Fain et al. 2010), it can produce 3-OH-11-*cis*-retinal (the derivative of 11-*cis*-retinal used as chromophore in flies) from dietary vitamin A (all-*trans*-retinol) or carotenoids and salvage chromophore from internalized, photolyzed rhodopsin via an enzymatic regeneration cycle (Montell 2012; Wang et al. 2010; Wang and Montell 2005). In the past few years, a retinoid-binding protein and several retinoid-metabolizing enzymes from *Drosophila* and other insects have been characterized that allow formulation of a regeneration cycle (Wang et al. 2012; Montell 2012; Wang et al. 2010) (Fig. 3.3). Based on the cellular localization of some of these components, enzymatic transformations of retinoids are likely to take place entirely within retinal pigment cells of the rhabdomer (Wang et al. 2012). The cycle begins with the release of 3-OH-all-*trans*-retinal from opsin as a result of internalization and degradation of the photolyzed receptor. This substrate is reduced to 3-OH-all-*trans*-retinol by a dehydrogenase (PDH, pigment cell dehydrogenase) specified by the *pdh* gene (Wang et al. 2010). 3-OH-all-*trans*-retinol is then isomerized by unknown enzyme(s) to 3-OH-11-*cis*-retinol. 3-OH-11-*cis*-retinol is oxidized to 3-OH-11-*cis*-retinal in a reaction catalyzed by a second dehydrogenase (RDHB, retinol dehydrogenase B), specified by the *rdhB* gene (Wang et al. 2012). Expression of RDHB has been demonstrated in retinal pigment cells. Conjugation of 3-OH-11-*cis*-retinal with opsins occurs spontaneously to complete the cycle. De novo synthesis of chromophore from carotenoids begins with cleavage of zeaxanthin, catalyzed by the *n*either *i*nactivation *n*or *a*fterpotential B (*NinaB*) gene product β,β′-carotene-15,15′-oxygenase, now called isomerooxygenase, which generates 3-OH-all-*trans*-retinal and 3-OH-11-*cis*-retinal (Oberhauser et al. 2008). Finally, retinal can be converted to 3-OH-retinal by the product of the *NinaG* gene, an oxidoreductase with hydroxylation activity (Sarfare et al. 2005).

One retinoid-binding protein has been characterized in the *Drosophila* regeneration cycle. The protein was isolated by screening chromosome 3 mutants whose phenotype showed an absence of a prolonged depolarization afterpotential (PDA)

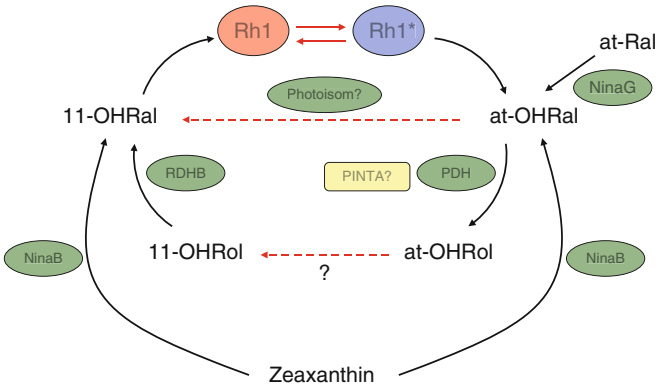

Fig. 3.3 Known reactions of a *Drosophila* visual cycle. Reactions are discussed in more detail in Sect. 3.5. *Rh1* rhodopsin 1, *Rh1** photolyzed rhodopsin 1, *at-OHRal* all-*trans*-3-hydroxy retinal, *at-OHRol* at-*trans*-3-hydroxy retinol, *11-OHRal* 11-*cis*-3-hydroxy retinal, *11-OHRol* 11-*cis*-3-hydroxy retinol, *NinaB* gene designation for isomerooxygense, aka BCDO, β-carotene dioxygenase; *NinaG* gene designation for an oxidoreductase in the GMC (glucose-methanol-choline) family, *PINTA* gene designation for a retinol-binding protein, *Photoisom?* uncharacterized photoisomerase, *RDHB* retinol dehydrogenase B, *PDH* photoreceptor dehydrogenase. Documented photoisomerization reactions are shown as *red arrows*. An uncharacterized photoisomerase is depicted as a *red dashed arrow*. (Constructed, in part, using the pathway proposed by Wang et al. 2012)

in the electroretinogram and named PINTA (*P*DA *i*s *n*ot *a*pparent). The *pinta* locus specifies a protein that shares amino acid sequences with CRALBP (23 % identical sequence over 196 residues) and other members of the CRAL_TRIO family of lipid-binding proteins (Wang and Montell 2005). The lipid-binding domain of CRAL_TRIO family members is that of yeast Sec14, a small protein required for protein secretion (Sha et al. 1998). PINTA bound all-*trans*-retinoids with high affinity and *cis*-retinoids with considerably lower affinity. The *pinta* mutation phenotype was rescued by expression of the gene in pigment cells of the compound eye, indicating that it participates in retinoid metabolism occurring in that tissue. The mechanism of PINTA function in insect eyes is not known. Its high affinity for all-*trans*-retinol suggests that it could be present in the substrate and accept the product of the PDH reaction.

3.5.2 Active Research Areas

3.5.2.1 Identity of a Photoisomerase

Photoisomerases have been noted in the visual systems of several insects including *Drosophila* (Pepe and Cugnoli 1992), blowfly (Schemer 1984), and honey bee

(Schwemer et al. 1984), and characterized in squid (see foregoing discussion). Their role in the regeneration cycle of bistable visual pigments has been clarified by studies of the visual system of the honey bee (*Apis mellifera*) (Smith and Goldsmith 1991; Cugnoli et al. 1989). A remarkable property of the honeybee retina is that 80 % of its retinal was extracted into an aqueous buffer where it was demonstrated to be bound to a photoisomerase (RalPI440) (Smith and Goldsmith 1991). Following illumination, the 11-*cis*-retinal formed could be either reduced to 11-*cis*-retinol by an uncharacterized dehydrogenase or delivered to opsin to regenerate rhodopsin. Honeybee photoisomerase has been proposed to supply 11-*cis*-retinal for rhodopsin biosynthesis in a slow renewal process involving membrane turnover (Pepe and Cugnoli 1992). However, given the large amount of photoisomerase (and perhaps other retinoid-binding proteins) (soluble retinal + retinol ~ 4–12 pmol/eye; visual pigment-associated retinal ~ 2.5 pmol/eye), it is conceivable that photoisomerase could serve as a reservoir of 11-*cis*-retinoid, which could fill up during daylight hours and deliver 11-*cis*-retinal to photolysed opsin in the dark (Smith and Goldsmith 1991), a role proposed for retinochrome in squid retina (see earlier). *Drosophila* has been reported to have "photoisomerase" activity (Pepe and Cugnoli 1992), but there is insufficient information available to determine whether it could function in a manner similar to that of the honey bee. The existence of an isomerase catalyzing a thermally driven isomerization reaction (dark isomerase), as opposed to a photoisomerase, remains unknown.

3.6 Intrinsically Photosensitive Retinal Ganglion Cells (ipRGC)

3.6.1 Description

Physiologists were long aware of a photosensory system in animals that was responsible for regulation of a number of nonvisual phenomena, including entrainment of the circadian clock and constriction of the pupil in response to light. For years it was assumed that functions were a subset of those of vertebrate rod and cone cells, even though it was clear that the requirements for visual phenomena, which respond primarily to spatial and temporal changes in contrast, were different from those of irradiance detection (Bailes and Lucas 2010). About a decade ago, the output of a small set of retinal ganglion cells was found to be driven by light even in animals that lacked rods and cones (Provencio et al. 2000). These cells, which were called intrinsically photosensitive retinal ganglion cells (ipRGC) (Hattar et al. 2002), contained a retinal-based pigment melanopsin (*Opn4*), which was more closely related to rhabdomeric opsins than ciliary opsins and that was coupled to Gq/11, similar to rhabdomeric phototransduction signaling cascades (Bailes and

Lucas 2013). High-intensity, nonphysiological illumination appears to deplete the system of dark-state photopigment. However, the system regained much of its photosensitivity following incubation in the dark (Sexton et al. 2012). During this period the dark state was regenerated either by thermal decay of the meta state (as observed in retinochrome) or by a dark regeneration cycle.

3.6.2 Active Research Areas

Many questions remain regarding the functions of this r-opsin-based photosensory system. In particular, the details regarding its regeneration remain to be specified.

3.6.2.1 Nature of a Regeneration Cycle

At present the evidence indicates that melanopsin is a bistable pigment whose regeneration is cell autonomous. Heterologously expressed melanopsin from amphioxus (*Branchiostoma belcheri*) generated a meta state when illuminated with blue light, which could be driven back to the original state by orange light, behavior typical of bistable pigments (Koyanagi et al. 2005). Illumination of mouse melanopsin, heterologously expressed and loaded with 11-*cis*-retinal, produced a meta state with an absorption maximum close to that of the original photopigment but with an elevated extinction coefficient (Matsuyama et al. 2012). Retinoid analysis revealed a ratio of 11-*cis*-retinal/all-*trans*-retinal whose values depended on the light history of the pigment (dark, 95/5; light, 34/60). Because the extinction coefficients of the dark state and meta state differed, reducing the intensity of the illumination would presumably shift the photoequilibrium back toward that of the dark state. Does melanopsin of ipRGC depend on the known 11-*cis*-retinal-generating systems described for rods and cones? The answer to this question remains unknown, but several features of CRALBP expression and function suggest that the binding protein could be involved in a nonvisual photosensory process (Saari and Crabb 2005). (1) CRALBP expression was evident by embryonic day 13 in rat retina (De Leeuw et al. 1990), long before the visual system becomes functional, around postnatal day 8 in the mouse (Carter-Dawson et al. 1986). (2) Müller cells are elongated radial glial of the retina, which extend from the inner retina, where they are in proximity with inner retinal neurons including RGCs, to the outer retina where they are in close proximity to cone inner segments (Newman and Reichenbach 1996). CRALBP was found not only in the apical region of Müller cells, where it could function in cone visual pigment regeneration (Kaylor et al. 2013), but throughout Müller cell cytoplasm (Bunt-Milam and Saari 1983; Saari et al. 2001). (3) CRALBP isolated from bovine retina was saturated with a mixture of 11-*cis*-retinal and 11-*cis*-retinol (Saari et al. 1982), suggesting that the retinoid-binding protein could be a source of 11-*cis*-retinoids in the inner retina, for instance, for regeneration of melanopsin in ipRGC. It would be

interesting to examine the pupillary light response or other circadian responses of CRALBP knockout mice (Saari et al. 2001).

3.7 Parallels in Vertebrate and Invertebrate Visual Cycles

In spite of the widely divergent ocular systems of insects with their compound, rhabdomeric eyes, squid with their simple refracting rhabdomeric eyes, and vertebrates with their refracting ciliary-based eyes, several similar themes are noted in the molecular apparatus of their retinoid regeneration cycles. Some parallels appear to be dictated by the common chemistry and toxicity of the shared retinoid chromophore. Others likely reflect a common ancestral origin of the components.

3.7.1 General Remarks

Visual systems are unusual in that they are based on a poorly soluble, chemically reactive, and toxic molecule, 11-*cis*-retinal, as their chromophore, which is present in high concentrations (~5 mM in the outer segments of rods and cones) and that must move through aqueous compartments filled with proteins and lipids. Toxicity of excess retinal has been demonstrated in several visual systems (for instance, see Chen et al. 2012; Voolstra et al. 2010). In addition, in vertebrates all-*trans*-retinal can be further processed to retinoic acid, a potent signaling molecule (Al Tanoury et al. 2013). Thus, the choice of chromophore dictates several mechanisms to minimize toxic effects during storage and processing of retinoids and to prevent inadvertent activation of other signaling pathways based on retinoic acid.

3.7.2 Parallels

3.7.2.1 Retinoid-Binding Proteins

In addition to the opsins, photosensory systems employing retinoids feature water-soluble binding proteins to solubilize the hydrophobic lipids, to protect them from oxidative damage, to act as substrate delivery vehicles, and to facilitate transport between compartments or cells. The vertebrate visual system employs four retinoid-binding proteins. RBP4 (retinol-binding protein) delivers all-*trans*-retinol from the liver to RPE via the bloodstream (Vogel et al. 2002). Within RPE cells, CRBP1 acts as a high-affinity acceptor for all-*trans*-retinol and may deliver the retinoid to LRAT for esterification (Bunt-Milam and Saari 1983; Bok et al. 1984). CRALBP, with high affinity for both 11-*cis*-retinal and 11-*cis*-retinol, is present in RPE, where it accepts the 11-*cis*-retinol from RPE65 and facilitates its oxidation to

11-*cis*-retinal (Winston and Rando 1998; Saari et al. 1994; Saari and Crabb 2005; Stecher et al. 1999) and in Müller cells where it could accept 11-*cis*-retinol from isomerase 2 (Kaylor et al. 2013). IRBP is present in the IPM (Bunt-Milam and Saari 1983), where it presumably facilitates transfer of all-*trans*-retinol or 11-*cis*-retinal/ol between RPE and photoreceptors (Liou et al. 1982; Lai et al. 1982). Numerous studies in vitro of IRBP function suggest this role for the protein in vertebrate physiology (Carlson and Bok 1992; Crouch et al. 1992; Wu et al. 2007; Qtaishat et al. 2005). However, studies of the role of IRBP in living mice have been more equivocal. Recently, an impaired rate of retinoid transfer between neural retina and RPE was demonstrated in IRBP$^{-/-}$ mice backcrossed to 129/sv mice expressing wild-type RPE65 (Jin et al. 2009). Earlier reports of a lack of affect on the rate of rhodopsin regeneration (Palczewski et al. 1999; Ripps et al. 2000) in IRBP$^{-/-}$ mice were attributed to the use of a strain of IRBP$^{-/-}$ mice carrying a $^{Leu}450^{Met}$ mutation in RPE65 (Kolesnikov et al. 2011). However, the effects of IRBP on cone regeneration remain controversial with conflicting reports of the effect of IRBP on cone visual pigment regeneration (Kolesnikov et al. 2011; Parker et al. 2009). Flies employ a retinoid-binding protein (PINTA) to solubilize retinols. Interestingly, the sec14 lipid-binding domain is present in both PINTA and in CRALBP, probably reflecting common ancestry of the fly and vertebrate retinoid-binding proteins. In squid and other mollusks, the retinoid-binding RLBP couples the two photoisomerization proteins of this retina, rhodopsin and retinochrome, as has been described earlier. Continued analysis of invertebrates is likely to reveal other retinoid-binding proteins.

3.7.2.2 The Isomerization Problem

Pauling pointed out years ago that 11-*cis*-retinoids are sterically hindered because of overlap between the C-10 hydrogen and the C-13 methyl atoms (Pauling 1949). Based on the proportions of geometric isomers of retinal at equilibrium (I_2 catalysis), isomerization of all-*trans*-retinoids to 11-*cis*-retinoids requires the input of approximately 4.1 kcal/mol (Rando 1990). Thus, energy must be put into the system to generate 11-*cis*-retinal. The vertebrate solution to this is to couple the endergonic isomerization with an exergonic reaction, hydrolysis of all-*trans*-retinyl ester (Deigner et al. 1989). Invertebrates (*Drosophila*) use a similar strategy to generate 11-*cis*-retinal from carotenoid/xanthophyll precursors via oxidative cleavage of a double bond (Oberhauser et al. 2008). The amino acid sequence of isomerooxygenase from *Galleria meilonella* (great wax moth) is related to that of the RPE65, CMO1, and CMO2 of vertebrates and retains the four histidine residues that are critical for binding Fe^{2+} (Oberhauser et al. 2008). Invertebrates also rely on photoisomerization to generate 11-*cis*-retinal in which the energy of the photon (e.g., ~ 65 kcal/mol, green light) is greatly in excess of that required to overcome the energetic barrier to isomerization. Rando has argued that regeneration of 11-*cis*-retinoid takes place in vertebrates at the oxidation level of retinol, not retinal, because retinal does not contain a group that can be activated by a group transfer

reaction that allows coupling of energy yielding and energy requiring reactions (Rando 1990). The reliance on photoisomerases and bistable visual pigments thus introduces a simplification. Retinal does not need to be reduced to retinol, esterified, isomerized, and then oxidized back to retinal if isomerization can take place at the aldehyde level of oxidation via photoisomerization. Photoisomerases have been discovered in other organisms, including peropsin (Sun et al. 1997) and retinal G protein-coupled receptor (RGR)-opsin (Shen et al. 1994) in vertebrates and photoisomerases in flies (Pepe and Cugnoli 1992) and honey bees (Cugnoli et al. 1989). Their functions remain to be determined.

3.7.2.3 Compartmentalization of Visual Cycle Reactions in RPE

In the visual systems that have been examined at the molecular level, regeneration of 11-*cis*-retinoid occurs in a cell or compartment adjacent to the photoreceptor cell, RPE cells for vertebrate rods, Müller cells for vertebrate cones, retinal pigment cells for *Drosophila* (Wang and Montell 2005), and inner retina for squid (Hara and Hara 1991; Terakita et al. 1989). The reason for separation of the two processes is not known with certainty, but in the vertebrate retina it is probably related to the "inverted" retina, in which the choroidal blood circulation, the main source of vitamin A from hepatic stores, bathes the basal surface of the RPE. In addition, the presence of the retinoid-metabolizing reactions occurring within a pigmented cell reduces the chances for photooxidative damage.

3.7.2.4 Scaffold Proteins

Organizing proteins are often employed in multiprotein networks to provide a scaffold for the constituent proteins. In *Drosophila*, INAD (inactivation, no afterpotential D), a protein with five PDZ (postsynaptic density, discs large, zonula occludens-1) domains, binds rhodopsin (Rh1) and several components of the phototransduction cascade to form a unit that has been termed a signalplex (Li and Montell 2000; Tsunoda et al. 1997). No similar scaffold protein has been demonstrated in the vertebrate rod or cone outer segments. However, CRALBP has been demonstrated to bind to a PDZ-domain protein called sodium–hydrogen exchanger regulatory factor type 1 (NHERF1), also known as ERM (ezrin, radixin, moesin)-binding protein of 50 kDa (EBP50) (Nawrot et al. 2004). NHERF1 has two PDZ domains toward its amino terminus and an ezrin-binding domain toward its carboxyl terminus. Ezrin is a multidomain adaptor protein with a regulated actin-binding domain at its carboxyl-terminal end (Bretscher et al. 2002; Shenolikar and Weinman 2001). CRALBP, NHERF1, ezrin, and actin are all found within apical processes of RPE cells (Nawrot et al. 2004; Huang et al. 2009), indicating the potential for complex formation in vivo. A complex of CRALBP and NHERF was isolated by gel filtration following incubation of the two recombinant proteins in vitro (Nawrot et al. 2006). It has been speculating that complex formation may

localize CRALBP in the vicinity of acidic phospholipids of the RPE cell apical membrane (Saari et al. 2009). However, there is no evidence in support of this hypothesis. It would be interesting to examine the phenotype of CRALBP$^{-/-}$ mice expressing CRALBP missing its carboxyl-terminal PDZ-binding motif.

3.8 Concluding Remarks

There appear to be clear differences in the mechanisms of regeneration of 11-*cis*-retinoid between retinas that rely on rhabdomeric or ciliary visual pigments. Those that employ c-opsins rely on a dark, enzymatically catalyzed isomerization process. Those that employ r-opsins rely on photoreversibility of a stable meta state of the visual pigment and an independent photoisomerase. Outside the vertebrate retina, no dark isomerase has been characterized except for isomerooxygenase (NinaB), which is involved in de novo generation of chromophore from carotenoids and xanthophylls in *Drosophila* (Oberhauser et al. 2008). Similarities in reaction types and use of chaperones are largely dictated by the chemical properties of the retinoid chromophore. The toxicity of retinal and its potential for oxidation and generation of other signaling molecules (retinoic acids) require that chaperones bind and sequester the retinoid and that dehydrogenases are available to reduce it to the less toxic retinol. The limited solubility of retinoids requires chaperones to bind and protect the retinoids during transit between cellular and extracellular compartments. Finally, the energy requirement for 11-*cis*-retinoid production from all-*trans* precursors likely requires that the reaction take place at the oxidation level of retinol, where a cleavable, energy-yielding bond can be generated or via photoisomerization where the energy of light can be harnessed.

References

Al Tanoury Z, Piskunov A, Rochette-Egly C (2013) Vitamin A and retinoid signaling: genomic and non-genomic effects. J Lipid Res 54:1761–1775

Ala-Laurila P, Cornwall MC, Crouch RK, Kono M (2009) The action of 11-*cis*-retinol on cone opsins and intact cone photoreceptors. J Biol Chem 284:16492–16500

Albalat R (2012) Evolution of the genetic machinery of the visual cycle: a novelty of the vertebrate eye? Mol Biol Evol 29:1461–1469

Bailes HJ, Lucas RJ (2010) Melanopsin and inner retinal photorecepton. Cell Mol Life Sci 67:99–111

Bailes HJ, Lucas RJ (2013) Human melanopsin forms a pigment maximally sensitive to blue light (max 479 nm) supporting activation of Gq/11 and Gi/o signalling cascades. Proc Roy Soc B 280:20122987

Barry RJ, Canada FJ, Rando RR (1989) Solubilization and partial purification of retinyl ester synthetase and retinoid isomerase from bovine ocular pigment epithelium. J Biol Chem 264:9231–9238

Beharry S, Zhong M, Molday RS (2004) *N*-Retinylidene-phosphatidylethanolamine is the preferred retinoid substrate for the photoreceptor-specific ABC transporter ABCA4 (ABCR). J Biol Chem 279:53972–53979

Bernstein PS, Rando RR (1986) In vivo isomerization of all-*trans*- to 11-*cis*-retinoids in the eye occurs at the alcohol oxidation state. Biochemistry 25:6473–6478

Bernstein PS, Law WC, Rando RR (1987) Isomerization of all-*trans*-retinoids to 11-*cis*-retinoids in vitro. Proc Natl Acad Sci USA 84:1849–1853

Bok D, Ong DE, Chytil F (1984) Immunocytochemical localization of cellular retinol binding protein in the rat retina. Invest Ophthalmol Vis Sci 25:877–883

Boll F (1877) Zur anatomie und physiologie der retina. Arch Anat Physiol (Physiol Abt) 4–35. English translation: Hubbard R (1977) On the anatomy and physiology of the retina. Vision Res 17:1253–1265

Bretscher A, Edwards K, Fehon RG (2002) ERM proteins and merlin: integrators at the cell cortex. Nat Rev Mol Cell Biol 3:586–599

Bunt-Milam AH, Saari JC (1983) Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. J Cell Biol 97:703–712

Burstedt MSI, Forsman-Semb K, Golovleva I et al (2001) Ocular phenotype of Bothnia dystrophy, an autosomal recessive retinitis pigmentosa associated with an R234W mutation in the *RLBP1* gene. Arch Ophthalmol 119:260–267

Burstedt MSI, Sandgren O, Golovleva I, Wachtmeister L (2003) Retinal function in Bothnia dystrophy. An electrophysiological study. Vis Res 43:2559–2571

Carlson A, Bok D (1992) Promotion of the release of 11-*cis*-retinal from cultured retinal pigment epithelium by interphotoreceptor-retinoid binding protein. Biochemistry 31:9056–9062

Carter-Dawson L, Alvarez RA, Fong S-L et al (1986) Rhodopsin, 11-*cis*-vitamin A, and interstitial retinol-binding protein (IRBP) during retinal development in normal and *rd* mutant mice. Dev Biol 116:431–438

Chen Y, Okano K, Maeda T et al (2012) Mechanism of all-*trans*-retinal toxicity with implications for Stargardt disease and age-related macular degeneration. J Biol Chem 287:5059–5069

Crouch RK, Hazard ES, Lind T et al (1992) Interphotoreceptor retinoid-binding protein and alpha-tocopherol preserve the isomeric and oxidation state of retinol. Photochem Photobiol 56:251–255

Cugnoli C, Mantovani R, Fioravanti R, Pepe I (1989) 11-*cis*-retinal formation in the light catalyzed by a retinal-binding protein from the honeybee retina. FEBS Lett 257:63–67

Das SR, Bhardwaj N, Kjeldbye H, Gouras P (1992) Müller cells of chicken retina synthesize 11-*cis*-retinol. Biochem J 285:907–913

De Leeuw AM, Gaur VP, Saari JC, Milam AH (1990) Immunolocalization of cellular retinol-, retinaldehyde-, and retinoic acid-binding proteins in rat retina during pre- and postnatal development. J Neurocytol 19:253–264

Deigner PS, Law WC, Canada FJ, Rando RR (1989) Membranes as the energy source in the endergonic transformations of vitamin A to 11-*cis*-retinol. Science 244:968–971

Dowling JE (1960) Chemistry of visual adaptation in the rat. Nature (Lond) 188:114–118

Fain GL, Hardie R, Laughlin SB (2010) Phototransduction and the evolution of photoreceptors. Curr Biol 20(3):R114–R124

Goldstein EB (1970) Cone pigment regeneration in the isolated frog retina. Vis Res 10:1065–1068

Golovleva I, Bhattacharya S, Wu Z et al (2003) Disease-causing mutations in the cellular retinaldehyde-binding protein tighten and abolish ligand interactions. J Biol Chem 278:12397–12402

Hara T, Hara R (1965) New photosensitive pigment found in the retina of the squid *Ommastrephes*. Nature (Lond) 206:1331–1334

Hara T, Hara R (1976) Distribution of rhodopsin and retinochrome in the squid retina. J Gen Physiol 67:791–805

Hara T, Hara R (1991) Retinal-binding protein: function in a chromophore exchange system in the squid visual cell. In: Osborne N, Chader J (eds) Progress in retinal research, vol 10. Pergamon Press, Oxford, pp 179–206

Hattar S, Liao HW, Takao M et al (2002) Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 295:1065–1070

He X, Lobsiger J, Stocker A (2009) Bothnia dystrophy is caused by domino-like rearrangements in cellular retinaldehyde-binding protein mutant R234W. Proc Natl Acad Sci USA 106:18545–18550

Hillman P, Hochstein S, Minke B (1983) Transduction in invertebrate photoreceptors: role of pigment bistability. Physiol Revs 63:669–772

Huang J, Possin DE, Saari JC (2009) Localizations of visual cycle components in retinal pigment epithelium. Mol Vision 15:223–234

Hubbard R, St. George RCC (1958) The rhodopsin system of the squid. J Gen Physiol 41:501–522

Illing M, Molday LL, Molday RS (1997) The 220 kDa rim protein of retinal rod outer segments is a member of the ABC transporter superfamily. J Biol Chem 272:10303–10310

Jin M, Li S, Moghrabi WN et al (2005) RPE65 is the retinoid isomerase in bovine retinal pigment epithelium. Cell 122:449–459

Jin M, Songhua L, Nusinowitz S et al (2009) The role of interphotoreceptor retinoid-binding protein on the translocation of visual retinoids and function of cone photoreceptors. J Neurosci 29:1486–1495

Jones GJ, Crouch RK, Wiggert B, Cornwall MC (1989) Retinoid requirements for recovery of sensitivity after visual pigment bleaching in isolated photoreceptors. Proc Natl Acad Sci USA 86:9606–9610

Katagiri N, Terakita A, Shichida Y, Katagiri Y (2001) Demonstration of a rhodopsin-retinochrome system in the stalk eye of a marine gastropod, *Onchidium*, by immunocytochemistry. J Comp Neurol 433:380–389

Kaylor JJ, Yuan Q, Cook J et al (2013) Identification of DES1 as a vitamin A isomerase in Müller glial cells of the retina. Nat Chem Biol 9:3036

Kefalov VJ (2012) Rod and cone visual pigments and phototransduction through pharmacological, genetic, and physiological approaches. J Biol Chem 287:1635–1641

Kiser PD, Golczak M, Lodowski DT et al (2009) Crystal structure of native RPE65, the retinoid isomerase of the visual cycle. Proc Natl Acad Sci USA 106:17325–17330

Kiser PD, Golczak M, Maeda A, Palczewski K (2011) Key enzymes of the retinoid (visual) cycle in vertebrate retina. Biochim Biophys Acta 1821:137–151

Kiser PD, Golczak M, Palczewski K (2014) Chemistry of the retinoid (visual) cycle. Chem Rev 114:194–232

Kolesnikov AV, Tang PH, Parker RO et al (2011) The mammalian cone visual cycle promotes rapid M/L-cone pigment regeneration independently of the interphotoreceptor retinoid-binding protein. J Neurosci 31:7900–7909

Koyanagi M, Kubokawa K, Tsukamoto H et al (2005) Cephalochrodate melanopsin: evolutionary linkage between invertebrate visual cells and vertebrate photosensitive retinal ganglion cells. Curr Biol 15:1065–1069

Kühne W (1879) Chemische vorgange in der netzhaut. In: Hermann L (ed) Handbuch der Physiologie, Erster Theil: Gesichtssinn. Leipzig. English translation: Hubbard R (1977) Chemical processes in the retina. Vision Res 17:1269–1316

Kusakabe TG, Takimoto N, Jin M, Tsuda M (2009) Evolution and the origin of the visual retinoid cycle in vertebrates. Philos Trans R Soc B 364:2897–2910

Lai YI, Wiggert B, Liu YP, Chader GJ (1982) Interphotoreceptor retinol-binding proteins: possible transport vehicles between compartments of the retina. Nature (Lond) 298:848–849

Lamb TD, Pugh EN Jr (2004) Dark adaptation and the retinoid cycle of vision. Prog Retinal Eye Res 23:307–380

Lamb TD, Pugh EN Jr (2006) Phototransduction, dark adaptation, and rhodopsin regeneration. The Proctor Lecture. Invest Ophthalmol Vis Sci 47:5138–5152

Li HS, Montell C (2000) TRP and the PDZ protein, INAD, form the core complex required for retention of the signalplex in *Drosophila* photoreceptor cells. J Cell Biol 150:1411–1422
Liou GI, Bridges CDB, Fong S-L et al (1982) Vitamin A transport between retina and pigment epithelium: an interstitital protein carrying endogenous retinol (interstitial retinol-binding protein). Vision Res 22:1457–1467
Mata NL, Radu RA, Clemmons R, Travis GH (2002) Isomerization and oxidation of vitamin A in cone-dominant retinas. A novel pathway for visual-pigment regeneration in daylight. Neuron 36:69–80
Matsuyama T, Yamashita T, Imamoto Y, Shichida Y (2012) Photochemical properties of mammalian melanopsin. Biochemistry 51:5454–5462
McBee J, Kuksa V, Alvarez R et al (2000) Isomerization of all-*trans*-retinol to *cis*-retinol in bovine retinal pigment epitheilial cells: dependence on the specificity of retinoid-binding proteins. Biochemistry 39:11370–11380
Moiseyev G, Crouch RK, Goletz P et al (2003) Retinyl esters are the substrate for isomerohydrolase. Biochemistry 42:2229–2238
Moiseyev G, Chen Y, Takahashi Y et al (2005) RPE65 is the isomerohydrolase in the retinoid visual cycle. Proc Natl Acad Sci USA 102:12414–12418
Moiseyev G, Takahashi Y, Chen Y et al (2008) RPE65 from cone-dominant chicken is a more efficient isomerohydrolase compared with that from rod-dominant species. J Biol Chem 283:8110–8117
Montell C (2012) *Drosophila* visual transduction. Trends Neurosci 35:356–363
Muniz A, Betts BS, Trevino AR et al (2009) Evidence for two retinoid cycles in the cone-dominated chicken eye. Biochemistry 48:6854–6863
Murakami M, Kouyama T (2008) Crystal structure of squid rhodopsin. Nature (Lond) 453:363–367
Nawrot M, West K, Huang J et al (2004) Cellular retinaldehyde-binding protein interacts with ERM-binding phosphoprotein 50 in retinal pigment epithelium. Invest Ophthalmol Vis Sci 45:393–401
Nawrot M, Liu T, Garwin GG et al (2006) Scaffold proteins and the regeneration of visual pigments. Photochem Photobiol 82:1482–1488
Newman E, Reichenbach A (1996) The Müller cell: a functional element of the retina. Trends Neurosci 19:307–312
Noy N (2000) Retinoid-binding proteins: mediators of retinoid action. Biochem J 348:481–495
Oberhauser V, Voolstra O, Banget A et al (2008) NinaB combines carotenoid oxygenase and retinoid isomerase activity in a single polypeptide. Proc Natl Acad Sci USA 105:19000–19005
Ozaki K, Hara R, Hara T, Kakitani T (1983) Squid retinochrome. Configurational changes of the retinal chromophore. Biophys J 44:127–137
Ozaki K, Terakita A, Ozaki M et al (1994) Molecular characterization and functional expression of squid retinal-binding protein. J Biol Chem 269:3838–3845
Palczewski K, Van Hooser JP, Garwin GG et al (1999) Kinetics of visual pigment regeneration in excised mouse eyes and in mice with a targeted disruption of the gene encoding interphotoreceptor retinoid-binding protein or arrestin. Biochemistry 38:12012–12019
Parker RO, Crouch RK (2010) Retinol dehydrogenases (RDHs) in the visual cycle. Exp Eye Res 91:788–792
Parker RO, Fan J, Nickerson JM et al (2009) Normal cone function requires the interphotoreceptor retinoid-binding protein. J Neurosci 29:1416–1421
Pauling L (1949) Zur *cis-trans*-Isomerisierung von Carotinoiden. Helv Chim Acta 32:2241–2246
Pepe IM, Cugnoli C (1992) Retinal photoisomerase: role in invertebrate visual cells. J Photochem Photobiol B 13:5–17
Poliakov E, Parikh T, Ayele M et al (2011) Aromatic lipophilic spin traps effectively inhibit RPE65 isomerohydrolase activity. Biochemistry 50:6739–6741
Poliakov E, Gubin AN, Stearn O, Li Y, Compos MM, Gentleman S, Rogozin IB, Redmond TM (2012) Origin and evolution of retinoid isomerization machinery in vertebrate visual cycle: hint from jawless vertebrates. PLOS One 7:e49976. doi:10.1371/journal.pone.0049975

Provencio I, Rodriguez IR, Jiang G et al (2000) A novel human opsin in the inner retina. J Neurosci 20:600–605

Qtaishat NM, Wiggert B, Pepperberg DR (2005) Interphotoreceptor retinoid-binding protein (IRBP) promotes the release of all-*trans*-retinol from the isolated retina following rhodopsin bleaching illumination. Exp Eye Res 81:455–463

Rando RR (1990) The chemistry of vitamin A and vision. Angew Chem Int Ed Engl 29:461–480

Redmond TM (2009) Focus on molecules: RPE65, the visual cycle retinol isomerase. Exp Eye Res 88:846–847

Redmond TM, Poliakov E, Yu S et al (2005) Mutation of key residues of RPE65 abolishes its enzymatic role as isomerohydrolase in the visual cycle. Proc Natl Acad Sci USA 102:13658–13663

Redmond TM, Poliakov E, Kuo S et al (2010) RPE65, visual cycle retinol isomerase, is not inherently 11-*cis*-specific. J Biol Chem 285:1919–1927

Ripps H, Peachey NS, Xu X et al (2000) The rhodopsin cycle is preserved in IRBP "knockout" mice despite abnormalities in retinal structure and function. Vis Neurosci 17:97–105

Rodieck RW (1998) The first steps in seeing. Sinauer, Sunderland

Saari JC (2012) Vitamin A metabolism in rod and cone visual cycle. Annu Rev Nutr 32:125–145

Saari JC, Bredberg L (1982) Enzymatic reduction of 11-*cis*-retinal bound to cellular retinal-binding protein. Biochim Biophys Acta 716:266–272

Saari JC, Bredberg DL (1987) Photochemistry and stereoselectivity of cellar retinaldehyde-binding protein from bovine retina. J Biol Chem 262:7618–7622

Saari JC, Bredberg DL (1988) CoA- and non-CoA-dependent retinol esterification in retinal pigment epithelium. J Biol Chem 263:8084–8090

Saari JC, Bredberg DL (1989) Lecithin:retinol acyltransferase in retinal pigment microsomes. J Biol Chem 264:8636–8640

Saari JC, Crabb JW (2005) Focus on molecules: cellular retinaldehyde-binding protein (CRALBP). Exp Eye Res 81:245–246

Saari JC, Bredberg L, Garwin GG (1982) Identification of the endogenous retinoids associated with three cellular retinoid-binding proteins from bovine retina and retinal pigment epithelium. J Biol Chem 257:13329–13333

Saari JC, Bunt-Milam AH, Bredberg DL, Garwin GG (1984) Properties and immunocytochemical localization of three retinoid-binding proteins from bovine retina. Vision Res 24:1595–1603

Saari JC, Bredberg DL, Noy N (1994) Control of substrate flow at a branch in the visual cycle. Biochemistry 33:3106–3112

Saari JC, Huang J, Asson-Batres MA et al (1995) Evidence of retinoid metabolism within cells of inner retina. Exp Eye Res 60:209–212

Saari JC, Nawrot M, Kennedy BN et al (2001) Visual cycle impairment in cellular retinaldehyde-binding protein (CRALBP) knockout mice results in delayed dark adaptation. Neuron 29:739–748

Saari JC, Nawrot M, Stenkamp RE et al (2009) Release of 11-*cis*-retinal from cellular retinaldehyde-binding protein by acidic lipids. Mol Vision 15:844–854

Sarfare S, Zhmad ST, Joyce MV et al (2005) The Drosophila *ninaG* oxidoreductase acts in visual pigment chromophore production. J Biol Chem 280:11895–11901

Schemer J (1984) Renewal of visual pigment in photoreceptors of the blowfly. J Comp Physiol A 154:535–547

Schertler GFX (2008) The rhodopsin story continued. Nature (Lond) 453:292–293

Schwemer J, Pepe IM, Paulsen R, Cugnoli C (1984) Light-induced *trans-cis* isomerization of retinal by a protein from honeybee retina. J Comp Physiol A 154:549–554

Sexton TJ, Golczak M, Palczewski K, Van Gelder RN (2012) Melanopsin is highly resistant to light and chemical bleaching *in vivo*. J Biol Chem 287:20888–20897

Sha B, Phillips SE, Bankaitis VA, Luo M (1998) Crystal structure of the *Saccharomyces cerevisiae* phosphatidyl-transfer protein. Nature (Lond) 391:506–510

Shen D, Jiang M, Hao W, Salazar M, Fong HKW (1994) A human opsin-related gene that encodes a retinaldehyde-binding protein. Biochemistry 33:13117–13125

Shenolikar S, Weinman J (2001) NHERF: targeting and trafficking membrane proteins. Am J Physiol 280:F389–F395

Smith WC, Goldsmith TH (1991) The role of retinal photoisomerase in the visual cycle of the honeybee. J Gen Physiol 97:143–165

Stecher H, Gelb MH, Saari JC, Palczewski K (1999) Preferential release of 11-*cis*-retinol from retinal pigment epithelial cells in the presence of cellular retinaldehyde-binding protein. J Biol Chem 274:8577–8585

Sun H, Nathans J (1997) Stargardt's ABCR is localized to the disc membrane of retinal rod outer segments. Nat Genet 17:15–16

Sun H, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J (1997) Peropsin, a novel visual pigment-like protein located in the apical microvilli. Proc Natl Acad Sci USA 94:9893–9898

Takahashi Y, Moiseyev G, Chen Y et al (2011) An alternative isomerohydrolase in the retinal Müller cells of a cone-dominant species. FEBS J 278:2913–2926

Takimoto N, Kusakabe T, Tsuda M (2007) Origin of the vertebrate visual cycle. Photochem Photobiol 83:242–247

Tang PH, Buhusi MC, Ma JX, Crouch RK (2011) RPE65 is present in human green/red cones and promotes photopigment regeneration in an in vitro cone cell model. J Neurosci 31:18618–18626

Tang PH, Kono M, Koutalos Y et al (2013) New insights into retinoid metabolism and cycling within the retina. Prog Retin Eye Res 32:48–63

Terakita A (2005) The opsins. Genome Biol 6:213

Terakita A, Hara R, Hara T (1989) Retinal-binding protein as a shuttle for retinal in the rhodopsin-retinochrome system of the squid visual cells. Vision Res 29:639–652

Thompson DA, Gal A (2003) Vitamin A metabolism in the retinal pigment epithelium: genes, mutations, and diseases. Prog Retin Eye Res 22:683–703

Travis GH, Golczak M, Moise AR, Palczewski K (2006) Diseases caused by defects in the visual cycle: retinoids as potential therapeutic agents. Annu Rev Pharmacol Toxicol 47:469–512

Trehan A, Cañada FJ, Rando RR (1990) Inhibitors of retinyl ester formation also oprevent the biosynthesis of 11-*cis*-retinol. Biochemistry 29:309–312

Tsukamoto H, Terakita A (2010) Diversity and functional properties of bistable pigments. Photochem Photobiol Sci 9:1435–1443

Tsunoda S, Sierralta J, Sun Y et al (1997) A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. Nature (Lond) 388:243–249

Vogel S, Piantedosi R, O'Byrne SM et al (2002) Retinol-binding protein-deficient mice: biochemical basis for impaired vision. Biochemistry 41:15360–15368

Voolstra O, Oberhauser V, Sumser E et al (2010) NinaB is essential for *Drosophila* vision but induces retinal degeneration in opsin-deficient photoreceptors. J Biol Chem 285:2130–2139

Wald G (1935) Carotenoids and the visual cycle. J Gen Physiol 19:351–371

Wald G (1968) Molecular basis of visual excitation. Science 162:230–239

Wang JS, Kefalov VJ (2011) The cone-specific visual cycle. Prog Retin Eye Res 30:115–128

Wang T, Montell C (2005) Rhodopson formation in *Drosophila* is dependent on the PINTA retinoid-binding protein. J Neurosci 25:5286–5194

Wang X, Wang T, Jiao Y, von Lintig J, Montell C (2010) Requirement for an enzymatic visual cycle in *Drosophila*. Curr Biol 20:93–102

Wang X, Wang T, Ni JD, von Lintig J, Montell C (2012) The *Drosophila* visual cycle and *de novo* chromophore synthesis depends on *rdhB*. J Neurosci 32:3485–3491

Winston A, Rando RR (1998) Regulation of isomerohydrolase activity in the visual cycle. Biochemistry 37:2044–2050

Wu Q, Blakeley LR, Cornwall MC et al (2007) Interphotoreceptor retinoid-binding protein is the physiologically relevant carrier that removes retinol from rod photoreceptor outer segments. Biochemistry 46:8669–8679

Yau K-W, Hardie RG (2009) Phototransduction motifs and variations. Cell 139:246–264

Chapter 4
Molecular Mechanism of Adaptation in Vertebrate Rods

Ala Morshedian and Gordon L. Fain

Abstract Steady background light and bright bleaching illumination produce changes in rod photoreceptor sensitivity and response waveform that are collectively known as adaptation. In this chapter, we review the phenomenology of both background and bleaching adaptation. We then describe the evidence for our present understanding of the molecular mechanisms of adaptation in vertebrate rods to both background light and bleaching, including the role of Ca^{2+} as a second messenger and modulation of guanylyl cyclase and phosphodiesterase. We also describe continuing areas of uncertainty awaiting resolution from future experimentation.

Keywords Adaptation • Calcium • Retina • Rod • Transduction • Vision

4.1 Introduction

Stimulation of a sensory receptor produces an electrical response that is communicated to higher levels of the nervous system to signal detection (Fain 2003). Receptor responses are graded, which means that their amplitudes increase in proportion to the intensity of the stimulus. The magnitude of the proportionality constant varies as the mean level of stimulation is altered. This process, known as sensory adaptation, is a time-dependent change in the sensitivity or responsiveness

A. Morshedian
Department of Integrative Biology and Physiology, University of California, 2045 Terasaki Life Sciences Building, Los Angeles 90095-7239, CA, USA

G.L. Fain (✉)
Department of Integrative Biology and Physiology, University of California, 2121 Terasaki Life Sciences Building, Los Angeles 90095-7239, CA, USA

Department of Ophthalmology and Jules Stein Eye Institute, University of California, Los Angeles 90095, CA, USA
e-mail: gfain@ucla.edu

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_4,

of the receptor in the presence of a constant stimulus. It produces an alteration in the amplitude of the response to constant stimulation as well as to any stimulus given in the presence of the maintained stimulus. Adaptation modulates the amount of neurotransmitter released onto postsynaptic neurons and regulates the message conveyed to the central nervous system (CNS).

Vertebrate photoreceptors respond to changes in light intensity over an enormous range of ambient light levels. Rod cells, which are responsible for detection of dim light, can signal the absorption of a single photon of visible light by their photopigment rhodopsin (Baylor et al. 1979). These same cells can adapt to brighter light and remain responsive at intensities that activate more than 1,000 rhodopsins per second (for example, see Mendez et al. 2001; Woodruff et al. 2008). The adaptation of photoreceptors in the presence of constant background illumination is called background adaptation. Photoreceptors also adapt after they are exposed to light bright enough to bleach a significant fraction of their visual pigment. The decrease in sensitivity after such bright light exposure is called "bleaching adaptation" and resembles in many respects the adaptation produced by background light. Intact rods slowly recover their sensitivity after bleaching as the photopigment is regenerated, a phenomenon known as dark adaptation. Background and bleaching adaptation are collectively referred to as light adaptation. The mechanisms of light adaptation have been extensively investigated, and many of these earlier studies have been previously reviewed (Fain et al. 2001; Lamb and Pugh 2004; Fain 2011; Reuter 2011; Arshavsky and Burns 2012; Kefalov 2012).

In this chapter, we summarize our present knowledge of the molecular mechanism of adaptation in rod photoreceptors, emphasizing more recent results on mammalian (mouse) rods. A summary of visual transduction, fundamental to our understanding of adaptation, is given first. We then describe the regulation of key components of the transduction cascade likely to be responsible for the modulation of sensitivity.

4.2 Molecular Mechanism Visual Transduction

One of the hallmarks of phototransduction in rods is its extreme sensitivity. This sensitivity is achieved through a highly regulated, multistep amplification process that results in a significant increase in signal gain (Fain 2003). Transduction begins with an opsin protein molecule that contains 11-*cis* retinal, a small organic molecule called the chromophore. The opsin and chromophore together make up the rhodopsin photopigment. This pigment has a broad absorption spectrum peaking, in a mammalian rod, at wavelengths near 500 nm. When a molecule of rhodopsin absorbs a photon of light, 11-*cis* retinal undergoes photoisomerization to all-*trans* retinal, which in turn causes a change in the conformation of the opsin molecule (Choe et al. 2011; Standfuss et al. 2011) to produce a photoproduct called meta II or Rh*, which triggers visual excitation (Fig. 4.1).

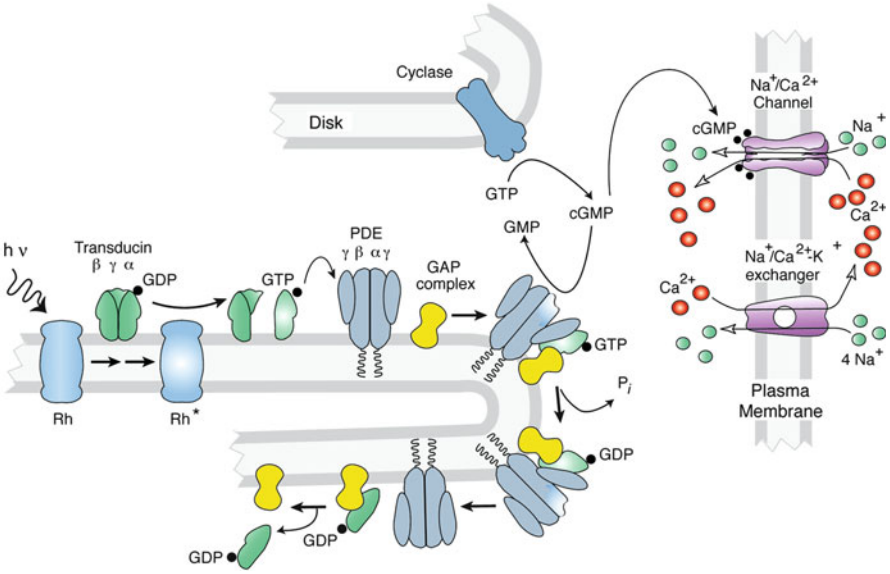

Fig. 4.1 G-protein transduction cascade of vertebrate rod (see text)

Rh* then binds to and activates a heterotrimeric G protein called transducin, whose α-subunit is a member of the α_i/α_o family. Binding of transducin to Rh* causes an exchange of GTP for GDP on the transducin-α guanosine-binding site, producing activated transducin-α or Tα·GTP. Each Rh* can produce many Tα·GTPs, which bind to and activate the effector enzyme phosphodiesterase 6 (PDE6). The PDE6 then hydrolyzes many cytoplasmic cGMP molecules in the next phase of amplification. PDE6 continues to hydrolyze and decrease the internal concentration of cGMP for as long as transducin remains in its active GTP-bound state. The reduction in concentration of cGMP via light-activated PDE6 results in the closure of cGMP-gated cation channels in the plasma membrane, which reduces the permeability of the rod membrane to Na^+, hyperpolarizes the membrane potential, and decreases the release of the synaptic transmitter glutamate from the photoreceptor synapse.

The recovery of the light response is achieved when all the activated components of the cascade are turned off and the concentration of intracellular cGMP is returned to its pre-stimulus level. Activated rhodopsin is extinguished by phosphorylation and binding of arrestin, which prevents further binding of transducin. Phosphodiesterase remains active until Tα·GTP is hydrolyzed to Tα·GDP. Although Tα can hydrolyze bound GTP without assistance, the rate is quite slow and is greatly accelerated by a complex of proteins called the GTPase-activating proteins or GAPs, of which there are three in photoreceptors: RGS9-1 (He et al. 1998), Gβ5L (Makino et al. 1999; He et al. 2000) and the R9AP protein, which anchors the GAP complex to the disc membrane (Hu and Wensel 2002). Tα·GDP then recombines with the G-protein beta and gamma subunits to form the inactive heterotrimer.

The reopening of the cGMP-gated channels requires restoration of the cGMP concentration, which is achieved by the activity of an outer segment, membrane-bound guanylyl cyclase. The activity of guanylyl cyclase is regulated by the cytoplasmic free Ca^{2+} concentration (Koch and Stryer 1988) by means of small molecular weight, Ca^{2+}-binding proteins called guanylyl cyclase-activating proteins (GCAPs) (Polans et al. 1996; Dizhoor 2000). This regulation occurs in the following way. The closing of the cGMP-gated channels in the light produces a decreases in Ca^{2+} concentration in the rod outer segment. In a mouse rod the concentration declines from about 250 nM in darkness to about 25 nM in light bright enough to close all the channels, a range of concentration of about tenfold (Woodruff et al. 2002). In the dark, when the free Ca^{2+} concentration is high, the Ca^{2+}-bound form of GCAP inhibits the cyclase. The decrease in free Ca^{2+} concentration produced by the closing of the cyclic nucleotide-gated channels causes Ca^{2+} to be released from the GCAPs, greatly increasing the rate of synthesis of cGMP. The Ca^{2+} dependence of the cyclase produces a feedback loop: light decreases cGMP, closes the channels, and decreases the Ca^{2+} concentration, and the decrease in Ca^{2+} then accelerates the cyclase and increases cGMP, counteracting the decrease in cGMP concentration produced by light. When the activity of the cyclase restores the cGMP concentration to its pre-stimulus level, the channels rapidly reopen (Karpen et al. 1988).

4.3 Background Adaptation

A single Rh* has been estimated to produce approximately 100 activated Tα·GTPs in an amphibian rod (Leskov et al. 2000; Makino et al. 2003) and perhaps closer to 20 in a mouse rod (Krispel et al. 2006). Each Tα·GTP activates a single catalytic subunit of PDE6, and each activated PDE6 catalytic subunit hydrolyzes several hundred molecules of cGMP per second. A single photon response at its peak, which occurs in a mouse rod about 250 ms after light absorption, can close about 5 % of the channels. Assuming photoreceptors simply summed the effects of each photon absorbed by rhodopsin molecules, fewer than 100 activated rhodopsins per second would lead to the closure of all the light-sensitive channels and would render the rods unresponsive to further illumination.

To prevent saturation, the rod response during exposure to continuous light immediately rises to a peak and then partially recovers. This relaxation is the result of reopening of some of the previously closed channels and is particularly evident at brighter intensities. Figure 4.2a demonstrates this phenomenon in suction-electrode recordings from the outer segments of mouse rods exposed to prolonged illumination. The rods adapt to the steady light; some of the channels begin to reopen, resulting in an increase in inward current. This process occurs in two phases: an initial rapid relaxation, followed by a more prolonged sag in current. Both the rapid and the slow decays can be fitted by exponential functions with time constants in mouse rods of a few hundred milliseconds and a few tens of seconds.

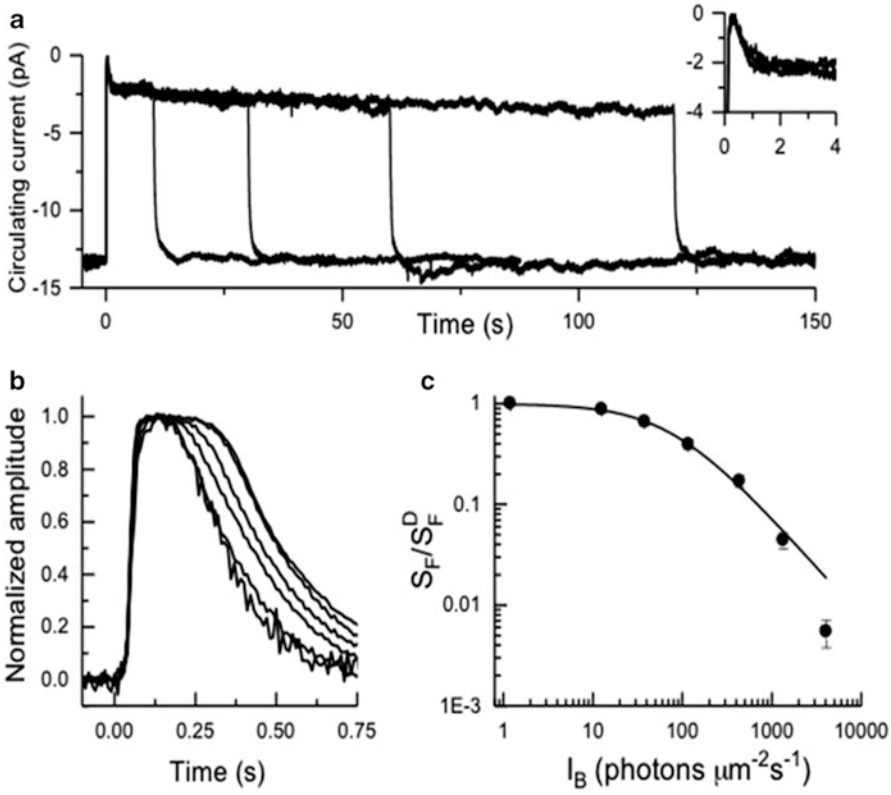

Fig. 4.2 Adaptation of wild-type (WT) mouse rods. (**a**) Superimposed suction-electrode recordings of means of currents of seven WT rods, each exposed three times to steps of 500-nm light beginning at $t = 0$, of intensity 440 photons μm^{-2} s^{-1} and 10, 30, 60, and 120 s in duration. *Inset*: Same data at faster time resolution showing only first 4 s. (**b**) Flash responses recorded in background light. Means from five presentations each of ten WT rods to 20-ms flashes of 500-nm light beginning at $t = 0$; intensity was kept at 453 photons μm^{-2} for flashes in darkness and in backgrounds of the following intensities (in photons μm^{-2} s^{-1}): 12, 38, 118, 438, and 1,354. Responses have been normalized cell by cell to the peak amplitude of the response and averaged. The most slowly decaying response is with no background; decay time course progressively accelerated as background intensity was increased. (**c**) Weber–Fechner plot. Sensitivity (S_F) in pA photon^{-1} μm^2 was calculated as the peak amplitude of the response in the linear range divided by the flash intensity. Graph gives mean sensitivity divided by sensitivity in the absence of a background (S_F^D) as a function of background intensity (I_B), averaged from ten WT rods. *Solid line* is given by Eq. (4.1) with a best-fitting value of $I_0 = 77$ photons μm^{-2} s^{-1}. [Reprinted from Fain (2011) with permission of Springer US]

When rods are stimulated with a brief flash, first in darkness and then in the presence of continuous background light, the amplitude of the response to the flash becomes smaller in the presence of the background, reflecting a decrease in photoreceptor sensitivity (e.g., Baylor and Hodgkin 1974; Woodruff et al. 2008). Moreover, the time course of decay is accelerated, such that the brighter the background, the greater the acceleration. Figure 4.2b shows normalized responses

to 20-ms flashes of constant intensity, which have been superimposed. The most slowly decaying response is the one recorded in the absence of a background light, and the other responses were recorded in progressively increasing background intensities. The decay of the flash response, at least for small-amplitude responses, follows a time course well fitted by a single exponential decay function, whose time constant is given as τ_{REC}. In a dark-adapted mouse rod, τ_{REC} has a value of about 200 ms, but this value decreases by a factor of 2 to about 100 ms in bright background light. A similar decrease occurs in the limiting time constant of response decay τ_D (Woodruff et al. 2008), indicating an acceleration in the rate of decay of light-activated PDE6 (Krispel et al. 2006; Tsang et al. 2006; Chen et al. 2010a). Response decay is slow in the dark to permit the cell to acquire and simultaneously sum as many photons as possible to maximize sensitivity. In brighter light, an abundance of photons allows rods to afford some reduction in the time over which the photoreceptors sum single photons, so that the photoreceptor can more readily detect change and motion. The acceleration of the time course of decay is partly responsible for the well-known increase in rod flicker-fusion frequency in background light.

For responses to brief flashes in the presence of a continuous background, the sensitivity decreases so that a brighter flash intensity is required to produce responses of same amplitude. In Fig. 4.2c, we show for mouse rods the sensitivity to a brief flash, S_F, divided by the sensitivity in darkness, S_F^D. Sensitivities were calculated by dividing the response to a flash (in pA of current) by the flash intensity (in photons μm^{-2}). The curve fitted to the data is known as the Weber–Fechner relationship:

$$\frac{S_F}{S_F^D} = \frac{I_0}{I_0 + I_B} \tag{4.1}$$

where I_B is the intensity of the background and I_0 is a constant called the dark light, which in Fig. 4.2c was 77 photons μm^{-2} s^{-1} or between 35 and 40 rhodopsin molecules bleached per second.

4.4 Bleaching Adaptation

In the experiments of Fig. 4.2, the changes in steady inward current, response decay time, and sensitivity all occurred in relatively dim light; the brightest light would have bleached much less than 1 % of the total amount of visual pigment. Once even the brightest background light used in the experiments of Fig. 4.2 was turned off, the rods rapidly recovered to their dark-adapted sensitivity. With brighter light exposures that bleach a significant fraction of the visual pigment, significant adaptation can occur even after the light has been extinguished. Some of the

decrease in sensitivity after bleaching is simply the result of a decrease in the concentration of the photopigment, which reduces the "quantum catch" or probability of absorption of a photon. A significant sensitivity decrease is, however, also produced by a mechanism much like the one that decreases sensitivity in background light.

Stiles and Crawford (1932) first suggested that light bright enough to produce significant bleaching can lead to photoreceptor desensitization by producing an "equivalent" background light generated by the bleached pigment, which would then persist until the pigment is regenerated (Barlow 1964). One way of exploring the effect of bleaching and the equivalent background is to expose a rod, isolated from the rest of the retina and pigment epithelium, to light bleaching a significant fraction of the rhodopsin; and then to wait from 30 to 60 min for the circulating current and sensitivity of the rod to come to steady state. A rod isolated in this way can regenerate only a small fraction of its rhodopsin; as a consequence, large bleaches produce a significant amount of opsin without chromophore, which can act as an equivalent background and stimulate the visual cascade (Cornwall and Fain 1994).

The responses in Fig. 4.3 (from Nymark et al. 2012) were recorded from mouse rods in darkness and in steady state after bleaching from 5 % to 90 % of the photopigment. Bleaching produces a sustained decrease in circulating current caused by the closing of the cGMP-gated channels in the outer segment, very similar to that produced by continuous background light exposure. This decrease in circulating current is reflected in the progressive decrease in the maximum amplitude of the response to flashes in the records of Fig. 4.3. Bleaching also produces an acceleration in the decay of the response. In darkness, a half-maximal response requires approximately 1 s to return to baseline (Fig. 4.3a), whereas a half-maximal response after a 90 % bleach returns to baseline in about half the time (Fig. 4.3f).

Bleaches also produce a decrease in sensitivity. In Fig. 4.4 (also from Nymark et al. 2012), we have plotted on the ordinate the sensitivity as a fraction of sensitivity in darkness, as in Fig. 4.2c but now from rods at steady state after exposure to bleaches; and we have plotted on the abscissa the fraction of pigment bleached. The dashed line is the theoretical decrease in sensitivity expected only from the loss of quantum catch: when 90 % of the pigment is bleached, the sensitivity is decreased by a factor of ten because the rod has only one-tenth of its pigment remaining. The data lie uniformly above the dashed line, indicating that some additional process is reducing the sensitivity of the rods.

For the solid line in Fig. 4.4, we assumed that the decrease in sensitivity consists of two components, one caused by the loss of quantum catch and an extra component from adaptation produced by activation of the cascade by the bleached pigment (Jones et al. 1996). Because the relative loss in sensitivity produced by the loss in quantum catch is equal simply to $(1-F)$, the fraction of pigment remaining, we calculated the component from cascade activation, $\Delta S_F/S_F^D$,

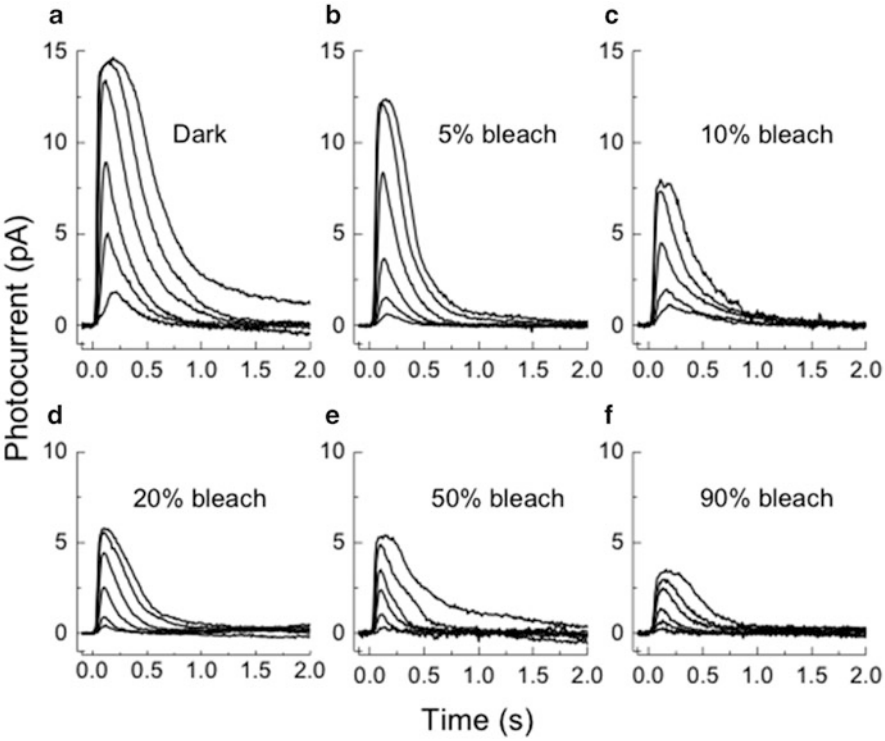

Fig. 4.3 Responses of mouse rods before and after exposure to bright bleaching light. Flashes were 20 ms in duration. (**a**) Averages of 18 dark-adapted rods. Flash intensities: 4, 17, 43, 159, 453, and 1,122 photons μm^{-2}. (**b**) Averages of 12 rods at steady state after bleach of 5 % of photopigment. Flash intensities: 4, 17, 43, 159, 453, and 1,122 photons μm^{-2}. (**c**) Averages of 8 rods at steady state after bleach of 10 % of photopigment. Flash intensities: 17, 43, 159, 453, and 1,122 photons μm^{-2}. (**d**) Averages of 28 rods at steady state after bleach of 20 % of photopigment. Flash intensities: 17, 43, 159, 453, 1,120, and 1,870 photons μm^{-2}. (**e**) Averages of 6 rods at steady state after bleach of 50 % of photopigment. Flash intensities: 43, 159, 453, 1,120, 3,250, and 10,500 photons μm^{-2}. (**f**) Averages of 7 rods at steady state after bleach of 90 % of photopigment. Flash intensities: 159, 453, 1,120, 2,430, 4,230, and 10,500 photons μm^{-2}. [Reprinted from Nymark et al. (2012) with permission of the Physiological Society]

by removing the component caused by loss in quantum catch from the total loss in sensitivity by dividing by $(1-F)$:

$$\frac{\Delta S_F}{S_F^D} = \frac{S_F}{S_F^D(1-F)} \tag{4.2}$$

Following Jones et al. (1996), we then assumed that bleached pigment activates the cascade as does background light, that is, according to the Weber–Fechner relationship of Eq. (4.1). Moreover, we assumed that activation of transduction is linearly proportional to the fraction bleached, that is, to the opsin concentration

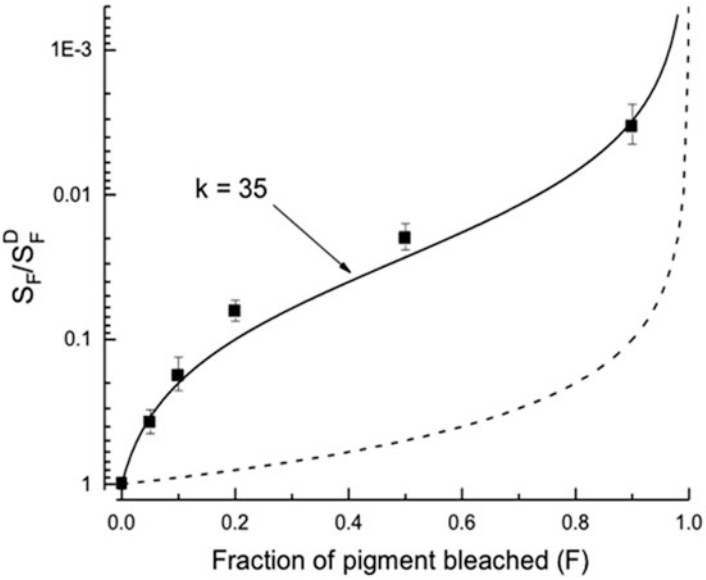

Fig. 4.4 Sensitivity of mouse rods as a function of percent pigment bleached (calculated as in Fig. 4.2). Means with SE are plotted as a function of percent bleached for rods that were dark adapted ($n = 38$) or at steady state after bleaches of 5 % ($n = 11$), 10 % ($n = 9$), 20 % ($n = 26$), 50 % ($n = 6$), and 90 % ($n = 7$). Continuous curve is Eq. (4.4) with best-fitting value of k of 35. *Dashed curve* is change in sensitivity predicted by loss of quantum catch ($S_F/S_D^F = 1 - F$). [Reprinted from Nymark et al. (2012) with permission of the Physiological Society]

(Cornwall and Fain 1994), with a proportionality constant given by k. From these assumptions and Eq. (4.1), the decrease in sensitivity ΔS_F should be given by

$$\frac{S_F^D}{\Delta S_F} - 1 = kF \tag{4.3}$$

Combining Eqs. (4.2) and (4.3), we have for the total decrease in sensitivity after a bleach

$$\frac{S_F}{S_F^D} = \frac{1 - F}{1 + kF} \tag{4.4}$$

This relationship is the solid curve in Fig. 4.4 with a best-fitting value of k of 35.

The results in Fig. 4.4 show that desensitization after bleaching is a linear combination of the loss in quantum catch and adaptation produced by an equivalent background whose intensity is proportional to the fraction of pigment bleached. In an isolated rod at steady state, the equivalent background is produced by opsin (Cornwall and Fain 1994), probably phosphorylated and bound to arrestin. In an intact rod, other intermediates of bleaching can also contribute to the equivalent

background, including (for small bleaches) reversion of the bleached pigment to Rh* and the binding of all-*trans* retinal to opsin (see Fain et al. 2001; Lamb and Pugh 2004). An additional component of desensitization can also be produced by translocation of transducin from the outer segment to the inner segment during bright light exposure, which reduces the gain of transduction (Sokolov et al. 2002; Majumder et al. 2013). The translocation of transducin is, however, unlikely to make any significant contribution to background adaptation, because translocation takes many minutes to complete and the light intensity required to produce significant migration of the protein is orders of magnitude brighter than the intensities used for the experiments of Fig. 4.2.

4.5 Calcium as the Second Messenger of Adaptation

Although it would be possible to imagine a mechanism of adaptation that was local and restricted, several observations indicate that the signal for the change in sensitivity can spread throughout the rod outer segment. Rods are desensitized by very dim illumination. The background intensity required to reduce sensitivity by half is given by I_0 in Eq. (4.1) and is only 35–40 Rh* per second in the mouse (see Fig. 4.2c) and 5–10 Rh* per second in amphibians (Fain 1986). The change in sensitivity occurs rather quickly after turning on the background light. Our experiments (in preparation) show that for background light even as dim as 12 photons $\mu m^{-2}\ s^{-1}$ the decay of the flash response begins to be accelerated within 500 ms of the beginning of the background exposure. Because only a few tens of excited pigment molecules are sufficient to reduce sensitivity by a factor of two, and rods have of the order of 1,000–2,000 disks, some molecule must diffuse between the disks so that a pigment molecule bleached in one disk can affect the response of pigment molecules subsequently bleached in other disks. Outer segment current measurements with spatially localized adapting lights and test stimuli also indicate that the change in sensitivity can migrate up and down the outer segment (Lamb et al. 1981; Cornwall et al. 1990; Gray-Keller et al. 1999).

Deric Bownds (1980) first proposed that steady light causes a reduction in intracellular calcium concentration, which in some way regulates components of the transduction cascade to produce desensitization of the photoreceptor response. The role of Ca^{2+} in adaptation was first tested by Torre and collaborators (Torre et al. 1986), who incorporated the Ca^{2+} chelator BAPTA into rods with whole-cell patch recording and showed that an increase in Ca^{2+} buffering resulted in an increase in the maximum amplitude of the light response and a slowing of its decay—the inverse of the effect of background light. Stronger evidence of a role of Ca^{2+} was obtained by perfusing rods with an external solution in which Ca^{2+} was buffered to a low level (to prevent Ca^{2+} influx) and Na^+ was substituted with Li^+ or guanidinium (to block Na^+/K^+–Ca^{2+} exchange and prevent Ca^{2+} efflux). In such a low-Ca^{2+}/zero-Na^+ solution, the outer segment Ca^{2+} concentration is kept nearly constant for at least about 10 s (Matthews and Fain 2001), and, within this

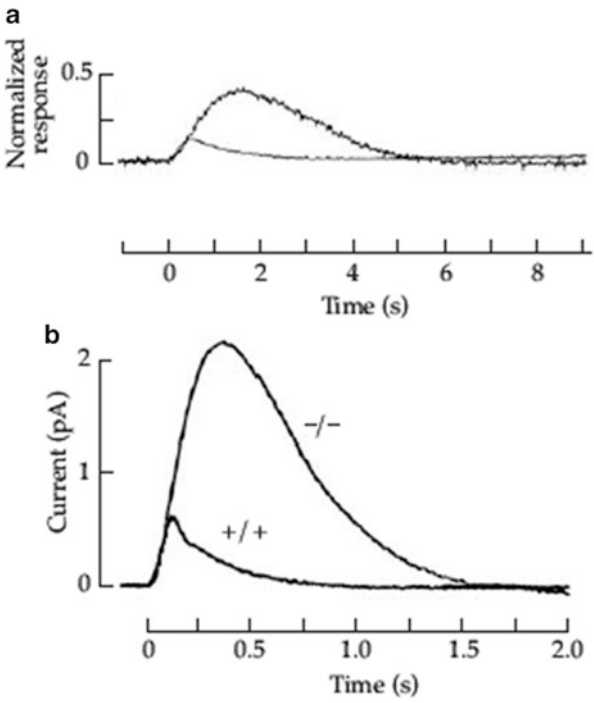

Fig. 4.5 Ca^{2+} and guanylyl cyclase-activating protein (GCAP) regulation of photoreceptor response. (**a**) Small-amplitude responses of a salamander rod to 20-ms flashes of intensity of 2.8 photons μm^{-2} in Ringer (smaller response) and in low-Ca^{2+}/zero-Na^{+} solution. (Modified from Fain et al. 1989.) (**b**) Averaged single-photon responses of wild-type mouse rod (+/+) and rod for which genes of GCAP proteins had been disrupted (−/−). (Modified from Mendez et al. 2001)

timeframe, responses to single photons sum linearly and adaptation is effectively eliminated (Matthews et al. 1988; Nakatani and Yau 1988).

Figure 4.5a compares responses of a salamander rod to a brief flash of light in Ringer solution and in low-Ca^{2+}/zero-Na^{+} solution. Exposure to low-Ca^{2+}/zero-Na^{+} produces an increase in the peak amplitude of the response and a marked prolongation of the time to peak and time course of decay, similar to incorporation of BAPTA in these cells. Other experiments show that the "sag" in current in response to steady background light no longer occurs, flash–response decay ceases to accelerate, and sensitivity declines with background intensity, not according to the Weber–Fechner relationship as in Fig. 4.2c but according to a theoretical curve predicted by the simple summation of responses to absorbed photons without any regulation of sensitivity (Matthews et al. 1988; Fain et al. 1989). Records similar to those in Fig. 4.5a have also been obtained from mammalian rods (unpublished data of M.L. Woodruff and G.L. Fain; see also Tamura et al. 1991), but more extensive investigations of the role of Ca^{2+} in mammalian photoreceptors have not yet been performed. The decrease in Ca^{2+} in a mouse rod occurs with two time constants of about 150 and 500–600 ms (Woodruff et al. 2002); in consequence, most of the reduction of Ca^{2+} will have taken place within 500 ms of turning on the background, a time course roughly consistent with the time course of background adaptation.

4.6 Regulation of Components of Signal Transduction by Ca^{2+}

Several components of the photoreceptor signal cascade have been reported to be altered by changes in the free Ca^{2+} concentration. One of the most important is guanylyl cyclase, which, as we explained earlier, is regulated by Ca^{2+} binding GCAP molecules. When the genes for the GCAP proteins are knocked out (Mendez et al. 2001), the rod response is larger and decays more slowly (Fig. 4.5b), much as in low-Ca^{2+}/zero-Na^{+} solution. When responses to flashes are recorded in the presence of background illumination in $GCAPs^{-/-}$ rods, sensitivity no longer declines according to the Weber–Fechner relationship (open circles in Fig. 4.6c), indicating that elimination of Ca^{2+} modulation of cyclase has a profound effect on adaptation. Adaptation is not however eliminated: the dotted and dashed lines in Fig. 4.6c are two alternative predictions of the relationship between sensitivity and background illumination in the absence of adaptation (see legend to figure). Both predictions give values of sensitivity that are orders of magnitude smaller than the sensitivity measured in $GCAPs^{-/-}$ rods (Mendez et al. 2001; Chen et al. 2010b), indicating that modulation of guanylyl cyclase cannot by itself explain the changes in rod response properties produced by background light.

Further evidence for this claim is given in the remainder of Fig. 4.6. Figure 4.6a shows responses of $GCAPs^{-/-}$ rods to prolonged exposure to continuous illumination, much as in Fig. 4.2a for wild-type rods. The rapid component of response "sag" is eliminated when the GCAPs are deleted, suggesting that this component is produced by cyclase modulation (see also Calvert et al. 2002). The slower phase of the "sag" seems however to be entirely intact (Burns et al. 2002). The records in Fig. 4.6b show that response decay continues to accelerate in background light even after the GCAPs have been deleted. This aspect of light adaptation also seems to depend upon some other mechanism of transduction regulation.

What other components of transduction are affected by changes in Ca^{2+}? Kawamura and Murakami (1991; Kawamura 1993) first described a small molecular weight protein that they called S-modulin but which is now more often referred to as recoverin (Dizhoor et al. 1991; Hurley et al. 1993; Kawamura et al. 1993). Recoverin has been shown to regulate the rate of rhodopsin kinase (Kawamura 1993; Chen et al. 1995) and modulate the lifetime of Rh* (Chen et al. 2010a). When the gene for recoverin is deleted, however, the relationship between sensitivity and background intensity is unaffected. The filled circles in Fig. 4.6c are from wild-type rods and the filled squares are from recoverin knockout animals (see Makino et al. 2004; Chen et al. 2010b). Regulation of rhodopsin lifetime seems to play little or no role in rod photoreceptor adaptation: recent experiments show that large increases and decreases in the expression level of rhodopsin kinase have minimal effects on rod transduction gain (Chen et al. 2012; Gross et al. 2012).

The channel can also be modulated: Ca^{2+} with calmodulin can alter the effective affinity of the rod cyclic nucleotide-gated channel for cGMP (Hsu and Molday 1993, 1994; Nakatani et al. 1995). The cyclic nucleotide-gated channel in a rod is a

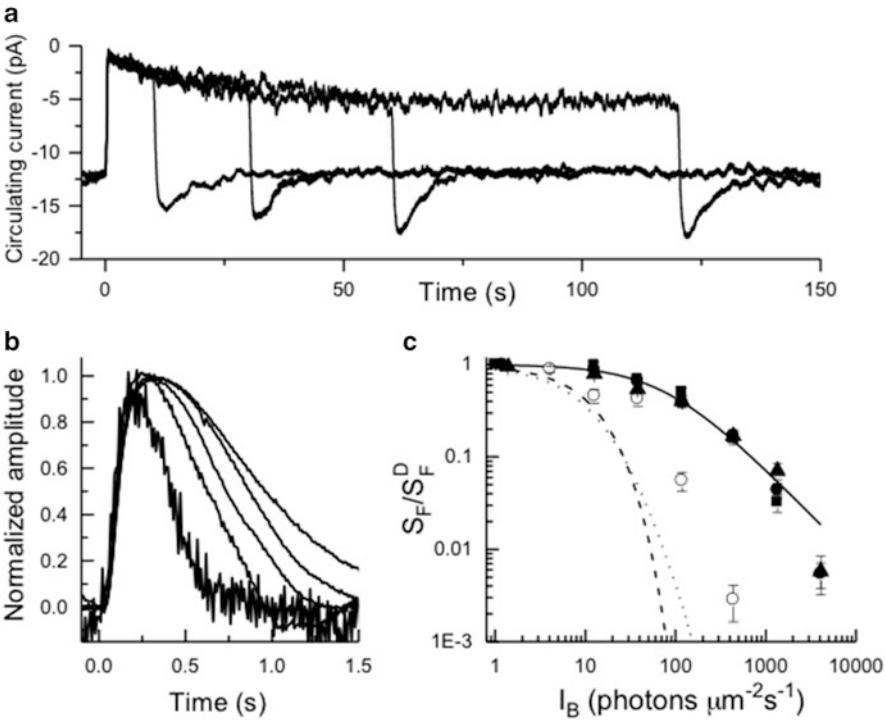

Fig. 4.6 Adaptation of $GCAP^{-/-}$ mouse rods. (**a**) Means of currents from three presentations each of 5 $GCAP^{-/-}$ rods to steps of light of intensity 38 photons μm^{-2} s^{-1} beginning at $t = 0$ for the following durations: 10, 30, 60, and 120 s. (**b**) Means from five presentations each of 7 $GCAPs^{-/-}$ rods to 20-ms flashes beginning at $t = 0$; intensity was kept at 17 photons μm^{-2} for flashes in darkness and backgrounds of the following intensities (in photons μm^{-2} s^{-1}): 4, 13, 38, and 118. Responses have been normalized cell by cell to peak amplitude of the response and averaged. The most slowly decaying response is with no background; decay time course progressively accelerated as background intensity was increased. (**c**) Weber–Fechner plot as in Fig. 4.2c. Graph gives mean relative sensitivity as a function of background intensity, averaged from 10 WT rods (*filled circles*), 14 rods lacking the protein recoverin (*filled squares*), 13 rods from which the Ca^{2+}-calmodulin-binding site of the cGMP-gated channel had been deleted (*filled triangles*), and 5 *GCAPs−/−* rods (*open circles*). *Solid line* is best-fitting Weber–Fechner function for WT rods given by Eq. (4.1) with $I_0 = 77$ photons μm^{-2} s^{-1}. *Black dashed* and *dotted curves* give theoretical predictions of change of sensitivity produced by simple saturation (*dashed*, from Mendez et al. 2001) or in the absence of cyclase and PDE feedback (*dotted*, from Chen et al. 2010b). [Reprinted from Fain (2011) with permission of Springer US]

tetramer composed of CNGA1 and CNGB1 subunits (Weitz et al. 2002; Zheng et al. 2002; Zhong et al. 2002), and the CNGB1 subunit has a binding site for Ca^{2+}-calmodulin (Grunwald et al. 1998; Weitz et al. 1998). When Ca^{2+} is relatively high in darkness, Ca^{2+}-calmodulin is bound to the channel, and the affinity of the channel for cGMP is comparatively low. As Ca^{2+} falls in the light, Ca^{2+}-calmodulin falls off of the CNGB1 subunit, increasing the affinity of the channel for cGMP and

counteracting the light-dependent fall in cGMP concentration. The change in channel affinity is in a direction to produce channel reopening as the cGMP level decreases during illumination, so channel modulation could potentially contribute to the regulation of steady current and sensitivity in background light. When however the Ca^{2+}-calmodulin binding site of the CNGB1 subunit was genetically deleted, there was a small change in the rate of decay of the photoreceptor response, especially to bright flashes, but little or no effect on the relationship between sensitivity and illumination in steady light (Fig. 4.6c, filled triangles; from Chen et al. 2010b).

4.7 Regulation of Phosphodiesterase

The results in Fig. 4.6 show that modulation of guanylyl cyclase by Ca^{2+} and the GCAPs makes a significant contribution to adaptation in background illumination, but that much of the adaptation nevertheless survives the genetic deletion of the GCAPs. Moreover, two further Ca^{2+}-dependent mechanisms of transduction regulation that have been discovered, namely, modulation of the rate of rhodopsin phosphorylation by rhodopsin kinase and recoverin and alteration of the effective affinity of the channel for cGMP by Ca^{2+}-calmodulin, have little role in the control of sensitivity during background illumination.

What is left? Four observations suggest that adaptation to background light in a mammalian rod can be produced in part by modulation of PDE6. These results are as follows. (1) Background light produces a decrease in the limiting time constant of response decay τ_D (Woodruff et al. 2008). As considerable evidence now indicates that the limiting time constant is a reflection of the rate of decay of light-activated PDE6 (Krispel et al. 2006; Tsang et al. 2006; Chen et al. 2010a), the rate of decay of PDE6 is likely to be accelerated in background light. (2) Mutations of the PDE6 γ-subunit can alter the relationship between sensitivity and background intensity (Woodruff et al. 2008). Although the interpretation of these experiments is not wholly free of difficulty, they do suggest that PDE6 modulation may play a role in sensitivity regulation. (3) The response of $GCAPs^{-/-}$ rods to prolonged illumination is followed by a pronounced current overshoot (see Fig. 4.6a). This overshoot cannot be produced by modulation of Rh*. Modulation of Rh* could not cause the current to become larger after the light is turned off than it was before the rod was illuminated; nor can the undershoots be produced by channel modulation, because they are even larger in $GCAPs^{-/-}$ rods that also lack the binding site for Ca^{2+}-calmodulin (Chen et al. 2010b). There is practically nothing left but regulation of PDE6. (4) A model incorporating modulation of both spontaneous and light-activated PDE6 activity by Ca^{2+} can successfully account for the changes in sensitivity and time course of rod responses in background light (Chen et al. 2010b).

Although none of these observations is definitive, in aggregate they make a relatively strong case for light-dependent modulation of PDE6. Recent experiments suggest that at least part of this modulation may be produced by rhodopsin

kinase (Chen et al. 2012). Both increases in kinase expression and the knocking out of recoverin can accelerate the limiting time constant of rod decay, which, as we have said, is apparently a measure of the rate of decay of PDE6. Moreover, the modulation of the time course of rod response decay in background light is nearly eliminated by deletion of the gene for recoverin, raising the interesting possibility that regulation of sensitivity and regulation of response decay are produced by somewhat different mechanisms.

4.8 Molecular Mechanism of Adaptation

The evidence that we presently have supports the following tentative conception of adaptation in mammalian rods. Background light and bleaches produce a steady decrease in circulating current, which reduces the outer segment free Ca^{2+} concentration. The decrease in Ca^{2+} increases the activity of guanylyl cyclase via the GCAP proteins, and activation of cyclase causes an increase in cGMP and a reopening of the channels. The modulation of cyclase is responsible for a rapid component of current recovery during maintained light exposure and some part of the modulation of sensitivity in background light, but it is not responsible for all the modulation of sensitivity, nor the slower component of current recovery, nor the acceleration of the time course of response decay in backgrounds or after bleaches. Neither modulation of rhodopsin lifetime nor regulation of the cGMP-gated channels by Ca^{2+}-calmodulin seems to make any significant contribution to adaptation. In contrast, there is increasing evidence for an important component produced by direct modulation of PDE6, perhaps in concert with rhodopsin kinase and recoverin. Rhodopsin kinase has the well known role of phosphorylating and turning off Rh*, but it may also phosphorylate PDE6 or some other protein that regulates PDE6. Translocation of transducin may have a small effect on sensitivity during recovery from bleaching but makes no contribution to adaptation in steady background light. The principal functions of transducin translocation seem to be conservation of energy, protection from photoreceptor degeneration, and modulation of the gain of the photoreceptor synapse (Fain 2006; Majumder et al. 2013).

Where do we go from here? One of the biggest difficulties that confronts us is the paucity of information about the biochemistry of PDE6. The rod PDE6 enzyme is a tetramer, consisting of catalytic α- and β-subunits and two inhibitory γ-subunits. All these proteins are potential sites of phosphorylation. Other mechanisms of regulation are also conceivable. Until we have a more detailed understanding of this intriguing molecule, we will have difficulty designing experiments to elucidate its role in photoreceptor physiology. The challenge for the future is to discover further mechanisms of regulation, and then to combine this knowledge with genetic and physiological approaches to discover the role of PDE6 and other possible modulators in background and bleaching adaptation. Ten years ago, we thought that adaptation was mostly solved. We now understand that there is still much work to do.

Acknowledgments Work in our laboratory would not have been possible without the assistance of many able (and congenial) collaborators, including (in alphabetical order) Jason Chen, Jeannie Chen, Carter Cornwall, Alex Dizhoor, Hugh Matthews, Dan Tranchina, and Steve Tsang. We are also grateful to Margery J. Fain for drawing Fig. 4.1. Our research has been generously supported during the last 37 years by NIH R01 EY01844.

References

Arshavsky VY, Burns ME (2012) Photoreceptor signaling: supporting vision across a wide range of light intensities. J Biol Chem 287:1620–1626

Barlow HB (1964) Dark-adaptation: a new hypothesis. Vision Res 4:47–58

Baylor DA, Hodgkin AL (1974) Changes in time scale and sensitivity in turtle photoreceptors. J Physiol (Lond) 242:729–758

Baylor DA, Lamb TD, Yau KW (1979) Responses of retinal rods to single photons. J Physiol (Lond) 288:613–634

Bownds MD (1980) Biochemical steps in visual transduction: roles for nucleotides and calcium ions. Photochem Photobiol 32:487–490

Burns ME, Mendez A, Chen J, Baylor DA (2002) Dynamics of cyclic GMP synthesis in retinal rods. Neuron 36:81–91

Calvert PD, Govardovskii VI, Arshavsky VY, Makino CL (2002) Two temporal phases of light adaptation in retinal rods. J Gen Physiol 119:129–145

Chen CK, Inglese J, Lefkowitz RJ, Hurley JB (1995) Ca(2+)-dependent interaction of recoverin with rhodopsin kinase. J Biol Chem 270:18060–18066

Chen CK, Woodruff ML, Chen FS, Chen D, Fain GL (2010a) Background light produces a recoverin-dependent modulation of activated-rhodopsin lifetime in mouse rods. J Neurosci 30:1213–1220

Chen J, Woodruff ML, Wang T, Concepcion F, Tranchina D, Fain GL (2010b) Channel modulation and the mechanism of light adaptation in mouse rods. J Neurosci 30:16232–16240

Chen CK, Woodruff ML, Chen FS, Chen Y, Cilluffo MC, Tranchina D, Fain GL (2012) Modulation of mouse rod response decay by rhodopsin kinase and recoverin. J Neurosci 32:15998–16006

Choe HW, Kim YJ, Park JH, Morizumi T, Pai EF, Krauss N, Hofmann KP, Scheerer P, Ernst OP (2011) Crystal structure of metarhodopsin II. Nature (Lond) 471:651–655

Cornwall MC, Fain GL (1994) Bleached pigment activates transduction in isolated rods of the salamander retina. J Physiol (Lond) 480:261–279

Cornwall MC, Fein A, MacNichol EF Jr (1990) Cellular mechanisms that underlie bleaching and background adaptation. J Gen Physiol 96:345–372

Dizhoor AM (2000) Regulation of cGMP synthesis in photoreceptors: role in signal transduction and congenital diseases of the retina. Cell Signal 12:711–719

Dizhoor AM, Ray S, Kumar S, Niemi G, Spencer M, Brolley D, Walsh KA, Philipov PP, Hurley JB, Stryer L (1991) Recoverin: a calcium sensitive activator of retinal rod guanylate cyclase. Science 251:915–918

Fain GL (1986) Evidence for a role of messenger substances in phototransduction. In: Stieve H (ed) The molecular mechanism of photoreception. Springer-Verlag, Berlin, pp 67–77

Fain GL (2003) Sensory transduction. Sinauer, Sunderland, MA

Fain GL (2006) Why photoreceptors die (and why they don't). Bioessays 28:344–354

Fain GL (2011) Adaptation of mammalian photoreceptors to background light: putative role for direct modulation of phosphodiesterase. Mol Neurobiol 44:374–382

Fain GL, Lamb TD, Matthews HR, Murphy RL (1989) Cytoplasmic calcium as the messenger for light adaptation in salamander rods. J Physiol (Lond) 416:215–243

Fain GL, Matthews HR, Cornwall MC, Koutalos Y (2001) Adaptation in vertebrate photoreceptors. Physiol Rev 81:117–151

Gray-Keller M, Denk W, Shraiman B, Detwiler PB (1999) Longitudinal spread of second messenger signals in isolated rod outer segments of lizards. J Physiol (Lond) 519 (pt 3):679–692

Gross OP, Pugh EN Jr, Burns ME (2012) Calcium feedback to cGMP synthesis strongly attenuates single-photon responses driven by long rhodopsin lifetimes. Neuron 76:370–382

Grunwald ME, Yu WP, Yu HH, Yau KW (1998) Identification of a domain on the beta-subunit of the rod cGMP-gated cation channel that mediates inhibition by calcium-calmodulin. J Biol Chem 273:9148–9157

He W, Cowan CW, Wensel TG (1998) RGS9, a GTPase accelerator for phototransduction. Neuron 20:95–102

He W, Lu L, Zhang X, El-Hodiri HM, Chen CK, Slep KC, Simon MI, Jamrich M, Wensel TG (2000) Modules in the photoreceptor RGS9-1.Gbeta 5L GTPase-accelerating protein complex control effector coupling, GTPase acceleration, protein folding, and stability. J Biol Chem 275:37093–37100

Hsu YT, Molday RS (1993) Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin [published erratum appears in Nature 1993 Sep 16;365(6443):279] [see comments]. Nature (Lond) 361:76–79

Hsu YT, Molday RS (1994) Interaction of calmodulin with the cyclic GMP-gated channel of rod photoreceptor cells. Modulation of activity, affinity purification, and localization. J Biol Chem 269:29765–29770

Hu G, Wensel TG (2002) R9AP, a membrane anchor for the photoreceptor GTPase accelerating protein, RGS9-1. Proc Natl Acad Sci 99:9755–9760

Hurley JB, Dizhoor AM, Ray S, Stryer L (1993) Recoverin's role: conclusion withdrawn [letter; comment]. Science 260:740

Jones GJ, Cornwall MC, Fain GL (1996) Equivalence of background and bleaching desensitization in isolated rod photoreceptors of the larval tiger salamander. J Gen Physiol 108:333–340

Karpen JW, Zimmerman AL, Stryer L, Baylor DA (1988) Gating kinetics of the cyclic-GMP-activated channel of retinal rods: flash photolysis and voltage-jump studies. Proc Natl Acad Sci USA 85:1287–1291

Kawamura S (1993) Rhodopsin phosphorylation as a mechanism of cyclic GMP phosphodiesterase regulation by S-modulin. Nature (Lond) 362:855–857

Kawamura S, Murakami M (1991) Calcium-dependent regulation of cyclic GMP phosphodiesterase by a protein from frog retinal rods. Nature (Lond) 349:420–423

Kawamura S, Hisatomi O, Kayada S, Tokunaga F, Kuo CH (1993) Recoverin has S-modulin activity in frog rods. J Biol Chem 268:14579–14582

Kefalov VJ (2012) Rod and cone visual pigments and phototransduction through pharmacological, genetic, and physiological approaches. J Biol Chem 287:1635–1641

Koch KW, Stryer L (1988) Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions. Nature (Lond) 334:64–66

Krispel CM, Chen D, Melling N, Chen YJ, Martemyanov KA, Quillinan N, Arshavsky VY, Wensel TG, Chen CK, Burns ME (2006) RGS expression rate-limits recovery of rod photoresponses. Neuron 51:409–416

Lamb TD, Pugh EN Jr (2004) Dark adaptation and the retinoid cycle of vision. Prog Retin Eye Res 23:307–380

Lamb TD, McNaughton PA, Yau KW (1981) Spatial spread of activation and background desensitization in toad rod outer segments. J Physiol (Lond) 319:463–496

Leskov IB, Klenchin VA, Handy JW, Whitlock GG, Govardovskii VI, Bownds MD, Lamb TD, Pugh EN Jr, Arshavsky VY (2000) The gain of rod phototransduction: reconciliation of biochemical and electrophysiological measurements. Neuron 27:525–537

Majumder A, Pahlberg J, Boyd KK, Kerov V, Kolandaivelu S, Ramamurthy V, Sampath AP, Artemyev NO (2013) Transducin translocation contributes to rod survival and enhances synaptic transmission from rods to rod bipolar cells. Proc Natl Acad Sci USA 25(1):341–348

Makino ER, Handy JW, Li T, Arshavsky VY (1999) The GTPase activating factor for transducin in rod photoreceptors is the complex between RGS9 and type 5G protein beta subunit. Proc Natl Acad Sci USA 96:1947–1952

Makino CL, Wen XH, Lem J (2003) Piecing together the timetable for visual transduction with transgenic animals. Curr Opin Neurobiol 13:404–412

Makino CL, Dodd RL, Chen J, Burns ME, Roca A, Simon MI, Baylor DA (2004) Recoverin regulates light-dependent phosphodiesterase activity in retinal rods. J Gen Physiol 123:729–741

Matthews HR, Fain GL (2001) A light-dependent increase in free Ca^{2+} concentration in the salamander rod outer segment. J Physiol (Lond) 532:305–321

Matthews HR, Murphy RL, Fain GL, Lamb TD (1988) Photoreceptor light adaptation is mediated by cytoplasmic calcium concentration. Nature (Lond) 334:67–69

Mendez A, Burns ME, Sokal I, Dizhoor AM, Baehr W, Palczewski K, Baylor DA, Chen J (2001) Role of guanylate cyclase-activating proteins (GCAPs) in setting the flash sensitivity of rod photoreceptors. Proc Natl Acad Sci USA 98:9948–9953

Nakatani K, Yau KW (1988) Calcium and light adaptation in retinal rods and cones. Nature (Lond) 334:69–71

Nakatani K, Koutalos Y, Yau KW (1995) Ca^{2+} modulation of the cGMP-gated channel of bullfrog retinal rod photoreceptors. J Physiol (Lond) 484:69–76

Nymark S, Frederiksen R, Woodruff ML, Cornwall MC, Fain GL (2012) Bleaching of mouse rods: microspectrophotometry and suction-electrode recording. J Physiol (Lond) 590:2353–2364

Polans A, Baehr W, Palczewski K (1996) Turned on by Ca^{2+}! The physiology and pathology of Ca^{2+}-binding proteins in the retina. Trends Neurosci 19:547–554

Reuter T (2011) Fifty years of dark adaptation: 1961–2011. Vision Res 51:2243–2262

Sokolov M, Lyubarsky AL, Strissel KJ, Savchenko AB, Govardovskii VI, Pugh EN Jr, Arshavsky VY (2002) Massive light-driven translocation of transducin between the two major compartments of rod cells: a novel mechanism of light adaptation. Neuron 34:95–106

Standfuss J, Edwards PC, D'Antona A, Fransen M, Xie G, Oprian DD, Schertler GF (2011) The structural basis of agonist-induced activation in constitutively active rhodopsin. Nature (Lond) 471:656–660

Stiles WS, Crawford BH (1932) Equivalent adaptation levels in localized retinal areas. In: Report of a joint discussion on vision, June 3, 1932, Imperial College of Science. Physical Society, London, pp 194–211

Tamura T, Nakatani K, Yau KW (1991) Calcium feedback and sensitivity regulation in primate rods. J Gen Physiol 98:95–130

Torre V, Matthews HR, Lamb TD (1986) Role of calcium in regulating the cyclic GMP cascade of phototransduction in retinal rods. Proc Natl Acad Sci USA 83:7109–7113

Tsang SH, Woodruff ML, Chen CK, Yamashita CY, Cilluffo MC, Rao AL, Farber DB, Fain GL (2006) GAP-independent termination of photoreceptor light response by excess gamma subunit of the c-GMP-phosphodiesterase. J Neurosci 26:4472–4480

Weitz D, Zoche M, Muller F, Beyermann M, Korschen HG, Kaupp UB, Koch KW (1998) Calmodulin controls the rod photoreceptor CNG channel through an unconventional binding site in the N-terminus of the beta-subunit. EMBO J 17:2273–2284

Weitz D, Ficek N, Kremmer E, Bauer PJ, Kaupp UB (2002) Subunit stoichiometry of the CNG channel of rod photoreceptors. Neuron 36:881–889

Woodruff ML, Sampath AP, Matthews HR, Krasnoperova NV, Lem J, Fain GL (2002) Measurement of cytoplasmic calcium concentration in the rods of wild-type and transducin knock-out mice. J Physiol (Lond) 542:843–854

Woodruff ML, Janisch KM, Peshenko IV, Dizhoor AM, Tsang SH, Fain GL (2008) Modulation of phosphodiesterase 6 turnoff during background illumination in mouse rod photoreceptors. J Neurosci 28:2064–2074

Zheng J, Trudeau MC, Zagotta WN (2002) Rod cyclic nucleotide-gated channels have a stoichiometry of three CNGA1 subunits and one CNGB1 subunit. Neuron 36:891–896

Zhong H, Molday LL, Molday RS, Yau KW (2002) The heteromeric cyclic nucleotide-gated channel adopts a 3A:1B stoichiometry. Nature (Lond) 420:193–198

Chapter 5
Energy Metabolism in the Vertebrate Retina

James B. Hurley, Andrei O. Chertov, Ken Lindsay, Michelle Giamarco, Whitney Cleghorn, Jianhai Du, and Susan Brockerhoff

Abstract The fundamental need for efficient and well-controlled energy metabolism in living organisms is ubiquitous. However, vertebrate retinas have many fascinating adaptations that give them the ability to regulate energy production and anabolic activity to meet a unique set of metabolic demands.

Retinas have

- An extraordinarily high rate of ATP consumption in darkness
- A localized and highly specialized signal transduction apparatus that has an overwhelming and direct influence on energy demand
- Exposure to light and O_2 that create a high demand for membrane renewal
- A higher rate of aerobic glycolysis than most other tissues
- A laminated structure that facilitates localization of metabolic enzymes and metabolites
- Synaptic terminals with a unique structure that allows recovery of neurotransmitter into presynaptic neurons at very high efficiency
- Unusual mitochondrial morphology and distribution
- Distinctive localization of creatine kinase and a specialized mechanism for distributing metabolic energy within the cell
- An extensive database of genes linked to retinal degeneration: many of these may cause metabolic dysregulation and failure
- Unique sensitivity to loss of mitochondrial isocitrate dehydrogenase activity

This review summarizes what is known about these extraordinary features in the context of the retina structure, its anabolic requirements, its metabolic requirements in light and darkness, the ways that it distributes metabolic energy, and, finally, the consequences of metabolic dysregulation.

J.B. Hurley (✉) • A.O. Chertov • K. Lindsay • M. Giamarco
W. Cleghorn • J. Du • S. Brockerhoff
Department of Biochemistry, University of Washington, Seattle, WA, USA
e-mail: jbhhh@uw.edu

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_5,

Keywords Energy metabolism • Glycolysis • Mitochondria • Retina

5.1 Introduction and Overview

Animals use their retinas to detect light in the environment. The signaling pathways by which a retina performs that function gives it unique set of anabolic and catabolic demands.

5.1.1 Anabolic Demands

Rods and cones are terminally differentiated. They do not undergo mitosis and they cannot be replaced, which is problematic because they are exposed to a nearly continuous stream of photons during daytime. Some of the energy absorbed from those photons can be dissipated in ways that damage proteins or phospholipids. Indeed, it has been suggested that even normal levels of illumination cause cumulative damage (Jarrett and Boulton 2012), and that above a threshold of illumination irreversible damage occurs (Organisciak and Vaughan 2010; Sykes et al. 1981).

Photoreceptors also are exposed to high levels of O_2 coming from the choriocapillaris (Jarrett and Boulton 2012). Metabolic production of NADPH is required to keep the primary antioxidant in the retina, glutathione, in its reduced state. Reduced glutathione may help to counteract oxidative damage caused by generation of retinaldehyde from rhodopsin or by direct effects of light on proteins and lipids. It has been proposed that photoreceptors also use an overriding mechanism that more directly replaces the most damaged membranes (Winkler 2008). Shedding of the oldest membranes from the tip of the outer segment and replacement with new membranes at the base ensures turnover of the most heavily damaged proteins and phospholipids. About 10 % of rod outer segment membranes are turned over per day (Basinger et al. 1976; LaVail 1976b; Young 1971), which requires synthesis of new proteins and phospholipids. Active anabolic pathways are needed to produce precursors for phospholipid and protein synthesis. Monkey retinas replace 80–90 discs per day for each rod cell (Young 1971).

5.1.2 Catabolic Demands

Energy consumption by photoreceptor neurons in the retina is greatest in darkness when there is a steady influx of Na^+ into photoreceptors through channels in their outer segments (Ames et al. 1992; Okawa et al. 2008). The flow of ions is compensated by Na^+/K^+ pumping activity in the inner segment that maintains the membrane potential. It has been estimated that this action consumes 10^8 ATP per

second in a mouse rod photoreceptor (Okawa et al. 2008). When light stimulates phototransduction, Na^+ influx slows and energy consumption decreases. Although phototransduction increases hydrolysis of GTP for cyclic GMP synthesis and for inactivation of transducin, the overall energy demand during illumination is only 20–30 % of the overall energy demand in darkness (Okawa et al. 2008).

How does the retina keep pace with these changes in energy demand? Several studies have measured metabolic flux and O_2 consumption: all reported an increase in O_2 consumption in darkness compared to light. However, the reported increases in darkness typically are only in the 30 % to 50 % range (Ames et al. 1992; Linsenmeier 1986; Medrano and Fox 1995), and there appears to be little change in the rate of glycolysis: that is, less than the approximately fivefold increase in metabolic demand required in darkness compared to light predicted by calculations based on electrophysiological analyses (Okawa et al. 2008). The mechanism by which the retina increases energy production to keep pace with the changes in energy demand in light versus darkness is not yet known.

5.1.3 The Polarized Morphology of Photoreceptor Neurons Creates Challenges for Energy Metabolism

Photoreceptors are highly polarized structures with an outer segment at one end optimized to transduce absorption of photons into chemical signals. At the opposite end is a synaptic terminal with a unique type of organization. In between are the nucleus, the cell body, and, at the apical end of the inner segment, the ellipsoid. Mitochondria are located in the ellipsoid, and their morphology and arrangement there show remarkable variation among species (Ishikawa and Yamada 1969). In vascularized retinas mitochondria also are present within the photoreceptor synaptic terminal. This structure creates localized domains of energy demand and energy production. Specific mechanisms have evolved to help distribute metabolic energy appropriately in light versus in darkness (Linton et al. 2010).

5.1.4 Aerobic Glycolysis

A remarkable feature of the mature vertebrate retina is that its metabolism is dominated by aerobic glycolysis. When cells grow under anaerobic conditions they rely heavily on glycolysis for energy production, a process referred to as anaerobic glycolysis. However, some cells rely mostly on glycolysis even when O_2 is abundant: this is referred to as aerobic glycolysis. Retinas perform aerobic glycolysis, that is, rapid production of lactate even when O_2 is abundant, as was noted first by Warburg in the 1920s while comparing metabolic features of cancer cells and a variety of tissues (Warbug 1927). Warburg found that the retina is

similar to cancer cells in the extent to which it uses aerobic glycolysis. The observation has been confirmed by many studies from many different laboratories. Photoreceptors are exceptionally susceptible to inhibition of glycolysis. Injection of iodoacetic acid into rabbits causes selective degeneration of the photoreceptor layer (Noell 1951).

The extraordinary capacity for aerobic glycolysis appears in the retina during development at about the same time as photoreceptor neurons mature (Graymore et al. 1959). There are two reasons to think that the increase in aerobic glycolytic activity comes from the differentiation of progenitors into photoreceptors. First, there is less aerobic glycolysis in retinas in which photoreceptors have degenerated (Graymore et al. 1959). Second, there is a decrease in aerobic glycolysis when retinal progenitors are induced to form only ganglion cells, that is, nonphotoreceptor types of neurons (Agathocleous et al. 2012)

5.1.5 Metabolism in the Retina Requires a Division of Labor

Light-sensing organs have at least two types of cells, light-sensitive cells and pigmented cells (Lamb et al. 2007). In the eyes of vertebrates these are two adjacent layers of tissue, the retina and the retinal pigment epithelium (RPE), with a matrix between them known as the interphotoreceptor matrix (IPM). The relationship between the retina and RPE seems more one sided than symbiotic. The RPE serves the retina by trimming the ends of the photoreceptor outer segments (Bok 1993; Strauss 2005). This daily phagocytosis by the RPE removes the oldest and most damaged part of the phototransduction apparatus so that it can be replaced by newly synthesized material at the base of the outer segment. Another way that the RPE serves the retina is by participating in metabolic activity needed to regenerate rhodopsin. The RPE also serves the retina by transporting nutrients from blood to the IPM and then to the retina (Strauss 2005). The presence of gradients of glucose and lactate within the IPM indicate that the RPE delivers glucose to the retina and that the retina delivers lactate to the RPE (Adler and Southwick 1992). It is generally considered that the latter provides a way to remove lactate from the retina through the IPM, then into the RPE, and ultimately to the blood.

Generally, photoreceptors do not survive long without a normally functioning RPE (Bok and Hall 1971; Strauss 2005) although with carefully controlled culture conditions in darkness photoreceptor morphology can be preserved (Zayas-Santiago and Kang Derwent 2009). However, in general, the RPE seems to do fine with or without the retina (Engelmann and Valtink 2004). There are indications that the retina may provide some support for the RPE. Because the RPE takes lactate from the IPM, it is possible that the RPE uses the lactate to fuel its own metabolic activity. Another suggestion that the RPE relies on the retina is that when light-induced damage occurs in the retina the RPE undergoes morphological and metabolic changes (Marc et al. 2008). However, it is hard to rule out the possibility that direct light damage to the RPE also contributes to those changes.

Müller cells, the glial cells that radially span the retina, play an important metabolic role (Reichenbach and Bringmann 2013). They have transporters for monocarboxylates, glucose, and amino acids, and they are the only cells in the retina that synthesize glutamine (Bringmann et al. 2009). Investigators studying metabolic relationships between glia and neurons in brain have proposed a model to explain the metabolic relationships between astrocytes, the glial cells in the brain, and neurons (Belanger et al. 2011; Pellerin and Magistretti 2012). According to this model, the astrocyte neuronal lactate shuttle (ANLS), astrocytes recover glutamate after it is released from neurons as a neurotransmitter. The uptake of glutamate and its conversion to glutamine in the astrocyte stimulates glycolysis and production of lactate. The lactate is released from the astrocyte and used to fuel metabolic activity in the neurons. There is considerable evidence to support this model in brain tissues, at least under specific sets of conditions (Pellerin and Magistretti 2012). Although there is evidence that supports the idea that the ANLS operates in the retina (Poitry-Yamate et al. 1995), there also are suggestions that the ANLS may not accurately represent metabolic relationships between Müller glia and photoreceptors. For example, the encapsulated structure of the photoreceptor synaptic terminal prevents glutamate from escaping the synapse before it is sequestered back into the photoreceptor through EAAT5 glutamate transporters (Hasegawa et al. 2006). Furthermore, inhibition of glutamine synthetase is more effective at causing glutamate loss in inner retinal neurons than in photoreceptors (Pow and Robinson 1994).

Nevertheless, the relationship between neurons and Müller glia does appear more symbiotic than the relationship between the retina and the RPE. The metabolic state of Müller cells changes dramatically when photoreceptors degenerate, suggesting that they are influenced by a signal or metabolite normally provided by the photoreceptors (Jones et al. 2011; Marc et al. 2008). When Müller cells are grown in culture in the absence of the rest of the retina, their characteristics change (Ueki et al. 2012).

5.1.6 General Importance of Understanding Retinal Metabolism

The retina is a specialized neuronal tissue in which specific metabolic and signaling pathways have become dominant and easily accessible to analysis. The retina therefore provides an opportunity to identify important metabolic pathways that are present but perhaps overshadowed and more difficult to recognize and analyze in other tissues.

Retinal degeneration occurs when photoreceptors are overwhelmed by metabolic dysfunction. Understanding retinal metabolism then may reveal mechanisms by which genetic and environmental stresses cause photoreceptors to degenerate. Many studies of retinal structure or signaling also require a basic understanding of retinal metabolism.

Biochemical and electrophysiological analyses of intact retinas rely on retinal neurons and glia performing their functions in a way that resembles their performance in a living animal, which requires some degree of understanding of the critical metabolic requirements for viability and function of the retina. For example, bicarbonate-based media are required by retinas for normal physiological activities (Besharse et al. 1980; Besharse and Dunis 1983; Winkler 1986; Winkler et al. 1977). In fact, a recent study showed that even the components of the media used for storing retinas in preparation for electrophysiology experiments can have significant effects on characteristics of responses of rod photoreceptor (Azevedo and Rieke 2011).

5.2 Functional Constraints on Retinal Structure

5.2.1 Retinal Function Dictates Structural Constraints

The layout of the retina is dictated by its function. In turn, the design of the metabolic system that fuels the visual process adheres to the function requirements. The eye evolved from a collection of photoreactive proteins localized to a part of a unicellular organism to indicate the presence of light (Salvini-Plawen and Mayr 1977).

One requirement for retinal function is an arrangement of cells that can detect spatial information. Multicellular organisms benefited from the evolution of light-sensitive cells on their surface as indicators of light. Later, the advantage of gauging the directionality of light led to the evolution of recessed light-sensitive patches. Over time the eye benefited from the evolution of a protective covering and a lens, which can focus light on photoreceptors for a sharper image. This structure evolved into a highly sophisticated visual system with photosensitive cells most concentrated in the fundus of the eye opposite from site of light entry.

Another requirement is high sensitivity, which requires highly concentrated visual pigment to guarantee that any photon that impinges on the photoreceptor is absorbed to initiate phototransduction. The outer segments meet this requirement by providing a large surface area in which to pack visual pigments at millimolar concentrations to maximize the probability of photon capture.

Another major role of photoreceptors is to pass on information to downstream neurons. These multiple functions led to the evolution of a cell with an elongated, highly polarized structure (Lamb et al. 2007). The highly specialized light sensor is at one end of the cell, the equally specialized synaptic terminal is at the other end, and most of the machinery needed to supply metabolic needs for all the compartments of the cell is located in the middle.

5.2.2 Metabolic Implications of Outer Segment Architecture

The shape of the outer segment also plays a role in the metabolic system of the retina. The high density of pigment-enriched membranes in the outer segment is an efficient way to ensure photon capture. However, the disc configuration results in a compartment with a low cytosolic volume, which hinders diffusion of molecules between the inner and outer segments. Thus, although the outer segments are the most proximal parts of the photoreceptors (PRs) to the choroid, the nutrients necessary for PR function and survival are absorbed at the ellipsoid. Recent studies showed that glucose enters through glucose transporters located in the ellipsoid (Gospe et al. 2010). This path is further confirmed by the fact that NADPH needed for the first steps of the recycling process of photo-activated pigment is generated in the ellipsoid (most likely by the pentose phosphate pathway), then diffuses to the outer segment, and then disperses more slowly throughout the highly membranous barriers within the outer segment (Miyagishima et al. 2009). Docosahexaenoic acid also is absorbed at the ellipsoid for biosynthesis of phospholipids: in the cones of some species it even accumulates there within oil droplets (Gordon and Bazan 1993).

5.2.3 Metabolic Implications of Photoreceptor Location

Photoreceptors are one of the most energetically ravenous cells in the body. They are the part of the retina that is closest to the choroidal blood flow. Photoreceptors are separated from the choroid only by the IPM and the RPE, which allow or facilitate transport of O_2, glucose, retinal, fatty acids, monocarboxylates, and other nutrients and waste products. O_2 consumption studies in vivo have shown that mitochondria located in the photoreceptor ellipsoid consume the majority of O_2 that enters the retina from the choroid. The inner layers of the retina come close to being hypoxic, except in those species that have vasculature deeper in the retina (summarized in Yu and Cringle (2001)). Thus, the inner retinal neurons from species with avascular retinas are reliant completely on glycolysis.

5.2.4 Metabolic Implications of Photoreceptor Structure

The compartmentalized nature of photoreceptor cells creates unique challenges for matching metabolic supply with demand. The greatest energy demand in darkness comes from Na^+/K^+ ATPase ion pumps that maintain the membrane potential of these neurons (Ames et al. 1992; Okawa et al. 2008; Wetzel et al. 1999). The high flux of ions through cGMP-gated cation channels in darkness places the most energy-demanding process in the cell adjacent to the mitochondria that provide

the energy to sustain it. Mitochondria in the ellipsoid also may help control local cellular [Ca^{2+}](Smaili et al. 2000; Simpson and Russell 1998; Nicholls 2005) by sequestering any Ca^{2+} that flows in from the outer segment (Krizaj et al. 2003). The amount of free Ca^{2+} in the outer segment is higher in darkness than in light (Sampath et al. 1999). When Ca^{2+} is taken up by the mitochondrial uniporter into the mitochondrial matrix, it can stimulate mitochondrial dehydrogenase activities (McCormack et al. 1990; Rutter et al. 1992; Denton 2009), mitochondrial protein levels, and mitochondrial biogenesis (Yeh et al. 2005). Thus, Ca^{2+} may help increase mitochondrial activity in darkness when energy demands are highest.

Although the central location of mitochondria is useful in some ways, it also creates an issue for ATP-dependent processes far away from the ellipsoid. In darkness ATP also is needed at the synaptic terminal to load neurotransmitter into synaptic vesicles. In light, at the other end of the cell, ATP is need for rhodopsin phosphorylation and synthesis of cGMP.

One solution to this problem is to have mitochondria at the synaptic terminal to provide ATP for neurotransmission. Photoreceptors from animals with vascularized retinas do solve the problem this way; they have mitochondria at their synaptic terminals (Bentmann et al. 2005; Johnson Jr et al. 2007; Stone et al. 2008). Photoreceptors from avascular retinas use a different solution. Because little O_2 can reach the synaptic terminals in these retinas, these photoreceptors use a phosphocreatine shuttle to transport energy from ellipsoid mitochondria to their synaptic terminal (Linton et al. 2010). Mitochondrial creatine kinase phosphorylates creatine to generate creatine phosphate (PCr). A different isoform of creatine kinase, CK-B, is localized specifically to the photoreceptor synaptic terminal. In these photoreceptors, PCr flows from the ellipsoid mitochondria down to the terminal where it is used by CK-B to generate ATP to fuel neurotransmission. In light, the pumps are not very active, so energy in the form of ATP diffuses to the outer segment to fuel phototransduction.

5.2.5 Metabolic Relationships Between Cell Types in the Retina

Retinas favor aerobic glycolysis (Cohen and Noell 1960; Winkler 1981). Even in the presence of O_2, they generate prodigious amounts of lactate and release it into the medium. Nevertheless, both glycolysis and oxidative phosphorylation are necessary for visual function (Ames et al. 1992). Removal of glucose rapidly diminishes the b-wave of the electroretinogram. Similar effects are produced either by inhibition of glycolysis or by removal of O_2 in the presence of glucose (Noell 1951; Wilkus et al. 1971; Winkler 1981). The b-wave represents responses originating in the inner retinal neurons stimulated by photoreceptors. These findings suggest that photoreceptors rely on oxidative phosphorylation for downstream signaling.

5.2.5.1 The Astrocyte Neuronal Lactate Shuttle

Such studies reveal characteristics of the overall metabolism of retinas, but they do not establish the identities of the cells that consume glucose and O_2 and which generate lactate. A series of studies on slices from honey bee retinas revealed that glucose is taken up only in glial cells surrounding the photoreceptors (Tsacopoulos et al. 1988), suggesting a model in which glial cells take up glucose, convert it to lactate, and then export it to photoreceptors. The photoreceptors then oxidize the lactate to pyruvate and use the pyruvate to fuel the TCA cycle and generate ATP aerobically. A similar relationship involving astrocytes and neurons in brain that has been identified and widely studied is often referred to as the astrocyte neuronal lactate shuttle (ANLS) (Pellerin and Magistretti 1994). The ANLS hypothesis has been investigated extensively, and much evidence has been presented both for and against it.

Evidence for ANLS activity in retina. Evidence for ANLS activity in mammalian retinas has been reported. The studies used isolated cells and cell clusters from dissociated guinea pig retinas. Müller cells in clusters with photoreceptors released less lactate into the medium than Müller cells alone (Poitry-Yamate et al. 1995; Poitry-Yamate and Tsacopoulos 1992). This finding suggested that photoreceptors in the clusters depleted lactate by using it for fuel, as predicted by the ANLS model. Later studies found that Müller cells from rat retinas rely on aerobic glycolysis and are resistant to anoxia (Winkler et al. 2000). The high rate of lactate production by Müller cells reported in that study also appears consistent with the ANLS theory. The discovery of abundant monocarboxylate (MCT) transporters on the surface of Müller cells provided further support for the idea that the ANLS plays a role in retina (Gerhart et al. 1999).

A more complete model for ANLS activity has emerged from studies of brain tissues. Early studies showed that glutamate released by neurons triggers uptake of glucose by astrocytes (Pellerin and Magistretti 1994). The glutamate is imported via a Na^+-glutamate cotransporter into astrocytes where it is converted to glutamine. The ATP expenditure needed to convert glutamate into glutamine and to pump out Na^+ stimulates glycolysis, lactate production, and glucose uptake. Lactate and glutamine are released from the astrocytes and taken up by the neurons. Glutamine is converted back to glutamate in the neurons and lactate is used to fuel their TCA cycle. Evidence that this type of cycle can occur in salamander retinas has been reported. Glutamate uptake into isolated Müller cells was shown to stimulate glycolysis. Subsequently lactate and glutamine were released when glutamine synthase was active (Poitry et al. 2000).

Evidence against ANLS in retina. There also is evidence that favors the classical view of glucose as the primary fuel for both neurons and glia in the retina. Autoradiography showed that ^{3}H-labeled 2-deoxyglucoseis taken up in all retinal cell layers with the highest concentration in photoreceptors (Winkler et al. 2003). The addition of exogenous lactate did not prevent deoxyglucose entry into the PRs,

and this was true for both vascular (rat) and avascular (guinea pig) retinas (Winkler et al. 2003). The site of glucose entry into rat and Xenopus photoreceptors has been identified as the Glut-1 transporter and localized to the photoreceptor ellipsoid (Gospe et al. 2010). The mitochondria-associated form of hexokinase also has been localized to the photoreceptor ellipsoid region of the retina (Reidel et al. 2011).

According to the ANLS model, photoreceptors would not use glucose directly but instead rely on lactate from Müller cells. Cultured mouse photoreceptors, Müller cells, and ganglion cells dissociated from rat retinas and isolated human RPE cells continue to produce lactate from glucose even in the presence of high concentrations of exogenous lactate (Winkler et al. 2004). This observation suggests that the lactate dehydrogenase reaction in these cells favors formation of lactate, a result that is inconsistent with predictions from the ANLS model.

The extent of ANLS activity in the photoreceptor layer of the retina appears to be limited by the presence of highly active glutamate transporters within the encapsulated structure of rod photoreceptor synaptic terminals (Hasegawa et al. 2006). These transporters sequester glutamate back into the photoreceptor before it can escape from the synapse. There also is evidence that Müller cells do not extend processes into cone photoreceptor terminals (Lasansky 1973), further limiting the ability of these glia to take up glutamate in the outer retina.

The importance of glucose metabolism for photoreceptors was highlighted in isolated mouse retinas as photoreceptors were found to die rapidly following glucose withdrawal (Chertov et al. 2011). Although exogenous mitochondrial fuels slowed photoreceptor death during glucose withdrawal, they could not halt it completely. Finally, mathematical models for brain energetics suggest that lactate utilization is so low that it may be irrelevant for contributing to neuronal or glial metabolism (DiNuzzo et al. 2011). A comprehensive analysis of the arguments for and against the ANLS in neuronal tissues can be found elsewhere (Aubert and Costalat 2007; Pellerin and Magistretti 2012; Stobart and Anderson 2013).

5.2.6 Müller Cell Function

Müller cells affect retinal metabolism through mechanisms outside the contested ANLS as well. These glial cells stretch the length of the retina with their distal ends ending next to photoreceptor inner segments. Müller cells express both Glut-1 and Glut-2 on their surface (Watanabe et al. 1994; Kumagai et al. 1994). Glut-2 is expressed in the same layer of the retina as photoreceptor glucose transporters, which allows the Müller cells to compete effectively with photoreceptors for glucose that flows from the choroid. Glucose uptake is very likely to be important for Müller cells as they rely primarily on glycolysis for their metabolic needs (Winkler et al. 2000). The presence of glycogen in Müller cells has been known since the 1960s (Kuwabara and Cogan 1961). The rapid loss of glycogen stores in response to ischemia also has been well characterized (Johnson 1977; Wasilewa et al. 1976). A recent study found that in rat retinas glycogen synthase in Müller

cells colocalizes with the synaptic layers in the retina (Perezleon et al. 2013), which could be another way to overcome the challenge of meeting the metabolic needs of the polarized photoreceptor. Although the rat retina is vascularized and contains mitochondria at the synapse, there still exists a need to obtain substrates for oxidative phosphorylation. The breakdown of glycogen stores and release of metabolic substrates from neighboring Müller cells could be a way to fuel photoreceptor neurotransmission during times of metabolic shortfall.

Müller cells also are the wardens of glutamine within the retina. Because neurons lack the enzyme glutamine-synthetase, all glutamine within the retina is produced within Müller cells. Glutamine is necessary to produce both the neurotransmitter (glutamate) and the neuroinhibitor (GABA) in the retina. Although it has been previously hypothesized that Müller cells recycle glutamate through the glutamate-glutamine cycle, that does not seem to be true. The low chance of glutamate escape from the photoreceptor synaptic cleft (Hasegawa et al. 2006) and the slow conversion rate of glutamate to glutamine in Müller cells (Winkler et al. 1999) suggest that glutamate recycling is not a paramount function of Müller cells. It appears that photoreceptors could be chiefly responsible for maintaining their own levels of glutamate. However, as the only glutamine-producing cell type in the retina, Müller cells are necessary to replace any small amounts of glutamate that are lost or oxidized by photoreceptors.

Müller cells are also major regulators of GABA. In the outer retina, Müller cells are responsible for all GABA removal, whereas in the inner retina, they share the task with amacrine cells (Marc 1992). Müller cells could be responsible for GABA signal termination (Biedermann et al. 2002).

Müller cells are responsible for clearance of ammonia from the retina. Blood-borne ammonium ions readily enter the brain and become fixed there by incorporation into glutamine (Cooper et al. 1979). If glutamine was not removed, its accumulation would result in an increase in osmolarity and cellular swelling. The endothelial cells of the blood–brain barrier actively import glutamine and allow it to be removed via the bloodstream (reviewed by Hawkins et al. 2006). Studies of Müller cells have shown that an increase in external concentrations of glutamine and ammonium ions result in cell swelling (Karl et al. 2011; Reichenbach et al. 1995). The retinal-spanning length of Müller cells and their ability to fix ammonia via glutamine synthesis would allow them to serve as the primary tool for exporting ammonia out of all retinal layers.

Another way that Müller cells can control photoreceptor metabolism is through release of neurotrophic factors. Release of growth factors from microglia leads to the release of basic fibroblast growth factor (bFGF) and glial cell line-derived neurotrophic factor (GDNF) (Harada et al. 2002). Another study showed that Müller cells also release nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 and -4 (Taylor et al. 2003). Nerve growth factor (NGF) activity regulates metabolism by activating the Akt pathway (Crowder and Freeman 1998) and the mTOR pathway that it stimulates.

5.3 Lipids and Anabolic Activity

5.3.1 Introduction

Anabolic activities in photoreceptors are primarily directed toward synthesis of outer segment discs, a highly membranous compartment of the photoreceptor and the site of the phototransduction cascade. Each day, up to 10 % of discs are shed at the tips of the outer segment and phagocytized by the adjoining retina pigment epithelium (RPE) (Basinger et al. 1976; LaVail 1976b). This turnover creates a significant demand for membrane and protein synthesis. Discs in the outer segment increase the overall cellular membrane surface area by 1,000-fold (Mayhew and Astle 1997), so that the overall demand for membrane synthesis must exceed that of a similarly sized more conventional neuron. Inner retina neurons and RPE are similar to other neurons and epithelial cells in that they do not undergo a similar amount of renewal, so we focus on the unique aspects of anabolic activity in photoreceptors.

5.3.2 Protein Synthesis

Early measurements of protein synthesis in photoreceptors focused on rhodopsin, the most abundant membrane protein in the cell (Young 1976). Incorporation of radiolabeled leucine and methionine revealed that photoreceptor disc formation begins in the ellipsoid where protein-loaded vesicles migrate to the base of the outer segment to begin the process of disc formation. Rhodopsin remains within the disc in which it is first incorporated into the outer segment (Fig. 5.1a). Newly formed discs made from proteins and phospholipids fill in behind the older discs as they migrate toward the end of the outer segment (Fig. 5.1a,b), a process that has been observed in fish (Willoughby and Jensen 2012), amphibians (Anderson et al. 1980b), and mammals (Besharse and Hollyfield 1979). RPE cells engulf the ends of the outer segments (Young and Bok 1969) and phagocytize the discs at the tip of the outer segment.

5.3.3 Membrane Synthesis

5.3.3.1 Lipids, Triglycerides, Phospholipids, and Cholesterol

Phospholipids turn over significantly faster than proteins (Anderson et al. 1980b). Although integral membrane proteins remain with the disc in which they were initially embedded, the components of phospholipids, fatty acids (Bibb and Young 1974a), and glycerol (Bibb and Young 1974b) exchange rapidly throughout the length of the outer segment. This exchange occurs either by transfer of intact

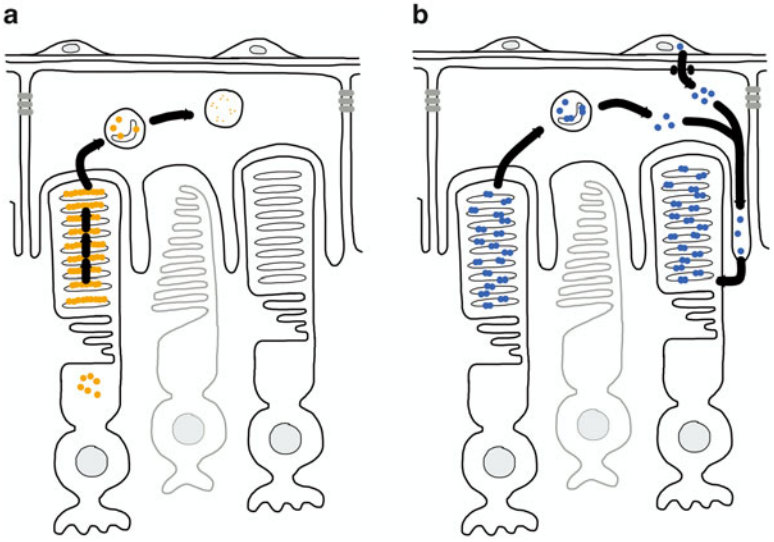

Fig. 5.1 (**a**) Movement of protein through a rod outer segment, subsequent phagocytosis, and degradation by the retinal pigment epithelium (RPE). (**b**) Movement of docosahexaenoic acid (DHA) through rod outer segment discs, subsequent phagocytosis, and recycling by the RPE. Additional DHA is taken up from the choriocapillaris by the RPE via high-affinity receptors at the membrane. *Double blue dots* represent phospholipid-bound DHA; *single blue dots* represent free DHA

phospholipids between discs (Bibb and Young 1974a; Dudley and Anderson 1978) or by exchange of fatty acyl groups (Anderson et al. 1980b). Radiolabeled glycerol appears initially at the base of the outer segment. However, with time the labeled glycerol becomes dispersed throughout the outer segment. In contrast, exchange of radiolabeled fatty acids into phospholipids by acyl transferases occurs throughout the length of the outer segment (Anderson et al. 1980b). In addition, base exchange reactions can catalyze rapid incorporation of serine (Mizuno 1976), choline (Anderson et al. 1980d), ethanolamine (Anderson et al. 1980a), and inositol (Anderson et al. 1980c) into retinal phospholipids. Turnover of these lipid components varies for glycerol, phospholipid headgroups, and fatty acids. β-oxidation of free fatty acids does occur in the retina (Keen and Chlouverakis 1965) and may play a significant role in modification of acyl chains, but it does not appear to be a significant source of energy for total retinal metabolism.

5.3.3.2 Polyunsaturated Fatty Acids and Docosahexaenoic Acid

There is a striking difference between fatty acid compositions of purified rod outer segments (ROS) and red blood cells (RBC) (Fig. 5.2a). The retina contains an unusually high concentration of polyunsaturated fatty acids (PUFAs), especially

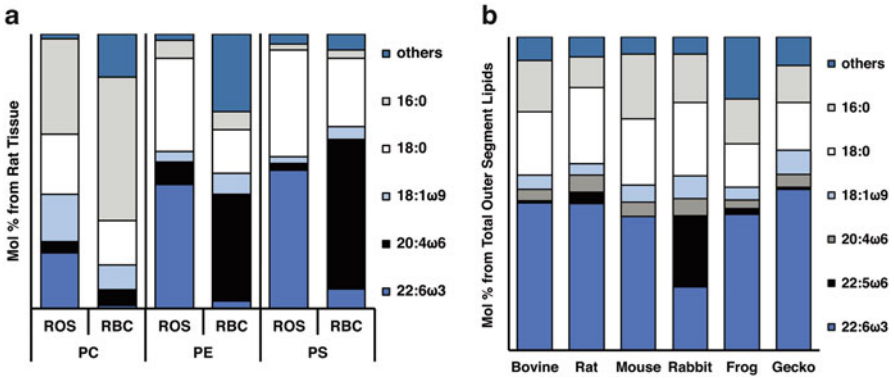

Fig. 5.2 (**a**) Fatty acid profiles of purified rod outer segments (ROS) and red blood cells (RBCs), shown as mole percent (*Mol%*) of three groups of phospholipids (Anderson and Maude 1972). (**b**) Fatty acid profiles of retinal outer segment lipids for six species: bovine (Stone et al. 1979), rat (Stone et al. 1979), mouse (Anderson et al. 2001), rabbit (Nielsen et al. 1986), frog (Stone et al. 1979), and gecko (Yuan et al. 1998). Amounts shown are mole percents of total lipids in the outer segment. *PC*: Phosphatidylcholine; *PE*: Phosphatidylethanolamine; *PS*: Phosphatidylserine

22:6ω3, docosahexaenoic acid (DHA). This type of enrichment of PUFAs in rod outer segments is conserved across species (Fig. 5.2b) (Anderson and Maude 1972; Anderson et al. 2001; Nielsen et al. 1986; Yuan et al. 1998). Remarkably, DHA comprises more than 50 % of all fatty acids in the outer segments of gecko retinas.

Radiolabeled DHA is absorbed from the intestine into the blood. It then crosses the blood–retina barrier through what appears to be a receptor-mediated process at the RPE. From there it becomes enriched in photoreceptor outer segments (Li et al. 1992). This mechanism must be the main source of retinal fatty acids, as the retina is unable to synthesize sufficient DHA to maintain outer segment disc turnover (SanGiovanni and Chew 2005).

DHA levels in the retina are maintained through retention of DHA within the retina. Total retinal DHA levels varied little over time in rats deprived of dietary DHA (Stinson et al. 1991), suggesting that there is a mechanism whereby PUFAs are recycled from the RPE to the photoreceptor following outer segment phagocytosis (Fig. 5.1b). Expression of apoproteins and the production of lipoprotein particles in the retina (Fliesler and Bretillon 2010) form a coordinated system whereby RPE uptakes fatty acids from the blood and repackages them with fatty acids recycled from phagocytosed discs for export to the photoreceptor and inner retina, through the interphotoreceptor matrix. However, the mechanism for recycling of DHA, although potentially similar to the exchange of retinal and retinol between the photoreceptor and the RPE, is not known.

DHA promotes several activities in the retina that can help protect photoreceptor cells from regular exposure to photooxidative damage. As a precursor for the neuroprotectin, NPD1, DHA helps to upregulate anti-apoptotic genes in the Bcl-2 family while downregulating pro-apoptotic and proinflammatory genes (Bazan 2007). DHA also activates the ERK/MAPK pathway to promote survival and

early differentiation of photoreceptors (German et al. 2006). Furthermore, DHA contributes significantly to the rhodopsin microenvironment (Feller and Gawrisch 2005), and in vitro analysis has revealed that DHA lends conformational stability to rhodopsin under both light and dark conditions (Sanchez-Martin et al. 2013).

Enzymes responsible for elongation and β-oxidation of PUFAs are present in the retina, and some have been implicated in human disease. For instance, the fatty acid elongase, ELOVL4, catalyzes the first rate-limiting step of PUFA elongation and is enriched in the inner segments of rods and cones. ELOVL4 is selective for PUFAs and specifically aids in synthesis of very long chain polyunsaturated fatty acids (VLCPUFAs; C24–C36). Several mutations in *STGD3*, the human gene encoding ELOVL4, are linked to autosomal dominant Stargardt type 3 disease, a heritable form of blindness (Molday and Zhang 2010). However, a recent study found that depletion of VLCPUFAs alone did not result in photoreceptor degeneration in a mouse model of Stargardt type 3 disease, suggesting that perhaps aggregation of the mutant ELOVL4 protein contributes to the disease phenotype (Barabas et al. 2013).

In addition to lipids and integral membrane proteins, cholesterol is essential for maintenance of membrane structure in mammalian cells. In an animal model of retinal degeneration, CYP27A1−/− (Omarova et al. 2012), disruption of cholesterol or isoprenoid metabolism can induce disruptive morphological changes in cell structure and retina vascularization. Abnormal accumulation of cholesterol and lipoprotein products in the back of the retina is associated with age-related macular degeneration. Although the photoreceptor appears capable of synthesizing cholesterol and isoprenoids from acetate groups (Zheng et al. 2012; Fliesler and Bretillon 2010), most retinal cholesterol appears to be recycled, following the general trend that outer segment renewal is a continuous cycle of buildup by the photoreceptors and breakdown by the RPE.

5.3.4 Nucleotide Synthesis

As a postmitotic cell, replication of DNA is not a major factor in anabolic processes of photoreceptor cells. Tracing of ^{3}H-labeled thymidine incorporation into newly synthesized DNA demonstrated that DNA in photoreceptors appears to undergo little change throughout the lifetime of the mammalian retina (Ishikawa 1986). However, messenger RNA is upregulated four- to tenfold in response to light, indicating a need for increased purine and pyrimidine synthesis during light adaptation (Schmidt et al. 1985; Hollyfield and Basinger 1980). This idea is corroborated by changes in the enzymes that catalyze de novo synthesis of purines (Etingof 2001) and indicates there is a regulated mechanism that can stimulate nucleotide synthesis. The relationship of purine synthesis to overall metabolism in the retina is also reflected in the increase of purine nucleotides and precursors that occurs during retina ischemia (Roth et al. 1997). Those findings suggest a possible role of nucleotides in preventing oxidative damage and progression of ischemia.

5.3.5 Circadian Regulation Versus Light and Dark Adaptation

Shedding of discs relies on contact between photoreceptors and RPE (Williams and Fisher 1987; Ruggiero et al. 2012). Renewal and shedding occur over about 10 days. The mechanism of disc shedding depends on light adaptation and appears to involve unique localization of phospholipids to the tip of the outer segments (Ruggiero et al. 2012). However, the coordinated pathway from light absorption to disc shedding is unknown.

Disc shedding and phagocytosis by the RPE is a process that is stimulated by light, but formation of new outer segment discs appears to be a constitutive ongoing anabolic process. Amino acid uptake into outer segment discs is unaffected by light or darkness (Hollyfield et al. 1982). Light stimulates uptake of inositol into the retina (Anderson and Hollyfield 1981), and exogenous inositol is rapidly incorporated into phospholipid headgroups (Anderson 1986). Inner retina neurons and photoreceptors have a similar circadian increase in inositol incorporation (Guido et al. 2001). Inositol incorporation into outer segments is stimulated by light (Ghalayini and Anderson 1995), but this may have more to do with signaling properties of phosphatidylinositols rather than a direct role in membrane synthesis. Ethanolamine and serine also incorporate rapidly into retina phospholipids, but their incorporation is not regulated by light (Anderson et al. 1980a, 1980d; Mizuno 1976).

Shedding of outer segment discs is regulated by light and darkness. The dependence on light exposure versus circadian rhythms varies from species to species. In amphibians, the process is regulated by light (Basinger et al. 1976; Hollyfield and Rayborn 1979) or exogenous factors such as heat (Basinger and Hollyfield 1980; Hollyfield and Rayborn 1979). In mice (Besharse and Hollyfield 1979) and rats (LaVail 1976a), disc shedding is circadian, but it can be adapted to light/dark stimuli and may rely on dark adaptation for normal shedding (LaVail 1976b; Currie et al. 1978). Disc shedding in mice is regulated by light levels, but formation of outer segments is unaffected by light. Ultimately, it appears to be shedding rather than synthesis that determine outer segment length and morphology (Besharse and Hollyfield 1979; Hollyfield and Rayborn 1979).

Retention of the outer segment structure in isolated photoreceptors in culture depends strongly on light versus darkness. Photoreceptors in culture retain their outer segments in darkness, but light causes the outer segments to disappear, leading to formation of a round cell with little evidence of the polarized morphology of the normal adult photoreceptor cell (Zayas-Santiago and Kang Derwent 2009). The mechanism behind this phenomenon is unknown, but it suggests that light-dependent interactions that alter synthesis or clearance of outer segment membranes may be of fundamental importance for photoreceptor function and morphology.

5.3.6 *Insulin Receptor*

Insulin receptors are present in photoreceptors (Rajala and Anderson 2010). The retinal insulin receptor has a higher basal level of phosphorylation than the insulin receptors in other tissues (Rajala 2010). Photoactivation of rhodopsin stimulates tyrosine phosphorylation of Grb14 by Src kinase (Basavarajappa et al. 2011). It also stimulates translocation of Grb14 from the inner segment to the outer segment (Rajala et al. 2009). In its phosphorylated state, Grb14 is a protein that inhibits PTP1B, the tyrosine phosphatase that removes phosphate from the insulin receptor (Basavarajappa et al. 2011). Consequently, the steady-state phosphorylation of the insulin receptor and perhaps other proteins normally dephosphorylated by PTP1B is higher in light than in darkness (Rajala and Anderson 2010). Increased phosphorylation of the insulin receptor during illumination appears to stimulate Akt and PI3K activity (Rajala 2010). This pathway promotes anabolic activity in many types of cells, and there is evidence that it has a neuroprotective role in the retina. Rod-specific knockout of the insulin receptor or deletion of an isoform of Akt cause increased photoreceptor degeneration in response to light-induced stress (Rajala 2010).

5.3.7 *Influence of RPE and Growth Factors on Outer Segment Maintenance*

Interactions between RPE and photoreceptors are crucial, not only to turnover of outer segment discs but also in maintenance of outer segment processes. Growth factors supplied to the retina may protect outer segment processes from the degenerative consequences of mutations (Wen et al. 2012). RPE provides numerous growth factors, such as BDNF, CNTF, and PEDF (Kay et al. 2013), that improve outer segment maintenance in models of photoreceptor degeneration. The secretion of these factors regulates neuronal differentiation and survival and as such may be critical to overall retinal metabolism (Jablonski et al. 2000).

5.4 Retinal Metabolism in Light Versus Dark

5.4.1 *Metabolic Demands in Darkness Are Different Than Demands in Light*

The different activities of photoreceptors in darkness versus light translate into qualitative, quantitative, and spatial differences in metabolic demand.

In darkness, photoreceptors behave as do conventional neurons. Photoreceptors in darkness use fuel to produce energy for ion exchange and require little anabolic

activity (Okawa et al. 2008). The ion pump activity is high in darkness to compensate for the constant entry of Na^+ into the cell. Photoreceptors are constitutively depolarized in darkness. The dark current is maintained by a steady influx of Na^+ through cGMP-gated channels. The energy to maintain the ion gradient comes from hydrolysis of ATP by the Na^+/K^+ pump in the inner segment.

Numerous studies have shown that both energy demand and energy production in retinas are greater in darkness than in light (Ames et al. 1992; Linsenmeier 1986; Medrano and Fox 1995; Wang et al. 1997; Winkler 1981; Xu et al. 2007). The most recent calculations indicate that photoreceptors consume 10^8 ATP per second in darkness, approximately fourfold more than in the light (Okawa et al. 2008). ATP expenditure was calculated for the three different energy-consuming compartments of the cell: the outer segment (OS), the inner segment (IS), and the synapse. Nearly all ATP consumption in darkness is used to maintain ion gradients: one ATP is hydrolyzed for every three Na^+ removed (Okawa et al. 2008). Neurotransmission (the steady release of glutamate from the synapse) puts significantly less energy demand on the cell.

When photoreceptors are illuminated, their metabolism transforms to become more like that of a cancer cell. Less energy is produced, less energy is consumed, and there is a shift to more anabolic metabolism. Influx of Na^+ stops because light stimulates the closure of cGMP-gated channels in the outer segment. The cell hyperpolarizes and slows release of glutamate into the synapse. Overall ATP consumption decreases by more than 75 % (Okawa et al. 2008), and metabolic priorities shift to support phototransduction (Ames et al. 1986), regeneration of visual pigment (Cornwall et al. 2003), and synthesis of new membranes (Rajala and Anderson 2003; Cornwall et al. 2003). Because light can cause cumulative damage to photoreceptors, anabolic activity also is needed to repair damage (Rapp and Ghalayini 1999).

The sites of energy consumption also are different in light versus darkness. In darkness, nearly all the activity is in the inner segment and terminal. The ion pumps and synaptic vesicle recycling and refilling machinery are the primary consumers of energy. In light, energy consumption occurs mainly in the outer segment to support phototransduction (Fig. 5.3).

How retinas provide energy for these enormous metabolic demands and how they control the distribution and pace of their metabolism to meet the varying needs of the cell under different illumination conditions are only beginning to be understood.

5.4.2 Influence of Light and Dark on O_2 Consumption

It has been reported that, per gram of tissue, retina consumes energy more rapidly than brain (Yu and Cringle 2001). Even though half the ATP synthesized in a retina comes from glycolysis, retinas still are prodigious consumers of O_2. Retinas are highly sensitive to hypoxia (Bui et al. 2003; Wilkus et al. 1971). Reducing O_2 delivery to the retina from 95 % to 10 % causes an 86 % drop in O_2 consumption and a 2.7-fold increase in lactate (Ames et al. 1992).

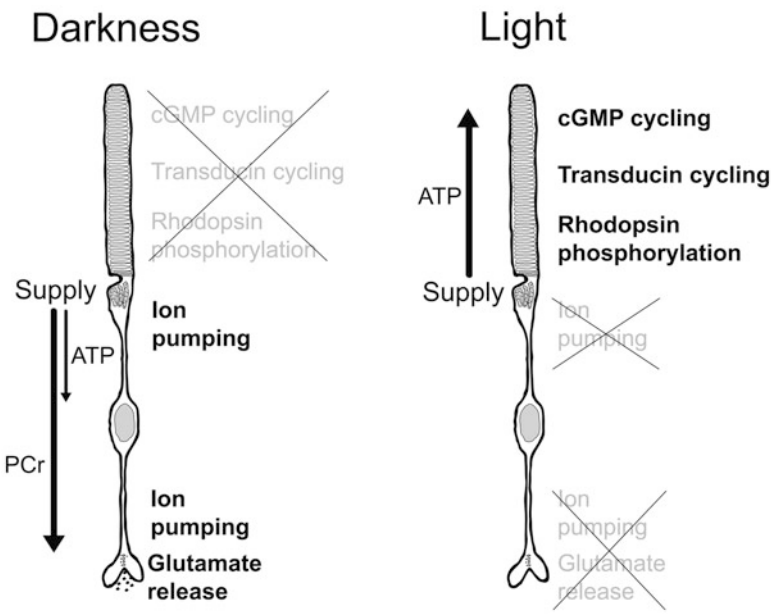

Fig. 5.3 Spatial differences of the energy demands for photoreceptors in darkness versus in light. In darkness, energy is used by the inner segment to pump ions to maintain ion gradients. Energy also is used at the synaptic terminal for refilling and recycling of synaptic vesicles. Overall, in darkness there is a net flow of metabolic energy from the ellipsoid toward the synaptic terminal. In some retinas (nonvascularized), the energy flow to the terminal must be carried in the form of creatine phosphate. During illumination, metabolic energy flows toward the outer segment to sustain phototransduction

Because there is no way to store O_2 in a retina, continuous supply is essential. Sources of O_2 vary between species. Rabbits and guinea pigs have avascular retinas, which rely mostly on the choroid to supply O_2. The inner retina in these species is predominantly glycolytic because most O_2 is consumed by photoreceptors before it has a chance to reach the inner retina (Yu and Cringle 2001). Mouse, rat, cat, and human retinas contain inner retinal vasculature. Even in these retinas, most of the O_2 supplied by the choroid is consumed by photoreceptor mitochondria before it can reach the inner retina (Wangsa-Wirawan and Linsenmeier 2003) In general, O_2 consumption is greatest at the photoreceptors and decreases toward the inner retina (Cringle et al. 1999).

5.4.3 Light Reduces O_2 Consumption

Light diminishes the energy cost of dark current (Ames et al. 1992; Okawa et al. 2008). In both vascular and avascular retinas, O_2 consumption decreases by 30 % to 60 % in the transition between dark and light (Ames et al. 1992; Linsenmeier 1986; Medrano and Fox 1995; Wang et al. 1997; Winkler 1981; Xu et al. 2007).

Light does not decrease lactate production under normoxia despite decreased demand for ATP production and large changes in O_2 consumption (Ames et al. 1992). When sufficient O_2 is present (normoxia), the decrease in ATP production during illumination must occur because of a decrease in oxidative phosphorylation and not a decrease in glycolysis. However, during hypoxia photoreceptors increase their capacity for glycolysis and changes in glycolysis contribute to light versus dark differences in energy production. Lactate production from rabbit retinas increased by 18 % during the light to dark transition when O_2 availability was reduced (Ames et al. 1992).

Mitochondria in photoreceptors are tightly packed in the inner segment and clustered near sites of ATP demand, that is, the ion pumps (Jones 1984; Steinberg 1987). In fact, O_2 consumption and lactate production fall by 49 % and 58 %, respectively, when Na^+/K^+ ATPase activity is blocked with strophanthidin (Ames et al. 1992). Studies using the strophanthidin analogue ouabain to block pump activity showed similar results (Winkler 1981).

5.4.4 O_2 Consumption in the Inner Retina

The inner and outer halves of the retina differ in oxygen tension and consumption (Wangsa-Wirawan and Linsenmeier 2003). In avascular retinas, for example, in guinea pigs, inner retina O_2 levels are extremely low. Neurons survive there in a nearly anoxic environment. Under normal conditions for these retinas, inner retinal O_2 consumption is less than 6 % of the outer retina rate (Yu and Cringle 2001).

In vascular retinas, as found in rats, the inner retina has access to O_2. Nevertheless, the outer retina still is the dominant O_2 consumer (Yu and Cringle 2001). The effect of light is to decrease O_2 consumption primarily in the photoreceptor layer. The inner part of the vascularized rat retina decreases O_2 consumption by only 4 %. In the outer retina, light causes a 60 % decrease in O_2 consumption (Medrano and Fox 1995; Wangsa-Wirawan and Linsenmeier 2003). Overall photoreceptors account for the majority of O_2 consumption in both light and dark conditions.

5.4.5 Light Can Be a Neuroprotectant

Because light adaptation lowers the metabolic demand on photoreceptors, then perhaps it could, in some situations, reduce the relative level of stress on photoreceptors. During hypoxia retinal function gradually declines. Light can delay that loss of function by lowering the metabolic drain on endogenous energy stores (Bui et al. 2003). Inhibition of creatine kinase blocks transport of metabolic energy to photoreceptor synaptic terminals. Light counteracts this effect by lowering metabolic demand (Linton et al. 2010). Hypoxia can stimulate a decrease in O_2

consumption that is similar to the reduction of O_2 consumption stimulated by light. It has been proposed that in both cases slowing ion pumping serves as a protective mechanism by suppressing metabolic demand (Steinberg 1987).

5.4.6 *Biochemical Mechanisms for Light–Dark Regulation of Photoreceptor Metabolism*

Energy production in retinas increases in darkness (Ames et al. 1992; Linsenmeier 1986; Medrano and Fox 1995; Wang et al. 1997; Winkler 1981; Xu et al. 2007). The concentration of cytosolic free Ca^{2+} in outer segments of photoreceptors is higher in darkness than in light (Gray-Keller and Detwiler 1994). This difference also may extend to mitochondria in the part of the cell body adjacent to the outer segment (Krizaj 2012). Therefore, differences in cytosolic free [Ca^{2+}] in photoreceptors could contribute to metabolic differences between light- and dark-adapted retinas. In other tissues it has been shown that elevated cytosolic Ca^{2+} stimulates import of glutamate into photoreceptor mitochondria by activating the aspartate glutamate carrier (AGC1). This transporter, also known as aralar, has Ca^{2+}-binding sites on its amino terminus (del Arco and Satrustegui 1998) and its activity is stimulated by physiological concentrations of Ca^{2+} (Contreras et al. 2007; Contreras and Satrustegui 2009; Gellerich et al. 2010; Gellerich et al. 2012; Palmieri et al. 2001). AGC1/aralar is present in photoreceptors in retina (Xu et al. 2007). Ca^{2+} also may stimulate respiration by entering into the matrix where it stimulates α-ketoglutarate oxidation by lowering the K_m of α-ketoglutarate dehydrogenase (Contreras and Satrustegui 2009; Gellerich et al. 2009; Gellerich et al. 2010; Gellerich et al. 2012). Investigations of these mechanisms in retina have not yet been reported, but they are likely to contribute to retinal metabolism because all the components required for this type of regulation are present in photoreceptors.

5.4.7 *Light–Dark Metabolism in Rods Versus Cones*

Rods and cones have similar physiological properties (Nikonov et al. 2006) but they also have quantitative and structural differences that can influence their metabolism. Rods are more sensitive to changes in glucose concentration (Macaluso et al. 1992) than cones. Rod photoreceptors can completely shut down the influx of Na^+ through light-sensitive channels whereas the influx of ions in cones does not fall as steeply or as completely (Matthews et al. 1990; Burkhardt 1994). In addition, cone photoreceptors may consume more energy than rods during illumination. Twice the number of transducin molecules are activated in cones, (Nikonov et al. 2008), and they have a higher activity of rhodopsin kinase (Kawamura and Tachibanaki 2008; Wada et al. 2006), so they may require faster ATP consumption to efficiently arrest signal transduction.

5.4.8 Disc Shedding in Light Versus Dark

Photoreceptors in rats and other mammals shed approximately 10 % of their outer segments at the onset of light each morning. Outer segments are completely replaced every 9 to 13 days (Young 1971). Older discs are shed from the apical end of the OS and are engulfed by the RPE into phagosomes. The outer segments maintain a uniform length, which suggests that photoreceptors replenish 10 % of their lipids each day. In mammals, this process is linked to circadian rhythm. Shedding occurs at the same time every day whether light is present or not (LaVail 1976b). However, in some species, such as frogs, disc shedding is regulated directly by light; if kept in the dark for multiple light cycles, discs begin to shed irregularly and randomly throughout the day (Basinger et al. 1976). Even circadian-entrained retinas begin to show a slowing of shedding as they are kept in the dark for extended periods of time, which suggests that light acts as a regulatory mechanism or primer for shedding. Exactly how photoreceptors synthesize enough lipids to meet this enormous daily demand, or how light initiates the shedding response, is unknown.

5.5 Distribution of Metabolic Activities in the Retina

5.5.1 Introduction

The laminated structure of the vertebrate retina influences the distribution of metabolic activities in the retina. It also influenced the types of experimental approaches investigators have used to analyze retinal metabolism. Specific types of neurons are confined to distinct layers in the retina. Proteins, metabolites, and physiological and biochemical activities can be localized to specific layers either by immunohistological methods (Marc et al. 1998) or by immunological or enzymatic assays of serial tangential sections (de Azeredo et al. 1981; Reidel et al. 2011).

5.5.2 The Blood–Retina Barrier

Nutrients from the blood must pass through the blood–retina barrier (BRB) to reach the retina. The outer BRB is formed by tight junctions between retinal pigment epithelial (RPE) cells. The inner BRB is created by tight junctions between endothelial cells of inner retina capillaries (Cunha-Vaz et al. 2011; Campbell and Humphries 2012). Glucose and other fuels pass through specific transporters or carriers to satisfy the high energy demands of the retina.

Table 5.1 Distributions of glucose transporter isoforms

Name	Species	Affinity	Distribution	References
GLUT1	Human, rat, mouse, *Xenopus*, monkey, cow	High	RPE, endothelial cells (EC), photoreceptors, Müller cells, ganglion cells	Harik et al. (1990), Takata et al. (1992, 1997), Hsu and Molday (1991), Gospe et al. (2010), Gerhart et al. (1999), Nihira et al. (1995), Tomi and Hosoya (2004), Mantych et al. (1993)
GLUT2	Rat	Low	Müller cells	Watanabe et al. (1994, 1999b)
GLUT3	Human, rat	High	IPL, OPL (Takata et al. 1997) amacrine cells	Watanabe et al. (1996, 1999a), Gerhart et al. (1999), Mantych et al. (1993)
GLUT4	Rat, frog, human	Insulin-regulated	Photoreceptors, INL ganglion cells	Sanchez-Chavez et al. (2012), Mantych et al. (1993)

5.5.3 Glucose Transporters

Glucose is essential for retinal metabolism (Fliesler et al. 1997; Chertov et al. 2011). It is imported into cells by facilitated diffusion through glucose transporters (GLUTs). GLUTs belong to the solute carrier 2A (SLC2A) family. There are 14 isoforms with a variety of properties and expression patterns (Thorens and Mueckler 2010). GLUT1, -2, -3, and -4, are expressed in retinas (Table 5.1).

GLUT1 carries glucose across both outer and inner BRBs. Its expression is highest in basal and apical RPE and vascular endothelial cells. During tight junction development, GLUT1 expression increases (Ban and Rizzolo 2000). GLUT1 appears to be the major path for glucose across blood–brain and blood–retina barriers (Takata et al. 1997).

GLUT1 also is expressed in photoreceptors, Müller cells, and ganglion cells. Its presence in the inner segment of photoreceptors is clear, but its distribution in the outer segment is less certain. GLUT1 immunoreactivity has been detected in outer segments of photoreceptors from various species (Hsu and Molday 1991; Lopez-Escalera et al. 1991; Mantych et al. 1993; Nihira et al. 1995). However, a recent study came to a different conclusion. Proteomic analyses of serial sections, immunofluorescence, and localization of GFP-fusion proteins led to the conclusion that GLUT1 is actively excluded from the outer segment (Gospe et al. 2010).

GLUT2 may regulate glucose homeostasis within the retina and in early stages of development. It is a low-affinity, high-capacity transporter and it can transport fructose with about sixfold-lower affinity than glucose (Olson and Pessin 1996). In adult rat retinas, GLUT2 is present at the apical ends of Müller cells that face the interphotoreceptor space. In neonatal rat retinas, it is at the boundary between the outer nuclear layer and photoreceptor layer (Watanabe et al. 1994; Watanabe et al. 1999b).

GLUT3 has higher affinity than GLUT1 and is distributed widely throughout the brain. Its high affinity maximizes glucose transport even when the substrate

Table 5.2 Distribution of monocarboxylate transporter isoforms

Name	Species	Distribution	References
MCT1	Rat, pig, human, mouse	RPE, EC, photoreceptors, IPL, OPL Müller cells	Philp et al. (1998, 2003a, b), Bergersen et al. (1999, 2002), Gerhart et al. (1999), Hamann et al. (2003), Chidlow et al. (2005)
MCT2	Rat, human	IPL, OPL, Müller cells	Gerhart et al. (1999), Chidlow et al. (2005)
MCT3	Chick, rat, human, mouse	RPE	Philp et al. (1998, 2003a, b), Bergersen et al. (1999), Chidlow et al. (2005)
MCT4	Rat, human, mouse	IPL, OPL, Müller cells	Philp et al. (2003a, b), Chidlow et al. (2005)

concentrations are relatively low. GLUT3 is present in the inner and outer plexiform and ganglion cell layers (Gerhart et al. 1999). In the rat, GLUT3 expresses in the inner plexiform layer near the time of birth and in the outer plexiform layer at postnatal day 14 (Watanabe et al. 1996). GLUT3 is also found on cholinergic amacrine cells (Watanabe et al. 1999b).

GLUT4 is the insulin-regulated glucose transporter. Both insulin and insulin receptor have been identified in retina (Waldbillig et al. 1987a, b; Rosenzweig et al. 1990). GLUT4 has been detected in rat and frog retinas in the photoreceptor, inner nuclear, and ganglion cell layers (Sanchez-Chavez et al. 2012). However, GLUT4 has not been detected in the human retina by immunoblot and immunohistochemistry (Mantych et al. 1993).

5.5.4 Monocarboxylate Transporters

Monocarboxylate transporters (MCTs) are integral membrane proteins that transport lactate, pyruvate, and ketone bodies across the plasma membrane. To date, 16 MCT isoforms have been identified by sequence similarity, inhibitor affinity, and activity measurements. Lactate is the primary substrate for proton-coupled MCT1-4 (Halestrap and Wilson 2012; Adijanto and Philp 2012).

MCT1 is the predominant isoform in most tissues and it facilitates movement of lactate across membranes (see Table 5.2). The direction of lactate flow depends on cell metabolic state. The distribution of MCT1 in retina is very similar to GLUT1: RPE, endothelium, photoreceptors, Müller cells, and the plexiform layers (Gerhart et al. 1999; Tomi and Hosoya 2004; Philp et al. 1998), except that MCT1 is present only at the apical membrane of the RPE and it is not detected in the outer segment.

MCT2 has higher affinity for lactate and pyruvate than MCT1. There are contradicting reports on its expression in retinas. One group reported no MCT2 expression in rat retina, based on polymerase chain reaction (PCR) analysis and immunohistochemistry (Bergersen et al. 1999). However, two other groups,

using similar methods, detected MCT2 in plexiform layers and in Müller cells in rat and human retinas.

MCT3 is expressed only in the RPE basolateral membrane. RPE may take up lactate from the subretinal space via MCT1 and release it into choroidal blood vessels via MCT3 (Philp et al. 1998; Bergersen et al. 1999).

MCT4 is present mostly in highly glycolytic cells, such as type II fast-twitch skeletal muscle cells, astrocytes, and immortalized mammalian cell lines (Dimmer et al. 2000; Halestrap and Wilson 2012). In rat, it is detected in the inner retina, especially the plexiform layers and Müller cells (Chidlow et al. 2005). MCT4 is not expressed in human RPE but it does appear in ARPE-19 cell lines (Philp et al. 2003b).

The functional importance of MCTs was demonstrated by analysis of CD147/basigin null mice, which have low and mislocalized expression of MCTs. In these mice photoreceptors gradually degenerate with accompanying loss of vision (Philp et al. 2003a; Deora et al. 2005). Knockout of MCT3 causes a threefold increase of lactate in the outer retina and impaired retinal function (Daniele et al. 2008).

In addition to proton-coupled MCTs, sodium-coupled MCTs (SMCT) also are present in the retina. SMCT1 is present in ganglion cells, plexiform layers, and photoreceptors, but SMCT2 is restricted in Müller glia (Martin et al. 2007).

5.5.5 Glutamate Transporters

Glutamate is the major excitatory neurotransmitter in the retina. It also is a critical metabolite for energy production and synthesis of glutathione and GABA. After release into the synapse, glutamate must be removed rapidly from the extracellular space to decrease the background signal as well as toxicity. Glutamate is transported by high-affinity transporters. Among them, five different sodium- and potassium-coupled glutamate transporters have been studied extensively. Each of these transports not only glutamate but also D- and L-aspartate.

- Excitatory amino acid transporter 1 (EAAT1), also named glutamate-aspartate transporter (GLAST), is present in glial cells (Rothstein et al. 1994).
- EAAT2 (also named glutamate transporter, GLT) has isoforms that are present in cone terminals, Müller cells, and 'OFF' bipolar cells (Eliasof et al. 1998; Rowan et al. 2010).
- EAAT3 (also named excitatory amino acid carrier, EAAC1) is localized to both glutamatergic and nonglutamatergic neurons (Danbolt 2001).
- EAAT4 is mainly in cerebellar Purkinje cells.
- EAAT5 was thought initially to be exclusively expressed in retina (Arriza et al. 1997), but a recent study using a new antibody shows it also is present in nonnervous tissues including liver, kidney, intestine, heart, lung, and skeletal muscle (Lee et al. 2013).

In vertebrate retinas, EAAT1 is expressed only in Müller cells (Rothstein et al. 1994). EAAT2 has an expression pattern in retina that is different from its expression in the central nervous system (CNS). It is not present in the Müller glia but instead expressed in cone and bipolar cells, amacrine cells, and ganglion cells (Eliasof et al. 1998; Rowan et al. 2010). EAAT3 is present in horizontal, amacrine, and ganglion cells (Fyk-Kolodziej et al. 2004). EAAT4 is in retinal astrocytes and RPE. EAAT5 is present specifically in photoreceptor terminals and in axon terminals of rod bipolar cells. The efficient removal of glutamate from the synaptic cleft by EAAT5 on photoreceptors suggests a smaller role for Müller cells than is exhibited by other CNS glial cells (Hasegawa et al. 2006). Müller cells from GLAST knockout mice can still take up glutamate. The activity is sodium dependent and has a high affinity for glutamate, indicating GLAST is not the only pathway for transporting glutamate into Müller cells (Sarthy et al. 2004; Sarthy et al. 2005).

5.5.6 Distributions of Metabolic Enzymes and Metabolites in Retina

5.5.6.1 Glycolysis

Retinas are extremely active in both aerobic and anaerobic glycolysis. Glycolysis converts glucose into pyruvate, ATP, and NADH through a well-characterized series of enzyme reactions. Hexokinase, phosphofructokinase, and pyruvate kinase are key regulatory steps in the pathway. In an invertebrate retina hexokinase activity is fivefold higher in glial cells than photoreceptors and very little is cytosolic (Veuthey et al. 1994). Hexokinase in the photoreceptors of vertebrate retinas is exclusively localized to the inner segment (Lowry et al. 1961; Simurda and Wilson 1980; Dick 1984; Reidel et al. 2011) where it is both cytosolic and mitochondrial. In contrast to most neurons that are rich in hexokinase I, photoreceptors also express hexokinase II. This isoform is normally associated with mitochondria and is enriched in metabolically active cells like cancer cells. In the retina, hexokinase I is primarily expressed in the plexiform and ganglion cell layers whereas hexokinase II is almost entirely confined to the photoreceptor inner segments (Reidel et al. 2011).

Glucose diffuses from the choroidal vessels through the RPE and IPM to the inner segments of photoreceptors where it is phosphorylated to glucose-6-phosphate. 2-Deoxyglucose, which becomes trapped in cells by hexokinase activity, labels all layers of vertebrate retinas (Winkler et al. 2003).

Phosphofructokinase and pyruvate kinase activities are present in all layers, especially the inner layers of the retina (Lowry et al. 1961). Avascular retinas such as in the rabbit have more phosphofructokinase activity in the plexiform layers and ganglion layers than vascularized retinas (Lowry et al. 1961; Dick 1984).

The aldolase step in glycolysis converts fructose-1,6-bisphosphate (F1,6BP) into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P). Triosephosphate isomerase (TPI) is the enzyme that interconverts DHAP and G3P. DHAP is a precursor for phospholipid synthesis. A recent proteomic analysis of serial sections from rat retina noted the absence of TPI in photoreceptors (Reidel et al. 2011). Consistent with this observation, F1,6BP is more concentrated in the inner retina, especially in the plexiform layers (Matschinsky et al. 1968). Low TPI activity in photoreceptors could raise the concentration of DHAP so that more glycerol phosphate is available for phospholipid synthesis (Reidel et al. 2011). DHAP is the substrate that is used to make glycerol-3-phosphoate. Paradoxically, the activity of glycerol-3-phosphate dehydrogenase (GPDH), the enzyme that makes glycerol-3-phosphate from DHAP, is about 20-fold lower in retina than brain (McGinnis and Leveille 1984; Adler and Klucznik 1982). It even has been suggested that GPDH might be absent from photoreceptors because the loss of photoreceptors by degeneration increases GPDH activity by twofold (McGinnis and Leveille 1984). Further analysis of these activities and the regulation of phospholipid synthesis in photoreceptors is required to resolve these apparent discrepancies.

Most neuronal tissues predominantly express the brain isoform of aldolase (aldolase C). However, retinas also express the muscle isoform (aldolase A) across all layers, especially in the outer nuclear layer (Caffe et al. 1994; Mamczur et al. 2010).

Immunoreactivity for glycolytic enzymes has been characterized in retina sections, and activities of glycolytic enzymes have been detected in purified outer segments of bovine retinas (Hsu and Molday 1991; Lopez-Escalera et al. 1991; Panfoli et al. 2011). Glycolytic enzymes also were identified in bovine and rat outer segments by proteomics (Kwok et al. 2008; Hajkova et al. 2010). An underlying concern with all such experiments is whether antibodies are used at the appropriate concentration and whether purified outer segment preparations can be made free of contamination from other layers of the retina. Approaches to specifically address this issue have been developed recently (Skiba et al. 2013).

Retina tissue produces lactate three times faster than brain tissue. Highly aerobic tissues such as heart and brain predominantly express lactate dehydrogenase (LDH) isoforms 1–3; LDH5 is found in tissues such as liver, muscle, and tumors. LDH5 is the major isoform in retinas (Saavedra and Anderson 1983; Li et al. 1988; Acosta et al. 2005). In chicken retinas, LDH5 is mainly in the inner retina and LDH1 is in photoreceptor inner segments and ganglion cells (Buono and Sheffield 1991). In mouse retinas LDH5 is the major isoform. It appears to be in photoreceptors because total LDH activity decreases when photoreceptors degenerate (Acosta et al. 2005). In both rabbit and monkey, LDH activity is higher in the plexiform layers (Lowry et al. 1956; Matschinsky et al. 1968), suggesting that the inner retina is more glycolytic.

5.5.6.2 TCA Cycle Enzymes and Intermediates

Despite its high glycolytic rate, the retina consumes O_2 more rapidly than many other tissues, including brain (Wangsa-Wirawan and Linsenmeier 2003). Oxidative metabolism of glucose by the classic TCA cycle has the potential to produce as much as 18-fold more ATP than is produced by glycolysis alone and it helps to generate intermediates for amino acid metabolism. O_2 profiles in retinas from cat, rat, pig, and monkey have been measured in vivo by inserting O_2-sensitive microelectodes through the retinas of intact living animals. pO_2 falls steeply from approximately 60 mm Hg in the choriocapillaris to approximately 20 mm Hg in the inner segment, and pO_2 decreases further toward the inner retina (Cringle et al. 2002; Linsenmeier 1986; Wangsa-Wirawan and Linsenmeier 2003; Yu and Cringle 2001).

Although the localization of malate dehydrogenase (MDH), succinate dehydrogenase, and fumarase in the retina is known, less is known about distributions of other TCA cycle enzymes or intermediates. The mitochondrial enzymes are enriched in the inner segment and poorly expressed in the ganglion cell layer (Lowry et al. 1956; Ross and Godfrey 1985; Newell and Kurimoto 1963; Ross et al. 1987; Andrews et al. 1999). There are weak activities in the plexiform layers and few to no activities in the outer segment. Proteomics analysis of rat retina serial sections also confirmed that TCA cycle enzymes colocalize with other mitochondrial proteins in the inner segment (Reidel et al. 2011).

Panfoli and colleagues have reported evidence for an extra-mitochondrial TCA cycle in retinal rod outer segments (Panfoli et al. 2011; Panfoli et al. 2012; Panfoli et al. 2013; Calzia et al. 2013). They found expression of oxidative enzymes, four mitochondrial complexes, and F_1F_0-ATPase by proteomic analyses of purified bovine rod outer segments (Panfoli et al. 2008). The cationic mitochondrial probes Mitotracker and JC-1 stained the outer segment (Bianchini et al. 2008). Outer segment preparations consume O_2, NADH and succinate to produce ATP, and this activity could be inhibited by respiration chain blockers (Panfoli et al. 2009). Activities for eight TCA cycle enzymes were found at levels comparable to those from inner segments (Panfoli et al. 2011). Based on those findings they proposed that the biochemical machinery needed for oxidative phosphorylation system docks on the discs and provides energy for activities in the outer segment. Further studies are needed to confirm this proposal. The biochemical studies rely on the purity of the rod outer segment preparations. For example, another group has used a type of enrichment analysis to show that there are only 11 major proteins in rod outer segment disc membranes: mitochondrial enzymes were not among them (Skiba et al. 2013). Other studies reported that malate dehydrogenase activities are extremely low in rod outer segments from monkey and rat retinas (Lowry et al. 1956; Ross et al. 1987), and there is no reported evidence that pyruvate dehydrogenase, a large protein complex, is present in the outer segment. Nevertheless, this is an interesting proposal with important implications for photoreceptor energetics that requires further investigation.

5.5.6.3 Enzymes and Metabolites in Glutamate/Glutamine Metabolism

Glutamate is not only an important neurotransmitter but it also is a critical metabolite in energy metabolism. Glutamate is produced from either glutamine or glucose, and it can convert into α-ketoglutarate to enter the TCA cycle. The exchange of glutamate with aspartate by the aspartate glutamate carrier (AGC) across the mitochondrial inner membrane is an essential component of the malate–aspartate shuttle (MAS). MAS activity transfers reducing equivalents from cytosolic NADH into the mitochondrial matrix.

Glutamate and glutamine are important components of the ANLS hypothesis. Glutamate converts into α-ketoglutarate either by glutamate dehydrogenase (GLDH) activity or by the activities of transaminases, primarily aspartate transaminase. Compared with brain, retinas have very low GLDH activity (Lowry et al. 1956). Transaminase activity is about 50- to 100-fold higher than GLDH in monkey retinas. The highest activity of GLDH is in photoreceptor inner segments, with evidence for some activity also in the inner plexiform layer and Müller cells (Gebhard 1992). The distribution of aspartate transaminase is almost exactly the same as MDH (Lowry et al. 1956; Ross et al. 1987). In fact, the cytosolic and mitochondrial isoforms of these two enzymes are essential components of the MAS.

There are two mammalian AGCs, AGC1 (also known as aralar) and AGC2 (also known as citrin). Only AGC1 has been detected in retina. It is located only in neuronal mitochondria (Xu et al. 2007) and is not present at significant levels in Müller cells. Consistent with the AGC1 localization, neither isoform of aspartate transaminase could be found in rat Müller cells by immunostaining (Gebhard 1991), suggesting either that Müller cells rely solely on lactate production to regenerate NAD^+ needed to support glycolysis or that there is an alternative pathway to transport reducing power from cytosolic NADH into mitochondria in these glial cells.

Müller cells are highly immunoreactive for glutamine, but glutamate levels in Müller cells are low compared to other cells in the retina (Marc et al. 1998). Besides Müller cells, glutamine also can be detected in horizontal cells and more weakly in bipolar and amacrine cells. Glutamate is most abundant in photoreceptor, bipolar, and ganglion cell neurons. It also is present in horizontal and amacrine cell neurons but it is minimally detected in Müller cells (Marc et al. 1998; Bui et al. 2009).

The glutamate-glutamine cycle may have only a small role in the outer retina, but it is likely to have a more dominant influence on the inner retina. In the glutamate-glutamine cycle, glutamate is converted to glutamine by glutamine synthetase in glia. Once it is exported to neurons, it is converted back into glutamate by glutaminase in neurons.

In retinas, glutamine synthetase is present only in Müller cells (Riepe and Norenburg 1977; Derouiche and Rauen 1995). Inhibition of glutamine synthetase by methionione sulfoximine decreases glutamine levels by 80 %, but glutamate and aspartate levels increase in Müller cells (Bui et al. 2009). The highest activity of glutaminase is in photoreceptor inner segments, followed by plexiform and

ganglion cell layers. Glutaminase signals are very low in photoreceptor outer segments and in the outer nuclear layer (Ross et al. 1987). Glutaminase immunoreactivity also has been detected in Müller cell processes, suggesting a possibility for this enzyme in Müller cells.

5.5.7 Future Directions

Most of the distribution studies are based on biochemical measurement of serial sections of retina and on immunostaining of sectioned retinas. Because Müller cells span almost the entire retina, enzyme activity measurements and proteomics analyses of serial sections could not distinguish Müller cells from neurons. The rapid development of markers for retinal neurons and Müller cells in transgenic mouse lines will help to clarify enzyme distributions in specific cell populations. There has been extensive analysis of transporters and enzymes in retina, but there has been relatively less analysis of metabolite distribution. The coupling of sensitive metabolomics techniques with serial sectioning of retinas may provide more information on metabolite distribution. Also, the application of fluorescent metabolite sensors together with transgenic cell markers may make in vivo imaging of metabolite traffic among cells possible. Furthermore, analyses of gain- or loss-of-function mutations should facilitate studies of transporters and enzymes in specific retinal cell types. The results of such studies, for example, conditional knockouts of MCTs in photoreceptors or glycolysis enzymes in Müller cells, will help resolve the extent to which the astrocyte neuron lactate shuttle or other not yet characterized metabolic pathways influence retinal metabolism.

5.6 Metabolic Dysregulation

Based on their high metabolic rates and demand for ATP one could predict that photoreceptors would be particularly sensitive to perturbations in metabolic supply or demand. They have dense aggregates of mitochondria in their inner segments, which cause formation of steep O_2 gradients, and this could make the retina particularly sensitive to small changes in O_2 availability. Psychophysical measurements have shown that reduced O_2 levels at high elevation increase the dark-adapted visual threshold (Steinberg 1987).

5.6.1 Mitochondrial Diseases

Mitochondrial diseases have been linked to retinal dysfunction. One of the hallmark features of Kearns–Sayre syndrome (KSS), a mitochondrial encephalomyopathy, is pigmentary retinopathy or photoreceptor degeneration. KSS patients have

abnormally large mitochondria as the result of genetic lesions either in mitochondrial (mt)DNA or in nuclear DNA (Phadke et al. 2012). Another example is Leber's hereditary optic neuropathy (LHON). LHON is associated with atrophy of the optic nerve and vision loss and is one of the most prevalent diseases caused by mutations in mtDNA. A mouse model for LHON has the hallmark features of the human disease; it harbors the missense mutation P25L in complex I protein ND6. The mutant mice have reduced ERG responses, age-related loss of small-caliber optic nerve fibers, fiber swelling, and abnormal mitochondria. Somewhat surprisingly, chronic oxidative stress rather than energy deficiency may be responsible for the mutant phenotype (Lin et al. 2012).

To determine a broad view of the prevalence of retinal abnormalities associated with mitochondrial dysfunction, a study reported in 2002 (Cooper et al. 2002) analyzed scotopic ERGs of 22 pediatric patients with mitochondrial disorders, some of which showed ophthalmic evidence of retinal disease. All patients had defects in the function of one or more mitochondrial enzyme complexes, although the specific genetic lesions were known in only 2 of the patients. Nineteen of these 22 patients showed differences in ERG components, with almost half (47 %) displaying reductions in the a-wave amplitude function, Rmp3.

A more recent study (Gronlund et al. 2010) used a variety of ophthalmological assays to analyze vision in 59 patients with mitochondrial disease associated with known mutations. Of these patients, 81 % had ophthalmological defects that included ptosis, reduced eye movement, external ophthalmoplegia, strabismus, nystagmus, low visual acuity, photophobia, optic atrophy, and refractive errors. Six genotypes, each associated with a different disease (KSS, LHON, Leigh syndrome, MELAS, mitochondrial myopathy, MERRF), caused retinal degeneration.

Subtle changes in mitochondrial function also appear correlated with prevalence toward retinal disease. In a recent study, mitochondrial haplotypes were determined for 162 patients with autosomal dominant macular degeneration (AMD). The analysis uncovered a strong association between AMD and the mitochondrial DNA haplotype cluster known as JTU, which is primarily found in northern Europeans (Kenney et al. 2013).

5.6.2 Genetic Strategies

Targeted gene knockout is a useful way to evaluate consequences of mutations in specific genes involved in metabolism for their effects on retinal function and morphology. Although this approach can provide information about the cause of degeneration associated with specific mutations, its limitations are that genes can be redundant and/or have a highly specialized function. For example, a study reported in 2010 (Phillips et al. 2010) analyzed the function and structure of retinas in mice deficient in the mitochondrial adenine nucleotide transporter, ANT1. Although mutations in ANT are often associated with myopathy and pronounced cardiac deficiencies, the authors found no differences in the function, structure, or general

morphology of the retinas, although they did note a slight increase in b-wave amplitude in mutants. Their conclusion is that compensation from other ANT isoforms may explain the lack of a retinal phenotype.

Genome-wide strategies also are a powerful approach to connect specific metabolic enzymes to photoreceptor degeneration. One study created cell lines from peripheral blood lymphocytes and analyzed them for reduced mRNA transcripts using Affimetrix arrays containing nearly 40,000 expressed sequence tags (Hartong et al. 2008). They identified loss-of-function mutations in IDH3B, encoding the β-subunit of the mitochondrial NAD-specific isocitrate dehydrogenase as causal in two families with retinitis pigmentosa. This work identified photoreceptor enzymes within the Krebs cycle as meeting a unique requirement of photoreceptors and highlighted the need for a better understanding of the specialized needs and adaptations of photoreceptor metabolism.

Forward genetic screens have highlighted the importance of mitochondria for visual function. Mutagenesis screens aimed at identifying behaviorally blind zebrafish identified mutations in both the E1 (Maurer et al. 2010) and the E2 (Taylor et al. 2004) subunits of the pyruvate dehydrogenase (PDH) complex. Because of defects in both the outer and inner retina, zebrafish PDH mutants have abnormal electroretinograms and are unable to visually track moving stripes. Mutant viability can be rescued with a ketogenic diet, which bypasses the need for PDH. Because human patients with deficiencies in PDH also are treated with a ketogenic diet (Prasad et al. 2011), the efficacy of this and other treatments can be analyzed in these mutants. Early developmental defects are not detected in zebrafish lacking PDH because of the presence of maternal RNA, which disappears during retinal maturation (Taylor et al. 2004). Zebrafish PDH mutants are therefore useful tools for studying the role of mitochondria during development of vision.

5.7 Indirect Links Between Dysregulation of Metabolism and Degeneration

The majority of genes that cause photoreceptor degeneration are not directly involved in energy metabolism. However, given the very strong correlation between mitochondrial function and photoreceptor malfunction, recent studies have focused on addressing whether secondary perturbations in metabolism may underlie degeneration caused by mutations in genes not directly involved in energy homeostasis. Punzo et al. analyzed changes in gene expression as a strategy for determining shared pathways underlying photoreceptor degeneration. Common changes in metabolism genes in four different mouse models of retinitis pigmentosa suggested that disruption of nutrient availability and disruption of normal regulation by insulin and the mTOR pathway might play key roles in degeneration of cones following rod degeneration. Inhibition of the mTOR pathway resulted in degeneration, and molecular indicators of nutritional imbalance such as HIF-1α and

GLUT1 upregulation appeared. Treatment with insulin delayed the degeneration and prevented HIF-1α upregulation. Although this study only started to examine the role that energy balance plays in photoreceptor survival, it showed how an understanding of this process may lead to strategies for treatments of retinal degenerative diseases (Punzo et al. 2009; Punzo et al. 2012).

The progressive nature of retinal disease can cause the extracellular environment to change in ways that affect retinal metabolism. Measurements of O_2 tension at different layers of the retina during mammalian photoreceptor degeneration show that dramatic increases in outer retinal O_2 occur with the loss of photoreceptor inner segments (Yu and Cringle 2005). A recent study with zebrafish demonstrate that growth factors released by dying retinal neurons stimulate regeneration (Nelson et al. 2013). Photoreceptor maintenance depends heavily on interactions with the RPE. Anabolic activity in photoreceptors functions primarily to resynthesize outer segment disc membranes and phototransduction components. Defective disc phagocytosis by the RPE caused by mutations in the receptor tyrosine kinase gene, Mertk, in the Royal College of Surgeons (RCS) rat causes photoreceptor degeneration (D'Cruz et al. 2000; Nandrot and Dufour 2010; Vollrath et al. 2001).

Perturbations in metabolism have profound consequences on the function and survival of retinal neurons. Currently 202 genes linked to retinal disease have been identified (https://sph.uth.edu/retnet/sum-dis.htm). Neuronal degeneration ultimately reflects a metabolic failure within the cell. It is important to identify the nature of these metabolic failures and their consequences, but this first requires a basic understanding of the metabolic demands and how the retina normally meets those demands. Understanding the metabolic needs of the retina could lead to effective nutritional approaches that could be used to slow progression of the diseases so that an intact retina is available for other treatments such as gene therapy. As this fundamentally important area of research moves toward center stage, it will exploit existing experimental strategies and combine them with sophisticated new approaches, such as metabolite biosensors, to allow real-time in vivo analyses of metabolism at the single-cell level (Hung et al. 2011; Tantama et al. 2012; Zhao et al. 2011).

References

Acosta ML, Fletcher EL, Azizoglu S, Foster LE, Farber DB, Kalloniatis M (2005) Early markers of retinal degeneration in rd/rd mice. Mol Vis 11:717–728

Adijanto J, Philp NJ (2012) The SLC16A family of monocarboxylate transporters (MCTs)–physiology and function in cellular metabolism, pH homeostasis, and fluid transport. Curr Top Membr 70:275–311. doi:10.1016/B978-0-12-394316-3.00009-0

Adler AJ, Klucznik KM (1982) Glycerol phosphate dehydrogenase in developing chick retina and brain. J Neurochem 38(4):909–915

Adler AJ, Southwick RE (1992) Distribution of glucose and lactate in the interphotoreceptor matrix. Ophthalmic Res 24(4):243–252

Agathocleous M, Love NK, Randlett O, Harris JJ, Liu J, Murray AJ, Harris WA (2012) Metabolic differentiation in the embryonic retina. Nat Cell Biol 14(8):859–864. doi:10.1038/ncb2531

Ames A III, Walseth TF, Heyman RA, Barad M, Graeff RM, Goldberg ND (1986) Light-induced Increases in cGMP metabolic flux correspond with electrical responses of photoreceptors. J Biol Chem 261:13034–13042

Ames A III, Li YY, Heher EC, Kimble CR (1992) Energy metabolism of rabbit retina as related to function: high cost of Na^+ transport. J Neurosci 12(3):840–853

Anderson RE (1986) Phosphoinositide metabolism in photoreceptor cells. Neurochem Int 9(2):235–237

Anderson RE, Hollyfield JG (1981) Light stimulates the incorporation of inositol into phosphatidylinositol in the retina. Biochim Biophys Acta 665(3):619–622

Anderson RE, Maude MB (1972) Lipids of ocular tissues. 8. The effects of essential fatty acid deficiency on the phospholipids of the photoreceptor membranes of rat retina. Arch Biochem Biophys 151(1):270–276

Anderson RE, Kelleher PA, Maude MB (1980a) Metabolism of phosphatidylethanolamine in the frog retina. Biochim Biophys Acta 620(2):227–235

Anderson RE, Kelleher PA, Maude MB, Maida TM (1980b) Synthesis and turnover of lipid and protein components of frog retinal rod outer segments. Neurochem Int 1C:29–42

Anderson RE, Maude MB, Kelleher PA (1980c) Metabolism of phosphatidylinositol in the frog retina. Biochim Biophys Acta 620(2):236–246

Anderson RE, Maude MB, Kelleher PA, Maida TM, Basinger SF (1980d) Metabolism of phosphatidylcholine in the frog retina. Biochim Biophys Acta 620(2):212–226

Anderson RE, Maude MB, Bok D (2001) Low docosahexaenoic acid levels in rod outer segment membranes of mice with rds/peripherin and P216L peripherin mutations. Invest Ophthalmol Vis Sci 42(8):1715–1720

Andrews RM, Griffiths PG, Johnson MA, Turnbull DM (1999) Histochemical localisation of mitochondrial enzyme activity in human optic nerve and retina. Br J Ophthalmol 83 (2):231–235

Arriza JL, Eliasof S, Kavanaugh MP, Amara SG (1997) Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proc Natl Acad Sci USA 94 (8):4155–4160

Aubert A, Costalat R (2007) Compartmentalization of brain energy metabolism between glia and neurons: insights from mathematical modeling. Glia 55(12):1272–1279. doi:10.1002/glia.20360

Azevedo AW, Rieke F (2011) Experimental protocols alter phototransduction: the implications for retinal processing at visual threshold. J Neurosci 31(10):3670–3682. doi:10.1523/JNEUROSCI.4750-10.2011

Ban Y, Rizzolo LJ (2000) Regulation of glucose transporters during development of the retinal pigment epithelium. Brain Res Dev Brain Res 121(1):89–95

Barabas P, Liu A, Xing W, Chen CK, Tong Z, Watt CB, Jones BW, Bernstein PS, Krizaj D (2013) Role of ELOVL4 and very long-chain polyunsaturated fatty acids in mouse models of Stargardt type 3 retinal degeneration. Proc Natl Acad Sci USA 110(13):5181–5186. doi:10.1073/pnas.1214707110

Basavarajappa DK, Gupta VK, Dighe R, Rajala A, Rajala RV (2011) Phosphorylated Grb14 is an endogenous inhibitor of retinal protein tyrosine phosphatase 1B, and light-dependent activation of Src phosphorylates Grb14. Mol Cell Biol 31(19):3975–3987. doi:10.1128/MCB.05659-11

Basinger SF, Hollyfield JG (1980) Control of rod shedding in the frog retina. Neurochem Int 1C:81–92

Basinger S, Hoffman R, Matthes M (1976) Photoreceptor shedding is initiated by light in the frog retina. Science 194(4269):1074–1076

Bazan NG (2007) Homeostatic regulation of photoreceptor cell integrity: significance of the potent mediator neuroprotectin D1 biosynthesized from docosahexaenoic acid: the Proctor Lecture. Invest Ophthalmol Vis Sci 48(11):4866–4881; biography 4864–4865. doi:10.1167/iovs.07-0918

Belanger M, Allaman I, Magistretti PJ (2011) Brain energy metabolism: focus on astrocyte–neuron metabolic cooperation. Cell Metab 14(6):724–738. doi:10.1016/j.cmet.2011.08.016

Bentmann A, Schmidt M, Reuss S, Wolfrum U, Hankeln T, Burmester T (2005) Divergent distribution in vascular and avascular mammalian retinae links neuroglobin to cellular respiration. J Biol Chem 280(21):20660–20665. doi:M501338200 [pii]10.1074/jbc.M501338200

Bergersen L, Johannsson E, Veruki ML, Nagelhus EA, Halestrap A, Sejersted OM, Ottersen OP (1999) Cellular and subcellular expression of monocarboxylate transporters in the pigment epithelium and retina of the rat. Neuroscience 90(1):319–331

Bergersen L, Rafiki A, Ottersen OP (2002) Immunogold cytochemistry identifies specialized membrane domains for monocarboxylate transport in the central nervous system. Neurochem Res 27(1-2):89–96

Besharse JC, Dunis DA (1983) Rod photoreceptor disc shedding in eye cups: relationship to bicarbonate and amino acids. Exp Eye Res 36(4):567–579

Besharse JC, Hollyfield JG (1979) Turnover of mouse photoreceptor outer segments in constant light and darkness. Invest Ophthalmol Vis Sci 18(10):1019–1024

Besharse C, Terrill RO, Dunis DA (1980) Light-evoked disc shedding by rod photoreceptors in vitro: relationship to medium bicarbonate concentration. Invest Ophthalmol Vis Sci 19(12):1512–1517

Bianchini P, Calzia D, Ravera S, Candiano G, Bachi A, Morelli A, Bruschi M, Pepe IM, Diaspro A, Panfoli I (2008) Live imaging of mammalian retina: rod outer segments are stained by conventional mitochondrial dyes. J Biomed Opt 13(5):054017. doi:10.1117/1.2982528

Bibb C, Young RW (1974a) Renewal of fatty acids in the membranes of visual cell outer segments. J Cell Biol 61(2):327–343

Bibb C, Young RW (1974b) Renewal of glycerol in the visual cells and pigment epithelium of the frog retina. J Cell Biol 62(2):378–389

Biedermann B, Bringmann A, Reichenbach A (2002) High-affinity GABA uptake in retinal glial (Müller) cells of the guinea pig: electrophysiological characterization, immunohistochemical localization, and modeling of efficiency. Glia 39(3):217–228. doi:10.1002/glia.10097

Bok D (1993) The retinal pigment epithelium: a versatile partner in vision. J Cell Sci Suppl 17:189–195

Bok D, Hall MO (1971) The role of the pigment epithelium in the etiology of inherited retinal dystrophy in the rat. J Cell Biol 49(3):664–682

Bringmann A, Pannicke T, Biedermann B, Francke M, Iandiev I, Grosche J, Wiedemann P, Albrecht J, Reichenbach A (2009) Role of retinal glial cells in neurotransmitter uptake and metabolism. Neurochem Int 54(3-4):143–160. doi:10.1016/j.neuint.2008.10.014

Bui BV, Kalloniatis M, Vingrys AJ (2003) The contribution of glycolytic and oxidative pathways to retinal photoreceptor function. Invest Ophthalmol Vis Sci 44:2708–2715

Bui BV, Hu RG, Acosta ML, Donaldson P, Vingrys AJ, Kalloniatis M (2009) Glutamate metabolic pathways and retinal function. J Neurochem 111(2):589–599. doi:10.1111/j.1471-4159.2009.06354.x

Buono RJ, Sheffield JB (1991) Changes in expression and distribution of lactate dehydrogenase isoenzymes in the developing chick retina. Exp Eye Res 53(2):199–204

Burkhardt DA (1994) Light adaptation and photopigment bleaching in cone photoreceptors in situ in the retina of the turtle. J Neurosci 14:1091–1105

Caffe AR, Von Schantz M, Szel A, Voogd J, Van Veen T (1994) Distribution of Purkinje cell-specific Zebrin-II/aldolase C immunoreactivity in the mouse, rat, rabbit, and human retina. J Comp Neurol 348(2):291–297. doi:10.1002/cne.903480210

Calzia D, Barabino S, Bianchini P, Garbarino G, Oneto M, Caicci F, Diaspro A, Tacchetti C, Manni L, Candiani S, Ravera S, Morelli A, Enrico Traverso C, Panfoli I (2013) New findings in

ATP supply in rod outer segments: insights for retinopathies. Biol Cell 105(8):345–358. doi:10.1111/boc.201300003

Campbell M, Humphries P (2012) The blood–retina barrier: tight junctions and barrier modulation. Adv Exp Med Biol 763:70–84

Chertov AO, Holzhausen L, Kuok IT, Couron D, Parker E, Linton JD, Sadilek M, Sweet IR, Hurley JB (2011) Roles of glucose in photoreceptor survival. J Biol Chem 286(40):34700–34711. doi:10.1074/jbc.M111.279752

Chidlow G, Wood JP, Graham M, Osborne NN (2005) Expression of monocarboxylate transporters in rat ocular tissues. Am J Physiol Cell Physiol 288(2):C416–C428. doi:10.1152/ajpcell.00037.2004

Cohen LH, Noell WK (1960) Glucose catabolism of rabbit retina before and after development of visual function. J Neurochem 5:253–276

Contreras L, Satrustegui J (2009) Calcium signaling in brain mitochondria: interplay of malate aspartate NADH shuttle and calcium uniporter/mitochondrial dehydrogenase pathways. J Biol Chem 284(11):7091–7099. doi:M808066200 [pii]10.1074/jbc.M808066200

Contreras L, Gomez-Puertas P, Iijima M, Kobayashi K, Saheki T, Satrustegui J (2007) Ca^{2+} activation kinetics of the two aspartate-glutamate mitochondrial carriers, aralar and citrin: role in the heart malate-aspartate NADH shuttle. J Biol Chem 282(10):7098–7106. doi:10.1074/jbc.M610491200

Cooper AJ, McDonald JM, Gelbard AS, Gledhill RF, Duffy TE (1979) The metabolic fate of ^{13}N-labeled ammonia in rat brain. J Biol Chem 254(12):4982–4992

Cooper LL, Hansen RM, Darras BT, Korson M, Dougherty FE, Shoffner JM, Fulton AB (2002) Rod photoreceptor function in children with mitochondrial disorders. Arch Ophthalmol 120(8):1055–1062

Cornwall MC, Tsina E, Crouch RK, Wiggert B, Chen C, Koutalos Y (2003) Regulation of the visual cycle: retinol dehydrogenase and retinol fluorescence measurements in vertebrate retina. Adv Exp Med Biol 533:353–360

Cringle SJ, Yu DY, Alder V, Su EN (1999) Light and choroidal PO_2 modulation of intraretinal oxygen levels in an avascular retina. Invest Opthalmol Vis Sci 40:2307–2313

Cringle SJ, Yu DY, Yu PK, Su EN (2002) Intraretinal oxygen consumption in the rat in vivo. Invest Ophthalmol Vis Sci 43(6):1922–1927

Crowder RJ, Freeman RS (1998) Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci 18(8):2933–2943

Cunha-Vaz J, Bernardes R, Lobo C (2011) Blood-retinal barrier. Eur J Ophthalmol 21(suppl 6):S3–S9. doi:10.5301/EJO.2010.6049

Currie JR, Hollyfield JG, Rayborn ME (1978) Rod outer segments elongate in constant light: darkness is required for normal shedding. Vis Res 18(8):995–1003

D'Cruz PM, Yasumura D, Weir J, Matthes MT, Abderrahim H, LaVail MM, Vollrath D (2000) Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rat. Hum Mol Genet 9(4):645–651

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65(1):1–105

Daniele LL, Sauer B, Gallagher SM, Pugh EN Jr, Philp NJ (2008) Altered visual function in monocarboxylate transporter 3 (Slc16a8) knockout mice. Am J Physiol Cell Physiol 295(2):C451–C457. doi:10.1152/ajpcell.00124.2008

de Azeredo FA, Lust WD, Passonneau JV (1981) Light-induced changes in energy metabolites, guanine nucleotides, and guanylate cyclase within frog retinal layers. J Biol Chem 256(6):2731–2735

del Arco A, Satrustegui J (1998) Molecular cloning of Aralar, a new member of the mitochondrial carrier superfamily that binds calcium and is present in human muscle and brain. J Biol Chem 273(36):23327–23334

Denton RM (2009) Regulation of mitochondrial dehydrogenases by calcium ions. Biochim Biophys Acta 1787(11):1309–1316. doi:10.1016/j.bbabio.2009.01.005

Deora AA, Philp N, Hu J, Bok D, Rodriguez-Boulan E (2005) Mechanisms regulating tissue-specific polarity of monocarboxylate transporters and their chaperone CD147 in kidney and retinal epithelia. Proc Natl Acad Sci USA 102(45):16245–16250. doi:10.1073/pnas.0504419102

Derouiche A, Rauen T (1995) Coincidence of L-glutamate/L-aspartate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: evidence for coupling of GLAST and GS in transmitter clearance. J Neurosci Res 42(1):131–143. doi:10.1002/jnr.490420115

Dick E (1984) Enzymes of energy metabolism in the mudpuppy retina. J Neurochem 43 (4):1124–1131

Dimmer KS, Friedrich B, Lang F, Deitmer JW, Broer S (2000) The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem J 350 (pt 1):219–227

DiNuzzo M, Gili T, Maraviglia B, Giove F (2011) Modeling the contribution of neuron–astrocyte cross talk to slow blood oxygenation level-dependent signal oscillations. J Neurophysiol 106 (6):3010–3018. doi:10.1152/jn.00416.2011jn.00416.2011 [pii]

Dudley PA, Anderson RE (1978) Phospholipid transfer protein from bovine retina with high activity towards retinal rod disc membranes. FEBS Lett 95(1):57–60

Eliasof S, Arriza JL, Leighton BH, Kavanaugh MP, Amara SG (1998) Excitatory amino acid transporters of the salamander retina: identification, localization, and function. J Neurosci 18 (2):698–712

Engelmann K, Valtink M (2004) RPE cell cultivation. Graefes Arch Clin Exp Ophthalmol 242 (1):65–67. doi:10.1007/s00417-003-0811-9

Etingof RN (2001) Purine biosynthesis de novo in the retina: evolutionary aspects. Zh Evol Biokhim Fiziol 37(1):3–9

Feller SE, Gawrisch K (2005) Properties of docosahexaenoic-acid-containing lipids and their influence on the function of rhodopsin. Curr Opin Struct Biol 15(4):416–422. doi:10.1016/j.sbi.2005.07.002

Fliesler SJ, Bretillon L (2010) The ins and outs of cholesterol in the vertebrate retina. J Lipid Res 51(12):3399–3413. doi:10.1194/jlr.R010538

Fliesler SJ, Richards MJ, Miller CY, McKay S, Winkler BS (1997) In vitro metabolic competence of the frog retina: effects of glucose and oxygen deprivation. Exp Eye Res 64(5):683–692. doi:10.1006/exer.1996.0281

Fyk-Kolodziej B, Qin P, Dzhagaryan A, Pourcho RG (2004) Differential cellular and subcellular distribution of glutamate transporters in the cat retina. Vis Neurosci 21(4):551–565. doi:10.1017/S0952523804214067

Gebhard R (1991) Cytochemical demonstration of aspartate aminotransferase activity in the rat retina. Brain Res 539(2):337–341

Gebhard R (1992) Histochemical demonstration of glutamate dehydrogenase and phosphate-activated glutaminase activities in semithin sections of the rat retina. Histochemistry 97 (1):101–103

Gellerich FN, Gizatullina Z, Arandarcikaite O, Jerzembek D, Vielhaber S, Seppet E, Striggow F (2009) Extramitochondrial Ca^{2+} in the nanomolar range regulates glutamate-dependent oxidative phosphorylation on demand. PloS One 4(12):e8181. doi:10.1371/journal.pone.0008181

Gellerich FN, Gizatullina Z, Trumbeckaite S, Nguyen HP, Pallas T, Arandarcikaite O, Vielhaber S, Seppet E, Striggow F (2010) The regulation of OXPHOS by extramitochondrial calcium. Biochim Biophys Acta 1797(6-7):1018–1027. doi:10.1016/j.bbabio.2010.02.005

Gellerich FN, Gizatullina Z, Trumbekaite S, Korzeniewski B, Gaynutdinov T, Seppet E, Vielhaber S, Heinze HJ, Striggow F (2012) Cytosolic Ca^{2+} regulates the energization of isolated brain mitochondria by formation of pyruvate through the malate-aspartate shuttle. Biochem J 443(3):747–755. doi:10.1042/BJ20110765

Gerhart DZ, Leino RL, Drewes LR (1999) Distribution of monocarboxylate transporters MCT1 and MCT2 in rat retina. Neuroscience 92(1):367–375. doi:S0306-4522(98)00699-X [pii]

German OL, Insua MF, Gentili C, Rotstein NP, Politi LE (2006) Docosahexaenoic acid prevents apoptosis of retina photoreceptors by activating the ERK/MAPK pathway. J Neurochem 98(5):1507–1520. doi:10.1111/j.1471-4159.2006.04061.x

Ghalayini AJ, Anderson RE (1995) Light adaptation of bovine retinas in situ stimulates phosphatidylinositol synthesis in rod outer segments in vitro. Curr Eye Res 14(11):1025–1029

Gordon WC, Bazan NG (1993) Visualization of [^{3}H]docosahexaenoic acid trafficking through photoreceptors and retinal pigment epithelium by electron microscopic autoradiography. Invest Ophthalmol Vis Sci 34(8):2402–2411

Gospe SM 3rd, Baker SA, Arshavsky VY (2010) Facilitative glucose transporter Glut1 is actively excluded from rod outer segments. J Cell Sci 123(pt 21):3639–3644. doi:10.1242/jcs.072389jcs.072389 [pii]

Gray-Keller MP, Detwiler PB (1994) The calcium feedback signal in the phototransduction cascade of vertebrate rods. Neuron 13(4):849–861

Graymore CN, Tansley K, Kerly M (1959) Metabolism of the developing retina. 2. The effect of an inherited retinal degeneration on the development of glycolysis in the rat retina. Biochem J 72:459–461

Gronlund MA, Honarvar AK, Andersson S, Moslemi AR, Oldfors A, Holme E, Tulinius M, Darin N (2010) Ophthalmological findings in children and young adults with genetically verified mitochondrial disease. Br J Ophthalmol 94(1):121–127. doi:10.1136/bjo.2008.154187

Guido ME, Garbarino Pico E, Caputto BL (2001) Circadian regulation of phospholipid metabolism in retinal photoreceptors and ganglion cells. J Neurochem 76(3):835–845

Hajkova D, Imanishi Y, Palamalai V, Rao KC, Yuan C, Sheng Q, Tang H, Zeng R, Darrow RM, Organisciak DT, Miyagi M (2010) Proteomic changes in the photoreceptor outer segment upon intense light exposure. J Proteome Res 9(2):1173–1181. doi:10.1021/pr900819k

Halestrap AP, Wilson MC (2012) The monocarboxylate transporter family–role and regulation. IUBMB Life 64(2):109–119. doi:10.1002/iub.572

Hamann S, Kiilgaard JF, la Cour M, Prause JU, Zeuthen T (2003) Cotransport of H^+, lactate, and H_2O in porcine retinal pigment epithelial cells. Exp Eye Res 76(4):493–504

Harada T, Harada C, Kohsaka S, Wada E, Yoshida K, Ohno S, Mamada H, Tanaka K, Parada LF, Wada K (2002) Microglia–Muller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration. J Neurosci 22(21):9228–9236

Harik SI, Kalaria RN, Whitney PM, Andersson L, Lundahl P, Ledbetter SR, Perry G (1990) Glucose transporters are abundant in cells with “occluding” junctions at the blood–eye barriers. Proc Natl Acad Sci USA 87(11):4261–4264

Hartong DT, Dange M, McGee TL, Berson EL, Dryja TP, Colman RF (2008) Insights from retinitis pigmentosa into the roles of isocitrate dehydrogenases in the Krebs cycle. Nat Genet 40(10):1230–1234. doi:10.1038/ng.223

Hasegawa J, Obara T, Tanaka K, Tachibana M (2006) High-density presynaptic transporters are required for glutamate removal from the first visual synapse. Neuron 50(1):63–74. doi:S0896-6273(06)00164-4 [pii]10.1016/j.neuron.2006.02.022

Hawkins RA, O’Kane RL, Simpson IA, Vina JR (2006) Structure of the blood–brain barrier and its role in the transport of amino acids. J Nutr 136(1 suppl):218S–226S

Hollyfield JG, Basinger SF (1980) RNA metabolism in the retina in relation to cyclic lighting. Vis Res 20(12):1151–1155

Hollyfield JG, Rayborn ME (1979) Photoreceptor outer segment development: light and dark regulate the rate of membrane addition and loss. Invest Ophthalmol Vis Sci 18(2):117–132

Hollyfield JG, Rayborn ME, Verner GE, Maude MB, Anderson RE (1982) Membrane addition to rod photoreceptor outer segments: light stimulates membrane assembly in the absence of increased membrane biosynthesis. Invest Ophthalmol Vis Sci 22(4):417–427

Hsu SC, Molday RS (1991) Glycolytic enzymes and a GLUT-1 glucose transporter in the outer segments of rod and cone photoreceptor cells. J Biol Chem 266(32):21745–21752

Hung YP, Albeck JG, Tantama M, Yellen G (2011) Imaging cytosolic NADH-NAD(+) redox state with a genetically encoded fluorescent biosensor. Cell Metab 14(4):545–554. doi:10.1016/j.cmet.2011.08.012

Ishikawa T (1986) On the stability of DNA in photoreceptor cells of rat retina. Cell Tissue Res 243 (2):445–448

Ishikawa T, Yamada E (1969) Atypical mitochondria in the ellipsoid of the photoreceptor cells of vertebrate retinas. Invest Ophthalmol 8(3):302–316

Jablonski MM, Tombran-Tink J, Mrazek DA, Iannaccone A (2000) Pigment epithelium-derived factor supports normal development of photoreceptor neurons and opsin expression after retinal pigment epithelium removal. J Neurosci 20(19):7149–7157

Jarrett SG, Boulton ME (2012) Consequences of oxidative stress in age-related macular degeneration. Mol Aspect Med 33(4):399–417. doi:10.1016/j.mam.2012.03.009

Johnson NF (1977) Retinal glycogen content during ischaemia. Albrecht Von Graefes Arch Klin Exp Ophthalmol 203(3-4):271–282

Johnson JE Jr, Perkins GA, Giddabasappa A, Chaney S, Xiao W, White AD, Brown JM, Waggoner J, Ellisman MH, Fox DA (2007) Spatiotemporal regulation of ATP and Ca^{2+} dynamics in vertebrate rod and cone ribbon synapses. Mol Vis 13:887–919. doi:v13/a97 [pii]

Jones DP (1984) Effect of mitochondrial clustering on O_2 supply in hepatocytes. Am J Physiol 247 (1 pt 1):C83–C89

Jones BW, Kondo M, Terasaki H, Watt CB, Rapp K, Anderson J, Lin Y, Shaw MV, Yang JH, Marc RE (2011) Retinal remodeling in the Tg P347L rabbit, a large-eye model of retinal degeneration. J Comp Neurol 519(14):2713–2733. doi:10.1002/cne.22703

Karl A, Wurm A, Pannicke T, Krugel K, Obara-Michlewska M, Wiedemann P, Reichenbach A, Albrecht J, Bringmann A (2011) Synergistic action of hypoosmolarity and glutamine in inducing acute swelling of retinal glial (Müller) cells. Glia 59(2):256–266. doi:10.1002/glia.21095

Kawamura S, Tachibanaki S (2008) Rod and cone photoreceptors: molecular basis of the difference in their physiology. Comp Biochem Physiol A Mol Integr Physiol 150:369–377

Kay P, Yang YC, Paraoan L (2013) Directional protein secretion by the retinal pigment epithelium: roles in retinal health and the development of age-related macular degeneration. J Cell Mol Med 17(7):833–843. doi:10.1111/jcmm.12070

Keen H, Chlouverakis C (1965) Metabolism of isolated rat retina. The role of non-esterified fatty acid. Biochem J 94:488–493

Kenney MC, Hertzog D, Chak G, Atilano SR, Khatibi N, Soe K, Nobe A, Yang E, Chwa M, Zhu F, Memarzadeh M, King J, Langberg J, Small K, Nesburn AB, Boyer DS, Udar N (2013) Mitochondrial DNA haplogroups confer differences in risk for age-related macular degeneration: a case control study. BMC Med Genet 14:4. doi:10.1186/1471-2350-14-4

Krizaj D (2012) Calcium stores in vertebrate photoreceptors. Adv Exp Med Biol 740:873–889. doi:10.1007/978-94-007-2888-2_39

Krizaj D, Lai FA, Copenhagen DR (2003) Ryanodine stores and calcium regulation in the inner segments of salamander rods and cones. J Physiol (Lond) 547(pt 3):761–774. doi:10.1113/jphysiol.2002.0356832002.035683 [pii]

Kumagai AK, Glasgow BJ, Pardridge WM (1994) GLUT1 glucose transporter expression in the diabetic and nondiabetic human eye. Invest Ophthalmol Vis Sci 35(6):2887–2894

Kuwabara T, Cogan DG (1961) Retinal glycogen. Arch Ophthalmol 66:680–688

Kwok MC, Holopainen JM, Molday LL, Foster LJ, Molday RS (2008) Proteomics of photoreceptor outer segments identifies a subset of SNARE and Rab proteins implicated in membrane vesicle trafficking and fusion. Mol Cell Proteomics 7(6):1053–1066. doi:10.1074/mcp.M700571-MCP200

Lamb TD, Collin SP, Pugh EN Jr (2007) Evolution of the vertebrate eye: opsins, photoreceptors, retina and eye cup. Nat Rev Neurosci 8(12):960–976. doi:10.1038/nrn2283

Lasansky A (1973) Organization of the outer synaptic layer in the retina of the larval tiger salamander. Philos Trans R Soc Lond B Biol Sci 265(872):471–489

LaVail MM (1976a) Rod outer segment disc shedding in relation to cyclic lighting. Exp Eye Res 23(2):277–280

LaVail MM (1976b) Rod outer segment disk shedding in rat retina: relationship to cyclic lighting. Science 194(4269):1071–1074

Lee A, Anderson AR, Stevens M, Beasley S, Barnett NL, Pow DV (2013) Excitatory amino acid transporter 5 is widely expressed in peripheral tissues. Eur J Histochem 57(1):e11. doi:10.4081/ejh.2013.e11

Li SS, Pan YE, Sharief FS, Evans MJ, Lin MF, Clinton GM, Holbrook JJ (1988) Cancer-associated lactate dehydrogenase is a tyrosylphosphorylated form of human LDH-A, skeletal muscle isoenzyme. Cancer Invest 6(1):93–101

Li J, Wetzel MG, O'Brien PJ (1992) Transport of n-3 fatty acids from the intestine to the retina in rats. J Lipid Res 33(4):539–548

Lin CS, Sharpley MS, Fan W, Waymire KG, Sadun AA, Carelli V, Ross-Cisneros FN, Baciu P, Sung E, McManus MJ, Pan BX, Gil DW, Macgregor GR, Wallace DC (2012) Mouse mtDNA mutant model of Leber hereditary optic neuropathy. Proc Natl Acad Sci USA 109 (49):20065–20070. doi:10.1073/pnas.1217113109

Linsenmeier RA (1986) Effects of light and darkness on oxygen distribution and consumption in the cat retina. J Gen Physiol 88(4):521–542

Linton JD, Holzhausen LC, Babai N, Song H, Miyagishima KJ, Stearns GW, Lindsay K, Wei J, Chertov AO, Peters TA, Caffe R, Pluk H, Seeliger MW, Tanimoto N, Fong K, Bolton L, Kuok DL, Sweet IR, Bartoletti TM, Radu RA, Travis GH, Zagotta WN, Townes-Anderson E, Parker E, Van der Zee CE, Sampath AP, Sokolov M, Thoreson WB, Hurley JB (2010) Flow of energy in the outer retina in darkness and in light. Proc Natl Acad Sci USA 107 (19):8599–8604. doi:10.1073/pnas.1002471071002471107 [pii]

Lopez-Escalera R, Li XB, Szerencsei RT, Schnetkamp PP (1991) Glycolysis and glucose uptake in intact outer segments isolated from bovine retinal rods. Biochemistry 30(37):8970–8976

Lowry OH, Roberts NR, Lewis C (1956) The quantitative histochemistry of the retina. J Biol Chem 220(2):879–892

Lowry OH, Roberts NR, Schulz DW, Clow JE, Clark JR (1961) Quantitative histochemistry of retina. II. Enzymes of glucose metabolism. J Biol Chem 236:2813–2820

Macaluso C, Onoe S, Niemeyer G (1992) Changes in glucose level affect rod function more than cone function in the isolated, perfused cat eye. Invest Opthalmol Vis Sci 33:2798–2808

Mamczur P, Mazurek J, Rakus D (2010) Ubiquitous presence of gluconeogenic regulatory enzyme, fructose-1,6-bisphosphatase, within layers of rat retina. Cell Tissue Res 341(2):213–221. doi:10.1007/s00441-010-1008-2

Mantych GJ, Hageman GS, Devaskar SU (1993) Characterization of glucose transporter isoforms in the adult and developing human eye. Endocrinology 133(2):600–607

Marc RE (1992) Structural organization of GABAergic circuitry in ectotherm retinas. Prog Brain Res 90:61–92

Marc RE, Murry RF, Fisher SK, Linberg KA, Lewis GP, Kalloniatis M (1998) Amino acid signatures in the normal cat retina. Invest Ophthalmol Vis Sci 39(9):1685–1693

Marc RE, Jones BW, Watt CB, Vazquez-Chona F, Vaughan DK, Organisciak DT (2008) Extreme retinal remodeling triggered by light damage: implications for age related macular degeneration. Mol Vis 14:782–806

Martin PM, Dun Y, Mysona B, Ananth S, Roon P, Smith SB, Ganapathy V (2007) Expression of the sodium-coupled monocarboxylate transporters SMCT1 (SLC5A8) and SMCT2 (SLC5A12) in retina. Invest Ophthalmol Vis Sci 48(7):3356–3363. doi:10.1167/iovs.06-0888

Matschinsky FM, Passonneau JV, Lowry OH (1968) Quantitative histochemical analysis of glycolytic intermediates and cofactors with an oil well technique. J Histochem Cytochem 16(1):29–39

Matthews HR, Fain GL, Murphy RL, Lamb TD (1990) Light adaptation in cone photoreceptors of the salamander: a role for cytoplasmic calcium. J Physiol (Lond) 420:447–469

Maurer CM, Schonthaler HB, Mueller KP, Neuhauss SC (2010) Distinct retinal deficits in a zebrafish pyruvate dehydrogenase-deficient mutant. J Neurosci 30(36):11962–11972. doi:10.1523/JNEUROSCI.2848-10.2010

Mayhew TM, Astle D (1997) Photoreceptor number and outer segment disk membrane surface area in the retina of the rat: stereological data for whole organ and average photoreceptor cell. J Neurocytol 26(1):53–61

McCormack JG, Longo EA, Corkey BE (1990) Glucose-induced activation of pyruvate dehydrogenase in isolated rat pancreatic islets. Biochem J 267(2):527–530

McGinnis JF, Leveille PJ (1984) Glycerol phosphate dehydrogenase in developing retina of normal and mutant mice. Curr Eye Res 3(2):363–367

Medrano CJ, Fox DA (1995) Oxygen consumption in the rat outer and inner retina: light- and pharmacologically-induced inhibition. Exp Eye Res 61(3):273–284

Miyagishima KJ, Cornwall MC, Sampath AP (2009) Metabolic constraints on the recovery of sensitivity after visual pigment bleaching in retinal rods. J Gen Physiol 134(3):165–175. doi:10.1085/jgp.200910267jgp.200910267 [pii]

Mizuno A (1976) Incorporation of serine and ethanolamine into the phospholipids in rabbit retina. J Biochem (Tokyo) 80(1):45–52

Molday RS, Zhang K (2010) Defective lipid transport and biosynthesis in recessive and dominant Stargardt macular degeneration. Prog Lipid Res 49(4):476–492. doi:10.1016/j.plipres.2010.07.002

Nandrot EF, Dufour EM (2010) Mertk in daily retinal phagocytosis: a history in the making. Adv Exp Med Biol 664:133–140. doi:10.1007/978-1-4419-1399-9_16

Nelson CM, Ackerman KM, O'Hayer P, Bailey TJ, Gorsuch RA, Hyde DR (2013) Tumor necrosis factor-alpha is produced by dying retinal neurons and is required for Müller glia proliferation during zebrafish retinal regeneration. J Neurosci 33(15):6524–6539. doi:10.1523/JNEUROSCI.3838-12.2013

Newell FW, Kurimoto S (1963) Histochemistry of the retina in the alloxan-diabetic Rat. Br J Ophthalmol 47:596–600

Nicholls DG (2005) Mitochondria and calcium signaling. Cell Calcium 38(3-4):311–317. doi:10.1016/j.ceca.2005.06.011

Nielsen JC, Maude MB, Hughes H, Anderson RE (1986) Rabbit photoreceptor outer segments contain high levels of docosapentaenoic acid. Invest Ophthalmol Vis Sci 27(2):261–264

Nihira M, Anderson K, Gorin FA, Burns MS (1995) Primate rod and cone photoreceptors may differ in glucose accessibility. Invest Ophthalmol Vis Sci 36(7):1259–1270

Nikonov SS, Kholodenko R, Lem J, Pugh EN Jr (2006) Physiological features of the S- and M-cone photoreceptors of wild-type mice from single-cell recordings. J Gen Physiol 127:547–557

Nikonov SS, Brown BM, Davis JA, Zuniga FI, Bragin A, Pugh EN Jr, Craft SM (2008) Mouse cones require an arrestin for normal inactivation of phototransduction. Neuron 59:462–474

Noell WK (1951) The effect of iodoacetate on the vertebrate retina. J Cell Physiol 37(2):283–307

Okawa H, Sampath AP, Laughlin SB, Fain GL (2008) ATP consumption by mammalian rod photoreceptors in darkness and in light. Curr Biol 18(24):1917–1921. doi:S0960-9822(08)01398-5 [pii]10.1016/j.cub.2008.10.029

Olson AL, Pessin JE (1996) Structure, function, and regulation of the mammalian facilitative glucose transporter gene family. Annu Rev Nutr 16:235–256. doi:10.1146/annurev.nu.16.070196.001315

Omarova S, Charvet CD, Reem RE, Mast N, Zheng W, Huang S, Peachey NS, Pikuleva IA (2012) Abnormal vascularization in mouse retina with dysregulated retinal cholesterol homeostasis. J Clin Invest 122(8):3012–3023. doi:10.1172/JCI63816

Organisciak DT, Vaughan DK (2010) Retinal light damage: mechanisms and protection. Prog Retinal Eye Res 29(2):113–134. doi:10.1016/j.preteyeres.2009.11.004

Palmieri L, Pardo B, Lasorsa FM, del Arco A, Kobayashi K, Iijima M, Runswick MJ, Walker JE, Saheki T, Satrustegui J, Palmieri F (2001) Citrin and aralar1 are Ca(2+)-stimulated aspartate/

glutamate transporters in mitochondria. EMBO J 20(18):5060–5069. doi:10.1093/emboj/20.18.5060

Panfoli I, Musante L, Bachi A, Ravera S, Calzia D, Cattaneo A, Bruschi M, Bianchini P, Diaspro A, Morelli A, Pepe IM, Tacchetti C, Candiano G (2008) Proteomic analysis of the retinal rod outer segment disks. J Proteome Res 7(7):2654–2669. doi:10.1021/pr7006939

Panfoli I, Calzia D, Bianchini P, Ravera S, Diaspro A, Candiano G, Bachi A, Monticone M, Aluigi MG, Barabino S, Calabria G, Rolando M, Tacchetti C, Morelli A, Pepe IM (2009) Evidence for aerobic metabolism in retinal rod outer segment disks. Int J Biochem Cell Biol 41 (12):2555–2565. doi:10.1016/j.biocel.2009.08.013

Panfoli I, Calzia D, Ravera S, Bruschi M, Tacchetti C, Candiani S, Morelli A, Candiano G (2011) Extramitochondrial tricarboxylic acid cycle in retinal rod outer segments. Biochimie 93(9):1565–1575. doi:10.1016/j.biochi.2011.05.020

Panfoli I, Calzia D, Ravera S, Morelli AM, Traverso CE (2012) Extra-mitochondrial aerobic metabolism in retinal rod outer segments: new perspectives in retinopathies. Med Hypotheses 78(4):423–427. doi:10.1016/j.mehy.2011.12.012

Panfoli I, Calzia D, Bruschi M, Oneto M, Bianchini P, Ravera S, Petretto A, Diaspro A, Candiano G (2013) Functional expression of oxidative phosphorylation proteins in the rod outer segment disc. Cell Biochem Funct 31(6):532–538. doi:10.1002/cbf.2943

Pellerin L, Magistretti PJ (1994) Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci USA 91(22):10625–10629

Pellerin L, Magistretti PJ (2012) Sweet sixteen for ANLS. J Cereb Blood Flow Metab 32 (7):1152–1166. doi:10.1038/jcbfm.2011.149

Perezleon JA, Osorio-Paz I, Francois L, Salceda R (2013) Immunohistochemical localization of glycogen synthase and GSK3beta: control of glycogen content in retina. Neurochem Res 38 (5):1063–1069. doi:10.1007/s11064-013-1017-0

Phadke M, Lokeshwar MR, Bhutada S, Tampi C, Saxena R, Kohli S, Shah KN (2012) Kearns Sayre syndrome: case report with review of literature. Ind J Pediatr 79(5):650–654. doi:10.1007/s12098-011-0618-3

Phillips MJ, Webb-Wood S, Faulkner AE, Jabbar SB, Biousse V, Newman NJ, Do VT, Boatright JH, Wallace DC, Pardue MT (2010) Retinal function and structure in Ant1-deficient mice. Invest Ophthalmol Vis Sci 51(12):6744–6752. doi:10.1167/iovs.10-5421

Philp NJ, Yoon H, Grollman EF (1998) Monocarboxylate transporter MCT1 is located in the apical membrane and MCT3 in the basal membrane of rat RPE. Am J Physiol 274(6 pt 2): R1824–R1828

Philp NJ, Ochrietor JD, Rudoy C, Muramatsu T, Linser PJ (2003a) Loss of MCT1, MCT3, and MCT4 expression in the retinal pigment epithelium and neural retina of the 5A11/basigin-null mouse. Invest Ophthalmol Vis Sci 44(3):1305–1311

Philp NJ, Wang D, Yoon H, Hjelmeland LM (2003b) Polarized expression of monocarboxylate transporters in human retinal pigment epithelium and ARPE-19 cells. Invest Ophthalmol Vis Sci 44(4):1716–1721

Poitry S, Poitry-Yamate C, Ueberfeld J, MacLeish PR, Tsacopoulos M (2000) Mechanisms of glutamate metabolic signaling in retinal glial (Müller) cells. J Neurosci 20(5):1809–1821

Poitry-Yamate CL, Tsacopoulos M (1992) Glucose metabolism in freshly isolated Müller glial cells from a mammalian retina. J Comp Neurol 320(2):257–266. doi:10.1002/cne.903200209

Poitry-Yamate CL, Poitry S, Tsacopoulos M (1995) Lactate released by Müller glial cells is metabolized by photoreceptors from mammalian retina. J Neurosci 15(7 pt 2):5179–5191

Pow DV, Robinson SR (1994) Glutamate in some retinal neurons is derived solely from glia. Neuroscience 60(2):355–366

Prasad C, Rupar T, Prasad AN (2011) Pyruvate dehydrogenase deficiency and epilepsy. Brain Dev 33(10):856–865. doi:10.1016/j.braindev.2011.08.003

Punzo C, Kornacker K, Cepko CL (2009) Stimulation of the insulin/mTOR pathway delays cone death in a mouse model of retinitis pigmentosa. Nat Neurosci 12(1):44–52. doi:nn.2234 [pii] 10.1038/nn.2234

Punzo C, Xiong W, Cepko CL (2012) Loss of daylight vision in retinal degeneration: are oxidative stress and metabolic dysregulation to blame? J Biol Chem 287(3):1642–1648. doi:10.1074/jbc.R111.304428

Rajala RV (2010) Phosphoinositide 3-kinase signaling in the vertebrate retina. J Lipid Res 51(1):4–22. doi:10.1194/jlr.R000232

Rajala RV, Anderson RE (2003) Light regulation of the insulin receptor in the retina. Mol Neurobiol 28:123–138

Rajala RV, Anderson RE (2010) Rhodopsin-regulated insulin receptor signaling pathway in rod photoreceptor neurons. Mol Neurobiol 42(1):39–47. doi:10.1007/s12035-010-8130-8

Rajala A, Daly RJ, Tanito M, Allen DT, Holt LJ, Lobanova ES, Arshavsky VY, Rajala RV (2009) Growth factor receptor-bound protein 14 undergoes light-dependent intracellular translocation in rod photoreceptors: functional role in retinal insulin receptor activation. Biochemistry 48(24):5563–5572. doi:10.1021/bi9000062

Rapp LM, Ghalayini AJ (1999) Influence of UVA light stress on photoreceptor cell metabolism: decreased rates of rhodopsin regeneration and opsin synthesis. Exp Eye Res 68(6):757–764. doi:10.1006/exer.1999.0662

Reichenbach A, Bringmann A (2013) New functions of Muller cells. Glia 61(5):651–678. doi:10.1002/glia.22477

Reichenbach A, Stolzenburg JU, Wolburg H, Hartig W, el-Hifnawi E, Martin H (1995) Effects of enhanced extracellular ammonia concentration on cultured mammalian retinal glial (Müller) cells. Glia 13(3):195–208. doi:10.1002/glia.440130306

Reidel B, Thompson JW, Farsiu S, Moseley MA, Skiba NP, Arshavsky VY (2011) Proteomic profiling of a layered tissue reveals unique glycolytic specializations of photoreceptor cells. Mol Cell Proteomics 10(3):M110 002469. doi:10.1074/mcp.M110.002469M110.002469 [pii]

Riepe RE, Norenburg MD (1977) Müller cell localisation of glutamine synthetase in rat retina. Nature (Lond) 268(5621):654–655

Rosenzweig SA, Zetterstrom C, Benjamin A (1990) Identification of retinal insulin receptors using site-specific antibodies to a carboxyl-terminal peptide of the human insulin receptor alpha-subunit. Upregulation of neuronal insulin receptors in diabetes. J Biol Chem 265 (29):18030–18034

Ross CD, Godfrey DA (1985) Distributions of aspartate aminotransferase and malate dehydrogenase activities in rat retinal layers. J Histochem Cytochem 33(7):624–630

Ross CD, Bowers M, Godfrey DA (1987) Distributions of the activities of aspartate aminotransferase isoenzymes in rat retinal layers. Neurosci Lett 74(2):205–210

Roth S, Rosenbaum PS, Osinski J, Park SS, Toledano AY, Li B, Moshfeghi AA (1997) Ischemia induces significant changes in purine nucleoside concentration in the retina-choroid in rats. Exp Eye Res 65(6):771–779. doi:10.1006/exer.1997.0391

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. Neuron 13(3):713–725

Rowan MJ, Ripps H, Shen W (2010) Fast glutamate uptake via EAAT2 shapes the cone-mediated light offset response in bipolar cells. J Physiol (Lond) 588(pt 20):3943–3956. doi:10.1113/jphysiol.2010.191437

Ruggiero L, Connor MP, Chen J, Langen R, Finnemann SC (2012) Diurnal, localized exposure of phosphatidylserine by rod outer segment tips in wild-type but not Itgb5$^{-/-}$ or Mfge8$^{-/-}$ mouse retina. Proc Natl Acad Sci USA 109(21):8145–8148. doi:10.1073/pnas.1121101109

Rutter GA, Pralong WF, Wollheim CB (1992) Regulation of mitochondrial glycerol-phosphate dehydrogenase by Ca^{2+} within electropermeabilized insulin-secreting cells (INS-1). Biochim Biophys Acta 1175(1):107–113. doi:0167-4889(92)90016-5 [pii]

Saavedra RA, Anderson GR (1983) A cancer-associated lactate dehydrogenase is expressed in normal retina. Science 221(4607):291–292

Salvini-Plawen LV, Mayr E (1977) On the evolution of photoreceptors and eyes. Evol Biol 10:207–263

Sampath AP, Matthews HR, Cornwall MC, Bandarchi J, Fain GL (1999) Light-dependent changes in outer segment free-Ca^{2+} concentration in salamander cone photoreceptors. J Gen Physiol 113(2):267–277

Sanchez-Chavez G, Pena-Rangel MT, Riesgo-Escovar JR, Martinez-Martinez A, Salceda R (2012) Insulin stimulated-glucose transporter Glut 4 is expressed in the retina. PloS One 7(12):e52959. doi:10.1371/journal.pone.0052959

Sanchez-Martin MJ, Ramon E, Torrent-Burgues J, Garriga P (2013) Improved conformational stability of the visual G protein-coupled receptor rhodopsin by specific interaction with docosahexaenoic acid phospholipid. ChemBioChem 14(5):639–644. doi:10.1002/cbic.201200687

SanGiovanni JP, Chew EY (2005) The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. Prog Retinal Eye Res 24(1):87–138. doi:10.1016/j.preteyeres.2004.06.002

Sarthy VP, Dudley VJ, Tanaka K (2004) Retinal glucose metabolism in mice lacking the L-glutamate/aspartate transporter. Vis Neurosci 21(4):637–643. doi:10.1017/S0952523804214122

Sarthy VP, Pignataro L, Pannicke T, Weick M, Reichenbach A, Harada T, Tanaka K, Marc R (2005) Glutamate transport by retinal Müller cells in glutamate/aspartate transporter-knockout mice. Glia 49(2):184–196. doi:10.1002/glia.20097

Schmidt SY, Blanks JC, Sandberg MA (1985) Enhancement of (polyA+)RNA synthesis in light in isolated intact photoreceptor cells of the rat. Exp Eye Res 41(2):159–170

Simpson PB, Russell JT (1998) Role of mitochondrial Ca^{2+} regulation in neuronal and glial cell signalling. Brain Res Brain Res Rev 26(1):72–81. doi:S0165017397000568 [pii]

Simurda M, Wilson JE (1980) Localization of hexokinase in neural tissue: immunofluorescence studies on the developing cerebellum and retina of the rat. J Neurochem 35(1):58–66

Skiba NP, Spencer WJ, Salinas RY, Lieu EC, Thompson JW, Arshavsky VY (2013) Proteomic identification of unique photoreceptor disc components reveals the presence of PRCD, a protein linked to retinal degeneration. J Proteome Res 12(6):3010–3018. doi:10.1021/pr4003678

Smaili SS, Hsu YT, Youle RJ, Russell JT (2000) Mitochondria in Ca^{2+} signaling and apoptosis. J Bioenerg Biomembr 32(1):35–46

Steinberg RH (1987) Monitoring communications between photoreceptors and pigment epithelial cells: effects of "mild" systemic hypoxia. Friedenwald lecture. Invest Ophthalmol Vis Sci 28(12):1888–1904

Stinson AM, Wiegand RD, Anderson RE (1991) Recycling of docosahexaenoic acid in rat retinas during n-3 fatty acid deficiency. J Lipid Res 32(12):2009–2017

Stobart JL, Anderson CM (2013) Multifunctional role of astrocytes as gatekeepers of neuronal energy supply. Front Cell Neurosci 7:38. doi:10.3389/fncel.2013.00038

Stone WL, Farnsworth CC, Dratz EA (1979) A reinvestigation of the fatty acid content of bovine, rat and frog retinal rod outer segments. Exp Eye Res 28(4):387–397

Stone J, van Driel D, Valter K, Rees S, Provis J (2008) The locations of mitochondria in mammalian photoreceptors: relation to retinal vasculature. Brain Res 1189:58–69. doi:S0006-8993(07)02586-3 [pii]10.1016/j.brainres.2007.10.083

Strauss O (2005) The retinal pigment epithelium in visual function. Physiol Rev 85(3):845–881. doi:10.1152/physrev.00021.2004

Sykes SM, Robison WG Jr, Waxler M, Kuwabara T (1981) Damage to the monkey retina by broad-spectrum fluorescent light. Invest Ophthalmol Vis Sci 20(4):425–434

Takata K, Kasahara T, Kasahara M, Ezaki O, Hirano H (1992) Ultracytochemical localization of the erythrocyte/HepG2-type glucose transporter (GLUT1) in cells of the blood-retinal barrier in the rat. Invest Ophthalmol Vis Sci 33(2):377–383

Takata K, Hirano H, Kasahara M (1997) Transport of glucose across the blood-tissue barriers. Int Rev Cytol 172:1–53

Tantama M, Hung YP, Yellen G (2012) Optogenetic reporters: fluorescent protein-based genetically encoded indicators of signaling and metabolism in the brain. Prog Brain Res 196:235–263. doi:10.1016/B978-0-444-59426-6.00012-4

Taylor S, Srinivasan B, Wordinger RJ, Roque RS (2003) Glutamate stimulates neurotrophin expression in cultured Muller cells. Brain Res Mol Brain Res 111(1-2):189–197

Taylor MR, Hurley JB, Van Epps HA, Brockerhoff SE (2004) A zebrafish model for pyruvate dehydrogenase deficiency: rescue of neurological dysfunction and embryonic lethality using a ketogenic diet. Proc Natl Acad Sci USA 101(13):4584–4589. doi:10.1073/pnas.0307074101 [pii]

Thorens B, Mueckler M (2010) Glucose transporters in the 21st century. Am J Physiol Endocrinol Metab 298(2):E141–E145. doi:10.1152/ajpendo.00712.2009

Tomi M, Hosoya K (2004) Application of magnetically isolated rat retinal vascular endothelial cells for the determination of transporter gene expression levels at the inner blood-retinal barrier. J Neurochem 91(5):1244–1248. doi:10.1111/j.1471-4159.2004.02842.x

Tsacopoulos M, Evequoz-Mercier V, Perrottet P, Buchner E (1988) Honeybee retinal glial cells transform glucose and supply the neurons with metabolic substrate. Proc Natl Acad Sci USA 85(22):8727–8731

Ueki Y, Karl MO, Sudar S, Pollak J, Taylor RJ, Loeffler K, Wilken MS, Reardon S, Reh TA (2012) P53 is required for the developmental restriction in Müller glial proliferation in mouse retina. Glia 60(10):1579–1589. doi:10.1002/glia.22377

Veuthey AL, Tsacopoulos M, Millan de Ruiz L, Perrottet P (1994) Cellular and subcellular localization of hexokinase, glutamate dehydrogenase, and alanine aminotransferase in the honeybee drone retina. J Neurochem 62(5):1939–1946

Vollrath D, Feng W, Duncan JL, Yasumura D, D'Cruz PM, Chappelow A, Matthes MT, Kay MA, LaVail MM (2001) Correction of the retinal dystrophy phenotype of the RCS rat by viral gene transfer of Mertk. Proc Natl Acad Sci USA 98(22):12584–12589. doi:10.1073/pnas.221364198

Wada Y, Sugiyama J, Okano T, Fukada Y (2006) GRK1 and GRK7: unique cellular distribution and widely different activities of opsin phosphorylation in the zebrafish rods and cones. J Neurochem 98:824–837

Waldbillig RJ, Fletcher RT, Chader GJ, Rajagopalan S, Rodrigues M, LeRoith D (1987a) Retinal insulin receptors. 1. Structural heterogeneity and functional characterization. Exp Eye Res 45(6):823–835

Waldbillig RJ, Fletcher RT, Chader GJ, Rajagopalan S, Rodrigues M, LeRoith D (1987b) Retinal insulin receptors. 2. Characterization and insulin-induced tyrosine kinase activity in bovine retinal rod outer segments. Exp Eye Res 45(6):837–844

Wang L, Kondo M, Bill A (1997) Glucose metabolism in cat outer retina. Effects of light and hyperoxia. Invest Ophthalmol Vis Sci 38(1):48–55

Wangsa-Wirawan ND, Linsenmeier RA (2003) Retinal oxygen: fundamental and clinical aspects. Arch Ophthalmol 121(4):547–557. doi:10.1001/archopht.121.4.547

Warbug O (1927) Über die Klassifizierung tierischer Gewebe nach ihrem Stoffwechsel. Biochem Z 184:484–488

Wasilewa P, Hockwin O, Korte I (1976) Glycogen concentration changes in retina, vitreous body and other eye tissues caused by disturbances of blood circulation. Albrecht Von Graefes Arch Klin Exp Ophthalmol 199(2):115–120

Watanabe T, Mio Y, Hoshino FB, Nagamatsu S, Hirosawa K, Nakahara K (1994) GLUT2 expression in the rat retina: localization at the apical ends of Müller cells. Brain Res 655(1-2):128–134

Watanabe T, Matsushima S, Okazaki M, Nagamatsu S, Hirosawa K, Uchimura H, Nakahara K (1996) Localization and ontogeny of GLUT3 expression in the rat retina. Brain Res Dev Brain Res 94(1):60–66

Watanabe T, Nagamatsu S, Matsushima S, Kirino T, Uchimura H (1999a) Colocalization of GLUT3 and choline acetyltransferase immunoreactivity in the rat retina. Biochem Biophys Res Commun 256(3):505–511. doi:10.1006/bbrc.1999.0369

Watanabe T, Nagamatsu S, Matsushima S, Kondo K, Motobu H, Hirosawa K, Mabuchi K, Kirino T, Uchimura H (1999b) Developmental expression of GLUT2 in the rat retina. Cell Tissue Res 298(2):217–223

Wen R, Tao W, Luo L, Huang D, Kauper K, Stabila P, LaVail MM, Laties AM, Li Y (2012) Regeneration of cone outer segments induced by CNTF. Adv Exp Med Biol 723:93–99. doi:10.1007/978-1-4614-0631-0_13

Wetzel RK, Arystarkhova E, Sweadner KJ (1999) Cellular and subcellular specification of Na, K-ATPase alpha and beta isoforms in the postnatal development of mouse retina. J Neurosci 19(22):9878–9889

Wilkus RJ, Chatrian GE, Lettich E (1971) The electroretinogram during terminal anoxia in humans. Electroencephalogr Clin Neurophysiol 31(6):537–546

Williams DS, Fisher SK (1987) Prevention of rod disk shedding by detachment from the retinal pigment epithelium. Invest Ophthalmol Vis Sci 28(1):184–187

Willoughby JJ, Jensen AM (2012) Generation of a genetically encoded marker of rod photoreceptor outer segment growth and renewal. Biology Open 1(1):30–36. doi:10.1242/bio.2011016

Winkler BS (1981) Glycolytic and oxidative metabolism in relation to retinal function. J Gen Physiol 77(6):667–692

Winkler BS (1986) Buffer dependence of retinal glycolysis and ERG potentials. Exp Eye Res 42(6):585–593

Winkler BS (2008) An hypothesis to account for the renewal of outer segments in rod and cone photoreceptor cells: renewal as a surrogate antioxidant. Invest Ophthalmol Vis Sci 49 (8):3259–3261. doi:10.1167/iovs.08-1785

Winkler BS, Simson V, Benner J (1977) Importance of bicarbonate in retinal function. Invest Ophthalmol Vis Sci 16(8):766–768

Winkler BS, Kapousta-Bruneau N, Arnold MJ, Green DG (1999) Effects of inhibiting glutamine synthetase and blocking glutamate uptake on b-wave generation in the isolated rat retina. Vis Neurosci 16(2):345–353. doi:S095252389916214X [pii]

Winkler BS, Arnold MJ, Brassell MA, Puro DG (2000) Energy metabolism in human retinal Müller cells. Invest Ophthalmol Vis Sci 41(10):3183–3190

Winkler BS, Pourcho RG, Starnes C, Slocum J, Slocum N (2003) Metabolic mapping in mammalian retina: a biochemical and ^{3}H-2-deoxyglucose autoradiographic study. Exp Eye Res 77(3):327–337. doi:S0014483503001477 [pii]

Winkler BS, Starnes CA, Sauer MW, Firouzgan Z, Chen SC (2004) Cultured retinal neuronal cells and Muller cells both show net production of lactate. Neurochem Int 45(2-3):311–320. doi:10.1016/j.neuint.2003.08.017S0197018603002948 [pii]

Xu Y, Ola MS, Berkich DA, Gardner TW, Barber AJ, Palmieri F, Hutson SM, LaNoue KF (2007) Energy sources for glutamate neurotransmission in the retina: absence of the aspartate/glutamate carrier produces reliance on glycolysis in glia. J Neurochem 101(1):120–131. doi:JNC4349 [pii]10.1111/j.1471-4159.2006.04349.x

Yeh TS, Ho JD, Yang VW, Tzeng CR, Hsieh RH (2005) Calcium stimulates mitochondrial biogenesis in human granulosa cells. Ann NY Acad Sci 1042:157–162. doi:1042/1/157 [pii] 10.1196/annals.1338.017

Young RW (1971) The renewal of rod and cone outer segments in the rhesus monkey. J Cell Biol 49(2):303–318

Young RW (1976) Visual cells and the concept of renewal. Invest Ophthalmol Vis Sci 15(9):700–725

Young RW, Bok D (1969) Participation of the retinal pigment epithelium in the rod outer segment renewal process. J Cell Biol 42(2):392–403

Yu DY, Cringle SJ (2001) Oxygen distribution and consumption within the retina in vascularized and avascular retinas and in animal models of retinal disease. Prog Retina Eye Res 20:175–208

Yu DY, Cringle SJ (2005) Retinal degeneration and local oxygen metabolism. Exp Eye Res 80(6):745–751. doi:10.1016/j.exer.2005.01.018

Yuan C, Chen H, Anderson RE, Kuwata O, Ebrey TG (1998) The unique lipid composition of gecko (Gekko Gekko) photoreceptor outer segment membranes. Comp Biochem Physiol B Biochem Mol Biol 120(4):785–789

Zayas-Santiago A, Kang Derwent JJ (2009) Preservation of intact adult rat photoreceptors in vitro: study of dissociation techniques and the effect of light. Mol Vis 15:1–9. doi:1 [pii]

Zhao Y, Jin J, Hu Q, Zhou HM, Yi J, Yu Z, Xu L, Wang X, Yang Y, Loscalzo J (2011) Genetically encoded fluorescent sensors for intracellular NADH detection. Cell Metab 14(4):555–566. doi:10.1016/j.cmet.2011.09.004

Zheng W, Reem RE, Omarova S, Huang S, DiPatre PL, Charvet CD, Curcio CA, Pikuleva IA (2012) Spatial distribution of the pathways of cholesterol homeostasis in human retina. PloS One 7(5):e37926. doi:10.1371/journal.pone.0037926

Chapter 6
Role and Mechanism of Ciliary Transport

Dusanka Deretic

Abstract Vertebrate rod photoreceptors are exceptionally adapted cells that convert light into image-forming signals, which are conveyed to the brain to create vision. The foundation for the optimal function of rod photoreceptors is the functional compartmentalization of the photosensitive membranes containing the light receptor rhodopsin to the uniquely modified primary cilia that form the specialized organelles, the rod outer segments (ROS). The ciliary transport machinery involved in ROS morphogenesis is composed of intersecting networks of macromolecular complexes that functionally link the small GTPases of the Arf and Rab families. This chapter summarizes the role and mechanisms of photoreceptor ciliary transport, with the main emphases on the ciliary targeting of rhodopsin, which is essential for photoreceptor function and viability.

Keywords Arfs • Cilium • Rabs • Retina • Rhodopsin • Trafficking

Abbreviations

ADRP	Autosomal dominant retinitis pigmentosa
CTS	Ciliary targeting signal
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
RIS	Rod inner segment(s)
ROS	Rod outer segment(s)
RTC(s)	Rhodopsin transport carrier(s)
TGN	Trans-Golgi network

D. Deretic (✉)
Departments of Surgery, Division of Ophthalmology, and Cell Biology and Physiology, University of New Mexico School of Medicine, Basic Medical Sciences Building, Rm. 377, 915 Camino de Salud, N. E., Albuquerque, NM 87131, USA
e-mail: dderetic@salud.unm.edu

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_6, © Springer Japan 2014

6.1 Introduction

Primary cilia are exquisitely organized membrane projections that emanate from the plasma membrane of almost all eukaryotic cells, where they function as environmental sensors that capture a wide range of extracellular signals (Singla and Reiter 2006). Sensory receptors and their associated signal transduction machineries are highly concentrated in the ciliary membrane, which is contiguous with, but distinctly different from, the surrounding plasma membrane. The compartmentalization of the sensory membranes is achieved by the directed transport of signaling molecules to the base of the cilia, followed by the selective admission through the periciliary diffusion barrier and retention of specific components in the ciliary membranes (Christensen et al. 2007; Emmer et al. 2010; Leroux 2007; Nachury et al. 2010; Rosenbaum and Witman 2002). Defects in primary cilia affect multiple tissues and organs and cause human diseases and disorders broadly classified as ciliopathies, which are manifested by both retinal degeneration and cystic kidneys, often accompanied by obesity, polydactyly, and sensory impairments (Blacque and Leroux 2006; Fliegauf et al. 2007; Gerdes et al. 2009; Sang et al. 2011).

Primary cilia of retinal rod photoreceptors elaborate specialized light sensing organelles known as the rod outer segments (ROS), which are continuously replenished throughout the lifetime of photoreceptor cells (Insinna and Besharse 2008). This membrane renewal depends on the precise ciliary targeting of light-sensing components followed by their confinement in the ROS membranes. Rod cell polarity is unmatched in other cells. Rod photoreceptors are distinctly divided into three different compartments: the cell body called the rod inner segment (RIS) that houses the biosynthetic and metabolic machinery, the ROS which is specialized for light capture, and the synapse that conveys the image-forming signal to the retinal neurons (Fig. 6.1). The light-sensing receptor rhodopsin that is highly concentrated in the ROS is a prototypic G protein-coupled receptor (GPCR). Because the ROS lack the biosynthetic machinery, rhodopsin is synthesized in the RIS. After the passage through the Golgi complex it is incorporated into rhodopsin transport carriers (RTCs) that bud from the trans-Golgi network (TGN) and deliver rhodopsin to the base of the cilia (Deretic 2006). This specialized RTC-mediated ciliary traffic that supports continuous ROS membrane renewal creates an unparalleled unidirectional membrane flow that represents an extreme case of ciliary receptor targeting. The volume of ROS membrane trafficking is much higher in amphibians than in mammals (Besharse 1986); thus, in amphibians the RTCs that carry the bulk of newly synthesized ROS membranes are reproducibly detected in the immediate proximity of the cilia where they presumably fuse with the plasma membrane (Deretic and Papermaster 1991; Papermaster et al. 1985; Papermaster et al. 1986) (Fig. 6.2). The biochemical characterization of isolated RTCs greatly facilitated identification of proteins that associate with newly synthesized rhodopsin during trafficking to the cilia and revealed the identity of important regulators of rhodopsin trafficking, including the small GTPases Rab6, Rab8, and Rab11 (Deretic et al. 1995; Deretic and Papermaster 1993; Deretic et al. 1996).

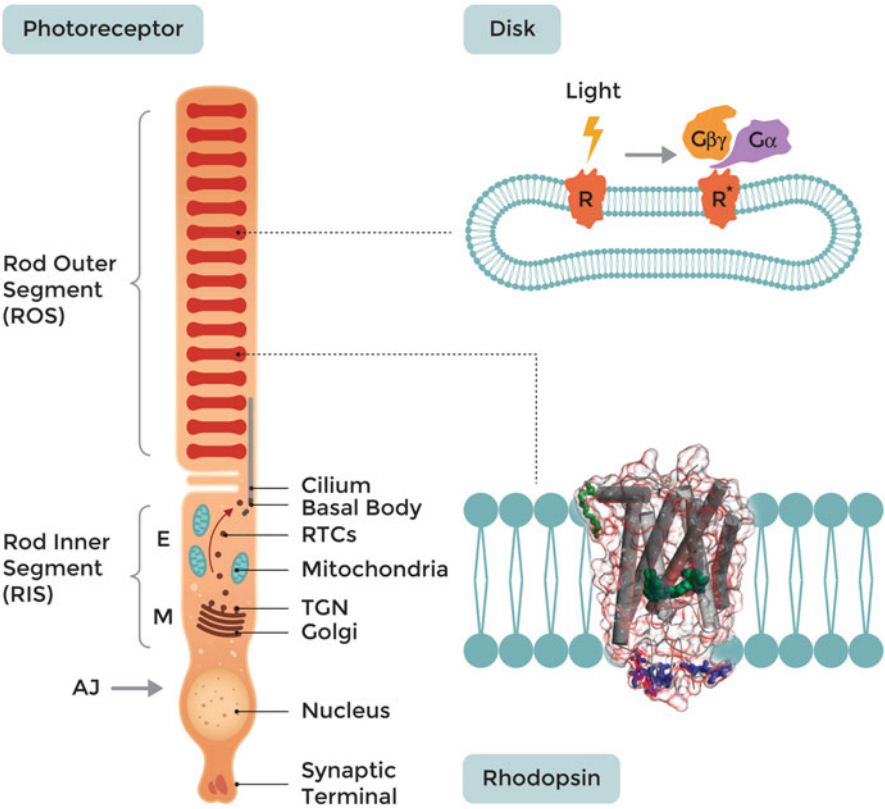

Fig. 6.1 Functional organization of retinal rod photoreceptors. Photoreceptor cells are divided into three distinct domains: the *ROS*, the *RIS*, and the synapse. RIS contain the biosynthetic and sorting machinery, including the Golgi, and the trans-Golgi network (*TGN*) that are localized in the myoid region (*M*), and a dense array of mitochondria involved in the energy metabolism that are localized in the ellipsoid region (*E*). Rhodopsin transport carriers (RTCs) bud from the TGN and traverse the ellipsoid on their way to the cilium, which emanates from the basal body. RIS are delineated by the adherens junctions (*AJ*). The synaptic terminal is at the opposite end of the cell from the ROS and contains synaptic ribbons that anchor synaptic vesicles involved in neurotransmitter release. The ROS houses approximately 1,000 stacked disk membranes that contain approximately 10^4–10^6 molecules of rhodopsin/disk. The phototransduction cascade is initiated in the disk membrane when light-activated rhodopsin (R*) encounters and activates the trimeric G-protein transducin, composed of Gα and Gβγ subunits. The crystal structure of rhodopsin (Palczewski et al. 2000) reveals seven transmembrane helices that shield the chromophore (*dark green*) and the cytoplasmic helix 8 that lies perpendicular to the membrane, anchored by two palmitates (*green*). The oligosaccharides are represented in *blue* (After Palczewski et al. 2000). In the ROS, the cytoplasmic surface of rhodopsin is a site of interaction for transducin and other regulators of phototransduction such as rhodopsin kinase and arrestin. In the RIS, the cytoplasmic surface of rhodopsin interacts with the regulators of ciliary targeting.

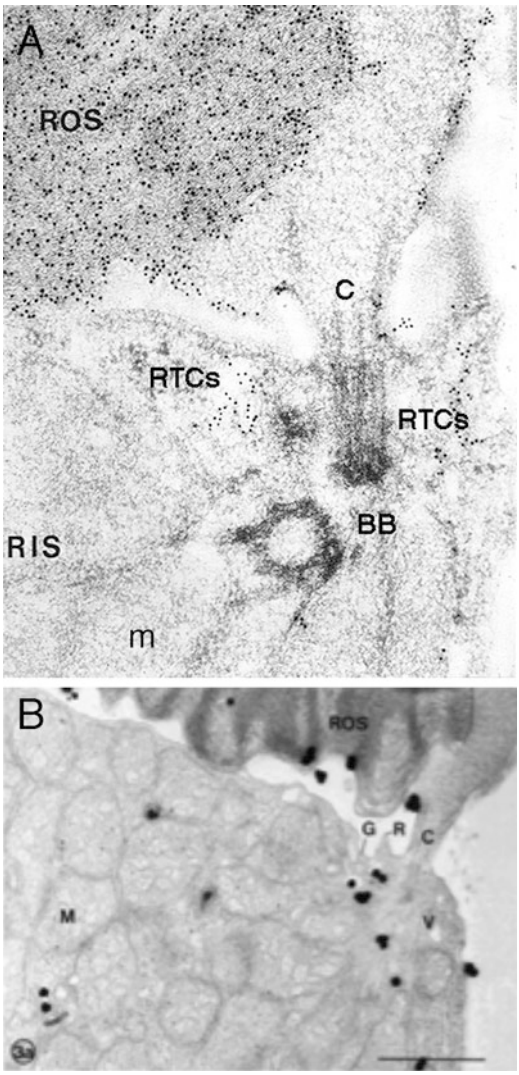

Fig. 6.2 RTCs are delivered to the base of the cilium. (**a**) Rhodopsin transport carriers (*RTCs*), detected by electron microscopy (EM) immunocytochemistry with anti-rhodopsin mAb 11D5, are clustered at the base of the cilium (*C*) in the *RIS*. Rhodopsin is also concentrated in the *ROS* disks and plasma membrane, but not in the RIS plasma membrane. *BB*, basal body; *m*, mitochondria. (Modified from Deretic and Papermaster 1991.) (**b**) Vesicular membrane structures at the base of the cilium carry newly synthesized rhodopsin, detected by EM autoradiography. Radiolabeled rhodopsin is also seen in the basal ROS disks. (Reproduced from Papermaster et al. 1986)

6.2 Role of Ciliary Transport

In the ROS, rhodopsin is tightly packed within the membranous disks, at concentrations high enough to capture a single photon and signal its absorption (Baylor et al. 1979). The ability of rods to function as perfect cellular machines that convert light absorbed by rhodopsin into changes in neurotransmitter release is based on the extraordinary magnitude of signal amplification through a succession of interactions that has been well documented and recently described in detail (Arshavsky and Burns 2012; Burns and Arshavsky 2005; Palczewski 2012). Briefly, within a millisecond of photon capture, activated rhodopsin (also known as metarhodopsin II or R*) initiates a G protein (transducin)-mediated cascade of

interactions that amplify the signal and convert it into electrical response that is relayed across synapses to other neurons in the retina. The first step of the cascade is transducin activation on membranous disks, which proceeds at the rate higher than any other G-protein activation by GPCRs. Further signal amplification is achieved through the subsequent activation of cGMP phosphodiesterase (PDE), an enzyme with an extraordinary catalytic activity that rapidly lowers cGMP concentration. The decrease in cGMP concentration results in closing of the cGMP channels in the ROS plasma membrane, which causes decrease in inward current that is further amplified by the intrinsic properties of the channels. These processes mediating fast signal propagation are perfectly orchestrated because the organelle that houses the components of the phototransduction cascade is built to optimize their performance. Components of the ROS are either a part of the well-integrated light-sensing network or provide the framework that supports it. Proteins and lipids that participate in phototransduction are efficiently sequestered in the ROS, away from those taking part in other cellular functions that would impede the propagation of the visual signal. The ROS architecture reflects the structure–functional organization of the organelle from which it is derived, the primary cilium. The entry into primary cilia is an extremely selective process that is regulated by the lipid and protein networks, which limit the ciliary access by forming the periciliary diffusion barrier, or the ciliary gate. Once the ciliary gate is established during ciliogenesis, only the selected proteins and lipids can gain ciliary access, thus allowing the exquisitely orchestrated interactions such as the phototransduction cascade to proceed at an optimal rate.

The signal amplification within ROS disk membranes is aided by the low viscosity resulting from the exceptionally high content of unsaturated long-chain phospholipids highly enriched in omega-3 docosahexaenoic acid [DHA, 22:6(n-3)]. However, these unsaturated phospholipids are exquisitely susceptible to light and oxidative damage, thus rendering the ROS environment toxic for its constituents (Anderson and Penn 2004; Winkler et al. 1999). To counteract the constant exposure to damaging agents, light-sensitive membranes are continuously produced and renewed, which creates a high-energy burden for photoreceptor cells. Newly synthesized ROS components have to traverse a maze of obstacles created by tightly packed mitochondria in the RIS and carry passwords that provide access into the cilia. Nevertheless, all these processes are essential for survival because disruptions of the regulatory networks at the entrance to the cilia lead to dysfunctions that cause human retinal dystrophies and syndromic diseases that affect multiple organs, including the eyes.

6.3 Mechanism of Ciliary Transport

6.3.1 Structure–Functional Organization of Primary Cilia and the ROS

Primary cilia are elaborated after the cell division in a process regulated by the centrosome, which is composed of two centrioles that dock at the plasma membrane and become the basal body (BB). During ciliogenesis, one (mother) centriole is

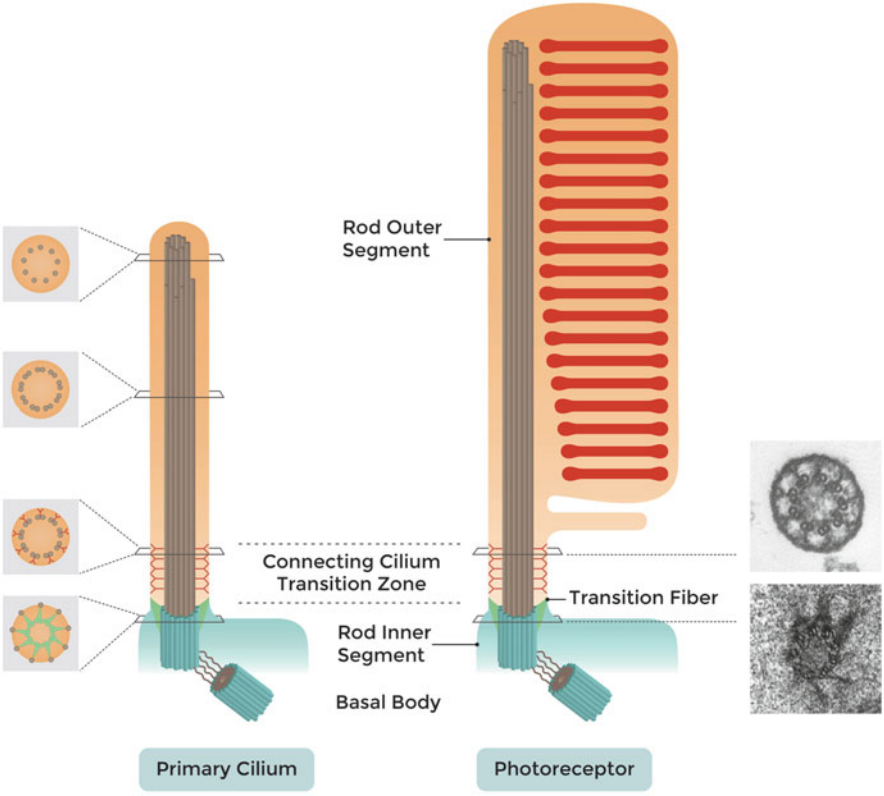

Fig. 6.3 ROS is a modified primary cilium. Parallel organization of the axoneme, transition zone, and the basal body of the primary cilia and the ROS is visualized through the alignment and color-coding that emphasizes the separation of cilia from the rest of the cell. Schematic cross sections on the *left* depict, from the *bottom*: the basal body and the transition fibers (*green*) that extend from the distal end of the basal body to the cell membrane; the transition zone with microtubule doublets and the Y-shaped cross-linkers (*red*); a circular array of microtubule doublets that fills the axoneme; and the singlets at the distal end of the axoneme. The electron micrographs on the *right* show the equivalent photoreceptor structures: the basal body and the transition fibers of mouse photoreceptor (reproduced from Sedmak and Wolfrum 2011) and the ciliary necklace in the transition zone (reproduced from Besharse et al. 1985)

modified and acquires appendages, accessory structures, and the pericentriolar material (PCM) that are necessary for the formation of the microtubule-organizing center (MTOC), and one (daughter) centriole remains unmodified (Bettencourt-Dias and Glover 2007) (Fig. 6.3). The mother centriole then elaborates the axoneme containing an orderly array of nine microtubule pairs, with two central single microtubules (structure 9C2) in motile cilia, or without the central pair (structure 9C0) in primary (nonmotile) cilia. The microtubule doublets extend into the axoneme and eventually become singlets. At the ciliary base, the basal body is connected to the ciliary membrane via transition fibers (Fig. 6.3), which are densely packed propeller-like sheets that leave only 60-nm openings severely restricting the passage into cilia

(Anderson 1972). Immediately adjacent to the basal body is the short and very narrow, approximately 300-nm, transition zone filled with the doublet microtubules and Y-shaped structures that link them to the ciliary membrane and form a unique organization termed the ciliary necklace (Gilula and Satir 1972). The Y-shaped structures are also observed in the so-called connecting cilium of photoreceptor cells (Besharse et al. 1985; Horst et al. 1987) (Fig. 6.3), which corresponds to the transition zone of primary cilia. The ROS is essentially a significantly enlarged cilium filled with a stacked array of membranous disks (Fig. 6.3). Microtubules are also connected to the ciliary membrane through the highly conserved intraflagellar transport complexes (IFTs) and molecular motors that move membrane proteins along the cilia (Insinna and Besharse 2008). The architecture of the mouse photoreceptor cilium was recently examined by cryo-electron tomography, which revealed tethered vesicles at the base of the cilium, which may correspond to RTCs, although they have not been identified as such (Gilliam et al. 2012). Nevertheless, in amphibian photoreceptors abundant RTCs are detected in the vicinity of the periciliary RIS plasma membrane (Deretic and Papermaster 1991; Papermaster et al. 1985; Papermaster et al. 1986), indicating that this is the site of delivery for the ciliary cargo. Thus, the rules of ciliary entry that restrict the access of non-cilia components have to apply to ROS membrane delivery. To gain access to the ROS, resident lipids and proteins have to pass the periciliary diffusion barrier, also called the ciliary gate.

6.3.2 The Periciliary Diffusion Barrier

The periciliary diffusion barrier, or the ciliary gate, is formed through specific lipid ordering and the formation of septin rings and multiprotein complexes that are located at the transition zone (Chih et al. 2012; Craige et al. 2010; Garcia-Gonzalo et al. 2011; Garcia-Gonzalo and Reiter 2012; Hu et al. 2010; Nachury et al. 2010; Sang et al. 2011; van Reeuwijk et al. 2011; Vieira et al. 2006; Williams et al. 2011). The periciliary diffusion barrier separates the ciliary membrane from the surrounding plasma membrane through the particularly high lipid ordering caused by the abundance of cholesterol and glycosphingolipids (Godi et al. 2004; Vieira et al. 2006). This increased lipid ordering has also been observed at the base of the photoreceptor cilium where cholesterol-enriched rings were detected by freeze-fracture analysis (Andrews and Cohen 1983). The ROS and the periciliary RIS membranes differ greatly in their composition and fluidity, which presumably limits the mixing of their contents. Septin rings may also participate in the barrier formation at the photoreceptor transition zone, but this has not yet been established.

The ciliary gate is generally formed through NPHP-JBTS-MKS protein complexes located at the centrosomes and the transition zone, which are composed of distinct functional modules linked into networks that build and maintain the primary cilium (Fig. 6.4) (Chih et al. 2012; Garcia-Gonzalo et al. 2011; Garcia-Gonzalo and Reiter 2012; Sang et al. 2011; van Reeuwijk et al. 2011; Williams et al. 2011). These molecular assemblies represent the largest group of the genes linked to photoreceptor degeneration. Mutations in genes encoding for NPHP-JBTS-MKS proteins cause ciliary defects that lead to retinal and neural

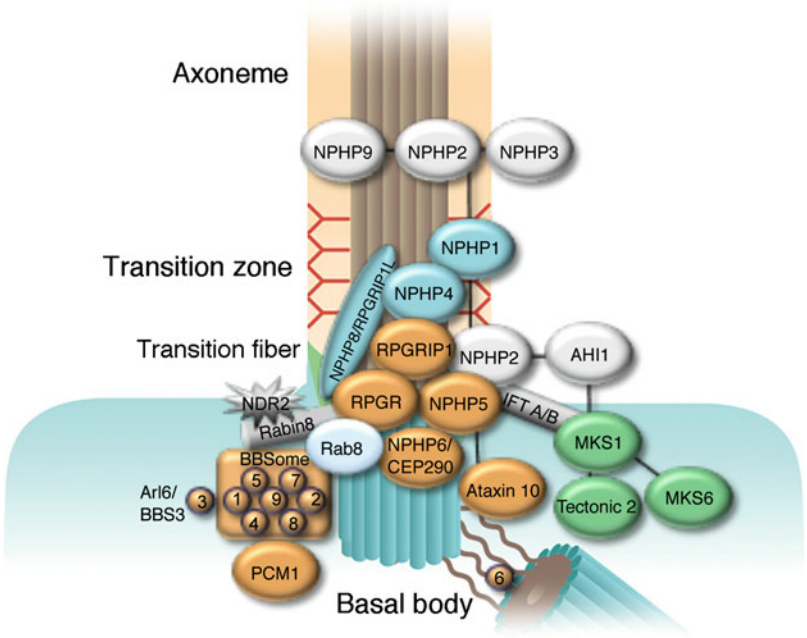

Fig. 6.4 The transition zone and the basal body house NPHP-JBTS-MKS and BBS multiprotein complexes that are linked to the small GTPase Rab8. The NPHP-JBTS-MKS network is presented in distinct modules as mapped by Sang et al. 2011, with transition zone proteins involved in cell polarization in *blue*, basal body network that regulates cilia integrity in *gold*, and the Hedgehog regulatory network in *green*. Inversins are shown in *gray*. BBSome is localized at the basal body and interacts with Arl6 (BBS3), which regulates its function, and with PCM1. BBS6 is located at the daughter centriole. Both the NPHP-JBTS-MKS network and the BBSome interact extensively with the small GTPase Rab8 and its GEF Rabin8, which regulate ciliogenesis and membrane trafficking to the cilia. The activity of Rabin8 is regulated through phosphorylation by the S/T kinase NDR2, which is also involved in ciliopathies

dysfunction, nephronophthisis, Joubert syndrome, and Meckel syndrome. Nephrocystin-5 (NPHP5) and nephrocystin-6 (NPHP6) form a module at the centrosomes that regulates ciliogenesis (Fig. 6.4). Mutations in NPHP5 cause combined retinitis pigmentosa and nephronophthisis, comprising the renal-retinal Senior-Loken syndrome (Otto et al. 2005). In retinal photoreceptors, NPHP5 functions in conjunction with NPHP2, and the MKS1-related protein B9d2, to support the ciliary transport of rhodopsin (Zhao and Malicki 2011).

NPHP6, also known as CEP290, is a multifunctional protein mutated in Joubert syndrome (Sayer et al. 2006) and truncated in early-onset retinal degeneration in the rd16 mouse (Chang et al. 2006). In retinal photoreceptors, NPHP6 has been localized to basal bodies and the cilium (Chang et al. 2006; Sayer et al. 2006). It was recently shown that CEP-290/NPHP6 interacts with CEP162, a microtubule-recognition protein that initiates the assembly of the transition zone (Wang et al. 2013). Of high relevance for ciliary membrane trafficking, at the transition zone CEP-290/NPHP6 links NPHP-JBTS-MKS complexes with the small GTPase Rab8

to promote ciliogenesis (Kim et al. 2008; Rachel et al. 2012; Sang et al. 2011; Tsang et al. 2008).

The small GTPase Rab8, which is described in detail later, is a central regulator of the final stages of polarized membrane traffic, carrier fusion, and ciliogenesis (Bryant et al. 2010; Deretic et al. 1995; Moritz et al. 2001; Murga-Zamalloa et al. 2010a; Nachury et al. 2007; Wang et al. 2012; Yoshimura et al. 2007). Rab8 is principally activated by the guanine nucleotide exchange factor (GEF) Rabin8 (Hattula et al. 2002). In rod photoreceptors, Rabin8 is associated with the TGN and the RTCs (Wang et al. 2012). However, in addition to Rabin8 retinal photoreceptors express another Rab8 GEF named retinitis pigmentosa GTPase regulator (RPGR). Mutations in RPGR that reduce its GEF activity cause photoreceptor degeneration in X-linked retinitis pigmentosa (XLRP)(Murga-Zamalloa et al. 2010a). The presence of multiple Rab8 GEFs in rod photoreceptors suggests that the additional or alternative control of Rab8 may be needed for the extraordinary ciliary traffic that supports continuous ROS membrane expansion. Moreover, given the multiple interactors of Rab8 during ciliogenesis and ciliary trafficking it is possible that some of the ciliopathy NPHP-JBTS-MKS proteins with presently unknown function actually perform regulatory roles in the Rab8 activity cycles.

The retina-specific Rab8 GEF RPGR is a key player in ciliary morphogenesis. It is homologous to RCC1, the nucleotide exchange factor for the small GTPase Ran (Meindl et al. 1996). Mutations in the ORF15 isoform of RPGR are linked to photoreceptor degeneration in XLRP (Kirschner et al. 1999). RPGR is anchored to the photoreceptor cilium through RPGRIP1, which is affected in patients with LCA (Dryja et al. 2001; Hong et al. 2000; Hong et al. 2001; Shu et al. 2005; Zhao et al. 2003). RPGR is intimately associated with the NPHP-JBTS-MKS protein network and interacts with NPHP5, NPHP6, and NPHP8, also known as RPGRIP1L (RPGRIP-like), mutations in which cause Joubert syndrome (Arts et al. 2007; Chang et al. 2006; Murga-Zamalloa et al. 2010b; Otto et al. 2005). RPGR is also a risk factor for retinal degeneration in patents with ciliopathies caused by mutations in other genes (Khanna et al. 2009). Given the high susceptibility of the RPGR regulatory network to disease-causing mutations, it is apparent that the activation of Rab8 by RPGR is subject to a tight spatiotemporal regulation at the rod photoreceptor transition zone.

The sheer number of retinopathy-associated ciliary proteins indicates that the malfunction of the ciliary gate has dire consequences for the photoreceptor polarity and health. Different functional networks involved in ciliogenesis and multiple ciliopathy complexes appear to be closely linked to the small GTPase Rab8. In addition to the NPHP-JBTS-MKS network, both Rab8 and Rabin8 also interact directly with the BBSome, a complex required for ciliogenesis that is composed of seven BBS proteins, mutations in which cause the Bardet Biedel syndrome (Jin et al. 2010; Nachury et al. 2007). Because of the BBsome connection to Rab8, some of the defects in Bardet Biedel syndrome may in fact be caused by defects in Rab8-mediated membrane transport to the cilium.

In addition to the universal components of the periciliary diffusion barrier, photoreceptors also have a structure called the periciliary ridge complex (PRC), a specialized domain involved in the fusion of incoming membrane carriers with the RIS plasma membrane in amphibians (Fig. 6.5) (Maerker et al. 2008; Peters

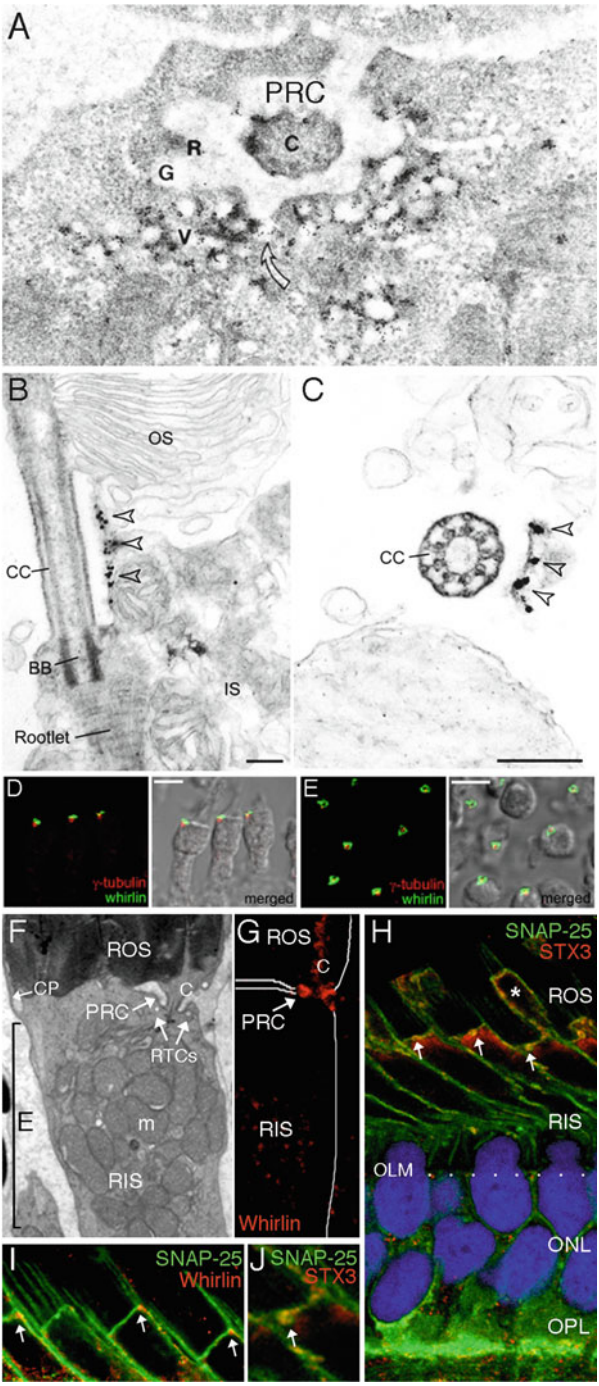

Fig. 6.5 The periciliary ridge complex (PRC) surrounds the base of the cilium and houses regulators of RTC fusion. (**a**) RTCs, referred to here as vesicles (*v*), fuse with the RIS plasma membrane at the periciliary ridge complex (*PRC*). (Reproduced form Peters et al. 1983.) (**b–e)**

et al. 1983). Mammalian photoreceptors have a functionally equivalent region called the periciliary membrane complex (PMC), which houses components of the protein network that is disrupted in Usher syndrome (USH), the most frequent cause of combined deafness and blindness (Jacobson et al. 2008; Maerker et al. 2008; Overlack et al. 2008; Rachel et al. 2012; Williams 2008; Yang et al. 2010). Through its multiprotein interactions, this network appears to support the unique specialization of the periciliary region and the linkage of the ciliary membrane to the surrounding RIS plasma membrane (Maerker et al. 2008).

PRC is the site of interaction of the RTC fusion executors syntaxin 3 and SNAP-25, the two plasma membrane SNAREs (soluble *N*-ethylmaleimide-ensitive factor attachment protein receptors) that control the Rab8-mediated delivery of rhodopsin to the cilium and the ROS (Fig. 6.5) (Mazelova et al. 2009b). The late-acting function of Rab8 in ciliary trafficking indicates that ROS membrane constituents have to enter the Rab8-regulated membrane pathway to gain ciliary access. This is precisely how rhodopsin gets to the cilia, and the way that it achieves that is through interactions with multiple small GTPases and the scaffold proteins that regulate their activation/inactivation cycles and collectively operate upstream of Rab8.

6.3.3 The Rab Family of Small GTPases

In its ciliary transport, rhodopsin interacts with the small GTPases of the Rab and Arf families that play a central role in organizing intracellular membrane trafficking as well as membrane delivery to primary cilia. The hallmark feature of all GTPases, including Rabs and Arfs, is their function as universal molecular switches whose on and off states are triggered by binding and hydrolysis of GTP. This tried-and-true mechanism gives directionality to cellular processes such as signal transduction, cytoskeleton dynamics, and membrane trafficking. The small GTPases undergo

Fig. 6.5 (continued) Usher protein whirlin is localized at the PMC in mouse photoreceptors (**b**, **c**) and at the PRC in frog photoreceptors (**d**, **e**). Longitudinal (**b**) and transverse (**c**) views of whirlin that was detected by immuno-EM. (**d**) Whirlin (*green*) was localized above the basal bodies marked by γ-tubulin (*red*) in the longitudinal views of frog photoreceptors. (**e**) Whirlin appears as *circles* surrounding the basal bodies in the transverse view. [(**b–e**) Reproduced from Yang et al. 2010.] (**f**) EM image detailing RIS structure of a frog photoreceptor. Ellipsoid region (*E*) is filled with densely packed mitochondria (*m*). RTCs (*arrows*) traverse this region and fuse at the base of the cilium (*C*), at the periciliary ridge complex (PRC, *arrow*). *CP*, calycal processes. (**g**) Anti-whirlin specifically detects the PRC (*arrow*). (**h**) SNAREs SNAP-25 (*green*) and syntaxin 3 (STX3, *red*) colocalize in the RIS at the RTC fusion sites (*yellow*, *arrows*). *Asterisk*, RIS of green rods. (**i**) SNAP-25 (*green*) and whirlin (*red*) colocalize at the same sites at the base of the cilium (*yellow*, *arrows*). (**j**) Magnified PRC from **h** shows RTC fusion sites. [(**f–j**) Reproduced from Mazelova et al. 2009b]

activation–inactivation cycles that are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) that cooperatively control membrane budding and tethering of membranes to other membranes and to cytoskeletal elements (Mizuno-Yamasaki et al. 2012). These regulatory functions are frequently performed by multifunctional proteins that act at the intersection of different cellular pathways and allow crosstalk between different families of small GTPases (Baschieri and Farhan 2012; Deretic 2013). Rabs regulate the delivery of cargo to their particular intracellular destinations through the recruitment of macromolecular complexes that organize membrane microdomains, thus inducing changes in membrane identity during cargo progression through intracellular compartments (Grosshans et al. 2006; Rink et al. 2005; Stenmark 2009; Zerial and McBride 2001). For instance, the succession of Rab6, Rab11, and Rab8 occurs on the photoreceptor biosynthetic membranes during progression of rhodopsin from the Golgi complex toward the cilia (Deretic et al. 1995; Deretic and Papermaster 1993; Deretic et al. 1996). The functional networks of Rab GEF and GAP cascades and positive-feedback loops generated by GEF–effector interactions link the different stages of a particular cellular transport pathway. A prime example of a GEF-effector network is the highly conserved Rab11-Rabin8-Rab8-Sec15 ciliogenesis cascade that is composed of the homologues of the Ypt32p-Sec2p-Sec4p-Sec15p proteins involved in yeast budding (Feng et al. 2012; Knodler et al. 2010; Wang et al. 2012; Westlake et al. 2011).

Rab8 is the linchpin of the Rab11-Rabin8-Rab8-Sec15 ciliogenesis cascade and a central regulator of intracellular membrane trafficking responsible for the final stages of the directed delivery of ciliary membrane components to the ciliary gate (Feng et al. 2012; Knodler et al. 2010; Murga-Zamalloa et al. 2010a; Nachury et al. 2007; Wang et al. 2012; Westlake et al. 2011; Yoshimura et al. 2007). In its active conformation, Rab8 performs multiple functions in ciliogenesis and ciliary targeting through an array of effectors and regulatory proteins. Consistent with its dynamic functions, Rab8 localizes to developing cilia but departs mature cilia (Westlake et al. 2011). The cilia and the ROS of mature photoreceptors are devoid of Rab8, which is present only in the RIS where it is predominantly associated with RTCs (Fig. 6.6) (Deretic et al. 1995; Moritz et al. 2001; Wang et al. 2012). The involvement of Rab8 in rhodopsin trafficking was established early on and subsequently demonstrated in transgenic photoreceptors expressing the dominant-negative GFP-Rab8T22N GTP-binding-deficient mutant that causes accumulation of RTCs below the cilium (Deretic et al. 1995; Moritz et al. 2001). Notably, Rab8 directly interacts with rhodopsin and other ciliary cargo, including polycystin 1 and fibrocystin (Follit et al. 2010; Wang et al. 2012; Ward et al. 2011).

Activation of Rab8 is mediated by Rab11a, through the activation of the Rab8 GEF Rabin8 (Feng et al. 2012; Knodler et al. 2010; Wang et al. 2012; Westlake et al. 2011). In rod photoreceptors, Rabin8 colocalizes with Rab11 at the Golgi/TGN (Wang et al. 2012). Rabin8 also associates with Rab11-containing carriers, including RTCs (Wang et al. 2012; Westlake et al. 2011), and with the basal body (Nachury et al. 2007). Rabin8 is recruited to the membrane through Rab11a, specific

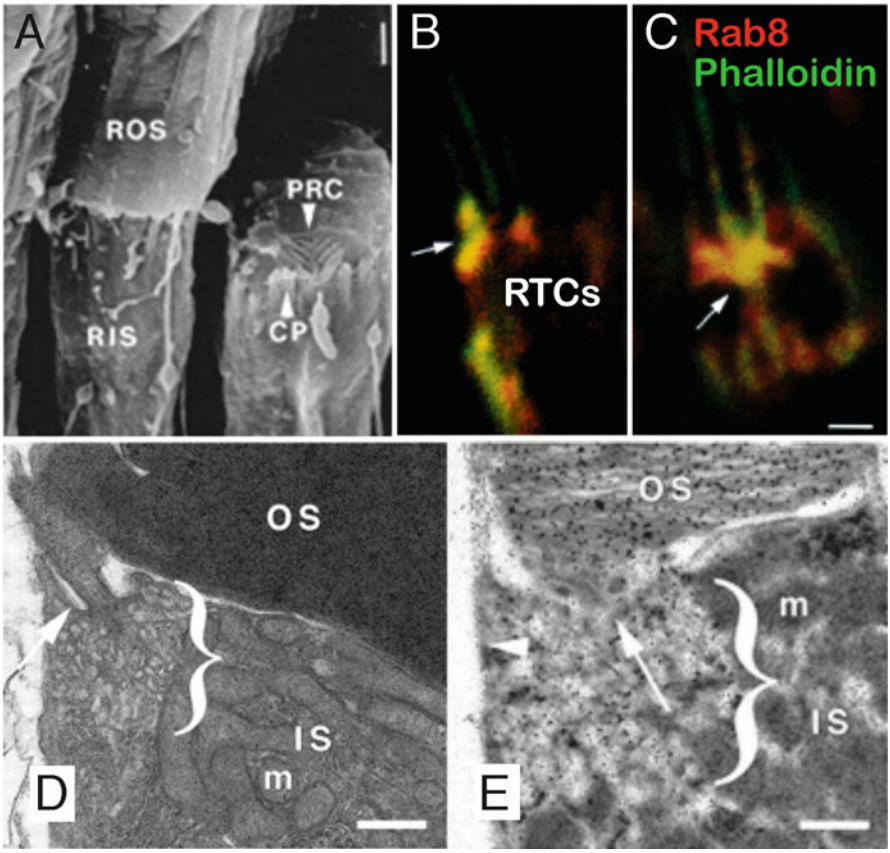

Fig. 6.6 Rab8 regulates RTC fusion at the base of the cilium. (**a**) Scanning EM of the frog retina reveals the PRC in a cell with the ROS broken off. *CP*, calycal processes. (**b**, **c**) RTCs cluster at the base of the cilium (*arrow*) where Rab8 (*red*) and actin detected by phalloidin staining (*green*) colocalize (*yellow*). (Reproduced from Deretic et al. 1995.) (**d**, **e**) Abundant membranous structures accumulate below the cilium in photoreceptors expressing GFP-Rab8T22N mutant. The accumulated membranes are RTCs as they are labeled with anti-rhodopsin mAb 11D5. (Reproduced from Moritz et al. 2001)

phospholipids, and a serine/threonine kinase NDR2 (also called STK38L) (Chiba et al. 2013) (see Fig. 6.4). NDR2 was identified as a canine retinal degeneration gene corresponding to the human ciliopathy Leber congenital amaurosis (LCA)(Berta et al. 2011; Goldstein et al. 2010). The exceptional conservation of the Rab11-Rabin8-Rab8 ciliogenesis cascade that also functions in photoreceptors implies that in its quest to reach the ROS rhodopsin engages the ancient molecular machinery that regulates the polarity and growth of baker's yeast. However, the road to the cilia is less straightforward than yeast budding and the additional complexity is imparted by Arf4 and its regulator, the Arf GAP ASAP1, which are not expressed in yeast.

6.3.4 The Arf Family of GTPases

The encounter with the small GTPase Arf4 at the Golgi/TGN endows rhodopsin with the initial information on its cellular destination (Deretic et al. 2005; Mazelova et al. 2009a). Arf4 is a member of the Arf family of small GTPases that includes Arf, Arf-like (Arl) and Sar proteins, which collectively regulate lipid metabolism, membrane trafficking, organelle morphology, and cytoskeleton dynamics (Donaldson 2005; Kahn et al. 2006). The mammalian Arfs consist of six isoforms (Arf1–Arf6) that are, with the exception of Arf6, associated with the Golgi. In addition to the distinctive function in the Golgi, Arf4, Arl3, Arl6, and Arl13b are also strongly implicated in specialized membrane transport to primary cilia (Donaldson and Jackson 2011; Mazelova et al. 2009a; Nachury et al. 2007). The precise site of action of Arf GTPases is determined by the intracellular localization of Arf GEFs, because Arf activation is directly coupled with membrane association by the N-terminal "myristoyl switch" that inserts the protein into the membrane. Arfs have very low catalytic activity, and Arf GAPs are absolutely critical for GTP hydrolysis and Arf inactivation providing directionality to membrane transport. Arf GAPs are often incorporated into protein coats that shape the membranes in preparation for budding of distinct transport carriers (Donaldson and Jackson 2011; Kahn et al. 2006). In rhodopsin trafficking, Arf4 and its GAP ASAP1 form the basis of the ciliary targeting complex that regulates its delivery to the cilia. ASAP1 is not only a GAP for Arf4, but is also its effector because a point mutation in Arf4 (I46D) that selectively abolishes ASAP1-mediated GTP hydrolysis disrupts RTC budding, causing rhodopsin mislocalization and rapid retinal degeneration in transgenic frogs (Mazelova et al. 2009a).

The Arf family GTPase Arl6 regulates the function of the BBSome, which is implicated in the delivery of ciliary cargo (Jin et al. 2010; Nachury et al. 2007). Arf GTPases Arl3 and Arl13b are also involved in ciliogenesis, and their impaired function is responsible for retinitis pigmentosa 2 (RP2) and Joubert syndrome, respectively. RP2 protein is a GAP that regulates GTP hydrolysis on Arl3 (Veltel et al. 2008). Arl3 controls the delivery of lipidated proteins to the ROS by specifically releasing lipid-modified ciliary cargo, including the N-acylated transducin α-subunit, from the lipid moiety-binding protein UNC119 at the base of the cilia (Gopalakrishna et al. 2011; Ismail et al. 2012; Schwarz et al. 2012; Veltel et al. 2008; Wright et al. 2011; Zhang et al. 2011). Remarkably, loss of RP2 also reduces rhodopsin content of the ROS, indicating that the transport of lipidated proteins may be functionally linked to the ciliary transport of rhodopsin (Li et al. 2013). Similar to Arl3, Arl13b is a part of a network that regulates ciliary targeting of lipid-modified proteins, which is affected in JBTS and NPHP (Humbert et al. 2012). Remarkably, even the limited number of ubiquitous proteins such as Arfs can regulate very specific cellular processes by being integrated into networks with distinct sets of regulators and effectors. Furthermore, Arfs act at distinct steps of cargo delivery to photoreceptor cilia: whereas Arf4 functions at the Golgi/TGN

to direct rhodopsin incorporation into ciliary-targeted RTCs, Arls are specifically localized to the cilia where they directly control the ciliary access of proteins that likely reach the cilia through diverse mechanisms.

6.3.5 *The Arf4-Based Ciliary Targeting Complex*

The Arf4-based ciliary targeting complex directs rhodopsin into the Rab11-Rabin8-Rab8-Sec15-regulated pathway, thus ensuring its ciliary access. In addition to small GTPases Arf4 and Rab11 and the Arf GAP ASAP1, the complex also contains the Rab11-Arf interacting protein FIP3 (Mazelova et al. 2009a; Wang et al. 2012). ASAP1 is a centerpiece of this complex because of its scaffolding properties that organize components acting sequentially en route to cilia (Fig. 6.7). At the Golgi/TGN, a specific Arf-GEF activates Arf4, which subsequently binds rhodopsin, initiating the assembly of the targeting complex (Deretic et al. 2005; Mazelova et al. 2009a). ASAP1 is recruited to the TGN through distinct lipid and protein interactions and forms a tripartite complex with activated Arf4 and rhodopsin (Wang et al. 2012). Concurrently, ASAP1 binds Rab11a and the Rab11-Arf effector FIP3, completing the initial targeting complex. ASAP1 likely causes membrane deformation through its specialized curvature-inducing BAR domain, which acts as the autoinhibitor of its GAP activity (Jian et al. 2009; Nie et al. 2006). The GTP hydrolysis on Arf4 by ASAP1 that completes this stage is likely assisted by FIP3, which acts as the regulator of ASAP1 GAP activity (Inoue et al. 2008). GTP hydrolysis and inactivation of Arf4 serve as the proofreading mechanism for rhodopsin incorporation into nascent RTCs. However, at this stage RTCs still do not posses the full information on their cellular destination, which is necessary to navigate their way to the cilium. This information is provided during budding of nascent RTCs from the TGN through the ASAP1- and Rab11a-mediated recruitment of Rab8 and its GEF Rabin8 (Wang et al. 2012). Because Rab8 acts at the site of fusion with the periciliary plasma membrane, RTCs marked with Rab8 are endowed with the distinct cellular address. Activation of Rab8 by Rabin8 likely takes place on RTCs, thus rendering them competent for fusion with the periciliary plasma membrane.

In the final stages of ciliary targeting, the Rab8a-positive membrane carriers are tethered to the plasma membrane by the conserved octameric complex called the exocyst (Fig. 6.7) (Das and Guo 2011; Heider and Munson 2012; Hsu et al. 2004; Novick et al. 2006). The exocyst, or the Sec6/8 complex, is localized at the base of the cilium in epithelial cells and photoreceptors (Mazelova et al. 2009b; Rogers et al. 2004). The exocyst undergoes dynamic assembly–disassembly cycles that regulate the polarized membrane delivery. The Sec15 subunit of the exocyst interacts directly with Rab8a, Rabin8, and Rab11a and travels to the plasma membrane on transport carriers (Bryant et al. 2010; Feng et al. 2012; Wu et al. 2005; Zhang et al. 2004). Sec15 is a component of the Rab11-Rabin8-Rab8-Sec15 cascade, which in fact constitutes a highly conserved Rab8

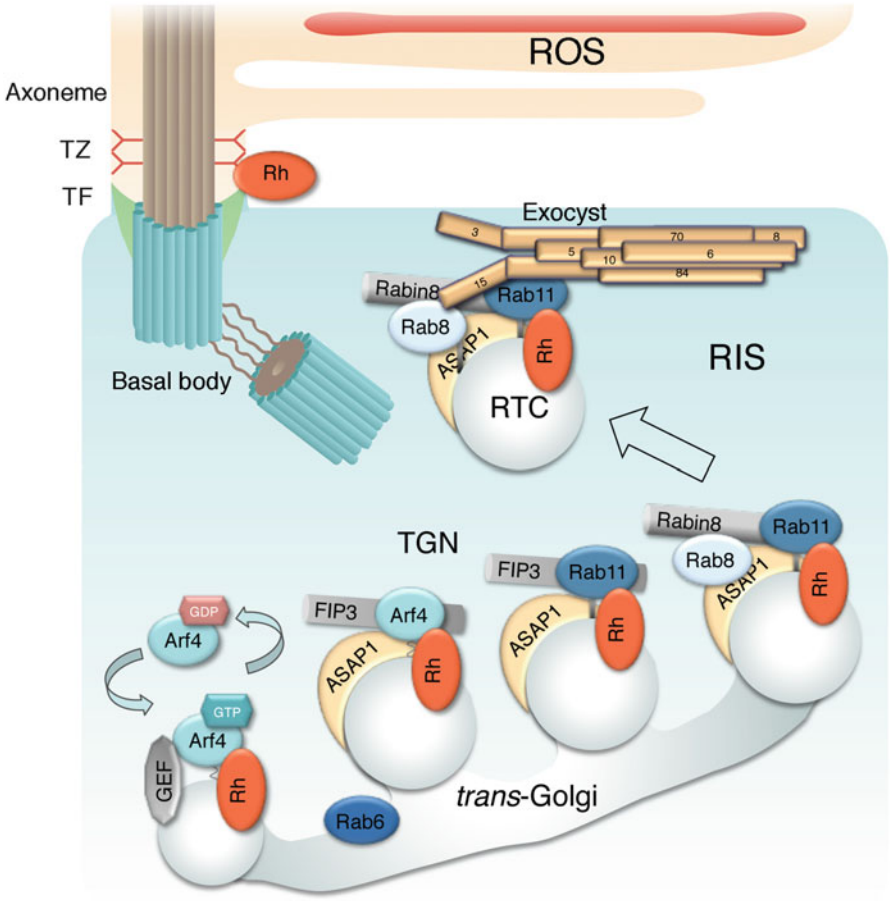

Fig. 6.7 Molecular interactions and the sequence of events taking place during rhodopsin trafficking to the cilia. At the *TGN*, rhodopsin forms a complex with activated Arf4 and ASAP1: this leads to the orderly formation of the Arf4-based ciliary targeting complex, which, in addition to Arf4, contains ASAP1, Rab11, and FIP3. Following GTP hydrolysis on Arf4, ASAP1 and Rab11 recruit Rabin8 and Rab8, which is a critical regulator of fusion with the plasma membrane. On RTCs, ASAP1 serves as a scaffold for activation of Rab8 by Rabin8. Activated Rab8 permits RTC fusion and cargo delivery across the diffusion barrier surrounding the cilium, based in part on its ability to interact with the Sec15 subunit of the exocyst, an octameric membrane tethering complex involved in ciliary targeting. Sec15 directly interacts with Rab8, Rab11, and Rabin8

GEF-effector interaction network that is essential for yeast budding and membrane trafficking to the cilium (Das and Guo 2011; Feng et al. 2012). In addition to Sec15, the Sec10 component of the exocyst is also involved in cargo delivery. Sec10 interacts directly with IFT20 and the ciliary receptor polycystin 2 and is required for its ciliary localization (Fogelgren et al. 2011).

The establishment of the sequence of events in the ciliary targeting of rhodopsin was recently facilitated through the use of the proximity ligation assay (PLA) for

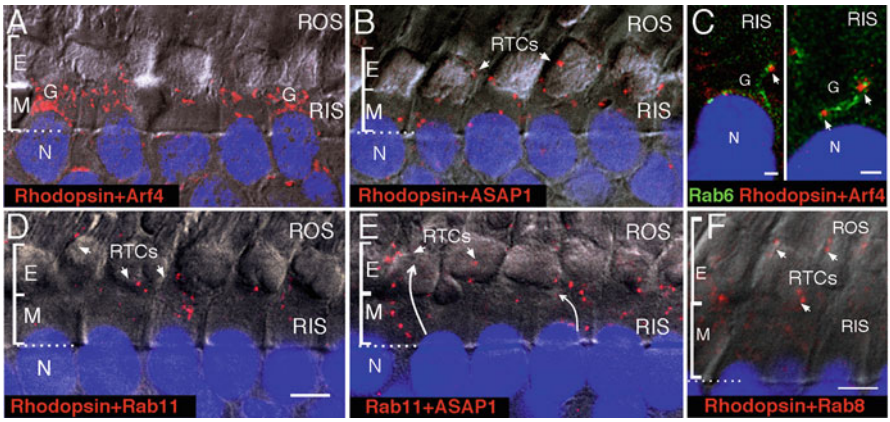

Fig. 6.8 Arf4, ASAP1, and the Rab11a–Rabin8–Rab8 ciliary-targeting complex sequentially interact with rhodopsin transiting from the Golgi/TGN into RTCs. Endogenous protein–protein interactions in situ were detected in a form of *fluorescent dots* by the proximity ligation assay (PLA). The signal is generated when the fluorescently labeled oligonucleotides are hybridized to the primers covalently linked to secondary antibodies, which recognize in coincidence binding of primary antibodies to two proteins interacting at a range <16 nm. Interaction sites (*red dots*) were detected by PLA for rhodopsin-Arf4 (**a**), rhodopsin-ASAP1 (**b**), rhodopsin-Rab11 (**d**), Rab11-ASAP1 (**e**), and rhodopsin-Rab8 (**f**). **c** Rhodopsin-Arf4 PLA (*red dots*) was subsequently stained with antibody to the trans-Golgi marker Rab6 conjugated to Alexa Fluor 488 (*green*). *Arrows* indicate rhodopsin–Arf4 interaction sites juxtaposed to the trans-Golgi cisternae (Rab6, *green*). (Modified from Wang et al. 2012)

analysis of protein–protein interactions in situ (Wang et al. 2012). The interaction sites of rhodopsin with the Arf4-based ciliary targeting complex and the Rab GTPases are illustrated in Fig. 6.8. Because of the unique geometry of the photoreceptors and the unidirectional progression of newly synthesized rhodopsin toward the cilia, quantification of the fluorescence signals generated by PLA provided invaluable information about the order of protein–protein interactions involved in ciliary targeting. For instance, more than 97 % of rhodopsin-Arf4 interaction sites are contained within the photoreceptor myoid at the Golgi complex, whereas only 3 % are found within the photoreceptor ellipsoid, where RTCs are localized. By contrast, 59 % of rhodopsin-Rab8 interaction sites are at the Golgi and 41 % are associated with RTCs (Fig. 6.8), pointing to the sequential interactions of rhodopsin with these GTPases, with Arf4 almost exclusively operating at the Golgi and upstream of Rab8 that functions on RTCs.

6.3.6 The Ciliary Targeting Signals

Clearly, to engage the correct sorting machinery and enter the ciliary pathway rhodopsin and other sensory receptors have to be equipped with adequate ciliary

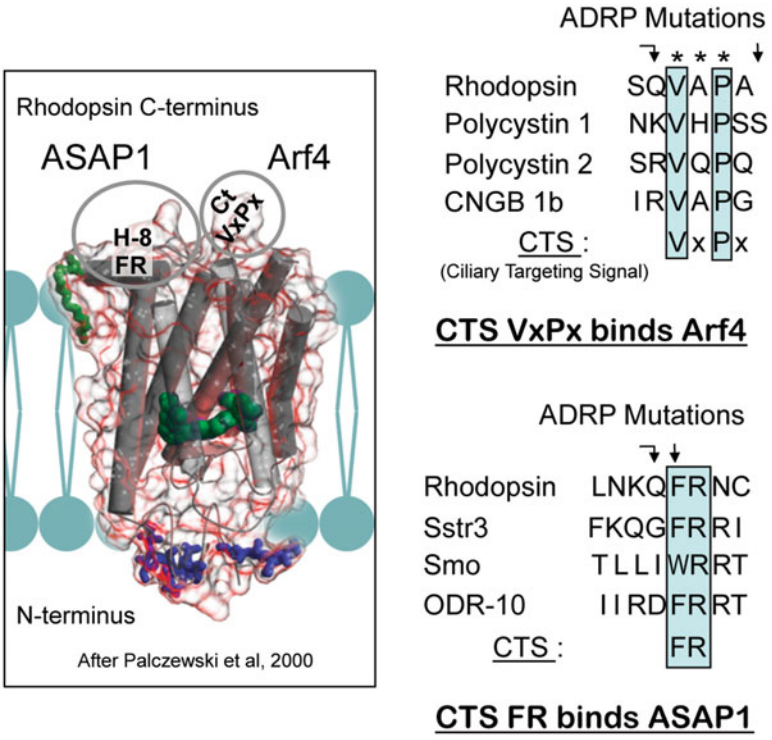

Fig. 6.9 Rhodopsin ciliary targeting signals (CTSs). The rhodopsin cytoplasmic C-terminal VxPx and H-8 FR are its functional ciliary targeting signals (CTSs) that are recognized by Arf4 and ASAP1, respectively. The VxPx and FR targeting motifs are highly conserved among other ciliary sensory receptors

targeting signals. The ciliary targeting sequences that have been identified to date are highly divergent and likely engage an array of binding partners that act at different stages of ciliary membrane traffic (Berbari et al. 2008; Corbit et al. 2005; Fan et al. 2007; Follit et al. 2010; Jin et al. 2010; Kizhatil et al. 2009; Mazelova et al. 2009a; Tao et al. 2009). The extremely conserved rhodopsin ciliary targeting signal (CTS) VxPx directly engages Arf4 to direct rhodopsin to the primary cilia (Deretic et al. 2005). The CTS VxPx is also conserved among other ciliary sensory receptors, including polycystins 1 and 2 and the CNGB1b subunit of the olfactory cyclic nucleotide-gated channel (Fig. 6.9) (Deretic et al. 1998; Deretic et al. 2005; Geng et al. 2006; Jenkins et al. 2006; Ward et al. 2011). Numerous mutations affecting the CTS VxPx cause the most severe forms of autosomal dominant retinitis pigmentosa (ADRP) (Berson et al. 2002), supporting the crucial role of this signal in the directed delivery of rhodopsin to the cilia and ROS. Transgenic expression of rhodopsin carrying mutations in the VxPx motif leads to retinal degeneration in various animal models as a result of rhodopsin mislocalization to multiple cellular compartments, including the synapse that is otherwise devoid of

rhodopsin (Concepcion and Chen 2010; Concepcion et al. 2002; Green et al. 2000; Lee and Flannery 2007; Li et al. 1996; Li et al. 1998; Ng et al. 2008; Sommer et al. 2011; Tam et al. 2000). Mislocalized rhodopsin likely exerts a dominant-negative effect on the regulators of functional compartmentalization in photoreceptor cells, even initiating abnormal neurite outgrowth that is observed in animal models and in patients with ADRP (Li et al. 1998).

The recently identified CTS of rhodopsin, the so-called CTS FR, is composed of amino acids phenylalanine and arginine (AA 313 and 314) localized within its cytoplasmic helix 8 (Wang et al. 2012). In addition to rhodopsin, CTS FR is conserved in ciliary-targeted GPCRs such as Smoothened and SSTR3 (Corbit et al. 2005) (Fig. 6.9). The CTS FR is a recognition motif for the Arf GAP ASAP1 (Wang et al. 2012). This signal is essential for the delivery, to primary cilia of mouse kidney IMCD3 cells, of a fusion protein composed of bovine rhodopsin and eGFP, followed by the C-terminal VxPx motif (Rh-GFP-VxPx). When the CTS FR is replaced with alanines, the fusion protein designated [FR-AA] Rh-GFP-VxPx does not interact with ASAP1, fails to connect with Rab8, and consequently does not localize to cilia (Wang et al. 2012). Thus, CTSs VxPx and FR engage two binding partners, Arf4 and ASAP1, which cooperatively assure the entry into the Rab8-regulated membrane pathway that provides ciliary access. During ciliary trafficking, rhodopsin is not just a passive cargo that is sorted into appropriate carriers but actively recruits the relevant sorting machinery involved in ciliary targeting, which is also shared by other ciliary sensory receptors.

Although the VxPx motif is a key to ciliary localization of rhodopsin, the only other known ROS constituent that has the functional VxPx targeting signal is a lipidated protein retinol dehydrogenase, but the mechanism of its targeting has not been examined (Luo et al. 2004). Other ROS membrane components have different targeting signals and employ different trafficking mechanisms. For example, targeting of peripherin/rds to ROS requires its C terminus, and particularly the valine at position 332 that is downstream of the conserved amphipathic helix, although the protein that recognizes this motif has not been identified (Salinas et al. 2013; Tam et al. 2004). Notably, NPHP and MKS proteins that regulate the ciliary transport of rhodopsin are not involved in the transport of peripherin/rds, supporting their different ciliary routes (Zhao and Malicki 2011). Guanylyl cyclase 1 (GC1) appears to have a diffuse targeting signal and may be cotransported to ROS with other proteins, possibly including rhodopsin (Bhowmick et al. 2009; Karan et al. 2011). Interestingly, in the absence of GC1, trafficking of lipidated ROS proteins is also affected, again suggesting the link between the integral and peripheral components of the ROS light-sensing membranes (Karan et al. 2008). The targeting of cyclic nucleotide-gated (CNG) channels that localize exclusively to the ROS plasma membrane is distinctly different from that of the disk membrane constituents and requires interaction with ankyrin-G (Kizhatil et al. 2009). Other ROS proteins are delivered to the cilia through a multitude of mechanisms that have been recently uncovered and reviewed in depth (Pearring et al. 2013).

6.4 Concluding Remarks

Functional compartmentalization of retinal rod photoreceptors endows these cells with qualities essential for the capture and propagation of visual signals. The underlying structure of the light-sensing organelle, the ROS, is that of a primary cilium, with specializations that include exceptional proliferation of sensory membranes that convert light absorbed by rhodopsin into changes in neurotransmitter release. Thus, the role of ciliary transport is to replenish the light-sensing membranes and deliver rhodopsin and the components of the phototransduction cascade to the exact periciliary location in the photoreceptor cell and nowhere else. The mechanism of ciliary membrane targeting employed by photoreceptors is highly conserved among ciliated cells and involves ordered recruitment and activation of small GTPases of the Rab and Arf families through scaffold proteins and multiprotein complexes, some of which are located at the entrance to the cilia. The disruption of the delicate balance of activities that coordinate the events in light-sensitive membrane renewal ultimately results in the loss of visual function.

Acknowledgments I thank past and present laboratory members, particularly Jing Wang, for their valuable contributions to the studies described here and many colleagues for their stimulating discussions. Supported by the NIH grant EY-12421.

References

Anderson RG (1972) The three-dimensional structure of the basal body from the rhesus monkey oviduct. J Cell Biol 54:246–265

Anderson RE, Penn JS (2004) Environmental light and heredity are associated with adaptive changes in retinal DHA levels that affect retinal function. Lipids 39:1121–1124

Andrews LD, Cohen AI (1983) Freeze-fracture studies of photoreceptor membranes: new observations bearing upon the distribution of cholesterol. J Cell Biol 97:749–755

Arshavsky VY, Burns ME (2012) Photoreceptor signaling: supporting vision across a wide range of light intensities. J Biol Chem 287:1620–1626

Arts HH, Doherty D, van Beersum SE, Parisi MA, Letteboer SJ, Gorden NT, Peters TA, Marker T, Voesenek K, Kartono A, Ozyurek H, Farin FM, Kroes HY, Wolfrum U, Brunner HG, Cremers FP, Glass IA, Knoers NV, Roepman R (2007) Mutations in the gene encoding the basal body protein RPGRIP1L, a nephrocystin-4 interactor, cause Joubert syndrome. Nat Genet 39:882–888

Baschieri F, Farhan H (2012) Crosstalk of small GTPases at the Golgi apparatus. Small GTPases 3:80–90

Baylor DA, Lamb TD, Yau KW (1979) Responses of retinal rods to single photons. J Physiol (Lond) 288:613–634

Berbari NF, Johnson AD, Lewis JS, Askwith CC, Mykytyn K (2008) Identification of ciliary localization sequences within the third intracellular loop of G protein-coupled receptors. Mol Biol Cell 19:1540–1547

Berson EL, Rosner B, Weigel-DiFranco C, Dryja TP, Sandberg MA (2002) Disease progression in patients with dominant retinitis pigmentosa and rhodopsin mutations. Invest Ophthalmol Vis Sci 43:3027–3036

Berta AI, Boesze-Battaglia K, Genini S, Goldstein O, O'Brien PJ, Szel A, Acland GM, Beltran WA, Aguirre GD (2011) Photoreceptor cell death, proliferation and formation of hybrid rod/S-cone photoreceptors in the degenerating STK38L mutant retina. PLoS One 6:e24074

Besharse JC (1986) Photosensitive membrane turnover: differentiated membrane domains and cell–cell interaction. In: Adler R, Farber D (eds) The retina: a model for cell biological studies. Academic, New York, pp 297–352

Besharse JC, Forestner DM, Defoe DM (1985) Membrane assembly in retinal photoreceptors. III. Distinct membrane domains of the connecting cilium of developing rods. J Neurosci 5:1035–1048

Bettencourt-Dias M, Glover DM (2007) Centrosome biogenesis and function: centrosomics brings new understanding. Nat Rev Mol Cell Biol 8:451–463

Bhowmick R, Li M, Sun J, Baker SA, Insinna C, Besharse JC (2009) Photoreceptor IFT complexes containing chaperones, guanylyl cyclase 1 and rhodopsin. Traffic 10:648–663

Blacque OE, Leroux MR (2006) Bardet-Biedl syndrome: an emerging pathomechanism of intracellular transport. Cell Mol Life Sci 63:2145–2161

Bryant DM, Datta A, Rodriguez-Fraticelli AE, Peranen J, Martin-Belmonte F, Mostov KE (2010) A molecular network for de novo generation of the apical surface and lumen. Nat Cell Biol 12:1035–1045

Burns ME, Arshavsky VY (2005) Beyond counting photons: trials and trends in vertebrate visual transduction. Neuron 48:387–401

Chang B, Khanna H, Hawes N, Jimeno D, He S, Lillo C, Parapuram SK, Cheng H, Scott A, Hurd RE, Sayer JA, Otto EA, Attanasio M, O'Toole JF, Jin G, Shou C, Hildebrandt F, Williams DS, Heckenlively JR, Swaroop A (2006) In-frame deletion in a novel centrosomal/ciliary protein CEP290/NPHP6 perturbs its interaction with RPGR and results in early-onset retinal degeneration in the rd16 mouse. Hum Mol Genet 15:1847–1857

Chiba S, Amagai Y, Homma Y, Fukuda M, Mizuno K (2013) NDR2-mediated Rabin8 phosphorylation is crucial for ciliogenesis by switching binding specificity from phosphatidylserine to Sec15. EMBO J 32(6):874–885

Chih B, Liu P, Chinn Y, Chalouni C, Komuves LG, Hass PE, Sandoval W, Peterson AS (2012) A ciliopathy complex at the transition zone protects the cilia as a privileged membrane domain. Nat Cell Biol 14:61–72

Christensen ST, Pedersen LB, Schneider L, Satir P (2007) Sensory cilia and integration of signal transduction in human health and disease. Traffic 8:97–109

Concepcion F, Chen J (2010) Q344ter mutation causes mislocalization of rhodopsin molecules that are catalytically active: a mouse model of Q344ter-induced retinal degeneration. PLoS One 5:e10904

Concepcion F, Mendez A, Chen J (2002) The carboxyl-terminal domain is essential for rhodopsin transport in rod photoreceptors. Vision Res 42:417–426

Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF (2005) Vertebrate Smoothened functions at the primary cilium. Nature (Lond) 437:1018–1021

Craige B, Tsao CC, Diener DR, Hou Y, Lechtreck KF, Rosenbaum JL, Witman GB (2010) CEP290 tethers flagellar transition zone microtubules to the membrane and regulates flagellar protein content. J Cell Biol 190:927–940

Das A, Guo W (2011) Rabs and the exocyst in ciliogenesis, tubulogenesis and beyond. Trends Cell Biol 21:383–386

Deretic D (2006) A role for rhodopsin in a signal transduction cascade that regulates membrane trafficking and photoreceptor polarity. Vision Res 46:4427–4433

Deretic D (2013) Crosstalk of Arf and Rab GTPases en route to cilia. Small GTPases 4(2):70–77

Deretic D, Papermaster DS (1991) Polarized sorting of rhodopsin on post-Golgi membranes in frog retinal photoreceptor cells. J Cell Biol 113:1281–1293

Deretic D, Papermaster DS (1993) Rab6 is associated with a compartment that transports rhodopsin from the trans-Golgi to the site of rod outer segment disk formation in frog retinal photoreceptors. J Cell Sci 106:803–813

Deretic D, Huber LA, Ransom N, Mancini M, Simons K, Papermaster DS (1995) rab8 in retinal photoreceptors may participate in rhodopsin transport and in rod outer segment disk morphogenesis. J Cell Sci 108:215–224

Deretic D, Puleo Scheppke B, Trippe C (1996) Cytoplasmic domain of rhodopsin is essential for post-Golgi vesicle formation in a retinal cell-free system. J Biol Chem 271:2279–2286

Deretic D, Schmerl S, Hargrave PA, Arendt A, McDowell JH (1998) Regulation of sorting and post-Golgi trafficking of rhodopsin by its C-terminal sequence QVS(A)PA. Proc Natl Acad Sci USA 95:10620–10625

Deretic D, Williams AH, Ransom N, Morel V, Hargrave PA, Arendt A (2005) Rhodopsin C terminus, the site of mutations causing retinal disease, regulates trafficking by binding to ADP-ribosylation factor 4 (ARF4). Proc Natl Acad Sci USA 102:3301–3306

Donaldson JG (2005) Arfs, phosphoinositides and membrane traffic. Biochem Soc Trans 33:1276–1278

Donaldson JG, Jackson CL (2011) ARF family G proteins and their regulators: roles in membrane transport, development and disease. Nat Rev Mol Cell Biol 12:362–375

Dryja TP, Adams SM, Grimsby JL, McGee TL, Hong DH, Li T, Andreasson S, Berson EL (2001) Null RPGRIP1 alleles in patients with Leber congenital amaurosis. Am J Hum Genet 68:1295–1298

Emmer BT, Maric D, Engman DM (2010) Molecular mechanisms of protein and lipid targeting to ciliary membranes. J Cell Sci 123:529–536

Fan S, Fogg V, Wang Q, Chen XW, Liu CJ, Margolis B (2007) A novel Crumbs3 isoform regulates cell division and ciliogenesis via importin beta interactions. J Cell Biol 178:387–398

Feng S, Knodler A, Ren J, Zhang J, Zhang X, Hong Y, Huang S, Peranen J, Guo W (2012) A Rab8 guanine nucleotide exchange factor-effector interaction network regulates primary ciliogenesis. J Biol Chem 287:15602–15609

Fliegauf M, Benzing T, Omran H (2007) When cilia go bad: cilia defects and ciliopathies. Nat Rev Mol Cell Biol 8:880–893

Fogelgren B, Lin SY, Zuo X, Jaffe KM, Park KM, Reichert RJ, Bell PD, Burdine RD, Lipschutz JH (2011) The exocyst protein Sec10 interacts with Polycystin-2 and knockdown causes PKD-phenotypes. PLoS Genet 7:e1001361

Follit JA, Li L, Vucica Y, Pazour GJ (2010) The cytoplasmic tail of fibrocystin contains a ciliary targeting sequence. J Cell Biol 188:21–28

Garcia-Gonzalo FR, Reiter JF (2012) Scoring a backstage pass: mechanisms of ciliogenesis and ciliary access. J Cell Biol 197:697–709

Garcia-Gonzalo FR, Corbit KC, Sirerol-Piquer MS, Ramaswami G, Otto EA, Noriega TR, Seol AD, Robinson JF, Bennett CL, Josifova DJ, Garcia-Verdugo JM, Katsanis N, Hildebrandt F, Reiter JF (2011) A transition zone complex regulates mammalian ciliogenesis and ciliary membrane composition. Nat Genet 43:776–784

Geng L, Okuhara D, Yu Z, Tian X, Cai Y, Shibazaki S, Somlo S (2006) Polycystin-2 traffics to cilia independently of polycystin-1 by using an N-terminal RVxP motif. J Cell Sci 119:1383–1395

Gerdes JM, Davis EE, Katsanis N (2009) The vertebrate primary cilium in development, homeostasis, and disease. Cell 137:32–45

Gilliam JC, Chang JT, Sandoval IM, Zhang Y, Li T, Pittler SJ, Chiu W, Wensel TG (2012) Three-dimensional architecture of the rod sensory cilium and its disruption in retinal neurodegeneration. Cell 151:1029–1041

Gilula NB, Satir P (1972) The ciliary necklace. A ciliary membrane specialization. J Cell Biol 53:494–509

Godi A, Di Campli A, Konstantakopoulos A, Di Tullio G, Alessi DR, Kular GS, Daniele T, Marra P, Lucocq JM, De Matteis MA (2004) FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P. Nat Cell Biol 6:393–404

Goldstein O, Kukekova AV, Aguirre GD, Acland GM (2010) Exonic SINE insertion in STK38L causes canine early retinal degeneration (erd). Genomics 96:362–368

Gopalakrishna KN, Doddapuneni K, Boyd KK, Masuho I, Martemyanov KA, Artemyev NO (2011) Interaction of transducin with uncoordinated 119 protein (UNC119): implications for the model of transducin trafficking in rod photoreceptors. J Biol Chem 286:28954–28962

Green ES, Menz MD, LaVail MM, Flannery JG (2000) Characterization of rhodopsin mis-sorting and constitutive activation in a transgenic rat model of retinitis pigmentosa. Invest Ophthalmol Vis Sci 41:1546–1553

Grosshans BL, Ortiz D, Novick P (2006) Rabs and their effectors: achieving specificity in membrane traffic. Proc Natl Acad Sci USA 103:11821–11827

Hattula K, Furuhjelm J, Arffman A, Peranen J (2002) A Rab8-specific GDP/GTP exchange factor is involved in actin remodeling and polarized membrane transport. Mol Biol Cell 13:3268–3280

Heider MR, Munson M (2012) Exorcising the exocyst complex. Traffic 13:898–907

Hong DH, Pawlyk BS, Shang J, Sandberg MA, Berson EL, Li T (2000) A retinitis pigmentosa GTPase regulator (RPGR)-deficient mouse model for X-linked retinitis pigmentosa (RP3). Proc Natl Acad Sci USA 97:3649–3654

Hong DH, Yue G, Adamian M, Li T (2001) Retinitis pigmentosa GTPase regulator (RPGRr)-interacting protein is stably associated with the photoreceptor ciliary axoneme and anchors RPGR to the connecting cilium. J Biol Chem 276:12091–12099

Horst CJ, Forestner DM, Besharse JC (1987) Cytoskeletal–membrane interactions: a stable interaction between cell surface glycoconjugates and doublet microtubules of the photoreceptor connecting cilium. J Cell Biol 105:2973–2987

Hsu SC, TerBush D, Abraham M, Guo W (2004) The exocyst complex in polarized exocytosis. Int Rev Cytol 233:243–265

Hu Q, Milenkovic L, Jin H, Scott MP, Nachury MV, Spiliotis ET, Nelson WJ (2010) A septin diffusion barrier at the base of the primary cilium maintains ciliary membrane protein distribution. Science 329:436–439

Humbert MC, Weihbrecht K, Searby CC, Li Y, Pope RM, Sheffield VC, Seo S (2012) ARL13B, PDE6D, and CEP164 form a functional network for INPP5E ciliary targeting. Proc Natl Acad Sci USA 109:19691–19696

Inoue H, Ha VL, Prekeris R, Randazzo PA (2008) Arf GTPase-activating protein ASAP1 interacts with Rab11 effector FIP3 and regulates pericentrosomal localization of transferrin receptor-positive recycling endosome. Mol Biol Cell 19:4224–4237

Insinna C, Besharse JC (2008) Intraflagellar transport and the sensory outer segment of vertebrate photoreceptors. Dev Dyn 237:1982–1992

Ismail SA, Chen YX, Miertzschke M, Vetter IR, Koerner C, Wittinghofer A (2012) Structural basis for Arl3-specific release of myristoylated ciliary cargo from UNC119. EMBO J 31:4085–4094

Jacobson SG, Cideciyan AV, Aleman TS, Sumaroka A, Roman AJ, Gardner LM, Prosser HM, Mishra M, Bech-Hansen NT, Herrera W, Schwartz SB, Liu XZ, Kimberling WJ, Steel KP, Williams DS (2008) Usher syndromes due to MYO7A, PCDH15, USH2A or GPR98 mutations share retinal disease mechanism. Hum Mol Genet 17(15):2405–2415

Jenkins PM, Hurd TW, Zhang L, McEwen DP, Brown RL, Margolis B, Verhey KJ, Martens JR (2006) Ciliary targeting of olfactory CNG channels requires the CNGB1b subunit and the kinesin-2 motor protein, KIF17. Curr Biol 16:1211–1216

Jian X, Brown P, Schuck P, Gruschus JM, Balbo A, Hinshaw JE, Randazzo PA (2009) Autoinhibition of Arf GTPase-activating protein activity by the BAR domain in ASAP1. J Biol Chem 284:1652–1663

Jin H, White SR, Shida T, Schultz S, Aguiar M, Nachury MV (2010) The conserved Bardet-Biedl syndrome proteins assemble a coat that traffics membrane proteins to cilia. Cell 141:1208–1219

Kahn RA, Cherfils J, Elias M, Lovering RC, Munro S, Schurmann A (2006) Nomenclature for the human Arf family of GTP-binding proteins: ARF, ARL, and SAR proteins. J Cell Biol 172:645–650

Karan S, Frederick JM, Baehr W (2008) Involvement of guanylate cyclases in transport of photoreceptor peripheral membrane proteins. Adv Exp Med Biol 613:351–359

Karan S, Tam BM, Moritz OL, Baehr W (2011) Targeting of mouse guanylate cyclase 1 (Gucy2e) to *Xenopus laevis* rod outer segments. Vision Res 51:2304–2311

Khanna H, Davis EE, Murga-Zamalloa CA, Estrada-Cuzcano A, Lopez I, den Hollander AI, Zonneveld MN, Othman MI, Waseem N, Chakarova CF, Maubaret C, Diaz-Font A, Macdonald I, Muzny DM, Wheeler DA, Morgan M, Lewis LR, Logan CV, Tan PL, Beer MA, Inglehearn CF, Lewis RA, Jacobson SG, Bergmann C, Beales PL, Attie-Bitach T, Johnson CA, Otto EA, Bhattacharya SS, Hildebrandt F, Gibbs RA, Koenekoop RK, Swaroop A, Katsanis N (2009) A common allele in RPGRIP1L is a modifier of retinal degeneration in ciliopathies. Nat Genet 41:739–745

Kim J, Krishnaswami SR, Gleeson JG (2008) CEP290 interacts with the centriolar satellite component PCM-1 and is required for Rab8 localization to the primary cilium. Hum Mol Genet 17:3796–3805

Kirschner R, Rosenberg T, Schultz-Heienbrok R, Lenzner S, Feil S, Roepman R, Cremers FPM, Ropers HH, Berger W (1999) RPGR transcription studies in mouse and human tissues reveal a retina-specific isoform that is disrupted in a patient with X-linked retinitis pigmentosa. Hum Mol Genet 8:1571–1578

Kizhatil K, Baker SA, Arshavsky VY, Bennett V (2009) Ankyrin-G promotes cyclic nucleotide-gated channel transport to rod photoreceptor sensory cilia. Science 323:1614–1617

Knodler A, Feng S, Zhang J, Zhang X, Das A, Peranen J, Guo W (2010) Coordination of Rab8 and Rab11 in primary ciliogenesis. Proc Natl Acad Sci USA 107(14):6346–6351

Lee ES, Flannery JG (2007) Transport of truncated rhodopsin and its effects on rod function and degeneration. Invest Ophthalmol Vis Sci 48:2868–2876

Leroux MR (2007) Taking vesicular transport to the cilium. Cell 129:1041–1043

Li T, Snyder WK, Olsson JE, Dryja TP (1996) Transgenic mice carrying the dominant rhodopsin mutation P347S: evidence for defective vectorial transport of rhodopsin to the outer segments. Proc Natl Acad Sci USA 93:14176–14181

Li ZY, Wong F, Chang JH, Possin DE, Hao Y, Petters RM, Milam AH (1998) Rhodopsin transgenic pigs as a model for human retinitis pigmentosa. Invest Ophthalmol Vis Sci 39:808–819

Li L, Khan N, Hurd T, Ghosh AK, Cheng C, Molday R, Heckenlively JR, Swaroop A, Khanna H (2013) Ablation of the X-linked rRetinitis pPigmentosa 2 (Rp2) gene in mice results in opsin mislocalization and photoreceptor degeneration. Invest Ophthalmol Vis Sci 54(7):4503–4511

Luo W, Marsh-Armstrong N, Rattner A, Nathans J (2004) An outer segment localization signal at the C terminus of the photoreceptor-specific retinol dehydrogenase. J Neurosci 24:2623–2632

Maerker T, van Wijk E, Overlack N, Kersten FF, McGee J, Goldmann T, Sehn E, Roepman R, Walsh EJ, Kremer H, Wolfrum U (2008) A novel Usher protein network at the periciliary reloading point between molecular transport machineries in vertebrate photoreceptor cells. Hum Mol Genet 17:71–86

Mazelova J, Astuto-Gribble L, Inoue H, Tam BM, Schonteich E, Prekeris R, Moritz OL, Randazzo PA, Deretic D (2009a) Ciliary targeting motif VxPx directs assembly of a trafficking module through Arf4. EMBO J 28:183–192

Mazelova J, Ransom N, Astuto-Gribble L, Wilson MC, Deretic D (2009b) Syntaxin 3 and SNAP-25 pairing, regulated by omega-3 docosahexaenoic acid, controls the delivery of rhodopsin for the biogenesis of cilia-derived sensory organelles, the rod outer segments. J Cell Sci 122:2003–2013

Meindl A, Dry K, Herrmann K, Manson F, Ciccodicola A, Edgar A, Carvalho MR, Achatz H, Hellebrand H, Lennon A, Migliaccio C, Porter K, Zrenner E, Bird A, Jay M, Lorenz B, Wittwer B, D'Urso M, Meitinger T, Wright A (1996) A gene (RPGR) with homology to the RCC1 guanine nucleotide exchange factor is mutated in X-linked retinitis pigmentosa (RP3). Nat Genet 13:35–42

Mizuno-Yamasaki E, Rivera-Molina F, Novick P (2012) GTPase networks in membrane traffic. Annu Rev Biochem 81:637–659

Moritz OL, Tam BM, Hurd LL, Peranen J, Deretic D, Papermaster DS (2001) Mutant rab8 impairs docking and fusion of rhodopsin-bearing post-Golgi membranes and causes cell death of transgenic *Xenopus* rods. Mol Biol Cell 12:2341–2351

Murga-Zamalloa CA, Atkins SJ, Peranen J, Swaroop A, Khanna H (2010a) Interaction of retinitis pigmentosa GTPase regulator (RPGR) with RAB8A GTPase: implications for cilia dysfunction and photoreceptor degeneration. Hum Mol Genet 19:3591–3598

Murga-Zamalloa CA, Desai NJ, Hildebrandt F, Khanna H (2010b) Interaction of ciliary disease protein retinitis pigmentosa GTPase regulator with nephronophthisis-associated proteins in mammalian retinas. Mol Vis 16:1373–1381

Nachury MV, Loktev AV, Zhang Q, Westlake CJ, Peranen J, Merdes A, Slusarski DC, Scheller RH, Bazan JF, Sheffield VC, Jackson PK (2007) A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. Cell 129:1201–1213

Nachury MV, Seeley ES, Jin H (2010) Trafficking to the ciliary membrane: how to get across the periciliary diffusion barrier? Annu Rev Cell Dev Biol 26:59–87

Ng YF, Chan HH, Chu PH, To CH, Gilger BC, Petters RM, Wong F (2008) Multifocal electroretinogram in rhodopsin P347L transgenic pigs. Invest Ophthalmol Vis Sci 49:2208–2215

Nie Z, Hirsch DS, Luo R, Jian X, Stauffer S, Cremesti A, Andrade J, Lebowitz J, Marino M, Ahvazi B, Hinshaw JE, Randazzo PA (2006) A BAR domain in the N terminus of the Arf GAP ASAP1 affects membrane structure and trafficking of epidermal growth factor receptor. Curr Biol 16:130–139

Novick P, Medkova M, Dong G, Hutagalung A, Reinisch K, Grosshans B (2006) Interactions between Rabs, tethers, SNAREs and their regulators in exocytosis. Biochem Soc Trans 34:683–686

Otto EA, Loeys B, Khanna H, Hellemans J, Sudbrak R, Fan S, Muerb U, O'Toole JF, Helou J, Attanasio M, Utsch B, Sayer JA, Lillo C, Jimeno D, Coucke P, De Paepe A, Reinhardt R, Klages S, Tsuda M, Kawakami I, Kusakabe T, Omran H, Imm A, Tippens M, Raymond PA, Hill J, Beales P, He S, Kispert A, Margolis B, Williams DS, Swaroop A, Hildebrandt F (2005) Nephrocystin-5, a ciliary IQ domain protein, is mutated in Senior-Loken syndrome and interacts with RPGR and calmodulin. Nat Genet 37:282–288

Overlack N, Maerker T, Latz M, Nagel-Wolfrum K, Wolfrum U (2008) SANS (USH1G) expression in developing and mature mammalian retina. Vision Res 48:400–412

Palczewski K (2012) Chemistry and biology of vision. J Biol Chem 287:1612–1619

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. Science 289:739–745

Papermaster DS, Schneider BG, Besharse JC (1985) Vesicular transport of newly synthesized opsin from the Golgi apparatus toward the rod outer segment. Ultrastructural immunocytochemical and autoradiographic evidence in *Xenopus* retinas. Invest Ophthalmol Vis Sci 26:1386–1404

Papermaster DS, Schneider BG, Defoe D, Besharse JC (1986) Biosynthesis and vectorial transport of opsin on vesicles in retinal rod photoreceptors. J Histochem Cytochem 34:5–16

Pearring JN, Salinas RY, Baker SA, Arshavsky VY (2013) Protein sorting, targeting and trafficking in photoreceptor cells. Prog Retin Eye Res 36:24–51

Peters KR, Palade GE, Schneider BG, Papermaster DS (1983) Fine structure of a periciliary ridge complex of frog retinal rod cells revealed by ultrahigh resolution scanning electron microscopy. J Cell Biol 96:265–276

Rachel RA, Li T, Swaroop A (2012) Photoreceptor sensory cilia and ciliopathies: focus on CEP290, RPGR and their interacting proteins. Cilia 1:22

Rink J, Ghigo E, Kalaidzidis Y, Zerial M (2005) Rab conversion as a mechanism of progression from early to late endosomes. Cell 122:735–749

Rogers KK, Wilson PD, Snyder RW, Zhang X, Guo W, Burrow CR, Lipschutz JH (2004) The exocyst localizes to the primary cilium in MDCK cells. Biochem Biophys Res Commun 319:138–143

Rosenbaum JL, Witman GB (2002) Intraflagellar transport. Nat Rev Mol Cell Biol 3:813–825

Salinas RY, Baker SA, Gospe SM 3rd, Arshavsky VY (2013) A single valine residue plays an essential role in peripherin/rds targeting to photoreceptor outer segments. PLoS One 8:e54292

Sang L, Miller JJ, Corbit KC, Giles RH, Brauer MJ, Otto EA, Baye LM, Wen X, Scales SJ, Kwong M, Huntzicker EG, Sfakianos MK, Sandoval W, Bazan JF, Kulkarni P, Garcia-Gonzalo FR, Seol AD, O'Toole JF, Held S, Reutter HM, Lane WS, Rafiq MA, Noor A, Ansar M, Devi AR, Sheffield VC, Slusarski DC, Vincent JB, Doherty DA, Hildebrandt F, Reiter JF, Jackson PK (2011) Mapping the NPHP-JBTS-MKS protein network reveals ciliopathy disease genes and pathways. Cell 145:513–528

Sayer JA, Otto EA, O'Toole JF, Nurnberg G, Kennedy MA, Becker C, Hennies HC, Helou J, Attanasio M, Fausett BV, Utsch B, Khanna H, Liu Y, Drummond I, Kawakami I, Kusakabe T, Tsuda M, Ma L, Lee H, Larson RG, Allen SJ, Wilkinson CJ, Nigg EA, Shou C, Lillo C, Williams DS, Hoppe B, Kemper MJ, Neuhaus T, Parisi MA, Glass IA, Petry M, Kispert A, Gloy J, Ganner A, Walz G, Zhu X, Goldman D, Nurnberg P, Swaroop A, Leroux MR, Hildebrandt F (2006) The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor ATF4. Nat Genet 38:674–681

Schwarz N, Novoselova TV, Wait R, Hardcastle AJ, Cheetham ME (2012) The X-linked retinitis pigmentosa protein RP2 facilitates G protein traffic. Hum Mol Genet 21:863–873

Sedmak T, Wolfrum U (2011) Intraflagellar transport proteins in ciliogenesis of photoreceptor cells. Biol Cell 103:449–466

Shu X, Fry AM, Tulloch B, Manson FD, Crabb JW, Khanna H, Faragher AJ, Lennon A, He S, Trojan P, Giessl A, Wolfrum U, Vervoort R, Swaroop A, Wright AF (2005) RPGR ORF15 isoform co-localizes with RPGRIP1 at centrioles and basal bodies and interacts with nucleophosmin. Hum Mol Genet 14:1183–1197

Singla V, Reiter JF (2006) The primary cilium as the cell's antenna: signaling at a sensory organelle. Science 313:629–633

Sommer JR, Wong F, Petters RM (2011) Phenotypic stability of Pro347Leu rhodopsin transgenic pigs as indicated by photoreceptor cell degeneration. Transgenic Res 20:1391–1395

Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10:513–525

Tam BM, Moritz OL, Hurd LB, Papermaster DS (2000) Identification of an outer segment targeting signal in the COOH terminus of rhodopsin using transgenic *Xenopus laevis*. J Cell Biol 151:1369–1380

Tam BM, Moritz OL, Papermaster DS (2004) The C terminus of peripherin/rds participates in rod outer segment targeting and alignment of disk incisures. Mol Biol Cell 15:2027–2037

Tao B, Bu S, Yang Z, Siroky B, Kappes JC, Kispert A, Guay-Woodford LM (2009) Cystin localizes to primary cilia via membrane microdomains and a targeting motif. J Am Soc Nephrol 20:2570–2580

Tsang WY, Bossard C, Khanna H, Peranen J, Swaroop A, Malhotra V, Dynlacht BD (2008) CP110 suppresses primary cilia formation through its interaction with CEP290, a protein deficient in human ciliary disease. Dev Cell 15:187–197

van Reeuwijk J, Arts HH, Roepman R (2011) Scrutinizing ciliopathies by unraveling ciliary interaction networks. Hum Mol Genet 20:R149–157

Veltel S, Gasper R, Eisenacher E, Wittinghofer A (2008) The retinitis pigmentosa 2 gene product is a GTPase-activating protein for Arf-like 3. Nat Struct Mol Biol 15:373–380

Vieira OV, Gaus K, Verkade P, Fullekrug J, Vaz WL, Simons K (2006) FAPP2, cilium formation, and compartmentalization of the apical membrane in polarized Madin-Darby canine kidney (MDCK) cells. Proc Natl Acad Sci USA 103:18556–18561

Wang J, Morita Y, Mazelova J, Deretic D (2012) The Arf GAP ASAP1 provides a platform to regulate Arf4- and Rab11-Rab8-mediated ciliary receptor targeting. EMBO J 31:4057–4071

Wang WJ, Tay HG, Soni R, Perumal GS, Goll MG, Macaluso FP, Asara JM, Amack JD, Bryan Tsou MF (2013) CEP162 is an axoneme-recognition protein promoting ciliary transition zone assembly at the cilia base. Nat Cell Biol 15(6):591–601

Ward HH, Brown-Glaberman U, Wang J, Morita Y, Alper SL, Bedrick EJ, Gattone VH 2nd, Deretic D, Wandinger-Ness A (2011) A conserved signal and GTPase complex are required for the ciliary transport of polycystin-1. Mol Biol Cell 22:3289–3305

Westlake CJ, Baye LM, Nachury MV, Wright KJ, Ervin KE, Phu L, Chalouni C, Beck JS, Kirkpatrick DS, Slusarski DC, Sheffield VC, Scheller RH, Jackson PK (2011) Primary cilia membrane assembly is initiated by Rab11 and transport protein particle II (TRAPPII) complex-dependent trafficking of Rabin8 to the centrosome. Proc Natl Acad Sci USA 108:2759–2764

Williams DS (2008) Usher syndrome: animal models, retinal function of Usher proteins, and prospects for gene therapy. Vision Res 48:433–441

Williams CL, Li C, Kida K, Inglis PN, Mohan S, Semenec L, Bialas NJ, Stupay RM, Chen N, Blacque OE, Yoder BK, Leroux MR (2011) MKS and NPHP modules cooperate to establish basal body/transition zone membrane associations and ciliary gate function during ciliogenesis. J Cell Biol 192:1023–1041

Winkler BS, Boulton ME, Gottsch JD, Sternberg P (1999) Oxidative damage and age-related macular degeneration. Mol Vision (Online) 5:32

Wright KJ, Baye LM, Olivier-Mason A, Mukhopadhyay S, Sang L, Kwong M, Wang W, Pretorius PR, Sheffield VC, Sengupta P, Slusarski DC, Jackson PK (2011) An ARL3-UNC119-RP2 GTPase cycle targets myristoylated NPHP3 to the primary cilium. Genes Dev 25:2347–2360

Wu S, Mehta SQ, Pichaud F, Bellen HJ, Quiocho FA (2005) Sec15 interacts with Rab11 via a novel domain and affects Rab11 localization in vivo. Nat Struct Mol Biol 12:879–885

Yang J, Liu X, Zhao Y, Adamian M, Pawlyk B, Sun X, McMillan DR, Liberman MC, Li T (2010) Ablation of whirlin long isoform disrupts the USH2 protein complex and causes vision and hearing loss. PLoS Genet 6:e1000955

Yoshimura S, Egerer J, Fuchs E, Haas AK, Barr FA (2007) Functional dissection of Rab GTPases involved in primary cilium formation. J Cell Biol 178:363–369

Zerial M, McBride H (2001) Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2:107–117

Zhang XM, Ellis S, Sriratana A, Mitchell CA, Rowe T (2004) Sec15 is an effector for the Rab11 GTPase in mammalian cells. J Biol Chem 279:43027–43034

Zhang H, Constantine R, Vorobiev S, Chen Y, Seetharaman J, Huang YJ, Xiao R, Montelione GT, Gerstner CD, Davis MW, Inana G, Whitby FG, Jorgensen EM, Hill CP, Tong L, Baehr W (2011) UNC119 is required for G protein trafficking in sensory neurons. Nat Neurosci 14:874–880

Zhao C, Malicki J (2011) Nephrocystins and MKS proteins interact with IFT particle and facilitate transport of selected ciliary cargos. EMBO J 30:2532–2544

Zhao Y, Hong DH, Pawlyk B, Yue G, Adamian M, Grynberg M, Godzik A, Li T (2003) The retinitis pigmentosa GTPase regulator (RPGR)-interacting protein: subserving RPGR function and participating in disk morphogenesis. Proc Natl Acad Sci USA 100:3965–3970

Chapter 7
Molecular Mechanisms of Photoreceptor Synaptic Transmission

Matthew J. Van Hook and Wallace B. Thoreson

Abstract Voltage changes resulting from the absorption of photons and triggering of a second-messenger cascade in outer segments of rod and cone photoreceptors are encoded at the synaptic terminal and transmitted to second-order bipolar and horizontal cells. In this chapter, we survey the unique structural, molecular, and functional features of photoreceptor synapses that enable them to encode and transmit light responses. We begin by describing the anatomy of the photoreceptor synapse, focusing on a unique structure called the synaptic ribbon, which is present in only a handful of other primary sensory synapses and plays an important role in priming glutamate-laden vesicles for release. We then discuss the locations and functional roles of many of the key proteins present at the synapse. Although many of these are shared in common with conventional nonribbon synapses, some important differences appear to contribute to the unique signaling capabilities of photoreceptors. We then explore the properties of synaptic transmission by rods and cones, noting the ways in which calcium-dependent regulation of the rate of vesicle fusion enables the synapse to encode different features of the visual scene. Although this chapter is focused on the molecular mechanisms of synaptic transmission in healthy retinas, we make note of how synaptic defects can lead to vision loss. For example, forms of X-linked congenital stationary night blindness can arise from mutations in the gene encoding the voltage-gated calcium channels present at rod and cone synapses.

M.J. Van Hook
Department of Ophthalmology & Visual Sciences, University of Nebraska Medical Center, Omaha, NE 68198, USA

W.B. Thoreson (✉)
Department of Ophthalmology & Visual Sciences, University of Nebraska Medical Center, Omaha, NE 68198, USA

Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198, USA
e-mail: wbthores@unmc.edu

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_7, © Springer Japan 2014

Keywords Cone photoreceptor • Retina • Ribbon synapse • Rod photoreceptor

7.1 Introduction

Vision begins with voltage changes arising from the absorption of photons in the outer segments of rod and cone photoreceptors. These voltage changes open or close voltage-gated calcium (Ca^{2+}) channels at the synaptic terminal, changing the rate of glutamate release onto second-order bipolar and horizontal cells. In this chapter, we survey the features of photoreceptor synapses that enable these cells to encode and transmit visual information. We begin by describing the unique structural features and molecular architecture of rod and cone synapses, highlighting those that functionally distinguish these synapses from synapses in other neurons. In the following sections, we draw on recent work from our laboratory and others to describe some of the basic properties of rod and cone synaptic transmission.

7.2 Ultrastructure of Photoreceptor Synapses

Dendrites of horizontal and bipolar cells contact rods and cones at invaginations in the photoreceptor synaptic terminals. A slight evagination, called the synaptic ridge, bulges out at the top of these invaginations (Fig. 7.1). The synaptic ridge is flanked on both sides by dendrites from horizontal cells. The cone synapse typically has a single invaginating 'On' bipolar cell dendrite flanked by two horizontal cell dendrites. In rods, there are typically two dendrites from rod bipolar cells running through the center of the invagination (Lasansky 1973; Raviola and Gilula 1975; Dowling and Werblin 1969; Ladman 1958; Evans 1966; Dowling and Boycott 1966). Near the edges of the invagination, other bipolar cells make flat contacts with the cone membrane (Dowling and Boycott 1966; Dowling and Werblin 1969).

Sitting within the synaptic ridge is the synaptic ribbon, a thin electron-dense organelle surrounded by a cloud of synaptic vesicles. The ribbon is the defining anatomical feature of photoreceptor synapses, and much recent work has focused on understanding the unique signaling capabilities that it confers to rods and cones. Rods in mammalian retinas typically possess only a single ribbon (Rao-Mirotznik et al. 1995), whereas those in amphibian retinas have about 7 ribbons (Townes-Anderson et al. 1985). Cone terminals have more ribbons. For example, amphibian cones have an average of 13 ribbons each (Bartoletti et al. 2010; Pang et al. 2008). In primate retina, foveal cones have about 20 ribbons and cones in the far periphery can have 50 ribbons (Haverkamp et al. 2001).

A host of electron microscopy studies have characterized the basic features of the photoreceptor synaptic ribbons in several species, including amphibians, rodents, and primates (Gray and Pease 1971; Townes-Anderson et al. 1985;

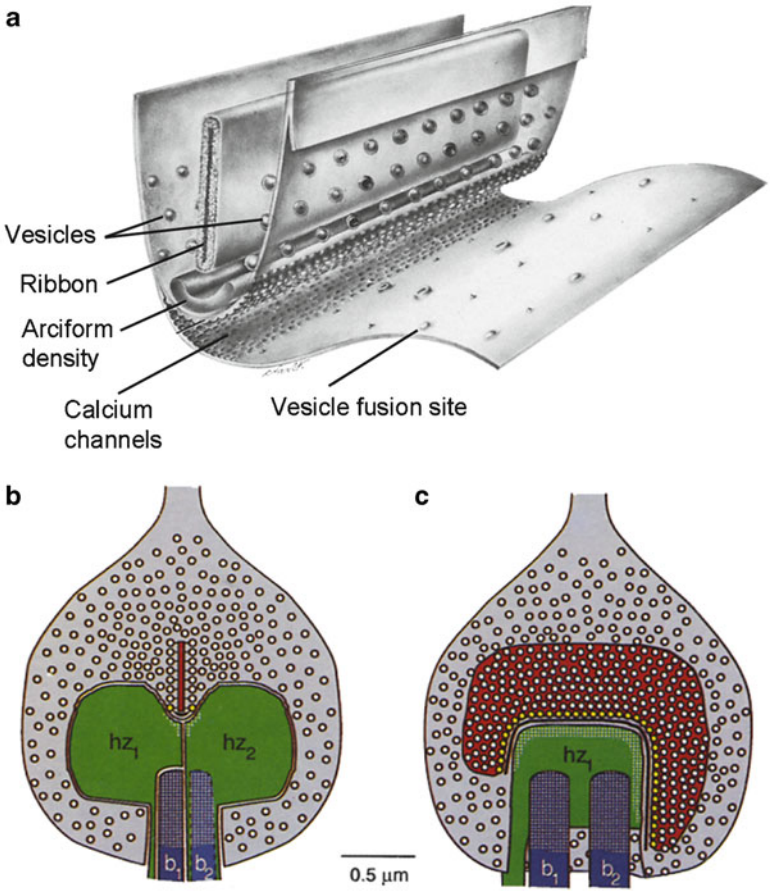

Fig. 7.1 Anatomy of photoreceptor ribbon synapses. (**a**) Schematic shows the structure of the photoreceptor ribbon synapse from freeze-fracture electron microscopy. The ribbon is seen running along the synaptic ridge atop the arciform density. Vesicles are tethered to the sides of the ribbon. Small particles beneath the arciform density that are present on both faces of the membrane split by the freeze-fracture are likely to be the voltage-gated Ca^{2+} channels. (**b**, **c**) Schematic of the rod synapse showing a single ribbon with vesicles tethered to the ribbon or distributed throughout the terminal. The ribbon appears as a thin strip in cross section (**b**) and as a crescent in oblique views (**c**). Processes from two horizontal cells (hz_1 and hz_2) as well as from two rod bipolar cells (b_1 and b_2) can be seen in the invaginating cleft. (**a**) Reproduced from Raviola and Gilula 1975 with permission from Rockefeller University Press. (**b**, **c**) Reproduced from Rao-Mirotznik et al. 1995, with permission from Elsevier, Inc.

Rao-Mirotznik et al. 1995; Lasansky 1973; Raviola and Gilula 1975; Zampighi et al. 2011; Sjöstrand 1958; Ladman 1958; Evans 1966; De Robertis and Franchi 1956; Dowling and Boycott 1966; Usukura and Yamada 1987). In cross section, the ribbon appears as a thin strip of electron-dense material approximately 35 nm thick and extending perpendicularly from the synaptic ridge about 0.5 µm into the

terminal (Sjöstrand 1958; Ladman 1958). Although fewer in number than cone ribbons, rod ribbons are typically longer. In mammalian rods, oblique sections show that the ribbon is a crescent-shaped, plate-like structure that extends 1–3 μm along the length of the synaptic ridge (Rao-Mirotznik et al. 1995; Ladman 1958). Cone ribbons range from 0.2 to 1 μm in length (Pierantoni and McCann 1981; Sterling and Matthews 2005; Pang et al. 2008). The base of the ribbon is separated about 62 nm from the plasma membrane at the apex of the synaptic ridge by the arciform density, a prominent electron-dense structure just beneath the ribbon (Gray and Pease 1971; Ladman 1958; Raviola and Gilula 1975; Lasansky 1973). In conical electron tomography images, it appears as a series of cage-shaped pentagonal columns (Zampighi et al. 2011). Small filaments arising from the arciform density attach to the base of the ribbon, supporting the notion that the arciform density is important in tethering the ribbon to the presynaptic membrane (Zampighi et al. 2011; Gray and Pease 1971; Usukura and Yamada 1987).

In images from freeze-fracture electron microscopy, an array of about 400 small polyhedral particles, each 9–11 nm in diameter, can be seen embedded in the plasma membrane beneath the arciform density (Raviola and Gilula 1975). These particles have a small dimple in their centers and are likely the voltage-gated Ca^{2+} channels responsible for transducing changes in voltage into a Ca^{2+} signal that triggers vesicle fusion (Pumplin et al. 1981).

The terminals of rods and cones are filled with an abundance of synaptic vesicles. Each vesicle is approximately 45 nm in diameter and spherical. Occasionally, vesicles may appear oblong or stellate (Lasansky 1973; Evans 1966) and vesicle diameters have been estimated in some studies to range from 25 to 60 nm (Thoreson et al. 2004; Lasansky 1973; Evans 1966; Ladman 1958; Sjöstrand 1958; Raviola and Gilula 1975; Zampighi et al. 2011; Townes-Anderson et al. 1985; De Robertis and Franchi 1956), but these differences likely result from artifacts of fixation with a possible contribution from species differences. Small differences in vesicle diameter between rods and cones have also been reported (Evans 1966; Lasansky 1973). Measurements of vesicle density indicate that cone synaptic pedicles from a variety of species (lizard, salamander, turtle) contain 170,000–250,000 synaptic vesicles (Pierantoni and McCann 1981; Rea et al. 2004; Choi et al. 2005; Sheng et al. 2007). Spherules from large rods in salamander and gecko retinas contain approximately 89,000 and approximately 156,000 vesicles (Sheng et al. 2007), respectively, whereas electron tomography studies of much smaller mouse rod spherules suggest that they have only 5,800–7,500 vesicles/terminal (Zampighi et al. 2011). The large supply of vesicles likely contributes to the ability of rods and cones to sustain high rates of synaptic transmission for long periods of time in darkness.

In addition to being present in the cytoplasm, vesicles also surround the ribbon. Ribbon-associated vesicles appear to be tethered to the face of the ribbon with small filaments (up to five tethers/vesicle) that are only barely visible in electron microscopy images (Gray and Pease 1971; Zampighi et al. 2011; Usukura and Yamada 1987). Vesicles on the bottom one to three rows of the ribbon adjacent to the synaptic ridge make contact with the plasma membrane. These lower rows of

vesicles, being closest to both Ca^{2+} channels and contacting the plasma membrane, are positioned for immediate fusion and comprise an immediately releasable pool of vesicles. The physiological characterization of anatomically defined vesicle pools is discussed in Sect. 7.6, following.

Other notable features of rod and cone terminals include a single prominent mitochondrion in rod spherules and several smaller mitochondria in cone pedicles of vascularized mouse retinas (Johnson et al. 2007a; Linton et al. 2010). Photoreceptor terminals of avascular retinas, such as those of zebrafish or salamander, do not contain mitochondria (Linton et al. 2010). Additionally, elements of smooth endoplasmic reticulum (ER) course down through the axon from the photoreceptor soma and can be seen in rod and cone terminals (Mercurio and Holtzman 1982; Townes-Anderson et al. 1985; Johnson et al. 2007a; De Robertis and Franchi 1956). In rods, high intraterminal [Ca^{2+}] can trigger Ca^{2+}-induced Ca^{2+} release (CICR) from intracellular stores in the ER, which is important for boosting synaptic transmission during sustained darkness (Krizaj et al. 1999; Babai et al. 2010a; Suryanarayanan and Slaughter 2006; Cadetti et al. 2006).

7.3 Molecular Components of the Photoreceptor Synapse

Despite striking differences in the structure of photoreceptor ribbon synapses compared to conventional synapses, many of the proteins that make up rod and cone synapses are shared with their conventional counterparts. Some differences, whether the isoform, amount, or spatial distribution of particular components, confer unique properties upon photoreceptor synapses that allow them to maintain high rates of tonic glutamate release, thereby encoding and transmitting visual information to downstream retinal neurons. In this section we describe some of the key molecular components of photoreceptor ribbon synapses (Fig. 7.2), highlighting their roles in synaptic transmission by rods and cones.

7.3.1 Key Synaptic Proteins

The most noteworthy molecule present at ribbon synapses is the protein RIBEYE, so named for being the major component of the synaptic *rib*bon and first studied in the *eye* of cows. RIBEYE antibodies localize to photoreceptor synaptic terminals at the level of light microscopy and to synaptic ribbons by immunogold electron microscopy (Schmitz et al. 2000). RIBEYE is composed of two distinct domains. The B-domain, which is identical to the transcriptional repressor protein CtBP2, is positioned on the surface of the ribbon, whereas a novel A-domain that is rich in serine and proline residues faces inward. A variety of RIBEYE–RIBEYE interactions, some of which are regulated by NAD(H) binding to the B-domain, appear to facilitate ribbon assembly (Magupalli et al. 2008). In goldfish bipolar cells, use of a

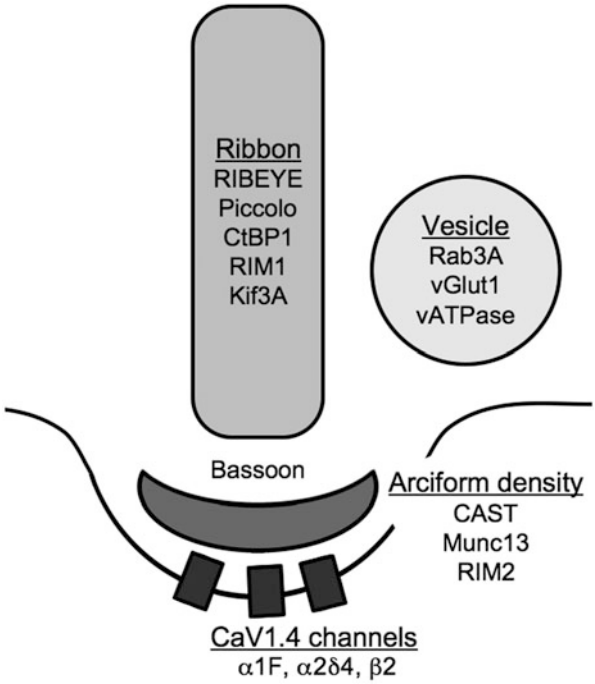

Fig. 7.2 Locations of some key presynaptic proteins at photoreceptor ribbon synapses. Piccolo, CtBP1, RIM1, and Kif3A are associated with the ribbon, which is composed mainly of RIBEYE. CAST, Munc13, and RIM2 localize to the arciform density, which sits above L-type Ca^{2+} channels composed of the pore-forming subunit α1F, together with α2δ4 and β2 accessory subunits. Bassoon is localized between the ribbon and the arciform density and tethers the ribbon in place. Rab3A, vGlut1, and a vesicular ATPase are located on the vesicle membrane

fluorescent RIBEYE binding peptide led to the estimate that each ribbon is assembled from about 4,000 RIBEYE molecules (Zenisek et al. 2004). Rod and cone ribbons are 11 to 22 times as large as bipolar cell ribbons (Heidelberger et al. 2005), suggesting that each photoreceptor ribbon is assembled from 44,000–88,000 RIBEYE molecules. CtBP1, a transcriptional cofactor related to the RIBEYE B-domain is also localized to the ribbon (tom Dieck et al. 2005), but the function and architecture of photoreceptor and bipolar cell synapses are largely unaffected in CtBP1-null mice and so its role is unclear (Vaithianathan et al. 2013a).

A component of the kinesin motor protein, Kif3A, also localizes to photoreceptor ribbons (Muresan et al. 1999; tom Dieck et al. 2005). Although loss of Kif3A leads to photoreceptor degeneration, its functional role is unclear (Jimeno et al. 2006). It is unlikely to enable ribbons to function as conveyor belts actively shuttling vesicles toward release sites, as this process would be too slow to account for the very rapid kinetics of exocytosis seen at ribbons (Parsons and Sterling 2003). Furthermore, release of ribbon-associated vesicles does not require ATP (Heidelberger et al. 2002). Instead, the fast kinetics of vesicle pool depletion may be a result of compound fusion (Parsons and Sterling 2003) of a chain of vesicles along the ribbon or simply a result of the ribbon constraining vesicular diffusion in two dimensions rather than three (Jackman et al. 2009). An additional motor protein, myosin Va, is localized to the photoreceptor terminal, and may be important for maintaining a supply of vesicles in the terminal (Libby et al. 2004).

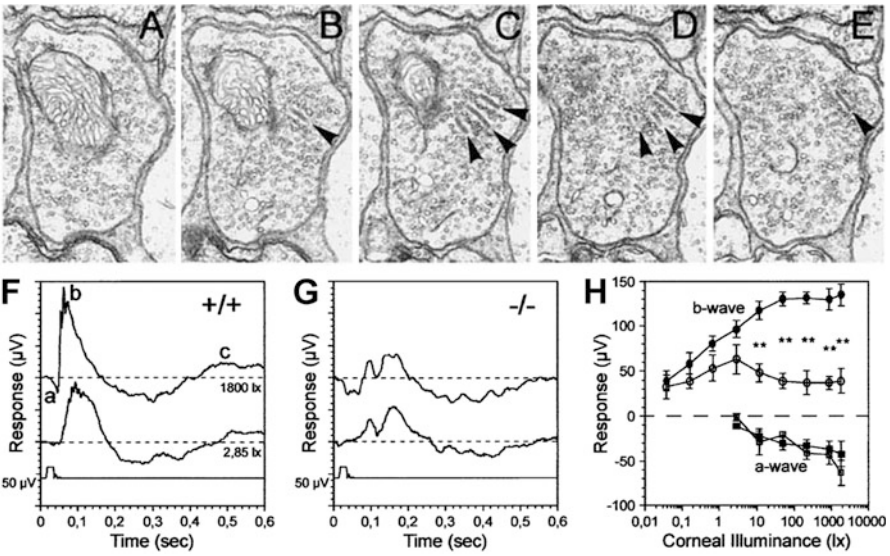

Fig. 7.3 Knockout of Bassoon disrupts ribbon morphology and photoreceptor synaptic transmission. (**a–e**) Series of electron micrographs through a rod terminal from a Bassoon knockout (−/−) mouse retina showing that ribbons are no longer tethered to the presynaptic membrane but are "free floating." (**f–h**) ERG recordings from wild-type (+/+; **f**) and Bassoon knockout (−/−; **g**) mouse at two flash intensities showing that the a-wave (denoted by the letter *a*) is unaffected (**h**) but the b-wave amplitude (denoted by the letter *b*) is reduced in the absence of Bassoon, indicative of a defect in photoreceptor synaptic transmission. (Reproduced from Dick et al. 2003, with permission from Elsevier, Inc.)

Bassoon is a large presynaptic protein (420 kDa) that is localized to the base of the ribbon (Garner et al. 2000; Brandstätter et al. 1999; Dick et al. 2003; tom Dieck et al. 2005) and interacts with RIBEYE (tom Dieck et al. 2005). In Bassoon knockout mice, photoreceptor ribbons are free floating and photoreceptor synaptic transmission, assessed with the ERG b-wave, is disrupted (Dick et al. 2003; Fig. 7.3). Bassoon also interacts directly with CAST, which is localized to the arciform density at the photoreceptor synapse, and disruption of Bassoon–CAST interactions disrupts synaptic transmission in cultured superior cervical ganglion neurons (Takao-Rikitsu et al. 2004; tom Dieck et al. 2005). In mouse retinas, genetic deletion of CAST causes several synaptic defects including a reduction in active zone size, neurite sprouting from second-order neurons to ectopic rod terminals in the outer nuclear layer, and reduced ERG b-wave (tom Dieck et al. 2012). It is possible that the thin fibers seen spanning from the arciform density to the base of the ribbon might be Bassoon tethers anchoring the ribbon in place through interactions with RIBEYE and CAST (Zampighi et al. 2011). However, in contrast to Bassoon knock-out mice, photoreceptor ribbons remain tethered to the presynaptic membrane in CAST knockouts (Dick et al. 2003; tom Dieck et al. 2012).

The protein Piccolo is another large presynaptic protein (520 kDa) that is structurally related to Bassoon (Garner et al. 2000). Similar to Bassoon, Piccolo is found in both conventional and ribbon-type synapses and can bind to CAST (Garner et al. 2000; Dick et al. 2001; Takao-Rikitsu et al. 2004). In photoreceptors, its location is distinct from that of Bassoon. Bassoon is located at the ribbon base, whereas Piccolo is found along the length of the ribbon (Dick et al. 2001; tom Dieck et al. 2005). The function of Piccolo is unknown. It can interact with prenylated Rab acceptor protein (PRA), a protein that binds the vesicle-associated Rab3A, suggesting it might play a role in tethering vesicles to the ribbon (Fenster et al. 2000; Garner et al. 2000). However, a mutation that truncates the Piccolo protein does not appear to affect synaptic structure or transmission in photoreceptors, leading to discovery of a prominent Piccolo splice isoform at ribbon synapses called Piccolino (Regus-Leidig et al. 2013). Piccolino has a molecular weight of about 350 kDa, smaller than the full-length Piccolo. Its function is also unclear, as Piccolino lacks the C-terminal binding domains for various synaptic proteins such as Bassoon, RIM proteins, Munc13, CAST, and Ca^{2+} channels (Regus-Leidig et al. 2013).

The Rab3 family of Rab GTPases is a group of neuronal, vesicle-associated small GTPases that play a role in regulating exocytosis (Fukuda 2008). Rab3A is the most abundant member of the Rab3 family found in the brain (Südhof 1995) and has been implicated in a host of functions at conventional synapses such as vesicle trafficking, tethering, priming, docking, regulation of fusion, and both short- and long-term plasticity (Geppert and Südhof 1998; Fukuda 2008; Geppert et al. 1994; Schlüter et al. 2006; Wang et al. 2011; Leitch et al. 2009; Castillo et al. 1997; Fukuda 2003; Leenders et al. 2001). Rab3A and other Rab3 family members, Rab3B and Rab3C, were initially reported to be absent from retinal ribbon synapses, although they were present at nonribbon amacrine cell synapses and in horizontal cell processes (Grabs et al. 1996). However, a recent study has shown that Rab3A can bind to rod and cone ribbons and is essential for vesicle replenishment of the ribbon (Tian et al. 2012).

In addition to possible interactions with PRA, the role of Rab3A in vesicle trafficking, tethering, and priming might involve interactions with RIM (*Rab3-i*nteracting *m*olecule) proteins (Schoch and Gundelfinger 2006). Two RIM proteins, RIM1 and RIM2, have been localized to the photoreceptor synapse, with RIM1 being found along the length of the ribbon and RIM2 concentrated at the base (tom Dieck et al. 2005). RIMs appear to be key scaffolding molecules at conventional presynaptic membranes and are important in vesicle priming, asynchronous release, and short- and long-term plasticity (Schoch and Gundelfinger 2006). In addition to interacting with Rab3 proteins, RIMs bind to several other synaptic molecules such as Bassoon, voltage-gated Ca^{2+} channels, ELKS, and Munc13 (Schoch and Gundelfinger 2006; Tian et al. 2012; Dulubova et al. 2005); this is consistent with a role in tethering vesicles to the ribbon and priming them for release. Mutations in the *RIM1* gene also cause changes in Ca^{2+} channel activity (Miki et al. 2007) and the cone-rod dystrophy CORD1 (Johnson et al. 2003).

Munc13 is found at the base of rod and cone ribbons (tom Dieck et al. 2005). It is reported to be essential for synaptic transmission at hippocampal glutamatergic

synapses and to play a role in vesicle priming, possibly by interaction with RIM proteins in a complex with Rab3A (Augustin et al. 1999; Betz et al. 2001; Rosenmund et al. 2002; Dulubova et al. 2005). However, knockout of ubMunc13-2, the Munc13 isoform found at mature photoreceptor synapses, has surprisingly modest effects on retinal synaptic transmission, with no change in the number of vesicles docked at the base of rod ribbons and only a slight reduction in the amplitude of the ERG b-wave (Cooper et al. 2012). One possible explanation for these modest effects is that Munc13 has more of a modulatory role, rather than an essential one, at photoreceptor synapses.

In addition to Rab3A, already discussed, vesicle membranes contain many other proteins important for synaptic transmission. Rods and cones are glutamatergic and vesicles express the vesicular glutamate transporter 1 (VGLUT1; Sherry et al. 2003). Although VGLUT2 immunoreactivity can be seen in some cones in the cat and mouse retina (Fyk-kolodziej et al. 2004; Wässle et al. 2006), VGLUT1 is necessary for photoreceptor synaptic transmission (Johnson et al. 2007b). Vesicles also express a vesicular ATPase, which acidifies the lumen of the vesicle, creating a proton gradient utilized by VGLUT to transport glutamate into the vesicle. In contrast to other synapses, rod and cone synapses do not contain synapsin, which acts to tether vesicles to the actin cytoskeleton (Mandell et al. 1990). Thus, the fine fibers that tether vesicles to ribbons (Gray and Pease 1971; Zampighi et al. 2011; Usukura and Yamada 1987) are not synapsin, but might be formed from a Rab3A/RIM complex (Tian et al. 2012).

7.3.2 SNARE Complex and Vesicle Fusion Machinery

Rod and cone synapses appear to contain a fairly standard complement of the major SNARE proteins responsible for synaptic vesicle exocytosis, although some differences in the subtypes in photoreceptor ribbon synapses versus conventional nonribbon synapses might contribute to the unique signaling capabilities of photoreceptors. Before release, a core complex of SNARE proteins (for *S*oluble *N*SF *A*ttachment Protein *Re*ceptor) draws the vesicle close to the presynaptic membrane, partially ascending the energy barrier to vesicle fusion.

This core SNARE complex is composed of the vesicle-associated VAMP (also known as Synaptobrevin), and membrane-associated SNAP-25 and Syntaxin (Südhof 2004). Two forms of VAMP, VAMP-1 and VAMP-2, are expressed in photoreceptor terminals but VAMP-2 is expressed more heavily (Sherry et al. 2003; von Kriegstein et al. 1999). VAMP-1 is also found extrasynaptically throughout the retina, making it unclear if it has a specific functional role at ribbon synapses (Sherry et al. 2003). Although initially thought to be absent (Grabs et al. 1996), SNAP-25 is present in photoreceptor terminals (Brandstätter et al. 1996; von Kriegstein et al. 1999; Ullrich and Südhof 2004). Retinal ribbon synapses contain Syntaxin 3 rather than the Syntaxin 1 isoform found at conventional retinal synapses (Morgans et al. 1996; von Kriegstein et al. 1999; Brandstätter

et al. 1996). This difference might relate to the presence of L-type Ca^{2+} channels at photoreceptor synapses (see following); whereas Syntaxin 1 can affect the gating of N-, P-, and Q-type channels (Bezprozvanny et al. 1995), Syntaxin 3 can alter gating of L-type channels (Kang et al. 2002).

Complexins are a family of proteins that bind to the assembled SNARE complex, where they appear to regulate neurotransmitter release by stabilizing an intermediate fusion state, simultaneously preventing the vesicle from undergoing spontaneous fusion and readying it to fuse in response to a surge in Ca^{2+} (Brose 2008; Südhof 2004). Conventional synapses use complexin 1 and 2, but ribbon synapses contain complexin 3 and 4 (Reim et al. 2005). Complexins have been shown to be important regulators of photoreceptor synaptic transmission because knockout of complexin 3 and 4 leads to disruptions in ribbon structure and defects in photoreceptor synaptic transmission (Reim et al. 2009). At retinal bipolar cell ribbon synapses, introduction of a complexin-inhibiting peptide triggered spontaneous vesicle fusion, suggesting that complexin is responsible for curtailing spontaneous release that might obscure meaningful changes in vesicle release rate (Vaithianathan et al. 2013b).

Synaptotagmin proteins are believed to be the Ca^{2+} sensors for exocytosis and catalyze the final step leading to full vesicle fusion (Südhof 2004). There are more than a dozen synaptotagmin proteins with a range of affinities for Ca^{2+} (Südhof 2002; Sugita et al. 2002; Geppert and Südhof 1998). Synaptotagmin I and II are typically associated with vesicles and thought to be responsible for catalyzing vesicle fusion at most synapses (Sugita et al. 2002; Südhof 2002; Geppert and Südhof 1998). Immunohistochemical labeling for synaptotagmin I has been observed at photoreceptor synapses in mammalian and chick retinas (Greenlee et al. 1996; Bergmann et al. 2000; von Kriegstein and Schmitz 2003; Berntson and Morgans 2003; Heidelberger et al. 2003; Lazzell et al. 2004; Fox and Sanes 2007; Wahlin et al. 2008), but synaptotagmin I antibodies do not label photoreceptor synapses in most lower vertebrates (Berntson and Morgans 2003; Heidelberger et al. 2003; Fox and Sanes 2007; but see Kreft et al. 2003). Immunohistochemical labeling for synaptotagmin II in synaptic terminals of photoreceptors has only been observed in the ray retina (Fox and Sanes 2007). Synaptotagmin III, which is typically associated with cell membranes rather than vesicle membranes, is also present at photoreceptor synapses (Ullrich and Südhof 2004; von Kriegstein et al. 1999; Berntson and Morgans 2003). Synaptotagmin III has a high affinity for Ca^{2+} similar to the high Ca^{2+} affinity exhibited by the Ca^{2+} sensor at photoreceptor synapses (Rieke and Schwartz 1996; Thoreson et al. 2004; Duncan et al. 2010).

7.4 Voltage-Gated Ca^{2+} Channels

Voltage changes in presynaptic terminals are transduced into a graded Ca^{2+} signal responsible for triggering vesicle fusion. Early studies from a variety of species showed that photoreceptors employ L-type voltage-gated Ca^{2+} channels to

accomplish this task (Bader et al. 1982; Corey et al. 1984; Barnes and Hille 1989; Maricq and Korenbrot 1988; Wilkinson and Barnes 1996). L-type channels are characterized as being dihydropyridine sensitive and having high-voltage activated (HVA), persistent (slowly activating or noninactivating) currents (Hille 2001; Catterall 2000; Catterall et al. 2003). These latter traits make them especially suitable for their role in photoreceptor synaptic transmission. Ca^{2+} currents found at conventional synapses exhibit fairly pronounced voltage-dependent and Ca^{2+}-dependent inactivation (VDI and CDI, respectively), leading to a transient Ca^{2+} signal that locks the timing of transmitter release to a single or a few action potentials. Photoreceptor Ca^{2+} currents, in contrast, exhibit relatively little inactivation, facilitating tonic, graded release (Corey et al. 1984; Bader et al. 1982; Barnes and Hille 1989; Rabl and Thoreson 2002; Taylor and Morgans 1998).

Ca^{2+} channel molecular identity is determined by the pore-forming α1-subunit. The family of L-type channels is composed of the subtypes $Ca_V1.1$–1.4 (α1S, α1C, α1D, and α1F, respectively). In photoreceptors, $Ca_V1.4$ appears to be the dominant channel subunit, although there is immunohistochemical evidence for other L-type α1-subunits localized to photoreceptor terminals in various species (Nachman-Clewner et al. 1999; Taylor and Morgans 1998; Morgans 1999; Firth et al. 2001; Kersten et al. 2010). A role for $Ca_V1.4$ in photoreceptor synaptic transmission was first identified in human patients with a form of X-linked incomplete congenital stationary night blindness (CSNB2), which is characterized by a normal ERG a-wave and reduced b-wave (Bech-Hansen et al. 1998; Strom et al. 1998). Patients with CSNB2 have mutations in a common gene, *CACNA1F*, which encodes the $Ca_V1.4$ subunit (Bech-Hansen et al. 1998; Strom et al. 1998; Wutz et al. 2002). These mutations can cause changes ranging from a lack of functional $Ca_V1.4$ protein to changes in voltage dependence of the Ca^{2+} current (Wutz et al. 2002; Hemara-Wahanui et al. 2005). $Ca_V1.4$ was later localized to photoreceptors in rodent retinas (Morgans et al. 2001; Morgans 2001; Morgans et al. 2005), and mutations in the *cacna1f* gene, such as the null mutation found in the *nob2* mouse mutant (Fig. 7.4), lead to disruptions in synaptic organization of the outer retina as well as to disruptions of the molecular organization of synaptic proteins in rod and cone terminals (Zabouri and Haverkamp 2013; Bayley and Morgans 2007; Chang et al. 2006). Specifically, the *cacna1f* null mutation leads to a thinning of the outer plexiform layer, extension of ectopic processes into the photoreceptor layer, detached synaptic ribbons, and progressive disorganization of synaptic scaffolding proteins (Zabouri and Haverkamp 2013; Bayley and Morgans 2007; Chang et al. 2006). The anatomical disruption is accompanied by a reduced ERG b-wave indicating a defect in synaptic transmission (Chang et al. 2006).

7.4.1 Accessory Ca^{2+} Channel Subunits

Accessory β and α2δ Ca^{2+} channel subunits are also important regulators of Ca^{2+} channel function (Catterall 2000; Dolphin 2012; Richards et al. 2004; Buraei and Yang 2010). The β-subunit is an intracellular protein that binds to an α1-interaction

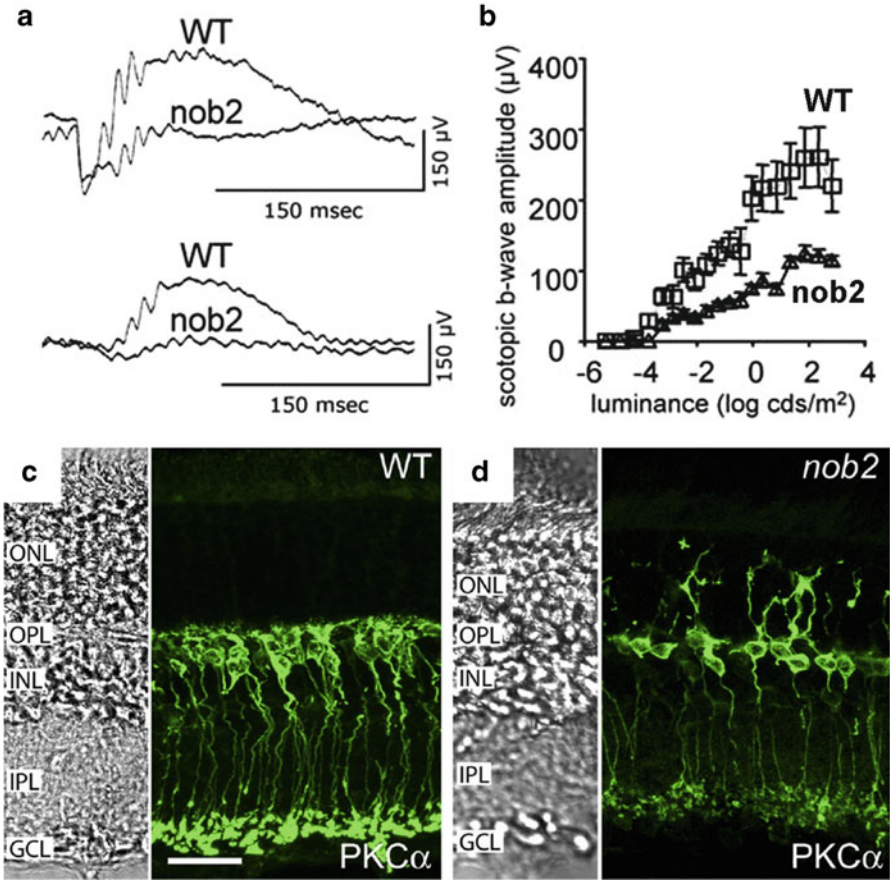

Fig. 7.4 Photoreceptor synaptic defects in mice with mutated $Ca_V1.4$ Ca^{2+} channels. (**a**, **b**) In the *nob2* mouse, insertion of a stop codon into the gene encoding the $Ca_V1.4$ Ca^{2+} channel subunit (*cacna1f*) leads to a reduction in the ERG b-wave with little change in the a-wave. Scotopic ERGs from two flash intensities: 1.89 log cd m^{-2} (*top*) and −0.81 log cd m^{-2} (*bottom*). (**c**, **d**) The *cacna1f* mutation also leads to several morphological changes including ectopic sprouting of bipolar cell postsynaptic processes into photoreceptor layers, seen in retinas stained for the rod bipolar cell marker PKCα (**b**). *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer. (**a**, **b**) Reproduced from Doering et al. 2008 under a Creative Commons Attribution License. (**c**, **d**) Reproduced from Bayley and Morgans 2007, with permission from Wiley

domain (AID) located between transmembrane domains I and II of the α1-subunit. This interaction shifts the voltage dependence to more hyperpolarized potentials and increases Ca^{2+} current density. The effect on current density is thought to be a result of the β-subunit blocking a domain that would otherwise signal the α1-subunit to be retained at the ER (Buraei and Yang 2010; Richards et al. 2004; Catterall 2000). The α2δ-subunit is membrane bound and expressed extracellularly.

It also shifts Ca^{2+} current activation to more hyperpolarized potentials and enhances membrane expression of functional channels (Dolphin 2012). Effects of α2δ- and β-subunits are seen in studies of heterologously expressed $Ca_V1.4$ channels (Baumann et al. 2004; McRory et al. 2004; Koschak et al. 2003). Varying the specific β-subtype has only small effects on $Ca_V1.4$ activation and inactivation parameters (McRory et al. 2004; Koschak et al. 2003).

In vivo, it appears that photoreceptors express β2- and α2δ4-subunits, as mutations to the genes encoding either subunit lead to defects in photoreceptor synaptic transmission and retinal morphology in mice. The β2 mutation causes a loss of the b-wave without any effect on the a-wave, a thinning of the outer plexiform layer (OPL), and a loss of $Ca_V1.4$ localization in the OPL (Ball et al. 2002). A similar thinning of the OPL is seen in mice with a mutation in α2δ4. This mutation also abolishes the b-wave, although in contrast to the β2 mutant, this is accompanied by a small reduction in a-wave amplitude (Wycsik et al. 2006). Antibodies to α2δ4 also label photoreceptor terminals (Wycsik et al. 2006; Mercer et al. 2011).

Interestingly, even with the coexpression of α2δ- and β-subunits, heterologously expressed $Ca_V1.4$ currents are activated at more positive membrane potentials than native currents (Baumann et al. 2004; McRory et al. 2004; Koschak et al. 2003; Taylor and Morgans 1998; Barnes and Hille 1989; Bader et al. 1982; Maricq and Korenbrot 1988; Corey et al. 1984). This difference appears to result, at least in part, from the absence of the Ca^{2+}-binding protein CaBP4 from expression systems used for studying $Ca_V1.4$ currents. When it is expressed along with $Ca_V1.4$, CaBP4 shifts the Ca^{2+} current activation to hyperpolarized potentials that more closely resemble the native current (Haeseleer et al. 2004). Mutations in CaBP4, whether in human patients or experimental models, cause ERG deficits resembling those of CSNB2 patients (Littink et al. 2009; Haeseleer et al. 2004; Maeda et al. 2005).

7.4.2 Regulation of Ca^{2+} Currents

Voltage-gated Ca^{2+} currents recorded from photoreceptors show no voltage-dependent inactivation (VDI) and only a modest amount of Ca^{2+}-dependent inactivation (CDI) (Corey et al. 1984; Rabl and Thoreson 2002). In heterologous expression systems, $Ca_V1.4$ currents show little or no CDI (Koschak et al. 2003; McRory et al. 2004; Baumann et al. 2004) whereas $Ca_V1.2$ or $Ca_V1.3$ currents show considerably more CDI. Calmodulin interaction with the α1-subunit C-terminal is the primary mechanism underlying CDI, although phosphorylation and dephosphorylation can also play a role (Budde et al. 2002; Cens et al. 2006). Calmodulin is preassociated with the C-terminal of the α1-subunit of all L-type Ca^{2+} channels. The absence of CDI in $Ca_V1.4$ is the result of a unique C-terminal autoinhibitory domain called ICDI (*i*nhibitor of CDI) that prevents CDI (Wahl-Schott et al. 2006; Griessmeier et al. 2009). Several mutations in the $Ca_V1.4$ C-terminal that preserve the voltage sensor and pore of the channel yet disrupt the ICDI domain have been found in CSNB2 patients (Strom et al. 1998; Boycott et al. 2001; Wutz et al. 2002).

Rod and cone Ca^{2+} currents are subject to modulation by a variety of additional mechanisms. One especially notable example is modulation of the amplitude and voltage dependence of photoreceptor Ca^{2+} currents that underlies lateral feedback from horizontal cells to rods and cones (reviewed by Thoreson and Mangel 2012). This lateral feedback is important for generating center-surround receptive fields and color opponency in the retina. Horizontal cell feedback appears to involve the actions of protons on photoreceptor Ca^{2+} currents (Hirasawa and Kaneko 2003; Vessey et al. 2005; Cadetti and Thoreson 2006). Extracellular protons inhibit Ca^{2+} influx and cause a positive shift in the Ca^{2+} current activation voltage (Barnes et al. 1993). Photoreceptor Ca^{2+} current gating can also be influenced by actions of potassium (Xu and Slaughter 2005), zinc (Chappell et al. 2008), and chloride ions (Thoreson and Miller 1996; Thoreson et al. 1997, 2000, 2002, 2003; Babai et al. 2010b) on both membrane surface charge and Ca^{2+} channel properties. Second messenger cascades arising from actions of various neuromodulators can also affect Ca^{2+} currents on a longer time scale (minutes). For instance, dopamine, which is a retinal signal for light adaptation (Witkovsky 2004) enhances rod Ca^{2+} currents but inhibits Ca^{2+} currents in most cones by actions on a D2-type dopamine receptor through a pathway involving a reduction in cAMP production and decrease in the activation of the cAMP-dependent protein kinase (PKA) (Stella and Thoreson 2000). Adenosine is released in darkness, in opposite phase to dopamine, and inhibits both rod and cone Ca^{2+} currents, ultimately reducing exocytosis (Blazynski and Perez 1991; Stella et al. 2002, 2003, 2007, 2009). Similar to dopamine, activation of the cannabinoid receptor CB1 enhances rod Ca^{2+} currents but reduces cone Ca^{2+} currents by acting through PKA (Straiker and Sullivan 2003). Nitric oxide, perhaps by mechanisms involving cGMP, also enhances rod Ca^{2+} currents but reduces cone Ca^{2+} currents (Kurenny et al. 1994; Kourennyi et al. 2004). Somatostatin acting through pertussis toxin-sensitive G proteins has opposite effects, reducing rod Ca^{2+} currents and enhancing cone Ca^{2+} currents (Akopian et al. 2000). Insulin acting through tyrosine kinase can also inhibit rod Ca^{2+} currents (Stella et al. 2001). Interpreting the physiological significance of many of these effects is complicated, as the conditions under which they might act on photoreceptors are largely unknown and other photoreceptor currents including I_h and I_K are also modulated by many of these agents. The existence of a variety of means by which rod and cone Ca^{2+} currents are regulated across a range of timescales suggests that transmitter release is not a simple report of photon absorption but is dynamically regulated to fine-tune the encoding of visual information transmitted to second-order neurons.

7.5 Ca^{2+} Buffering and Extrusion

In addition to regulation of Ca^{2+} at the point of entry, photoreceptors employ several mechanisms to regulate the timing and spread of Ca^{2+} ions after they enter the terminal: these include a combination of endogenous buffers and extrusion

mechanisms such as plasma membrane Ca^{2+} ATPase (PMCA), a sodium-Ca^{2+} exchanger (NCX), and sequestration into the ER. Endogenous Ca^{2+}-binding molecules such as calbindin, calretinin, parvalbumin, CaBP4, and calmodulin can buffer Ca^{2+} ions. Photoreceptors in tetrapods generally contain the low mobility buffer calbindin whereas the high mobility buffer calretinin appears largely absent (Morona et al. 2011). Immunohistochemical labeling for parvalbumin has been observed in some but not other studies (Endo et al. 1986; Nag and Wadhwa 1999; Deng et al. 2001; Cuenca et al. 2002).

Ca^{2+} extrusion in photoreceptors appears to be largely mediated by PMCA (Morgans et al. 1998; Zenisek and Matthews 2000) whereas NCX plays a dominant role in Ca^{2+} extrusion by photoreceptor outer segments (Krizaj and Copenhagen 1998). In tree shrew cones, the decay of a Ca^{2+}-activated chloride current ($I_{Cl(Ca)}$), which serves as an indicator of intraterminal [Ca^{2+}], was slowed by dialyzing cone with orthovanadate, an inhibitor of PMCA ATPase activity, yet was largely unchanged when extracellular Na^+ was replaced with Li^+, which inhibits NCX (Morgans et al. 1998). Interestingly, in cones, PMCA proteins are located some distance away from the photoreceptor active zones on the flanks of the synapse (Morgans et al. 1998; Johnson et al. 2007a). There is also immunohistochemical evidence that NCX is located closer to cone ribbons (Johnson et al. 2007a). NCX transporters have a lower Ca^{2+} affinity than PMCAs but are considerably faster. Rods stain very strongly for PMCA throughout their spherules and display only weak NCX labeling (Johnson et al. 2007a). There are four varieties of PMCA (PMCA1-4) that differ in Ca^{2+} affinity, kinetics, and strength of calmodulin-dependent regulation. Three of these, PMCA1, PMCA2, and PMCA4, have been localized to photoreceptor terminals (Krizaj et al. 2002; Duncan et al. 2006), and in the *deafwaddler* mouse, loss of functional PMCA2 protein leads to a reduction in the rod-mediated ERG b-wave amplitude (Duncan et al. 2006).

In addition to extrusion, Ca^{2+} can be removed from the cytoplasm by sequestration into the ER and mitochondria. Ca^{2+} is taken up into the ER by actions of SERCA proteins, which have a fairly low Ca^{2+} affinity and slow kinetics. Mitochondria play a greater role in sequestering Ca^{2+} in the ellipsoid than in the terminal (Szikra and Krizaj 2007). Mitochondria provide a source of ATP to fuel both PMCA and SERCA (Zenisek and Matthews 2000) and, at the ultrastructural level, PMCA is localized near the mitochondria found in photoreceptor terminals of vascularized retinas (Johnson et al. 2007a). The combination of spatially segregated pumps with differing affinities and kinetics may generate a gradient of [Ca^{2+}] across the terminal that can differentially regulate transmitter release, vesicle replenishment, $I_{Cl(Ca)}$, and CICR (Johnson et al. 2007a).

7.6 Vesicle Properties and Pools

As described previously, photoreceptor terminals contain large numbers of vesicles in the cytoplasm and attached to the ribbon. Anatomical vesicle pools have been correlated with physiologically characterized pools (Fig. 7.5). In salamander cones,

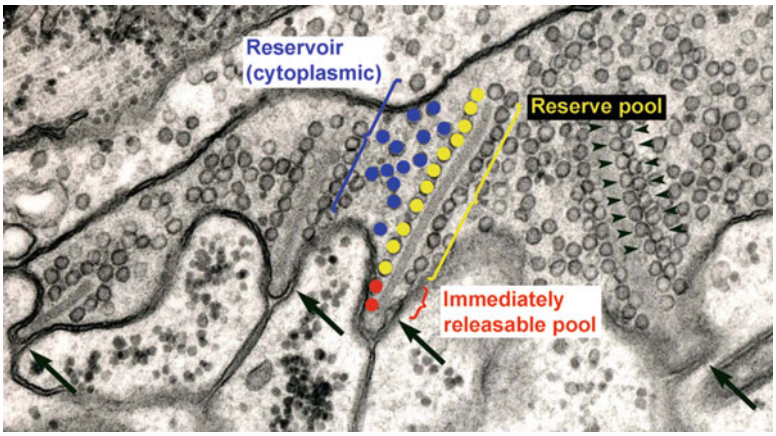

Fig. 7.5 Correlation of physiological and anatomical vesicle pools at the ribbon synapse. Transmission electron micrograph of a rod terminal from a salamander retina shows four ribbons with tethered vesicles. Several vesicles at one ribbon have been color coded to illustrate the anatomical identities of physiologically defined vesicle pools. The immediately releasable pool (IRP) is formed by the bottom 1–3 rows of vesicles (*red*) that sit on the ribbon docked adjacent to the synaptic ridge. A second pool of ribbon-tethered vesicles, a reserve pool (*yellow*), travel down the ribbon, refilling spots vacated when vesicles from the IRP undergo exocytosis. The ribbon is resupplied with vesicles from a cytoplasmic reservoir pool (*blue*). (Modified from Thoreson et al. 2004, with permission from Elsevier, Inc.)

ultrastructural studies reveal that about 20 vesicles tethered along the bottom two rows of the ribbon are also in contact with the plasma membrane (Pang et al. 2008; Lasansky 1973). Careful physiological measurements have shown that the immediately releasable pool (IRP), which is composed of the vesicles released within a few milliseconds upon strong depolarization, also consists of about 20 vesicles (Bartoletti et al. 2010). Maintained depolarization releases about 90 additional vesicles, forming a reserve pool that is emptied with a time constant of a few hundred milliseconds. The size of this pool matches the number of vesicles tethered to the remainder of the cone ribbon (Bartoletti et al. 2010; Rabl et al. 2005; Innocenti and Heidelberger 2008). The combined IRP and reserve pools are thus about five times larger than the IRP alone, similar to ultrastructural findings in primate cones that the total number of vesicles on the entire ribbon is about five times greater than the number of vesicles contacting the plasma membrane at the base of the ribbon (Sterling and Matthews 2005). The number of vesicles tethered to the ribbon in a salamander rod (~700 vesicles per ribbon) is also similar to the number of vesicles in the combined IRP and reserve pool (Thoreson et al. 2004). After depleting the ribbon-tethered pool, release can be maintained indefinitely at a linear rate that involves movement of vesicles from the cytoplasm (a reservoir pool) onto the ribbon (Bartoletti et al. 2010; Snellman et al. 2011).

As discussed earlier, photoreceptor terminals can contain hundreds of thousands of cytoplasmic vesicles. In contrast to conventional synapses where most vesicles are tethered to the cytoskeleton by synapsin (Cesca et al. 2010), synapsins are

absent from ribbon synapses (Mandell et al. 1990), and fluorescence recovery after photobleaching experiments showed that most (~85 %) of cytoplasmic vesicles are freely mobile (Rea et al. 2004). Thus, photoreceptors contain an exceedingly large cytoplasmic reservoir pool of vesicles available for replenishment of ribbon-related vesicle pools.

7.7 Properties of Transmitter Release

Light-dependent changes in photoreceptor membrane potential produced by the phototransduction process are transmitted to second-order bipolar and horizontal cells in the retina by changes in the rate at which glutamate-laden synaptic vesicles are released from photoreceptor synaptic terminals. The information about light intensity contained within the graded changes in photoreceptor membrane potential is therefore encoded into a series of discrete synaptic vesicle release events.

As discussed earlier, synaptic release from photoreceptors is a Ca^{2+}-dependent process involving L-type Ca^{2+} channels, not N or P/Q channels as at most other CNS synapses. Light-evoked changes in the membrane potential of photoreceptors alter the likelihood that L-type Ca^{2+} channels clustered beneath the base of synaptic ribbons will open. The resulting changes in Ca^{2+} influx alter release of synaptic vesicles from cone terminals. At a membrane potential of −40 mV, near the resting dark potential, photoreceptor Ca^{2+} currents attain roughly one-third of their maximum activation (Fig. 7.6). The influx of Ca^{2+} ions into photoreceptor terminals accompanying the activation of Ca^{2+} channels by sustained depolarization at −40 mV leads to a significant depletion of extracellular Ca^{2+} ions from the synaptic

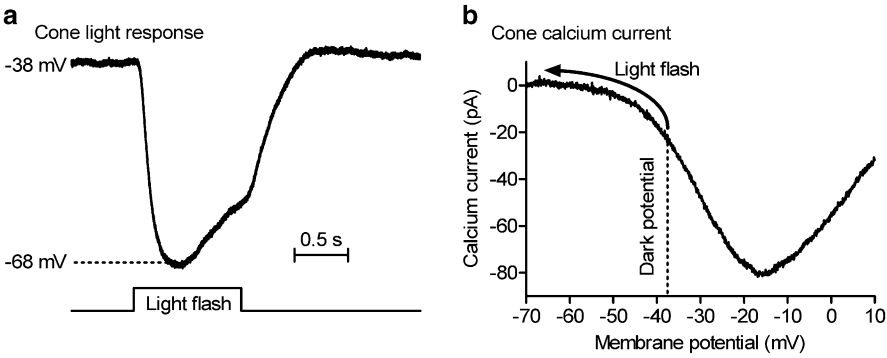

Fig. 7.6 Influence of light-evoked changes in membrane potential on cone Ca^{2+} currents. (**a**) A bright flash of light strongly hyperpolarizes the cone from its resting potential of −38 mV. (**b**) Ca^{2+} current averaged from eight salamander cones evoked by a ramp voltage protocol (0.5 mV/ms). The dark potential of the cone in (**a**) is denoted by the *dashed line* and the reduction in I_{Ca} caused by the hyperpolarizing light response is shown by the *arrow*. (Reproduced from Thoreson 2010, with permission from Elsevier, Inc.)

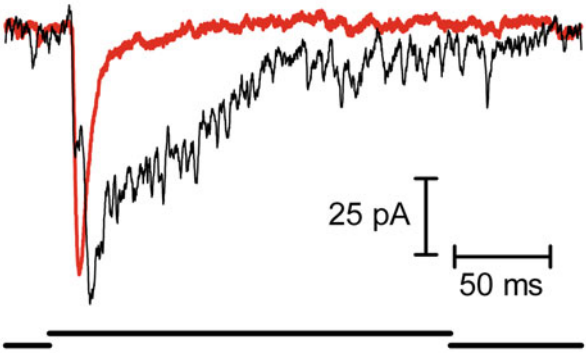

Fig. 7.7 Cone-driven postsynaptic responses are faster than rod-driven postsynaptic responses. EPSCs recorded in a single off bipolar cell triggered by depolarization (−70 to −10 mV, 200 ms) of either a cone (*red*) or a rod (*black*). The cone-evoked response is composed of a large fast component and smaller slow component. The rod-evoked response also has an initial fast component but is dominated by slower components. (Modified from Cadetti et al. 2005, with permission from Wiley)

cleft that contributes to a significant reduction in Ca^{2+} current amplitude (Rabl and Thoreson 2002). As discussed in Sect. 7.4.2, photoreceptor Ca^{2+} channels show almost no voltage-dependent inactivation. However, in contrast to heterologously expressed $Ca_V1.4$ channels, Ca^{2+} channels in photoreceptor terminals exhibit modest, slow Ca^{2+}-dependent inactivation (Corey et al. 1984; Rabl and Thoreson 2002). The combination of Ca^{2+}-dependent inactivation with the depletion of extracellular Ca^{2+} causes a substantial reduction in the Ca^{2+} current amplitude of rods (Rabl and Thoreson 2002), reducing it by as much as fivefold (Thoreson et al. 2003). Limiting the intracellular Ca^{2+} load may help to limit cytotoxic effects of excessive Ca^{2+}.

Rods exhibit slower release kinetics than cones (Schnapf and Copenhagen 1982; Rabl et al. 2005; Sheng et al. 2007). For example, Fig. 7.7 shows that depolarizing stimulation of cones produces faster responses in second-order retinal neurons than stimulation of rods, which evokes slower, more complex responses. Depolarizing a cone evokes a large and fast postsynaptic current followed by a considerably smaller slow component in an 'Off' bipolar or horizontal cell (Cadetti et al. 2005). Postsynaptic responses evoked by stimulating rods also have a fast component (Li et al. 2010), but the response is often dominated by larger slow components (Cadetti et al. 2005). In salamander cones, damaging the synaptic ribbon by fluorophore-assisted laser inactivation (FALI) reduces both fast and slow components of release suggesting that most, if not all, release from cones involves the synaptic ribbon (Snellman et al. 2011). In rods, damaging ribbons by FALI causes a greater reduction in the initial burst of release than slower components of release (Chen et al. 2013). Similarly, mutant Rab3A peptides that disrupt vesicle attachment to the ribbon also selectively inhibited fast but not slow release components (Tian et al. 2012). Furthermore, direct visualization of vesicle fusion events by total

internal reflectance microscopy showed that maintained depolarization stimulates release preferentially from nonribbon sites (Chen et al. 2013). Thus, in rods, while the initial fast component of release appears to be caused by release of ribbon-associated vesicles, slower release involves nonribbon sites. Additional slow components in the depolarization-evoked responses of rod-driven cells can result from synaptic release by neighboring rods activated by the spread of current through gap junctions (Attwell et al. 1984; Cadetti et al. 2005).

Slow release from rods is amplified by activation of Ca^{2+} -induced Ca^{2+} release (CICR) from intracellular stores (Krizaj et al. 1999; Suryanarayanan and Slaughter 2006; Cadetti et al. 2006; Babai et al. 2010a). In addition to amplifying the rate of release in darkness, CICR also appears to increase the amplitude of individual miniature synaptic currents by promoting multivesicular release at rod synapses (Suryanarayanan and Slaughter 2006). ER extends from the synaptic terminal to the soma (Mercurio and Holtzman 1982), and so it is possible that Ca^{2+} diffusing through the ER (Choi et al. 2006; Petersen and Verkhratsky 2007) may sustain localized release of Ca^{2+} from stores in the terminal and thereby help to sustain synaptic release.

7.7.1 Different Mechanisms Dominate Release from Cones in Light and Dark

Studies on the cone synapse show differences in the mechanisms that govern release under dim and bright light conditions. In response to bright light, cones hyperpolarize to a negative membrane potential approaching −65 mV (Fig. 7.6). From the plot of the current–voltage relationship in Fig. 7.6, one can see that at this membrane potential, virtually all the Ca^{2+} channels are closed. The rate of vesicle release therefore diminishes to very low basal levels in bright light. With little or no ongoing release, the IRP at the base of the ribbon can be replenished (Jackman et al. 2009). The membrane depolarization accompanying a subsequent decrement in light intensity will trigger the opening of Ca^{2+} channels and rapidly stimulate release of the recently replenished IRP. Ca^{2+} channels are very close (<100 nm) to vesicles in the IRP (Mercer et al. 2011), and release of these vesicles requires the opening of only about two Ca^{2+} channels per vesicle (Bartoletti et al. 2011). Thus, in bright light conditions, the depolarizing membrane potential changes that accompany decrements in light intensity (darkness) can be converted to vesicle release with high efficiency and great temporal precision.

The opening of a few Ca^{2+} channels at the base of the terminal in sustained darkness allows $[Ca^{2+}]_i$ to rise to 600–800 nM near the ribbon (Jackman et al. 2009). As noted earlier, the exocytotic Ca^{2+} sensor responsible for triggering vesicle fusion in rods and cones has an unusually high sensitivity to Ca^{2+} (Rieke and Schwartz 1996; Thoreson et al. 2004; Duncan et al. 2010), and so 600 nM Ca^{2+} is sufficient to trigger fusion at this synapse (although this level of Ca^{2+} would not stimulate release

at most other synapses). Thus, in darkness, the IRP at the base of the synaptic ribbon is soon emptied of vesicles. Once this pool has been emptied, the sustained release of vesicles in darkness is no longer regulated by individual Ca^{2+} channel openings but is instead governed by the rate at which release-competent vesicles can replenish release sites at the base of the synaptic ribbon (Jackman et al. 2009). This replenishment-driven sustained release enables the synapse to encode luminance whereas bursts of release at light offset are responsible for temporally precise encoding of contrast (Jackman et al. 2009; Oesch and Diamond 2011).

The rate of vesicle replenishment, and thus sustained release, is controlled by two principal mechanisms (Babai et al. 2010c). First, replenishment is limited by the number of available release sites (i.e., occupancy state of the IRP) (Babai et al. 2010c; Oesch and Diamond 2011). By making a large number of release sites available for replenishment, release of a large number of vesicles by strong depolarization allows a high rate of replenishment. Second, vesicle replenishment is accelerated by Ca^{2+}, a process that occurs approximately 300 nm away from sites of Ca^{2+} entry (Babai et al. 2010c).

The different release mechanisms favored by cones in light and dark offer different advantages for signaling light intensity in light and dark conditions. In light, the occurrence of individual Ca^{2+} channel openings is closely correlated with depolarizing changes in membrane potential caused by decrements in light intensity. Vesicle release events are thus tightly correlated with individual channel openings. However, in darkness, when Ca^{2+} channel openings occur largely at random intervals, coupling release to individual channel openings will simply increase noise. The visual system is capable of detecting single photons that evoke responses of only 1 mV in rods (reviewed by Pahlberg and Sampath 2011). The random occurrence of release events associated with stochastic channel openings can obscure postsynaptic detection of small changes in the rate of release (Schein and Ahmad 2005). However, the replenishment mechanisms that govern sustained release appear to be regulated by spatially averaged Ca^{2+} levels and are therefore linked less directly to the activity of individual channels than release under bright light conditions (Babai et al. 2010c). Thus, one proposed benefit of this mechanism is that it may reduce the noise introduced by the stochastic nature of individual channel openings (Jackman et al. 2009).

7.8 Properties of Endocytosis

Retrieval of vesicular membrane after exocytosis, a process called endocytosis, is important for allowing synapses to replenish a finite pool of reserve vesicles, enabling them to maintain synaptic transmission over a sustained timeframe (Wu et al. 2007). The rate at which synaptic vesicles are released from rods and cones in darkness has been estimated to range from 10 to 80 v/s/ribbon (Choi et al. 2005; Berntson and Taylor 2003; Sheng et al. 2007; Rieke and Schwartz 1996; Ashmore and Copenhagen 1983). At a rate of 20 v/s/ribbon, the entire

cytoplasmic pool of vesicles would be depleted from a rod within 5–10 min without compensatory endocytosis.

Work in various types of neurons, especially retinal bipolar cells, indicates that there are a few main mechanisms by which synaptic vesicles are endocytosed after release. As in the retrieval of many other surface proteins, a major mechanism by which vesicle membrane and proteins are retrieved is by assembly of clathrin-coated vesicles. This mechanism appears to operate slowly, with a time constant of many seconds. In bipolar cells, it involves amphiphysin and the adapter complex AP2 (Jockusch et al. 2005). There are also more rapid forms of endocytosis that are clathrin independent. In bipolar cells, fast clathrin-independent endocytosis has been shown to require endophilin (Llobet et al. 2011). The scission step in both fast and slow endocytosis typically involves the GTPase dynamin (Dittman and Ryan 2009). In a third mechanism of endocytosis, vesicles can be retrieved rapidly by a mechanism known as "kiss-and-run" fusion (Harata et al. 2006). In this mechanism, vesicles form a transient fusion pore allowing release of small neurotransmitter molecules, but the vesicle is retrieved before collapsing fully into the plasma membrane. This mechanism contributes significantly to release of large dense core vesicles at neuroendocrine cells, but its contribution to release of small clear synaptic vesicles is more controversial (Harata et al. 2006; LoGiudice and Matthews 2007; Wu et al. 2007). Finally, following strong stimulation, vesicle membrane can be retrieved by bulk endocytosis whereby large endosomes containing the membrane of many vesicles can be retrieved (Wu et al. 2007).

The mechanisms of endocytosis in photoreceptors are not well characterized. There is immunohistochemical evidence for the presence of clathrin in photoreceptor terminals (Sherry and Heidelberger 2005), and coated vesicles can be seen in regions of membrane adjacent to the synaptic ribbon (Gray and Pease 1971; Schaeffer and Raviola 1978). Endocytic proteins appear to cluster just beyond the active zone, in a region called the periactive zone, suggesting that it is a region of high endocytic activity (Wahl et al. 2013). When incubated with peroxidase in darkness, vesicles throughout the terminal can be seen to have taken up tracer (Ripps et al. 1976; Schacher et al. 1976; Schaeffer and Raviola 1978; Cooper and McLaughlin 1983; Townes-Anderson et al. 1985). Labeled vesicles randomly populate the ribbon in proportion to the number of labeled cytoplasmic vesicles, consistent with the idea that a highly mobile population of cytoplasmic vesicles resupplies the ribbon. Although prolonged incubation in tracers can sometimes produce labeling of large endosomes (Ripps et al. 1976; Schacher et al. 1976), experiments involving sequential loading with two different fluorescent dyes showed little mixing of retrieved vesicles suggesting that recycling through endosomal intermediates or bulk retrieval of large endosomes are not major contributors to the synaptic vesicle cycle in cones (Rea et al. 2004).

From the changes in membrane capacitance that accompany changes in membrane surface area, rods and cones have been found to exhibit both fast and slow components of endocytosis (Rieke and Schwartz 1996; Rabl et al. 2005; Innocenti and Heidelberger 2008; Van Hook and Thoreson 2012). In cones,

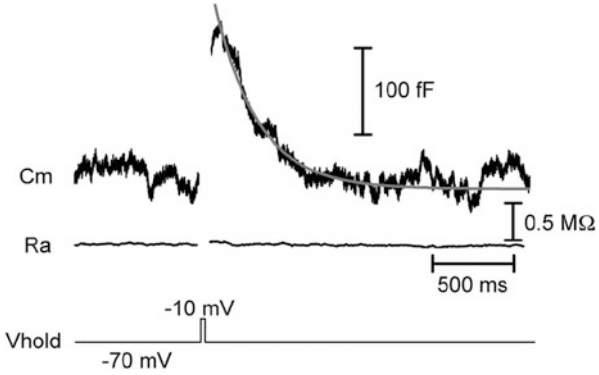

Fig. 7.8 Fast endocytosis recorded from a salamander cone. A whole-cell capacitance recording showing an exocytotic increase in membrane capacitance (*Cm*) evoked by a step depolarization (−70 to −10 mV, 25 ms, Vhold). The capacitance response declines back to baseline as membrane is retrieved by endocytosis. The time course of endocytosis was measured by fitting the capacitance decline with a single exponential function with a time constant of 257 ms (*gray line*). There was no change in access resistance (Ra), indicating that the capacitance response is not an artifact of recording changes and represents true exocytosis and endocytosis. (Reproduced with permission from Van Hook and Thoreson 2012)

the fast component has a very rapid time constant of about 250 ms (Van Hook and Thoreson 2012; Fig. 7.8). With depolarizing stimulation for 0.5 to 5 s, a slower component lasting many seconds can also be observed (Innocenti and Heidelberger 2008). Studies with Ca^{2+} buffers suggest that the trigger for the rapid form of endocytosis involves sites within 100 nm of Ca^{2+} channels (Van Hook and Thoreson 2012), closer than clathrin-coated vesicles observed by ultrastructure (Gray and Pease 1971; Schaeffer and Raviola 1978). Immunohistochemical labeling for dynamin at rod and cone synapses is weaker and more diffuse than labeling at conventional synapses (Sherry and Heidelberger 2005) and the fast endocytic component in cones is insensitive to dynamin inhibitors or the GTPase inhibitor, GTPγS (Van Hook and Thoreson 2012). One possible explanation for dynamin- and GTPase-independent endocytosis could be contributions from kiss-and-run retrieval. However, it has also been suggested that dynamin may not be absolutely required for endocytosis but may simply accelerate the process (Dittman and Ryan 2009). An interesting possibility is that the ribbon itself may play a role in endocytosis because RIBEYE contains a CtBP domain homologous to CtBP1/BAR proteins that are thought to help induce membrane curvature during endocytosis (Hansen and Nichols 2009). However, damaging RIBEYE molecules by FALI did not significantly alter the rate of endocytosis observed in cones (Snellman et al. 2011). Endocytosis in cones appears to rely heavily on the PIP2 phosphatase synaptojanin (Van Epps et al. 2004; Holzhausen et al. 2009). Dephosphorylation of PIP2 by synaptojanin is thought to decrease phospholipid binding to BAR domain proteins and thereby slow endocytosis (Chang-Ileto et al. 2011).

Endocytosis in rod terminals appears to involve different mechanisms than cones. In contrast to cones, inhibition of dynamin or introduction of GTPγS significantly slows membrane retrieval in rods (Van Hook and Thoreson 2012). Furthermore, rods lack synaptojanin 1 (Holzhausen et al. 2009) but show stronger immunohistochemical labeling for amphiphysin and clathrin than cone pedicles (Sherry and Heidelberger 2005).

7.9 Conclusion

At the first synapse in seeing, rod and cone light responses are encoded and transmitted to second-order bipolar and horizontal cells as discrete packets of the neurotransmitter glutamate. The distinct features of synaptic transmission by photoreceptors shape the signals available for processing by downstream neurons in the visual pathway and thereby constitute an early-stage filter on visual information. As we have described here, the manner in which rods and cones encode and transmit visual information arises from the unique capabilities conferred on photoreceptor synapses by a variety of features such as the synaptic ribbon, the contribution of persistent and dynamically regulated L-type Ca^{2+} currents, and multiple levels of Ca^{2+}-dependent regulation at various stages of the vesicle cycle, from release to endocytosis to replenishment. Present and future work is focused on obtaining greater understanding of the molecular details and functions of photoreceptor synapses. Several avenues for future exploration include further elucidating the various protein interactions at the ribbon, the ways in which rod and cone light responses dynamically alter Ca^{2+} channel gating and glutamate release, and how various disease states might originate in rod and cone synaptopathies or impinge on the function of the synapse.

References

Akopian A, Johnson J, Gabriel R et al (2000) Somatostatin modulates voltage-gated K^+ and Ca^+ currents in rod and cone photoreceptors of the salamander retina. J Neurosci 20:929–936

Ashmore JF, Copenhagen DR (1983) An analysis of transmission from cones to hyperpolarizing bipolar cells in the retina of the turtle. J Physiol (Lond) 340:569–597

Attwell D, Wilson M, Wu SM (1984) A quantitative analysis of interactions between photoreceptors in the salamander (Ambystoma) retina. J Physiol 352:703–737

Augustin I, Rosenmund C, Südhof TC et al (1999) Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. Nature (Lond) 400:457–461

Babai N, Morgans CW, Thoreson WB (2010a) Calcium-induced calcium release contributes to synaptic release from mouse rod photoreceptors. Neuroscience 165:1447–1456

Babai N, Kanevsky N, Dascal N et al (2010b) Anion-sensitive regions of L-type CaV1.2 calcium channels expressed in HE293 cells. PLoS One 5:e8602

Babai N, Bartoletti TM, Thoreson WB (2010c) Calcium regulates vesicle replenishment at the cone ribbon synapse. J Neurosci 30:15866–15877

Bader CR, Bertrand D, Schwartz EA (1982) Voltage-activated and calcium-activated currents studies in solitary rod inner segments from the salamander retina. J Physiol 331:253–284

Ball SL, Powers PA, Shin HS et al (2002) Role of β_2 subunit of voltage-dependent calcium channels in the retinal outer plexiform layer. Invest Ophthalmol Vis Sci 43:1595–1603

Barnes S, Hille B (1989) Ionic channels of the inner segment of tiger salamander cone photoreceptors. J Gen Physiol 94:719–743

Barnes S, Merchant V, Mahmud F (1993) Modulation of transmission gain by protons at the photoreceptor output synapse. Proc Natl Acad Sci USA 90:10081–10085

Bartoletti TM, Babai N, Thoreson WB (2010) Vesicle pool size at the salamander cone ribbon synapse. J Neurophysiol 103:419–428

Bartoletti TM, Jackman SL, Babai N et al (2011) Release from the cone ribbon synapse under bright light conditions can be controlled by the opening of only a few Ca^{2+} channels. J Neurophysiol 106:2922–2935

Baumann L, Gerstner A, Zong X et al (2004) Functional characterization of the L-type Ca^{2+} channel $Ca_v1.4\alpha1$ from mouse retina. Invest Ophthalmol Vis Sci 45:708–713

Bayley PR, Morgans CW (2007) Rod bipolar cells and horizontal cells form displaced synaptic contacts with rods in the outer nuclear layer of the *nob2* retina. J Comp Neurol 500:586–298

Bech-Hansen NT, Naylor MJ, Maybaum TA et al (1998) Loss-of-function mutations in a calcium channel α_1-subunit gene in Xp11.23 cause incomplete X-linked congenital stationary night blindness. Nat Genet 19:264–267

Bergmann M, Grabs D, Rager G (2000) Expression of presynaptic proteins is closely correlated with the chronotopic pattern of axons in the retinotectal system of the chick. J Comp Neurol 418:361–372

Berntson A, Taylor WR (2003) The unitary event amplitude of mouse retinal on-cone bipolar cells. Vis Neurosci 20:621–626

Berntson AK, Morgans CW (2003) Distribution of the presynaptic calcium sensors, synaptotagmin I/II and synaptotagmin III, in the goldfish and rodent retinas. J Vis 3:274–280

Betz A, Thakur P, Junge HJ et al (2001) Functional interaction of the active zone proteins Munc13-1 and RIM1 in synaptic vesicle priming. Neuron 30:183–196

Bezprozvanny I, Scheller RH, Tsien RW (1995) Functional impact of syntaxin on gating of N-type and Q-type calcium channels. Nature (Lond) 378:623–626

Blazynski C, Perez MT (1991) Adenosine in vertebrate retina: localization, receptor characterization, and function. Cell Mol Neurobiol 11:463–484

Boycott KM, Maybaum TA, Naylor MJ et al (2001) A summary of 20 CACNA1F mutations identified in 36 families with incomplete x-linked congenital stationary night blindness, and characterization of splice variants. Hum Genet 108:91–97

Brandstätter JH, Wässle H, Betz H et al (1996) The plasma membrane protein SNAP-25, but not syntaxin, is present at photoreceptor and bipolar cell synapses in the rat retina. Eur J Neurosci 8:823–828

Brandstätter JH, Fletcher EL, Garner CC et al (1999) Differential expression of the presynaptic cytomatric protein bassoon among ribbon synapses in the mammalian retina. Eur J Neurosci 11:3683–3693

Brose N (2008) For better or for worse: complexins regulate SNARE function and vesicle fusion. Traffic 9:1403–1413

Budde T, Meuth S, Pape HC (2002) Calcium-dependent inactivation of neuronal calcium channels. Nat Rev Neurosci 3:873–883

Buraei Z, Yang J (2010) The β subunit of voltage-gated Ca^{2+} channels. Physiol Rev 90:1461–1506

Cadetti L, Tranchina D, Thoreson WB (2005) A comparison of release kinetics and glutamate receptor properties in shaping rod-cone differences in EPSC kinetics in the salamander retina. J Physiol (Lond) 569:773–788

Cadetti L, Bryson EJ, Ciccone CA et al (2006) Calcium-induced calcium release in rod photoreceptor terminals boosts synaptic transmission during maintained depolarization. Eur J Neurosci 23:2983–2990

Cadetti L, Thoreson WB (2006) Feedback effects of horizontal cell membrane potential on cone calcium currents studied with simultaneous recordings. J Neurophysiol 95:1992–1995
Castillo PE, Janz R, Südhof TC (1997) Rab3A is essential for mossy fiber long-term potentiation in the hippocampus. Nature (Lond) 388:590–593
Catterall WA (2000) Structure and regulation of voltage-gated Ca^{2+} channels. Annu Rev Cell Dev Biol 16:521–555
Catterall WA, Striessnig J, Snutch TP et al (2003) International union of pharmacology. XL. Compendium of voltage-gated ion channels: calcium channels. Pharmacol Rev 55:579–581
Cens T, Rousset M, Leyris J-P et al (2006) Voltage- and calcium-dependent inactivation in high voltage-gated Ca^{2+} channels. Prog Biophys Mol Biol 90:104–117
Cesca F, Baldelli P, Valtorta F et al (2010) The synapsins: key actors of synapse function and plasticity. Prog Neurobiol 91:313–348
Cuenca N, Deng P, Linberg KA (2002) The neurons of the ground squirrel retina as revealed by immunostains for calcium binding proteins and neurotransmitters. J Neurocytol 31:649–666
Chang B, Heckenlively JR, Bayley PR et al (2006) The *nob2* mouse, a null mutation in *Cacna1f*: anatomical and functional abnormalities in the outer retina and their consequences on ganglion cell visual responses. Vis Neurosci 23:11–24
Chang-Ileto B, Frere SG, Chan RB et al (2011) Synaptojanin 1-mediated $PI(4,5)P_2$ hydrolysis is modulated by membrane curvature and facilitates membrane fission. Dev Cell 20:206–218
Chappell RL, Anastassov I, Lugo P et al (2008) Zinc-mediated feedback at the synaptic terminal of vertebrate photoreceptors. Exp Eye Res 87:394–397
Chen M, Van Hook MJ, Zenisek D et al (2013) Properties of ribbon and non-ribbon release from rod photoreceptors revealed by visualizing individual synaptic vesicles. J Neurosci 33:2071–2086
Choi SY, Barghuis B, Rea R et al (2005) Encoding light intensity by the cone photoreceptor synapse. Neuron 48:555–562
Choi YM, Kim SH, Chung S et al (2006) Regional interaction of endoplasmic reticulum Ca^{2+} signals between soma and dendrites through rapid luminal Ca^{2+} diffusion. J Neurosci 26:12127–12136
Cooper B, Hemmerlein M, Ammermüller J et al (2012) Munc13-independent vesicle priming at mouse photoreceptor ribbon synapses. J Neurosci 32:8040–8052
Cooper NGF, McLaughlin BJ (1983) Tracer uptake by photoreceptor synaptic terminals. J Ultrastruct Res 84:252–267
Corey DP, Dubinsky JM, Schwartz EA (1984) The calcium current in inner segments of rods from the salamander (*Ambystoma tigrinum*) retina. J Physiol (Lond) 354:557–575
De Robertis E, Franchi CM (1956) Electron microscope observations on synaptic vesicles in synapses of the retinal rods and cones. J Biophys Biochem Cytol 2:307–318
Deng P, Cuenca N, Doerr T et al (2001) Localization of neurotransmitters and calcium binding proteins to neurons of salamander and mudpuppy retinas. Vis Res 41:1771–1783
Dick O, Hack I, Altrock WD et al (2001) Localization of the presynaptic cytomatrix protein Piccolo at ribbon and conventional synapses in the rat retina: comparison with Bassoon. J Comp Neurol 439:224–234
Dick O, tom Dieck S, Altrock WD et al (2003) The presynaptic active zone protein bassoon is essential for photoreceptor ribbon synapse formation in the retina. Neuron 37:775–786
Dittman J, Ryan TA (2009) Molecular circuitry of endocytosis at nerve terminals. Annu Rev Cell Dev Biol 25:133–160
Dolphin AC (2012) Calcium channel auxiliary $\alpha_2\delta$ and β subunits: trafficking and one step beyond. Nat Rev Neurosci 13:542–555
Dowling JE, Boycott BB (1966) Organization of the primate retina: electron microscopy. Proc R Soc Lond B Biol Sci 166:80–111
Dowling JE, Werblin FS (1969) Organization of the retina of the mudpuppy *Necturus maculosus*. I. Synaptic structure. J Neurophysiol 32:315–338

Dulubova I, Lou X, Lu J et al (2005) A Munc13/RIM/Rab3 tripartite complex: from priming to plasticity? EMBO J 25:2839–2850
Duncan G, Rabl K, Gemp I et al (2010) Quantitative analysis of synaptic release at the photoreceptor synapse. Biophys J 98:2102–2110
Duncan JL, Yang H, Doan T et al (2006) Scotopic visual signaling in the mouse retina is modulated by high-affinity plasma membrane calcium extrusion. J Neurosci 26:7201–7211
Endo T, Kobayashi M, Kobayashi S et al (1986) Immunocytochemical and biochemical localization of parvalbumin in the retina. Cell Tissue Res 243:213–217
Evans EM (1966) On the ultrastructure of the synaptic region of visual receptors in certain vertebrates. Z Zellforsch Mikrosk Anat 71:499–516
Fenster SD, Chung WJ, Zhai R et al (2000) Piccolo, a presynaptic zinc finger protein structurally related to bassoon. Neuron 25:203–214
Firth SI, Morgan IG, Boelen MK et al (2001) Localization of voltage-sensitive L-type calcium channels in the chicken retina. Clin Exp Ophthalmol 29:183–187
Fox MA, Sanes JR (2007) Synaptotagmin I and II are present in distinct subsets of central synapses. J Comp Neurol 503:280–296
Fukuda M (2003) Distinct Rab binding specificity of Rim1, Rim2, Rabphilin, and Noc2. J Biol Chem 278:15373–15380
Fukuda M (2008) Regulation of secretory vesicle traffic by Rab small GTPases. Cell Mol Life Sci 65:2801–2813
Fyk-kolodziej B, Dzhagaryan A, Qin P et al (2004) Immunocytochemical localization of three vesicular glutamate transporters in the cat retina. J Comp Neurol 475:518–530
Garner CC, Kindler S, Gundelfinger ED (2000) Molecular determinants of presynaptic active zones. Curr Opin Neurobiol 10:321–327
Geppert M, Bolshakov VY, Siegelbaum SA et al (1994) The role of Rab3A in neurotransmitter release. Nature (Lond) 369:493–497
Geppert M, Südhof TC (1998) Rab3 and synaptotagmin: the yin and yang of synaptic membrane fusion. Annu Rev Neurosci 21:75–95
Grabs D, Bergmann M, Urban M (1996) Rab3 proteins and SNAP-25, essential components of the exocytosis machinery in conventional synapses, are absent from ribbon synapses of the mouse retina. Eur J Neurosci 8:162–168
Gray EG, Pease HL (1971) On understanding the organisation of the retinal receptor synapses. Brain Res 35:1–15
Greenlee MH, Swanson JJ, Simon JJ et al (1996) Postnatal development and the differential expression of presynaptic terminal-associated proteins in the developing retina of the Brazilian opossum, *Monodelphis domestica*. Brain Res Dev Brain Res 96:159–172
Griessmeier K, Cuny H, Rötzer K et al (2009) Calmodulin is a functional regulator of Cav1.4 L-type Ca^{2+} channels. J Biol Chem 284:29809–29816
Haeseleer F, Imanishi Y, Maeda T et al (2004) Essential role of Ca^{2+}-binding protein 4, a $Ca_V1.4$ channel regulator, in photoreceptor synaptic function. Nat Neurosci 7:1079–1087
Hansen CG, Nichols BJ (2009) Molecular mechanisms of clathrin-independent endocytosis. J Cell Sci 122:1713–1721
Harata NC, Aravanis AM, Tsien RW (2006) Kiss-and-run and full-collapse fusion as modes of exo-endocytosis in neurosecretion. J Neurochem 97:1546–1570
Haverkamp S, Grünert U, Wässle H (2001) The synaptic architecture of AMPA receptors at the cone pedicle of the primate retina. J Neurosci 21:2488–2500
Heidelberger R, Sterling P, Matthews G (2002) Role of ATP in depletion and replenishment of the releasable pool of synaptic vesicles. J Neurophysiol 88:98–106
Heidelberger R, Wang MM, Sherry DM (2003) Differential distribution of synaptotagmin immunoreactivity among synapses in the goldfish, salamander, and mouse retina. Vis Neurosci 20:37–49
Heidelberger R, Thoreson WB, Witkovsky P (2005) Synaptic transmission at retinal ribbon synapses. Prog Retin Eye Res 24:682–720

Hemara-Wahanui A, Berjukow S, Hope CI et al (2005) A *CACNA1F* mutation identified in an X-linked retinal disorder shifts the voltage-dependence of Cav1.4 channel activation. Proc Natl Acad Sci USA 102:7553–7558

Hille B (2001) Ion channels of excitable membranes, 3rd edn. Sinauer, Sunderland

Hirasawa H, Kaneko A (2003) pH changes in the invaginating synaptic cleft mediate feedback from horizontal cells to cone photoreceptors by modulating Ca^{2+} channels. J Gen Physiol 122:657–671

Holzhausen LC, Lewis AA, Cheong KK et al (2009) Differential role for synaptojanin 1 in rod and cone photoreceptors. J Comp Neurol 517:633–644

Innocenti B, Heidelberger R (2008) Mechanisms contributing to tonic release at the cone photoreceptor ribbon synapse. J Neurophysiol 99:26–36

Jackman SL, Choi SY, Thoreson WB et al (2009) Role of the synaptic ribbon in transmitting the cone light response. Nat Neurosci 12:303–310

Jimeno D, Lillo C, Roberts EA (2006) Kinesin-2 and photoreceptor cell death: requirement for motor subunits. Invest Ophthalmol Vis Sci 47:5039–5046

Jockusch WJ, Mraefcke GJK, McMahon HT et al (2005) Clathrin-dependent and clathrin-independent retrieval of synaptic vesicles in retinal bipolar cells. Neuron 46:869–878

Johnson S, Halford S, Morris AG (2003) Genomic organisation and alternative splicing of RIM1, a gene implicated in autosomal dominant cone-rod dystrophy (CORD7). Genomics 81:304–314

Johnson JE, Perkins GA, Giddabasappa A et al (2007a) Spatiotemporal regulation of ATP and Ca^{2+} dynamics in vertebrate rod and cone ribbon synapses. Mol Vis 13:887–919

Johnson J, Fremeau RT Jr, Duncan JL et al (2007b) Vesicular glutamate transporter 1 is required for photoreceptor synaptic signaling but not for intrinsic visual functions. J Neurosci 27:7245–7255

Kang Y, Huang X, Pasyk EA et al (2002) Syntaxin-3 and syntaxin-1A inhibit L-type calcium channel activity, insulin biosynthesis and exocytosis in beta-cell lines. Diabetologia 45:231–241

Kersten FFJ, van Wijk E, van Reeuwijk J et al (2010) Association of whirlin with $Ca_V1.3$ (α_{1D}) channels in photoreceptors, defining a novel member of the Usher protein network. Invest Ophthalmol Vis Sci 51:2338–2346

Koschak A, Reimer D, Walter D et al (2003) $Ca_v1.4\alpha1$ subunits can form slowly inactivating dihydropyridine-sensitive L-type Ca^{2+} channels lacking Ca^{2+}-dependent inactivation. J Neurosci 23:6041–6049

Kourennyi DW, Liu X, Hart J et al (2004) Reciprocal modulation of calcium dynamics at rod and cone photoreceptor synapses by nitric oxide. J Neurophysiol 92:477–483

Kreft M, Krizaj D, Grilc S et al (2003) Properties of exocytotic response in vertebrate photoreceptors. J Neurophysiol 90:218–225

Krizaj D, Copenhagen DR (1998) Compartmentalization of calcium extrusion mechanisms in the outer and inner segments of photoreceptors. Neuron 21:249–256

Krizaj D, Bao JX, Schmitz Y et al (1999) Caffeine-sensitive calcium stores regulate synaptic transmission from retinal rod photoreceptors. J Neurosci 19:7259–7261

Krizaj D, DeMarco SJ, Johnson J (2002) Cell-specific expression of plasma membrane calcium ATPase isoforms in retinal neurons. J Comp Neurol 451:1–21

Kurenny DE, Moroz LL, Turner RW et al (1994) Modulation of ion channels in rod photoreceptors by nitric oxide. Neuron 13:315–324

Ladman AJ (1958) The fine structure of the rod-bipolar cell synapse in the retina of the albino rat. J Biophys Biochem Cytol 4:459–466

Lasansky A (1973) Organization of the outer synaptic layer in the retina of the larval tiger salamander. Philos Trans R Soc Lond B Biol Sci 265:471–489

Lazzell DR, Belizaire R, Thakur P et al (2004) SV2B regulates synaptotagmin 1 by direct interaction. J Biol Chem 279:52124–52131

Leenders AG, Lopes da Silva FH, Ghijsen WE et al (2001) Rab3A is involved in transport of synaptic vesicles to the active zone in mouse brain nerve terminals. Mol Biol Cell 12:3095–3102

Leitch B, Shevtsova O, Kerr RJ (2009) Selective reduction in synaptic proteins involved in vesicle docking and signaling at synapses in the ataxic mutant mouse *stargazer*. J Comp Neurol 512:52–73

Li W, Chen S, DeVries SH (2010) A fast rod photoreceptor signaling pathway in the mammalian retina. Nat Neurosci 13:414–416

Libby RT, Lillo C, Kitamoto J et al (2004) Myosin Va is required for normal photoreceptor synaptic activity. J Cell Sci 117:4509–4515

Linton JD, Holzhausen LC, Babai N et al (2010) Flow of energy in the outer retina in darkness and in light. Proc Natl Acad Sci USA 107:8599–8604

Littink KW, van Gengeren MM, Collin RWJ et al (2009) A novel homozygous nonsense mutation in *CABP4* causes congenital cone-rod synaptic disorder. Invest Ophthalmol Vis Sci 50:2344–2350

Llobet A, Gallop JL, Burden JJE et al (2011) Endophilin drives the fast mode of vesicle retrieval in a ribbon synapse. J Neurosci 31:8512–8519

LoGiudice L, Matthews G (2007) Endocytosis at ribbon synapses. Traffic 8:1123–1128

Maeda T, Lem J, Palczewski K et al (2005) A critical role of CaBP4 in the cone synapse. Invest Ophthalmol Vis Sci 46:4320–4327

Magupalli VG, Schwarz K, Alpadi K et al (2008) Multiple RIBEYE–RIBEYE interactions create a dynamic scaffold for the formation of synaptic ribbons. J Neurosci 28:7954–7967

Mandell JW, Townes-Anderson E, Czernik AJ et al (1990) Synapsins in the vertebrate retina: absence from ribbon synapses and heterogeneous distribution among conventional synapses. Neuron 5:19–33

Maricq AV, Korenbrot JI (1988) Calcium and calcium-dependent chloride currents generate action potential in solitary cone photoreceptors. Neuron 1:503–515

McRory JE, Hamid J, Doering CJ et al (2004) The *CACNA1F* gene encodes an L-type calcium channel with unique biophysical properties and tissue distribution. J Neurosci 24:1707–1708

Mercer AJ, Rabl K, Riccardi GE et al (2011) Location of release sites and calcium-activated chloride channels relative to calcium channels at the photoreceptor ribbon synapse. J Neurophysiol 105:321–335

Mercurio AM, Holtzman E (1982) Smooth endoplasmic reticulum and other agranular reticulum in frog retinal photoreceptors. J Neurocytol 11:263–293

Miki T, Kiyonaka S, Uriu Y (2007) Mutation associated with an autosomal dominant cone-rod dystrophy CORD7 modifies RIM1-mediated modulation of voltage-dependent Ca^{2+} channels. Channels 1:144–147

Morgans CW, Brandstätter JH, Kellerman J et al (1996) A SNARE complex containing syntaxin 3 is present in ribbon synapses of the retina. J Neurosci 16:6713–6721

Morgans CW, El Far O, Berntson A et al (1998) Calcium extrusion from mammalian photoreceptor terminals. J Neurosci 18:2467–2474

Morgans CW (1999) Calcium channel heterogeneity among cone photoreceptors in the tree shrew retina. Eur J Neurosci 11:2989–2993

Morgans CW (2001) Localization of the α_{1F} calcium channel subunit in the rat retina. Invest Ophthalmol Vis Sci 42:2414–2418

Morgans CW, Gaughwin P, Maleszka R (2001) Expression of the α_{1F} calcium channel subunit by photoreceptors in the rat retina. Mol Vis 7:202–209

Morgans CW, Bayley PR, Oesch NW et al (2005) Photoreceptor calcium channels: insight from night blindness. Vis Neurosci 22:561–568

Morona R, Northcutt RG, González A (2011) Immunohistochemical localization of calbindin D28k and calretinin in the retina of two lungfishes, *Protopterus dolloi* and *Neoceratodus forsteri*: colocalization with choline acetyltransferase and tyrosine hydroxylase. Brain Res 1368:28–43

Muresan V, Lyass A, Schnapp BJ (1999) The kinesin motor KIF3A is a component of the presynaptic ribbon in vertebrate photoreceptors. J Neurosci 19:1027–2037
Nachman-Clewner M, St. Jules R, Townes-Anderson E (1999) L-type calcium channels in the photoreceptor ribbon synapse: localization and role in plasticity. J Comp Neurol 415:1–16
Nag TC, Wadhwa S (1999) Developmental expression of calretinin immunoreactivity in the human retina and a comparison with two other EF-hand calcium binding proteins. Neuroscience 91:41–50
Oesch NW, Diamond JS (2011) Ribbon synapses compute temporal contrast and encode luminance in retinal rod bipolar cells. Nat Neurosci 14:1555–1561
Pahlberg J, Sampath AP (2011) Visual threshold is set by linear and nonlinear mechanisms in the retina that mitigate noise. Bioessays 33:438–447
Pang JJ, Gao F, Barrow A et al (2008) How do tonic glutamatergic synapses evade receptor desensitization? J Physiol (Lond) 586:2889–2902
Parsons TD, Sterling P (2003) Synaptic ribbon: conveyor belt or safety belt? Neuron 37:379–382
Petersen OH, Verkhratsky A (2007) Endoplasmic reticulum calcium tunnels integrate signaling in polarised cells. Cell Calcium 42:373–378
Pierantoni RL, McCann GD (1981) A quantitative study on synaptic ribbons in the photoreceptors of turtle and frog. In: Borsellino A, Cervetto L (eds) Photoreceptors. Plenum, New York, pp 381–386
Pumplin DW, Reese TS, Llinas R (1981) Are the presynaptic membrane particles the calcium channels? Proc Natl Acad Sci USA 78:7210–7213
Rabl K, Thoreson WB (2002) Calcium-dependent inactivation and depletion of synaptic cleft calcium ions combine to regulate rod calcium currents under physiological conditions. Eur J Neurosci 16:2070–2077
Rabl K, Cadetti L, Thoreson WB (2005) Kinetics of exocytosis is faster in cones than in rods. J Neurosci 25:4633–4640
Rao-Mirotznik R, Harkins AB, Buchsbaum G et al (1995) Mammalian rod terminal: architecture of a binary synapse. Neuron 14:561–569
Raviola E, Gilula NB (1975) Intramembrane organization of specialized contacts in the outer plexiform layer of the retina: a freeze-fracture study in monkeys and rabbits. J Cell Biol 65:192–222
Rea R, Li J, Dharia A et al (2004) Streamlined synaptic vesicle cycle in cone photoreceptor terminals. Neuron 41:755–766
Regus-Leidig H, Ott C, Löhner M et al (2013) Identification and immunocytochemical characterization of Piccolino, a novel Piccolo splice variant selectively expressed at sensory ribbon synapses of the eye and ear. PLoS One. 8:e70737
Reim K, Wegmeyer H, Brandstätter JH et al (2005) Structurally and functionally unique complexins at retinal ribbon synapses. J Cell Biol 169:669–680
Reim K, Regus-Leidig H, Ammermüller J et al (2009) Aberrant function and structure of retinal ribbon synapses in the absence of complexin 3 and complexin 4. J Cell Sci 122:1352–1361
Richards MW, Butcher AJ, Dolphin AC (2004) Ca^{2+} channel β-subunits: structural insights AID our understanding. Trends Pharmacol Sci 25:626–632
Rieke F, Schwartz EA (1996) Asynchronous transmitter release: control of exocytosis and endocytosis at the salamander rod synapse. J Physiol (Lond) 493:1–8
Ripps H, Shakib M, MacDonald ED (1976) Peroxidase uptake by photoreceptor terminals of the skate retina. J Cell Biol 70:86–96
Rosenmund C, Sigler A, Augustin I et al (2002) Differential control of vesicle priming and short-term plasticity by Munc13 isoforms. Neuron 33:411–424
Schacher S, Holtzman E, Hood DC (1976) Synaptic activity of frog retinal photoreceptors: a peroxidase uptake study. J Cell Biol 70:178–192
Schaeffer SF, Raviola E (1978) Membrane recycling in the cone cell endings of the turtle retina. J Cell Biol 79:802–825

Schein S, Ahmad KM (2005) A clockwork hypothesis: synaptic release by rod photoreceptors must be regular. Biophys J 89:3931–3949
Schlüter OM, Basu J, Südhof TC et al (2006) Rab3 superprimes synaptic vesicles for release: implications for short-term synaptic plasticity. J Neurosci 26:1239–1246
Schmitz F, Königstorfer A, Südhof TC (2000) RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. Neuron 28:857–872
Schnapf JL, Copenhagen DR (1982) Differences in the kinetics of rod and cone synaptic transmission. Nature (Lond) 296:862–864
Schoch S, Gundelfinger ED (2006) Molecular organization of the presynaptic active zone. Cell Tissue Res 326:379–391
Sheng Z, Choi SY, Dharia A et al (2007) Synaptic Ca^{2+} in darkness is lower in rods than cones, causing slower tonic release of vesicles. J Neurosci 27:5033–5042
Sherry DM, Wang MM, Frishman LJ (2003) Differential distribution of vesicle associated membrane protein isoforms in the mouse retina. Mol Vis 9:673–688
Sherry DM, Heidelberger R (2005) Distribution of proteins associated with synaptic vesicle endocytosis in the mouse and goldfish retina. J Comp Neurol 484:440–457
Sjöstrand FS (1958) Ultrastructure of retinal rod synapses of the guinea pig eye as revealed by three-dimensional reconstructions from serial sections. J Ultrastruct Res 2:122–170
Snellman J, Mehta B, Babai N et al (2011) Acute destruction of the synaptic ribbon reveals a role for the ribbon in vesicle priming. Nat Neurosci 14:1135–1141
Stella SL Jr, Thoreson WB (2000) Differential modulation of rod and cone calcium currents in tiger salamander retina by D2 dopamine receptors and cAMP. Eur J Neurosci 12:3537–3548
Stella SL Jr, Bryson EJ, Thoreson WB (2001) Insulin inhibits voltage-dependent calcium influx into rod photoreceptors. Neuroreport 12:947–951
Stella SL Jr, Bryson EJ, Thoreson WB (2002) A2 adenosine receptors inhibit calcium influx through L-type calcium channels in rod photoreceptors of the salamander retina. J Neurophysiol 87:351–360
Stella SL Jr, Bryson EJ, Cadetti L et al (2003) Endogenous adenosine reduces glutamatergic output from rods through activation of A2-like adenosine receptors. J Neurophysiol 90:165–174
Stella SL Jr, Hu WD, Vila A et al (2007) Adenosine inhibits voltage-dependent Ca^{2+} influx in cone photoreceptor terminals of the tiger salamander retina. J Neurosci Res 85:1126–1137
Stella SL Jr, Hu WD, Brechna NC (2009) Adenosine suppresses exocytosis from cone terminals of the tiger salamander retina. Neuroreport 20:923–929
Sterling P, Matthews G (2005) Structure and function of ribbon synapses. Trends Neurosci 28:20–29
Straiker A, Sullivan JM (2003) Cannabinoid receptor activation differentially modulates ion channels in photoreceptors of the tiger salamander. J Neurophysiol 89:2647–3654
Strom TM, Nyakatura G, Apfelstedt-Sylla E et al (1998) An L-type calcium channel gene mutated in incomplete X-linked congenital stationary night blindness. Nat Genet 19:260–263
Südhof TC (1995) The synaptic vesicle cycle: a cascade of protein–protein interactions. Nature (Lond) 375:645–653
Südhof TC (2002) Synaptotagmins: why so many? J Biol Chem 277:7629–7632
Südhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509–547
Sugita S, Shin OH, Han W et al (2002) Synaptotagmins form a hierarchy of exocytotic Ca^{2+} sensors with distinct Ca^{2+} affinities. EMBO J 21:270–280
Suryanarayanan A, Slaughter MM (2006) Synaptic transmission mediated by internal calcium stores in rod photoreceptors. J Neurosci 26:1759–1766
Szikra T, Krizaj D (2007) Intracellular organelles and calcium homeostasis in rods and cones. Vis Neurosci 24:733–743
Takao-Rikitsu E, Mochida S, Inoue E et al (2004) Physical and functional interaction of the active zone proteins, CAST, RIM1, and Bassoon in neurotransmitter release. J Cell Biol 164:301–311

Taylor WR, Morgans CW (1998) Localization and properties of voltage-gated calcium channels in cone photoreceptors of *Tupaia belangeri*. Vis Neurosci 15:541–552

Thoreson WB, Miller RF (1996) Removal of extracellular chloride suppresses transmitter release from photoreceptor terminals in the mudpuppy retina. J Gen Physiol 107:631–642

Thoreson WB, Nitzan R, Miller RF (1997) Reducing extracellular Cl^- suppresses dihydropyridine-sensitive Ca^{2+} currents and synaptic transmission in amphibian photoreceptors. J Neurophysiol 77:2175–2190

Thoreson WB, Mitzan R, Miller RF (2000) Chloride efflux inhibits single calcium channel open probability in vertebrate photoreceptors: chloride imaging and cell-attached patch-clamp recordings. Vis Neurosci 17:197–206

Thoreson WB, Stella SL Jr, Bryson EL et al (2002) D2-like dopamine receptors promote interactions between calcium and chloride channels that diminish rod synaptic transfer in the salamander retina. Vis Neurosci 19:235–247

Thoreson WB, Bryson EJ, Rabl K (2003) Reciprocal interactions between calcium and chloride in rod photoreceptors. J Neurophysiol 90:1747–1753

Thoreson WB, Rabl K, Townes-Anderson E et al (2004) A highly Ca^{2+}-sensitive pool of vesicles contributes to linearity at the rod photoreceptor ribbon synapse. Neuron 42:595–605

Thoreson WB (2010) The physiology of photoreceptor synapses and other ribbon synapses. In: Hodges R, Dartt D (eds) Encyclopedia of the eye. Elsevier, New York, chap. 155

Thoreson WB, Mangel SC (2012) Lateral interactions in the outer retina. Prog Retin Eye Res 31:407–441

Tian M, Xu S, Montpetit R et al (2012) Rab3A mediates vesicle delivery at photoreceptor ribbon synapses. J Neurosci 32:6931–6936

tom Dieck S, Altrock WD, Kessels MM et al (2005) Molecular dissection of the photoreceptor ribbon synapse: physical interaction of Bassoon and RIBEYE is essential for the assembly of the ribbon complex. J Cell Biol 168:825–836

tom Dieck S, Specht D, Strenzke N et al (2012) Deletion of the presynaptic scaffold CAST reduces active zone size in rod photoreceptors and impairs visual processing. J Neurosci 32:12192–12203

Townes-Anderson E, MacLeish PR, Raviola E (1985) Rod cells dissociated from mature salamander retina: ultrastructure and uptake of horseradish peroxidase. J Cell Biol 100:175–188

Ullrich B, Südhof TC (2004) Distribution of synaptic markers in the retina: implications for synaptic vesicle traffic in ribbon synapses. J Physiol (Lond) 88:249–257

Usukura J, Yamada E (1987) Ultrastructure of the synaptic ribbons in photoreceptor cells of *Rana catesbeiana* revealed by freeze-etching and freeze substitution. Cell Tissue Res 247:483–488

Vaithianathan T, Akmentin W, Henry D et al (2013a) The ribbon-associated protein C-terminal-binding protein 1 is not essential for the structure and function of retinal ribbon synapses. Mol Vis 19:917–926

Vaithianathan T, Zanazzi G, Henry D et al (2013b) Stabilization of spontaneous neurotransmitter release at ribbon synapses by ribbon-specific subtypes of complexin. J Neurosci 33:8216–8226

Van Epps HA, Hayashi M, Lucast L et al (2004) The zebrafish *nrc* mutant reveals a role for the phosphoinositide phosphatase synaptojanin 1 in cone photoreceptor ribbon anchoring. J Neurosci 24:8641–8650

Van Hook MJ, Thoreson WB (2012) Rapid synaptic vesicle endocytosis in cone photoreceptors of salamander retina. J Neurosci 32:18112–18123

Vessey JP, Stratis AK, Daniels BA et al (2005) Proton-mediated feedback inhibition of presynaptic calcium channels at the cone photoreceptor synapse. J Neurosci 25:4108–4117

von Kriegstein K, Schmitz F, Link E et al (1999) Distribution of synaptic vesicle proteins in the mammalian retina identifies obligatory and facultative components of ribbon synapses. Eur J Neurosci 11:1335–1348

von Kriegstein K, Schmitz F (2003) The expression pattern and assembly profile of synaptic membrane proteins in ribbon synapses of the developing mouse retina. Cell Tissue Res 311:159–173

Wahlin KJ, Moreira EF, Huang H et al (2008) Molecular dynamics of photoreceptor synapse formation in the developing chick retina. J Comp Neurol 506:822–837
Wahl-Schott C, Baumann L, Cuny H et al (2006) Switching off calcium-dependent inactivation in L-type calcium channels by an autoinhibitory domain. Proc Natl Acad Sci USA 103:15657–15662
Wahl S, Katiyar R, Schmitz F (2013) A local, periactive zone endocytic machinery at photoreceptor synapses in close vicinity to synaptic ribbons. J Neurosci 33:10278–10300
Wang X, Wang Q, Yang S et al (2011) Impaired activity-dependent plasticity of quantal amplitude at the neuromuscular junction of Rab3A deletion and Rab3A earlybird mutant mice. J Neurosci 31:3580–3588
Wässle H, Regus-Leidig H, Haverkamp S (2006) Expression of the vesicular glutamate transporter vGlut2 in a subset of cones of the mouse retina. J Comp Neurol 496:544–555
Wilkinson MF, Barnes S (1996) The dihydropyridine-sensitive calcium channel subtype in cone photoreceptors. J Gen Physiol 107:621–630
Witkovsky P (2004) Dopamine and retinal function. Doc Ophthalmol 108:17–40
Wu LG, Ryan TA, Lagnado L (2007) Modes of vesicle retrieval at ribbon synapses, calyx-type synapses and small central synapses. J Neurosci 27:11793–11802
Wutz K, Sauer C, Zrenner E et al (2002) Thirty distinct *CACNA1F* mutations in 33 families with incomplete type of XLCSNB and *Cacna1f* expression profiling in mouse retina. Eur J Hum Genet 10:449–456
Wycsik KA, Budde B, Feil S et al (2006) Structural and functional abnormalities of retinal ribbon synapses due to *Cacna2d4* mutation. Invest Ophthalmol Vis Sci 47:3523–3530
Xu JW, Slaughter MM (2005) Large-conductance calcium-activated potassium channels facilitate transmitter release in salamander rod synapse. J Neurosci 17:7660–7668
Zabouri N, Haverkamp S (2013) Calcium channel-dependent molecular maturation of photoreceptor synapses. PLoS One 8:e63853
Zampighi GA, Schietroma C, Zampighi LM et al (2011) Conical tomography of a ribbon synapse: structural evidence for vesicle fusion. PLoS One 6:e16944
Zenisek D, Matthews G (2000) The role of mitochondria in presynaptic calcium handling at a ribbon synapse. Neuron 25:229–237
Zenisek D, Horst NK, Merrifield C et al (2004) Visualizing synaptic ribbons in the living cell. J Neurosci 24:9752–9759

Chapter 8
Structure and Development of the Photoreceptor Ribbon Synapse

Yoshihiro Omori and Takahisa Furukawa

Abstract Photoreceptor cells are sensory neurons and does not possess dendrites, whereas photoreceptor axonal terminals develop a specialized synaptic structure called ribbon synapses that contains electro-dense horseshoe-like ribbons. Photoreceptor ribbon synapses have connections with dendritic terminals of bipolar cells and tips of horizontal cell processes, and therefore are critical for visual transduction. In both humans and genetically modified mice, loss of function in photoreceptor synaptic components and its regulators often causes visual impairment and retinal diseases including retinitis pigmentosa and night blindness. In this chapter, we review and discuss the development of photoreceptor ribbon synapses and the functions of ribbon synapse key components.

Keywords Bipolar cell dendrite • Cabp4 • CASK • Crx • Dystroglycan • Dystrophin • ERG • Horizontal cell process • L-type Ca^{2+} channel • Night blindness • Nrl • Photoreceptor axonal terminal • Pikachurin • Retinitis pigmentosa • Ribbon synapses

8.1 Synaptic Connection of Photoreceptor Cells with Other Neurons in the Retina

In the vertebrate retina, synaptic connections are formed between five different types of neurons, including photoreceptor, bipolar, horizontal, amacrine, and ganglion cells. These synapses are organized into distinct laminae: the outer plexiform layer (OPL) and the inner plexiform layer (IPL) (Masland 2001). Photoreceptor axonal terminals contact both horizontal cell processes and bipolar cell dendrites in the OPL.

Y. Omori • T. Furukawa (✉)
Laboratory for Molecular and Developmental Biology, Institute for Protein Research, Osaka University, Osaka, Japan
e-mail: takahisa.furukawa@protein.osaka-u.ac.jp

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_8, © Springer Japan 2014

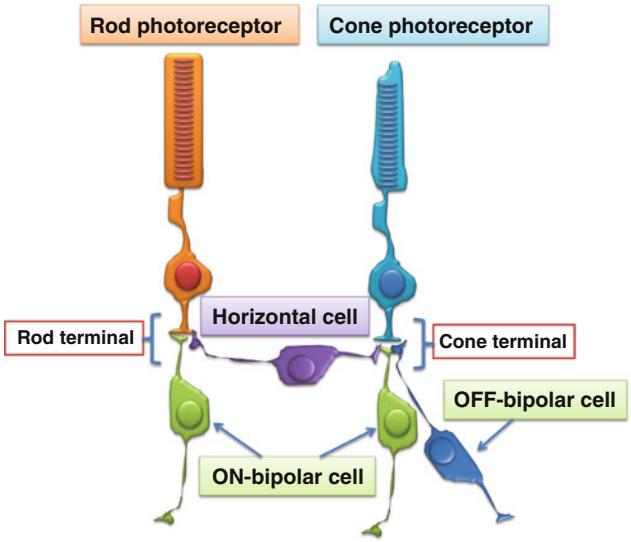

Fig. 8.1 Rod and cone photoreceptor synapses in the outer plexiform layer (OPL). Rod photoreceptor axonal terminals connect with rod ON-bipolar cell dendrites and horizontal cell processes. Cone photoreceptor terminals connect with cone ON- and cone OFF-bipolar cell dendrites as well as horizontal cell processes. These synapses are located in the outer plexiform layer (OPL)

Synaptic connections of bipolar cell axonal terminals and ganglion cell dendrites to amacrine cell processes are localized in the IPL. Rod photoreceptor cells connect with rod ON-bipolar cells and horizontal cells, whereas cone photoreceptor cells have connections with cone ON- and cone OFF bipolar cells as well as horizontal cells (Fig. 8.1).

In the OPL, photoreceptors and bipolar cells transfer information at specialized synapses, the ribbon synapses, which transmit signals tonically and in a graded fashion (tom Dieck and Brandstatter 2006). In vertebrates, several types of neurons in sensory organs form ribbon synapses including retinal bipolar cells, photoreceptor-like neurons in the pineal grand, and auditory and vestibular hair cells (Sterling and Matthews 2005). Graded synaptic output in photoreceptors requires that a large number of synaptic vesicles be released. To accomplish this, photoreceptors possess large pools of regularly aligned, fast-release vesicles tethered to the synaptic ribbons (Sterling and Matthews 2005).

A synaptic ribbon is an electron-dense organelle in the photoreceptor presynaptic cytoplasm, observable by electron microscopy. A photoreceptor synaptic ribbon has a plate-like structure that anchors it to the presynaptic membrane of an axon terminal. The photoreceptor synaptic ribbons are associated with invaginations present along the base of the photoreceptor terminals (Fig. 8.2). Horizontal cell processes and bipolar cell dendrites are inserted into photoreceptor invaginations and terminate near the ridge in which the synaptic ribbon lies. The synaptic structures of rod and cone photoreceptors are different. The rod photoreceptor terminals

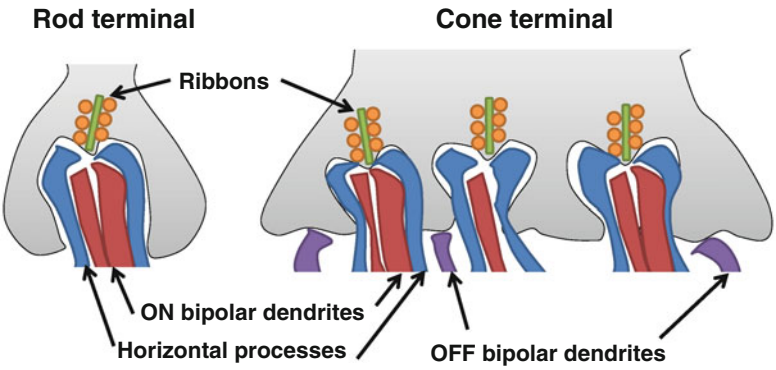

Fig. 8.2 Structure of rod and cone photoreceptor synaptic terminals. The photoreceptor ribbons (*green bars*) and synaptic vesicles (*yellow circles*) are associated with invaginations present at the base of the rod (*left panel*) and cone (*right panel*) photoreceptor synapses. In both rod and cone photoreceptors, horizontal cell processes (*blue*) and ON-bipolar cell dendrites (*red*) penetrate into photoreceptor invaginations. OFF-bipolar dendrites (*purple*) contact cone photoreceptor terminals on the surface of the synaptic terminals. The rod photoreceptor terminals contain a single ribbon. The cone photoreceptor terminals contain multiple ribbons and more horizontal and bipolar connections than those of rod photoreceptor cells

contain a single ribbon that has a horseshoe-shaped structure (Rao-Mirotznik et al. 1995). Rod photoreceptor synapses are called spherules. The cone photoreceptor terminals are larger than those of rods and contain multiple smaller synaptic ribbons in a single cone photoreceptor axonal terminal. The cone photoreceptor synapses are called pedicles. In the vertebrate rod synaptic invaginations, the rod ON-bipolar dendrites terminate as the central element. In the cone synaptic invaginations, the cone ON-bipolar dendrites end as the central element. The dendrites of cone OFF-bipolar cells contact the basal surfaces of cone terminals. In the invaginations of both rod and cone terminals, horizontal cell processes end laterally and deeper, and synaptic ribbons are located at the apex of invaginations.

8.2 Development of Photoreceptor Synapses

In vertebrates, retinal circuit development occurs in a sequential manner that is common across species (Olney 1968; Blanks et al. 1974; McLaughlin 1976; Fisher 1979; Schmitt and Dowling 1999). In the developing mouse retina, synaptic connections are mainly formed after birth (Blanks et al. 1974). Photoreceptor synaptic maturation occurs in three different stages of configuration termed apposition, dyad, and triad configurations (Fig. 8.3).

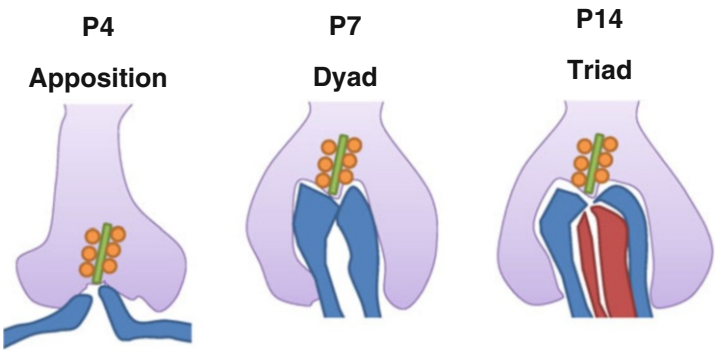

Fig. 8.3 Developmental stages of photoreceptor synapses. Photoreceptor synaptic formation in the mouse retina occurs postnatally (Blanks et al. 1974). Mouse photoreceptor synaptic formation occurs in three different stages referred to as apposition, dyad, and triad configurations. *Left panel:* After P4, the synaptic processes from horizontal cells are juxtaposed with the membrane of the photoreceptor synaptic terminal, but have not yet penetrated into the photoreceptor synaptic terminal. *Middle panel:* At around P7, the dyad configuration in the photoreceptor synaptic terminal is formed with two lateral horizontal processes and a synaptic ribbon within the photoreceptor synaptic terminal. *Right panel:* Around P14, a bipolar central process enters the photoreceptor synaptic invagination and forms the triad configuration

8.2.1 Apposition Configuration (P4–P5)

Soon after birth in the mouse, retinal progenitor cells continue proliferating. The synaptic layer in the OPL is difficult to recognize at postnatal day 3 (P3). The first sign of synaptic formation in the OPL is observable at P4 by electron microscopy analysis. Immature photoreceptor axonal synaptic terminals contain clusters of developing ribbons. The synaptic processes from horizontal cells are juxtaposed with the membrane of the photoreceptor synaptic terminal in the OPL but have not yet penetrated into the photoreceptor synaptic terminals. The synaptic contacts of horizontal cell processes are located on the base of the photoreceptor terminal, usually near developing synaptic ribbons. This stage of photoreceptor synapse formation is referred to as apposition (Blanks et al. 1974). The photoreceptor dystrophin complex containing pikachurin and dystroglycan is a useful marker for synaptic connections between photoreceptor presynapses and bipolar dendritic postsynapses (Schmitz and Drenckhahn 1997; Sato et al. 2008; Omori et al. 2012). At this stage, weak pikachurin signals are observed in the OPL adjacent to immature CtBP2-positive ribbons. Although dystroglycan and dystrophin signals are less intense than the pikachurin signal, these signals are weakly concentrated at the OPL. This finding suggests that the photoreceptor presynaptic complex including dystrophin, dystroglycan, and pikachurin begins forming on the surface of synaptic terminals before the photoreceptor terminus is connected with bipolar dendrites.

8.2.2 *Dyad Configuration (P6–P9)*

After the formation of synaptic contacts with horizontal cell processes on the surface of photoreceptor axonal terminals, horizontal cell processes penetrate the invagination of the photoreceptor synaptic terminal lateral to a synaptic ribbon at around P7 (Blanks et al. 1974). The dyad configuration is two lateral horizontal cell processes aligned with a synaptic ribbon within the photoreceptor synaptic terminal. At this stage, bipolar processes have not yet penetrated; however, strong dystroglycan and pikachurin signals are observed adjacent to the synaptic ribbon, suggesting that pikachurin, dystroglycan, and dystrophin form a complex in the photoreceptor synaptic terminals before the insertion of bipolar dendrites. At this stage, the complexity of the OPL increases, and a row of photoreceptor terminals with several synaptic dyads as well as a layer of neuropil with horizontal cell processes and bipolar cell dendrites is formed.

8.2.3 *Triad Configuration (After P10)*

Around P10, a bipolar central process enters the photoreceptor synaptic invagination to yield a triad configuration, which is a synaptic ribbon with two lateral processes from horizontal cells and with at least one central dendrite from bipolar cells in a rod photoreceptor synaptic terminal. The formation of synaptic triads at photoreceptor synaptic terminals is complete by P14. At this stage, photoreceptor synapses with dystrophin, dystroglycan, and pikachurin puncta are formed in the vicinity of a horseshoe-like ribbon in the OPL of adult mice.

8.3 Components of Synaptic Ribbons and Ribbon-Associated Proteins

In the presynaptic region of conventional synapses, neurotransmitter release is restricted to an electron-dense cytoskeletal meshwork, the cytomatrix at the active zone (CAZ), usually observable by electron microscopy. The photoreceptor synaptic ribbon represents a specialization of the CAZ of conventional synapses (tom Dieck et al. 2005) (Fig. 8.4). Presynaptic CAZ is situated opposite the postsynaptic neurotransmitter reception regions, an electron-dense cytoskeletal matrix called the postsynaptic density (Ziff 1997; Dresbach et al. 2001). At photoreceptor ribbons, CAZ proteins segregate into two compartments. The first compartment associated with the ribbon contains Piccolo, Ctbp1, Ctbp2, RIM1, and KIF3A. The plasma membrane-associated second compartment contains RIM2, Munc13-1, a Ca^{2+}

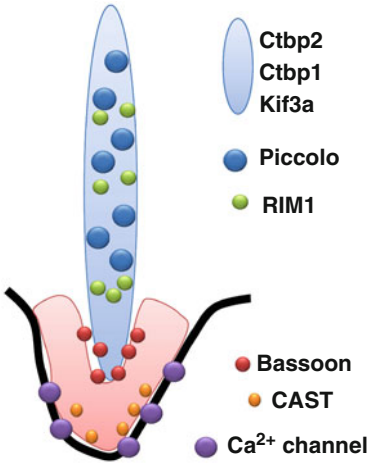

Fig. 8.4 Schematic diagram of photoreceptor ribbon compartments. Localization of CAZ proteins defines two compartments of the ribbon synaptic complex in photoreceptor synaptic terminals. The ribbon-associated compartment includes Ctbp2, Ctbp1, KIF3A, Piccolo, and RIM1. The plasma membrane-associated compartment includes CAST and an L-type Ca^{2+} channel α1-subunit. Bassoon localizes at the border and connects these two compartments. (Diagram modified from tom Dieck et al. 2005)

channel α1-subunit, and CAST/ERC2. Ctbp2 and Bassoon interact directly and link these two compartments. These proteins are important for signal transmission between photoreceptor and bipolar cells.

8.3.1 Bassoon

Bassoon is a presynaptic protein and a major component of the photoreceptor ribbon. Bassoon is an approximately 4,000-amino-acid protein containing a zinc finger motif similar to another ribbon component, Piccolo (tom Dieck et al. 1998). In *Bassoon*-deficient photoreceptor cells, synaptic ribbons are not anchored to the presynaptic active zones, resulting in impaired photoreceptor synaptic transmission (Dick et al. 2003). Most of the ribbons in *Bassoon*-deficient mice are not docked at the synaptic site but float freely in the cytoplasm (free-floating ribbons) (Fig. 8.5). More than half of the rod photoreceptor terminals are empty, without presynaptic ribbons and postsynaptic invaginating elements, in *Bassoon* mutant mice. In a normal cone synaptic terminal, multiple ribbons with the horizontal cell processes and bipolar dendrites are formed (Boycott and Wassle 1999); however, in *Bassoon*-deficient cone terminals, free-floating ribbons without invaginations dominate. The free-floating presynaptic ribbons in *Bassoon* mutants aggregate and form "ribbon fields." Fewer postsynaptic processes penetrate into the cone terminals of *Bassoon* mutants. In addition, abnormal dendritic branching of horizontal and bipolar cells to

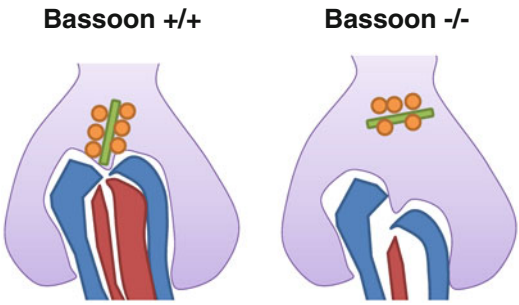

Fig. 8.5 Formation of aberrant ribbons and invaginations in *Bassoon*-deficient photoreceptor terminals. Ribbons in the *Bassoon*-deficient mice are not docked at the synaptic site and float freely in the cytoplasm. Empty rod photoreceptor terminals without presynaptic ribbons and postsynaptic invaginating elements are often observed in the *Bassoon*-deficient retina

photoreceptors and ectopic synapses form in the ONL of *Bassoon* mutant mice. These results suggest that Bassoon is essential for the proper positioning of cone and rod photoreceptor synaptic ribbons and proper connections between photoreceptors and horizontal or bipolar cells (Dick et al. 2003).

8.3.2 CAST

CAST/ERC2 is a component of the photoreceptor CAZ, a site of synaptic vesicle fusion (Ohtsuka et al. 2002). CAST contains multiple coiled-coil domains and a C-terminal PDZ-binding motif that interacts with RIM1, a Rab3a effector protein (Ohtsuka et al. 2002; Takao-Rikitsu et al. 2004). In the *CAST*-null retina, the size of the rod presynaptic active zones and the extension of the OPL are diminished (Fig. 8.6) (tom Dieck et al. 2012). Previous studies reported that sproutings of postsynaptic elements into the ONL with ectopic synapse formation were found in the retinas of mutant mice with impaired photoreceptor synaptic transmission (Mansergh et al. 2005; Haeseleer et al. 2004; Specht et al. 2009). Similar to these mutants, *CAST*-null mouse horizontal cells extended processes into the ONL. These horizontal cell sproutings in *CAST*-deficient mice contain a ribbon marker, CtBP2, in the ONL. Electron micrographs of *CAST*-deficient retinas also showed the presence of ectopic neurites and ectopic synapses with ribbons in the ONL. In addition, *CAST*-deficient mice show diminished b-wave amplitudes in scotopic ERGs, indicating that synaptic transmissions from rod photoreceptors to rod bipolar cells are impaired. Thus, in rod photoreceptor ribbon synapses, CAST regulates proper CAZ formation and effective synaptic transmission between photoreceptor terminals and bipolar dendrites.

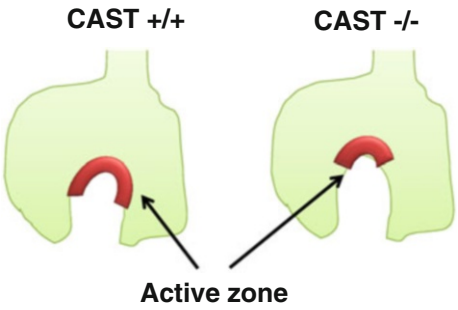

Fig. 8.6 CAST is required for proper photoreceptor CAZ formation. The size of the active zone is reduced in *CAST*-deficient photoreceptor synaptic terminals. *CAST*-deficient mice show ectopic synaptic formation in the ONL and diminished ERG b-waves. CAST is essential for proper photoreceptor CAZ formation in the OPL

8.3.3 Endocytotic Regulators

At photoreceptor ribbon synapses, continuous exocytosis of vesicles needs to be balanced by compensatory endocytosis to replenish the vesicle pools. The rate of vesicle recycling is essential for signaling at photoreceptor ribbon synapses (Jackman et al. 2009; Babai et al. 2010). Synaptic ribbons are required for the resupply of release-ready synaptic vesicles (Frank et al. 2010; Schnee et al. 2011; Snellman et al. 2011; Tian et al. 2012). Recently, by super-resolution structured illumination microscopy and electron microscopy analysis, the precise localizations of major components of the endocytotic membrane retrieval machinery in photoreceptor synapses were determined (Wahl et al. 2013). Local endocytic machinery is enriched at the periactive zone in the vicinity of the synaptic ribbon. This periactive zone endocytic machinery is ideally placed to replenish the exocytotic machinery of the continuously active photoreceptor ribbon synapse. Dynamin, syndapin, amphiphysin, and calcineurin are enriched around the active zone and the synaptic ribbon in photoreceptor terminals. Clathrin heavy chain variant 1 (CHC-V1) is enriched in the periactive zone of photoreceptor synapses, and clathrin heavy chain variant 2 (CHC-V2) is located in an endosomal compartment proximal to the presynaptic terminal. Concentration of endocytic proteins around the ribbon is consistent with the focal uptake of endocytic markers at that site. The photoreceptor periactive zone surrounding the ribbons seems to be a hotspot of endocytosis.

8.3.4 L-Type Ca^{2+} Channel and Its Regulator Cabp4

At ribbon synapses, Ca^{2+} influx through L-type Ca^{2+} channels triggers neurotransmitter release (Schmitz et al. 2000). The L-type Cav1.4 channel α1-subunit (Cacna1f) is expressed in photoreceptor cells and is localized in photoreceptor synaptic terminals (Barnes and Kelly 2002). Null mutations in the *Cav1.4* gene

are responsible for an X-linked disorder, congenital stationary night blindness type 2A (CSNB2A) (Strom et al. 1998; Bech-Hansen et al. 2000). The lack of an ERG observed in these patients suggests that Cav1.4 is essential for synaptic signal transmission between rod photoreceptor and bipolar cells. In the animal model, mutation in the *Cacna1f* gene causes a similar phenotype to that observed in human patients, including abnormal ERG and diminished photoreceptor calcium signals (Mansergh et al. 2005; Specht et al. 2009).

Cabp4, a Ca^{2+}-binding protein similar to calmodulin, directly interacts with Cav1.4 α1-subunit and modulates Cav1.4 activity in cells. Cabp4 is also specifically expressed in retinal photoreceptors. Cabp4 is localized to both rod and cone photoreceptor synaptic terminals in the OPL. *Cabp4*-deficient mice showed a thinner OPL than in control mice (Haeseleer et al. 2004). In the *Cabp4*-deficient retina, ectopic photoreceptor synapses are formed in the ONL. In addition, processes of rod bipolar and horizontal cells aberrantly extend into the ONL in *Cabp4*-deficient mice. Rod bipolar cell responses are remarkably reduced in sensitivity, and the ERG indicated a reduction in both cone and rod synaptic function in *Cabp4*-deficient mice. Thus, Cabp4 regulates Ca^{2+} influx and neurotransmitter release in photoreceptor synaptic terminals. In humans, mutations in the *CABP4* gene were reported to cause congenital stationary night blindness type 2B (CSNB2B) (Zeitz et al. 2006). These findings show that the Cav1.4 channel and its regulator Cabp4 are essential factors for ribbon synapse function in photoreceptor cells.

8.4 Dystrophin Complex in Photoreceptor Synapses

Dystrophin, one of the genes responsible for muscular dystrophy, plays an essential role in the formation of synapses including neuromuscular junctions (NMJ) and retinal photoreceptor synapses. Dystroglycan, a transmembrane proteoglycan, forms a dystrophin–glycoprotein complex and connects the extracellular matrix (ECM) with the actin cytoskeleton in the NMJ and in non-muscle tissues such as the brain and retina (Henry and Campbell 1996). Dystroglycan consists of an extracellular α-subunit and a transmembrane β-subunit. In both rod and cone photoreceptor synapses, dystrophin forms a complex with the α-dystroglycan subunit and ECM protein pikachurin (Fig. 8.7) (Kanagawa et al. 2010; Howard et al. 1998; Sato et al. 2008; Omori et al. 2012). The dystrophin-dystroglycan-pikachurin (DGP) complex is required for the formation of proper synaptic connections between photoreceptor axon terminals and dendritic tips of bipolar cells.

8.4.1 Dystrophin

In humans, mutations in the *dystrophin* gene cause Duchenne–Becker muscular dystrophy (DMD/BMD), an X-linked recessive disease (Burghes et al. 1987; Kunkel et al. 1986; Monaco et al. 1986; Hoffman et al. 1987). Loss of the

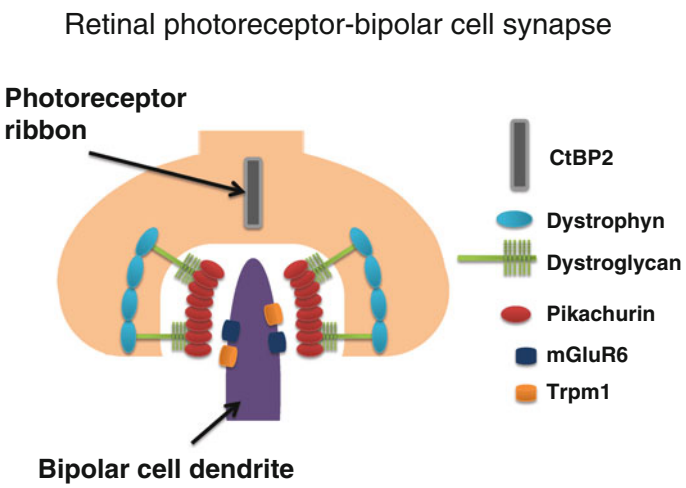

Fig. 8.7 Dystrophin-dystroglycan-pikachurin (DGP) complex in photoreceptor synaptic terminals. In photoreceptor synapses, dystrophin, a protein responsible for muscular dystrophy, forms a complex with dystroglycan, a transmembrane proteoglycan, and pikachurin, an extracellular matrix (ECM) protein. These components are essential for the connection between photoreceptor axon terminals and bipolar dendrites. Defects of either of these genes cause abnormal electroretinograms (ERGs) in mammals

dystrophin protein in the NMJ causes progressive abnormalities of muscular functions. Aberrant ERGs are observed in both human patients (Pillers et al. 1993; Fitzgerald et al. 1994) and mice with mutations in dystrophin (Pillers et al. 1995; Kameya et al. 1997). Analysis of ERGs from allelic variants of muscular dystrophic (mdx) mutant mice and DMD/BMD patients with mutations in different regions of the *dystrophin* gene indicates a correlation between the position of the mutation and the severity of the ERG abnormality (Schmitz and Drenckhahn 1997).

In rod and cone photoreceptor cells, dystrophin localizes to the microdomain of the photoreceptor synaptic plasma membrane that forms the lateral wall of the synaptic cavity and projects with finger-like extensions into the postsynaptic dendritic complex. The DGP complex of the cavital plasma membrane appears to stabilize the elaborate synaptic morphology.

8.4.2 Pikachurin

Pikachurin, an ECM protein, is essential for proper synaptic connection between photoreceptor cells and bipolar cells (Sato et al. 2008). Pikachurin was first identified as a photoreceptor-specific gene. Pikachurin contains fibronectin-3 repeats and EGF-like and Laminin-G domains with N-terminal signal sequences.

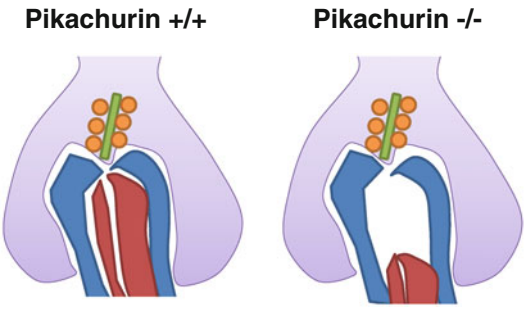

Fig. 8.8 Photoreceptor synaptic terminals in control and *pikachurin*-deficient mice. *Pikachurin*-deficient mice show aberrant ERGs as well as mislocalization of dystroglycan in the OPL. Pikachurin interacts with dystroglycan on the surface of the photoreceptor synaptic membrane and is anchored by dystroglycan. Glycosylation of dystroglycan is important for the interaction between pikachurin and dystroglycan

Pikachurin is localized to the photoreceptor synaptic cleft in the vicinity of the synaptic ribbon. Ultrastructural analysis revealed that *pikachurin*-deficient mice exhibited improper apposition of the bipolar dendritic tip to the photoreceptor synaptic invagination (Fig. 8.8). Consistent with these observations, *pikachurin*-deficient mice showed an increase in implicit times of both scotopic and photopic b-waves compared with those of control mice. In addition, *pikachurin*-deficient mice exhibited impaired optokinetic responses (OKR) (Sato et al. 2008; Omori et al. 2012). Pikachurin directly binds to the extracellular domain of dystroglycan, indicating that pikachurin is a ligand for dystroglycan in the retina (Kanagawa et al. 2010).

8.4.3 Dystroglycan

In contrast to the predominantly retinal expression of pikachurin, dystroglycan is broadly expressed in various tissues, including the central nervous system (CNS) (Ibraghimov-Beskrovnaya et al. 1992; Ibraghimov-Beskrovnaya et al. 1993), and *dystroglycan*-deficient embryos exhibit gross developmental abnormalities beginning at around 6.5 days of gestation with early embryonic lethality (Williamson et al. 1997). This lethality prevents analyses of dystroglycan roles in organ development or maintenance at postnatal stages. To investigate the function of dystroglycan in photoreceptor cells, retinal photoreceptor-specific *dystroglycan* conditional knockout mice (CKO) were analyzed (Sato et al. 2008; Omori et al. 2012). *Dystroglycan* CKO mice show a reduced ERG amplitude and a prolonged ERG b-wave implicit time (Fig. 8.9). Electron microscopic analysis shows that bipolar cell dendritic penetration into the photoreceptor terminus is perturbed in the *dystroglycan*-deficient retina. In the *dystroglycan*-deficient retina, the pikachurin signal is markedly decreased at photoreceptor synapses.

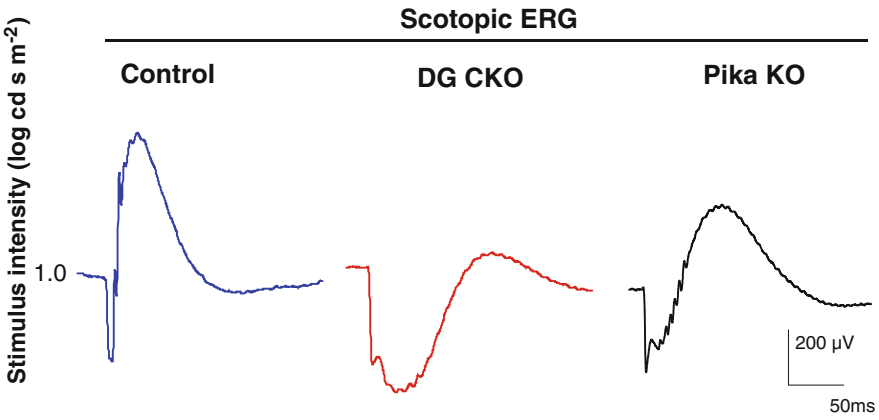

Fig. 8.9 Electroretinograms recorded from *dystroglycan*-null and *pikachurin*-null retina: scotopic ERGs of a control mouse (*left*), a *dystroglycan* CKO mouse (*middle*), and *pikachurin*-deficient mouse (*right*). Implicit times of scotopic ERG b-waves are elongated in the *dystroglycan* CKO and *pikachurin*-deficient mice, and the amplitude of scotopic ERG b-waves is severely decreased in *dystroglycan*-CKO mice and mildly reduced in *pikachurin*-deficient mice

Conversely, in the *pikachurin*-deficient retina, the dystroglycan signals at the ribbon synaptic terminus are severely reduced. These findings suggest that pikachurin is required for the presynaptic accumulation of dystroglycan at the photoreceptor synaptic terminus and vice versa. Thus, the presynaptic interaction of pikachurin with dystroglycan at photoreceptor terminals is essential for both the formation of proper photoreceptor ribbon synaptic structures and normal retinal electrophysiology.

8.5 Synaptic Formation Is Regulated by Photoreceptor-Specific Transcription Factors

Photoreceptor synaptic abnormalities are reported in mice with defects in transcription factors Crx and Nrl, which control photoreceptor differentiation (Morrow et al. 2005; Strettoi et al. 2004). These transcription factors control synaptogenesis through regulating the expression of genes involved in photoreceptor synapse formation.

8.5.1 Crx

The *Crx* gene encodes an Otx-family homeodomain transcription factor that is expressed predominantly in cone and rod photoreceptors and is essential for photoreceptor development (Furukawa et al. 1997; Furukawa et al. 1999).

Mutations in the human *CRX* gene are associated with retinal diseases including retinitis pigmentosa, LCA, and cone-rod dystrophy 2 (CRD2) (Freund et al. 1997; Swain et al. 1997; Sohocki et al. 1998; Swaroop et al. 1999). An ultrastructural study of *Crx*-deficient mice demonstrated that both rod and cone photoreceptor terminals are highly disorganized at P21 in the *Crx*-deficient OPL (Morrow et al. 2005). In *Crx*-deficient mice, processes with synaptic vesicles and ribbon-like structures are observed, showing that synapse components are at least generated in the mutant mice. A normal rod photoreceptor contains a single ribbon in the wild-type retina, however, many of photoreceptor synaptic terminals contain multiple ribbons in the *Crx*-null retina. In addition, aberrant ribbons are not tethered to the plasma membrane and are occasionally observed in perinuclear positions in the *Crx*-deficient mice. These finding suggest that Crx regulates expression of an essential factor for photoreceptor synaptogenesis. Microarray study of the *Crx*-deficient retina revealed that the expressions of many photoreceptor-specific genes including *Cabp4* are markedly decreased in the *Crx*-deficient retina compared to the wild type (Livesey et al. 2000; Hsiau et al. 2007).

8.5.2 Nrl

Nrl, a basic leucine zipper transcription factor, is expressed in rods, but not cone photoreceptors, and is necessary for rod photoreceptor differentiation (Swaroop et al. 1992; Rehemtulla et al. 1996; Bessant et al. 1999; Swain et al. 2001; Zhu et al. 2003). In the *Nrl*-null retina, photoreceptor precursors do not develop into rods but generate an excess number of cone-like photoreceptors (Strettoi et al. 2004). Electron microscopy analysis revealed that the *Nrl*-deficient synaptic terminals are smaller than cone synaptic pedicles, and larger than typical rod synaptic spherules. Normal cone synaptic terminals form a single layer in the OPL, however, *Nrl*-deficient synaptic terminals occupy at least two layers. In *Nrl*-deficient mice, horizontal and bipolar processes form invaginations in photoreceptor terminals as they do in wild-type mice. Microarray study of the *Nrl*-deficient retina showed that the expressions of many rod photoreceptor-specific genes are markedly decreased (Yoshida et al. 2004); however, it is unknown which gene is responsible for the rod photoreceptor synaptic phenotypes observed in the *Nrl*-deficient retina.

8.6 Open Questions/Perspectives

Recently, marked progress has been achieved in numerous studies of the molecular components of the photoreceptor synaptic ribbon and associated proteins; however, the molecular mechanisms that create the differences between rod and cone photoreceptor synapses are still poorly understood. How are the numbers of ribbons in rod and cone regulated? How are the specific connections between the special types

of bipolar cells and proper invaginations in multiple types of photoreceptor cells formed? Which molecules regulate the complex structures of photoreceptor ribbon synapses? These questions will be addressed using new techniques involving genetically manipulated animals including tissue-specific and/or developmental stage-specific gene targeting, as well as an increasing bounty of animal resources, including thousands of systematically generated floxed mouse lines (https://www.komp.org/). Microarray and proteomic analysis of retinal components using genetically modified animal tissues will provide valuable information in finding new factors. Research answering these questions will provide new knowledge not only for basic visual science but also for better understanding of the pathogenesis underlying retinal diseases such as retinitis pigmentosa, cone-rod dystrophy, and Leber's congenital amaurosis.

References

Babai N, Bartoletti TM, Thoreson WB (2010) Calcium regulates vesicle replenishment at the cone ribbon synapse. J Neurosci 30:15866–15877

Barnes S, Kelly ME (2002) Calcium channels at the photoreceptor synapse. Adv Exp Med Biol 514:465–476

Bech-Hansen NT, Naylor MJ, Maybaum TA, Sparkes RL, Koop B, Birch DG, Bergen AA, Prinsen CF, Polomeno RC, Gal A, Drack AV, Musarella MA, Jacobson SG, Young RS, Weleber RG (2000) Mutations in NYX, encoding the leucine-rich proteoglycan nyctalopin, cause X-linked complete congenital stationary night blindness. Nat Genet 26:319–323

Bessant DA, Payne AM, Mitton KP, Wang QL, Swain PK, Plant C, Bird AC, Zack DJ, Swaroop A, Bhattacharya SS (1999) A mutation in NRL is associated with autosomal dominant retinitis pigmentosa. Nat Genet 21:355–356

Blanks JC, Adinolfi AM, Lolley RN (1974) Synaptogenesis in the photoreceptor terminal of the mouse retina. J Comp Neurol 156:81–93

Boycott B, Wassle H (1999) Parallel processing in the mammalian retina: the Proctor Lecture. Invest Ophthalmol Vis Sci 40:1313–1327

Burghes AH, Logan C, Hu X, Belfall B, Worton RG, Ray PN (1987) A cDNA clone from the Duchenne/Becker muscular dystrophy gene. Nature 328:434–437

Dick O, tom Dieck S, Altrock WD, Ammermuller J, Weiler R, Garner CC, Gundelfinger ED, Brandstatter JH (2003) The presynaptic active zone protein bassoon is essential for photoreceptor ribbon synapse formation in the retina. Neuron 37:775–786

Dresbach T, Qualmann B, Kessels MM, Garner CC, Gundelfinger ED (2001) The presynaptic cytomatrix of brain synapses. Cell Mol Life Sci 58:94–116

Fisher LJ (1979) Development of synaptic arrays in the inner plexiform layer of neonatal mouse retina. J Comp Neurol 187:359–372

Fitzgerald KM, Cibis GW, Giambrone SA, Harris DJ (1994) Retinal signal transmission in Duchenne muscular dystrophy: evidence for dysfunction in the photoreceptor/depolarizing bipolar cell pathway. J Clin Invest 93:2425–2430

Frank T, Rutherford MA, Strenzke N, Neef A, Pangrsic T, Khimich D, Fejtova A, Gundelfinger ED, Liberman MC, Harke B, Bryan KE, Lee A, Egner A, Riedel D, Moser T (2010) Bassoon and the synaptic ribbon organize Ca^{2+} channels and vesicles to add release sites and promote refilling. Neuron 68:724–738

Freund CL, Gregory-Evans CY, Furukawa T, Papaioannou M, Looser J, Ploder L, Bellingham J, Ng D, Herbrick JA, Duncan A, Scherer SW, Tsui LC, Loutradis-Anagnostou A, Jacobson SG,

Cepko CL, Bhattacharya SS, McInnes RR (1997) Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (CRX) essential for maintenance of the photoreceptor. Cell 91:543–553

Furukawa T, Morrow EM, Cepko CL (1997) Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell 91:531–541

Furukawa T, Morrow EM, Li T, Davis FC, Cepko CL (1999) Retinopathy and attenuated circadian entrainment in Crx-deficient mice. Nat Genet 23:466–470

Haeseleer F, Imanishi Y, Maeda T, Possin DE, Maeda A, Lee A, Rieke F, Palczewski K (2004) Essential role of Ca^{2+}-binding protein 4, a Cav1.4 channel regulator, in photoreceptor synaptic function. Nat Neurosci 7:1079–1087

Henry MD, Campbell KP (1996) Dystroglycan: an extracellular matrix receptor linked to the cytoskeleton. Curr Opin Cell Biol 8:625–631

Hoffman EP, Brown RH Jr, Kunkel LM (1987) Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 51:919–928

Howard PL, Dally GY, Wong MH, Ho A, Weleber RG, Pillers DA, Ray PN (1998) Localization of dystrophin isoform Dp71 to the inner limiting membrane of the retina suggests a unique functional contribution of Dp71 in the retina. Hum Mol Genet 7:1385–1391

Hsiau TH, Diaconu C, Myers CA, Lee J, Cepko CL, Corbo JC (2007) The cis-regulatory logic of the mammalian photoreceptor transcriptional network. PLoS One 2:e643

Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW, Campbell KP (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature 355:696–702

Ibraghimov-Beskrovnaya O, Milatovich A, Ozcelik T, Yang B, Koepnick K, Francke U, Campbell KP (1993) Human dystroglycan: skeletal muscle cDNA, genomic structure, origin of tissue specific isoforms and chromosomal localization. Hum Mol Genet 2:1651–1657

Jackman SL, Choi SY, Thoreson WB, Rabl K, Bartoletti TM, Kramer RH (2009) Role of the synaptic ribbon in transmitting the cone light response. Nat Neurosci 12:303–310

Kameya S, Araki E, Katsuki M, Mizota A, Adachi E, Nakahara K, Nonaka I, Sakuragi S, Takeda S, Nabeshima Y (1997) Dp260 disrupted mice revealed prolonged implicit time of the b-wave in ERG and loss of accumulation of beta-dystroglycan in the outer plexiform layer of the retina. Hum Mol Genet 6:2195–2203

Kanagawa M, Omori Y, Sato S, Kobayashi K, Miyagoe-Suzuki Y, Takeda S, Endo T, Furukawa T, Toda T (2010) Post-translational maturation of dystroglycan is necessary for Pikachurin binding and ribbon synaptic localization. J Biol Chem 285:31208–31216

Kunkel LM et al (1986) Analysis of deletions in DNA from patients with Becker and Duchenne muscular dystrophy. Nature 322:73–77

Livesey FJ, Furukawa T, Steffen MA, Church GM, Cepko CL (2000) Microarray analysis of the transcriptional network controlled by the photoreceptor homeobox gene Crx. Curr Biol 10:301–310

Mansergh F, Orton NC, Vessey JP, Lalonde MR, Stell WK, Tremblay F, Barnes S, Rancourt DE, Bech-Hansen NT (2005) Mutation of the calcium channel gene Cacna1f disrupts calcium signaling, synaptic transmission and cellular organization in mouse retina. Hum Mol Genet 14:3035–3046

Masland RH (2001) The fundamental plan of the retina. Nat Neurosci 4:877–886

McLaughlin BJ (1976) A fine structural and E-PTA study of photoreceptor synaptogenesis in the chick retina. J Comp Neurol 170:347–364

Monaco AP, Neve RL, Colletti-Feener C, Bertelson CJ, Kurnit DM, Kunkel LM (1986) Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. Nature 323:646–650

Morrow EM, Furukawa T, Raviola E, Cepko CL (2005) Synaptogenesis and outer segment formation are perturbed in the neural retina of Crx mutant mice. BMC Neurosci 6:5

Ohtsuka T, Takao-Rikitsu E, Inoue E, Inoue M, Takeuchi M, Matsubara K, Deguchi-Tawarada M, Satoh K, Morimoto K, Nakanishi H, Takai Y (2002) Cast: a novel protein of the cytomatrix at the active zone of synapses that forms a ternary complex with RIM1 and munc13-1. J Cell Biol 158:577–590

Olney JW (1968) An electron microscopic study of synapse formation, receptor outer segment development, and other aspects of developing mouse retina. Invest Ophthalmol 7:250–268

Omori Y, Araki F, Chaya T, Kajimura N, Irie S, Terada K, Muranishi Y, Tsujii T, Ueno S, Koyasu T, Tamaki Y, Kondo M, Amano S, Furukawa T (2012) Presynaptic dystroglycan-pikachurin complex regulates the proper synaptic connection between retinal photoreceptor and bipolar cells. J Neurosci 32:6126–6137

Pillers DA, Weleber RG, Woodward WR, Green DG, Chapman VM, Ray PN (1995) mdxCv3 mouse is a model for electroretinography of Duchenne/Becker muscular dystrophy. Invest Ophthalmol Vis Sci 36:462–466

Pillers DA, Bulman DE, Weleber RG, Sigesmund DA, Musarella MA, Powell BR, Murphey WH, Westall C, Panton C, Becker LE et al (1993) Dystrophin expression in the human retina is required for normal function as defined by electroretinography. Nat Genet 4:82–86

Rao-Mirotznik R, Harkins AB, Buchsbaum G, Sterling P (1995) Mammalian rod terminal: architecture of a binary synapse. Neuron 14:561–569

Rehemtulla A, Warwar R, Kumar R, Ji X, Zack DJ, Swaroop A (1996) The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. Proc Natl Acad Sci USA 93:191–195

Sato S, Omori Y, Katoh K, Kondo M, Kanagawa M, Miyata K, Funabiki K, Koyasu T, Kajimura N, Miyoshi T, Sawai H, Kobayashi K, Tani A, Toda T, Usukura J, Tano Y, Fujikado T, Furukawa T (2008) Pikachurin, a dystroglycan ligand, is essential for photoreceptor ribbon synapse formation. Nat Neurosci 11:923–931

Schmitt EA, Dowling JE (1999) Early retinal development in the zebrafish, *Danio rerio*: light and electron microscopic analyses. J Comp Neurol 404:515–536

Schmitz F, Drenckhahn D (1997) Localization of dystrophin and beta-dystroglycan in bovine retinal photoreceptor processes extending into the postsynaptic dendritic complex. Histochem Cell Biol 108:249–255

Schmitz F, Konigstorfer A, Sudhof TC (2000) RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. Neuron 28:857–872

Schnee ME, Santos-Sacchi J, Castellano-Munoz M, Kong JH, Ricci AJ (2011) Calcium-dependent synaptic vesicle trafficking underlies indefatigable release at the hair cell afferent fiber synapse. Neuron 70:326–338

Snellman J, Mehta B, Babai N, Bartoletti TM, Akmentin W, Francis A, Matthews G, Thoreson W, Zenisek D (2011) Acute destruction of the synaptic ribbon reveals a role for the ribbon in vesicle priming. Nat Neurosci 14:1135–1141

Sohocki MM, Sullivan LS, Mintz-Hittner HA, Birch D, Heckenlively JR, Freund CL, McInnes RR, Daiger SP (1998) A range of clinical phenotypes associated with mutations in CRX, a photoreceptor transcription-factor gene. Am J Hum Genet 63:1307–1315

Specht D, Wu SB, Turner P, Dearden P, Koentgen F, Wolfrum U, Maw M, Brandstatter JH, tom Dieck S (2009) Effects of presynaptic mutations on a postsynaptic Cacna1s calcium channel colocalized with mGluR6 at mouse photoreceptor ribbon synapses. Invest Ophthalmol Vis Sci 50:505–515

Sterling P, Matthews G (2005) Structure and function of ribbon synapses. Trends Neurosci 28:20–29

Strettoi E, Mears AJ, Swaroop A (2004) Recruitment of the rod pathway by cones in the absence of rods. J Neurosci 24:7576–7582

Strom TM, Nyakatura G, Apfelstedt-Sylla E, Hellebrand H, Lorenz B, Weber BH, Wutz K, Gutwillinger N, Ruther K, Drescher B, Sauer C, Zrenner E, Meitinger T, Rosenthal A, Meindl

A (1998) An L-type calcium-channel gene mutated in incomplete X-linked congenital stationary night blindness. Nat Genet 19:260–263

Swain PK, Chen S, Wang QL, Affatigato LM, Coats CL, Brady KD, Fishman GA, Jacobson SG, Swaroop A, Stone E, Sieving PA, Zack DJ (1997) Mutations in the cone-rod homeobox gene are associated with the cone-rod dystrophy photoreceptor degeneration. Neuron 19:1329–1336

Swain PK, Hicks D, Mears AJ, Apel IJ, Smith JE, John SK, Hendrickson A, Milam AH, Swaroop A (2001) Multiple phosphorylated isoforms of NRL are expressed in rod photoreceptors. J Biol Chem 276:36824–36830

Swaroop A, Wang QL, Wu W, Cook J, Coats C, Xu S, Chen S, Zack DJ, Sieving PA (1999) Leber congenital amaurosis caused by a homozygous mutation (R90W) in the homeodomain of the retinal transcription factor CRX: direct evidence for the involvement of CRX in the development of photoreceptor function. Hum Mol Genet 8:299–305

Swaroop A, Xu JZ, Pawar H, Jackson A, Skolnick C, Agarwal N (1992) A conserved retina-specific gene encodes a basic motif/leucine zipper domain. Proc Natl Acad Sci USA 89:266–270

Takao-Rikitsu E, Mochida S, Inoue E, Deguchi-Tawarada M, Inoue M, Ohtsuka T, Takai Y (2004) Physical and functional interaction of the active zone proteins, CAST, RIM1, and Bassoon, in neurotransmitter release. J Cell Biol 164:301–311

Tian M, Xu CS, Montpetit R, Kramer RH (2012) Rab3A mediates vesicle delivery at photoreceptor ribbon synapses. J Neurosci 32:6931–6936

tom Dieck S, Brandstatter JH (2006) Ribbon synapses of the retina. Cell Tissue Res 326:339–346

tom Dieck S, Altrock WD, Kessels MM, Qualmann B, Regus H, Brauner D, Fejtova A, Bracko O, Gundelfinger ED, Brandstatter JH (2005) Molecular dissection of the photoreceptor ribbon synapse: physical interaction of Bassoon and RIBEYE is essential for the assembly of the ribbon complex. J Cell Biol 168:825–836

tom Dieck S, Sanmarti-Vila L, Langnaese K, Richter K, Kindler S, Soyke A, Wex H, Smalla KH, Kampf U, Franzer JT, Stumm M, Garner CC, Gundelfinger ED (1998) Bassoon, a novel zinc-finger CAG/glutamine-repeat protein selectively localized at the active zone of presynaptic nerve terminals. J Cell Biol 142:499–509

tom Dieck S, Specht D, Strenzke N, Hida Y, Krishnamoorthy V, Schmidt KF, Inoue E, Ishizaki H, Tanaka-Okamoto M, Miyoshi J, Hagiwara A, Brandstatter JH, Lowel S, Gollisch T, Ohtsuka T, Moser T (2012) Deletion of the presynaptic scaffold CAST reduces active zone size in rod photoreceptors and impairs visual processing. J Neurosci 32:12192–12203

Wahl S, Katiyar R, Schmitz F (2013) A local, periactive zone endocytic machinery at photoreceptor synapses in close vicinity to synaptic ribbons. J Neurosci 33:10278–10300

Williamson RA, Henry MD, Daniels KJ, Hrstka RF, Lee JC, Sunada Y, Ibraghimov-Beskrovnaya-O, Campbell KP (1997) Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag1-null mice. Hum Mol Genet 6:831–841

Yoshida S, Mears AJ, Friedman JS, Carter T, He S, Oh E, Jing Y, Farjo R, Fleury G, Barlow C, Hero AO, Swaroop A (2004) Expression profiling of the developing and mature $Nrl^{-/-}$ mouse retina: identification of retinal disease candidates and transcriptional regulatory targets of Nrl. Hum Mol Genet 13:1487–1503

Zeitz C, Kloeckener-Gruissem B, Forster U, Kohl S, Magyar I, Wissinger B, Matyas G, Borruat FX, Schorderet DF, Zrenner E, Munier FL, Berger W (2006) Mutations in CABP4, the gene encoding the Ca^{2+}-binding protein 4, cause autosomal recessive night blindness. Am J Hum Genet 79:657–667

Zhu X, Brown B, Li A, Mears AJ, Swaroop A, Craft CM (2003) GRK1-dependent phosphorylation of S and M opsins and their binding to cone arrestin during cone phototransduction in the mouse retina. J Neurosci 23:6152–6160

Ziff EB (1997) Enlightening the postsynaptic density. Neuron 19:1163–1174

Chapter 9
Cell Fate Determination of Photoreceptor Cells

Constance Cepko

Abstract Rods and cones are highly related, sharing many morphological and functional features. Early lineage studies showed that they are produced by mitotic retinal progenitor cells (RPCs) that produce not only photoreceptor cells, but other retinal cell types as well, even in a terminal division (Holt et al., Neuron 1(1):15–26, 1988; Turner and Cepko, Nature 328 (6126):131–136, 1987; Turner et al., Neuron 4(6):833–845, 1990; Wetts and Fraser, Science 239(4844):1142–1145, 1988). More recent lineage studies have added more depth to the conclusions of these early findings, showing that there are specific and distinct RPCs that produce specific retinal cell types in a terminal division (Godinho et al., Neuron 56(4):597–603, 2007; Hafler et al., Proc Natl Acad Sci USA 109(20):7882–7887, 2012; Rompani and Cepko, Proc Natl Acad Sci USA 105(1):192–197, 2008; Suzuki et al., Proc Natl Acad Sci USA 110(37):15109–15114, 2013). Rather surprisingly, one type of RPC makes cones and horizontal cells (HCs), whereas another makes rods and amacrine cells, and still others make only rods, rods and bipolar cells, or rods and Muller glial cells (Emerson et al., Dev Cell 154(4):928–939, 2013; Hafler et al., Proc Natl Acad Sci USA 109(20):7882–7887, 2012). The genetic networks that are operating in these different types of RPCs have similarities and differences, with one of the differences leading to the cone fate and indirectly repressing the rod fate. The studies that lead to these conclusions are described in this chapter.

Keywords Cone • Retinal development • Retinal progenitor cell • Rod

C. Cepko (✉)
Departments of Genetics and Ophthalmology, Howard Hughes Medical Institute, 77 Avenue Louis Pasteur, Boston, MA 02115, USA
e-mail: Cepko@genetics.med.harvard.edu

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_9, © Springer Japan 2014

9.1 Introduction

There are a number of fascinating questions concerning the development of photoreceptor cells. One question concerns the cell fate determination mechanisms that direct the irreversible acquisition of the photoreceptor fate, as well as the rod versus cone fate. Another question concerns the intersection between these fate determination events and the patterning machinery that regulates the distribution of rods and cones across the retina: for example, why does the fovea have only cones and no rods? A third question revolves around the mechanistic aspects of the morphogenesis of the highly evolved photoreceptor structure, for example, how is the outer segment built and maintained? Some of these questions are covered within several chapters in this book, and will be of interest for some time, as we know very little about most of these processes, although efforts are being made to learn more. Here, the focus is on the cell fate determination events for rods and cones.

The birthdates of retinal cells are defined as the day when a cell undergoes its last S-phase. Birthdating studies across many vertebrates, including mammals, birds, fish, and amphibians, have shown that the birth order of retinal neurons is conserved (Altshuler et al. 1991). In the mouse, the cone photoreceptors are born early, starting at embryonic day 10 (E10), which is about the same time as the other early-born neurons, the ganglion cells and horizontal cells (HCs) (Fig. 9.1) (Carter-Dawson and LaVail 1979; Young 1985). The last cones are born in the

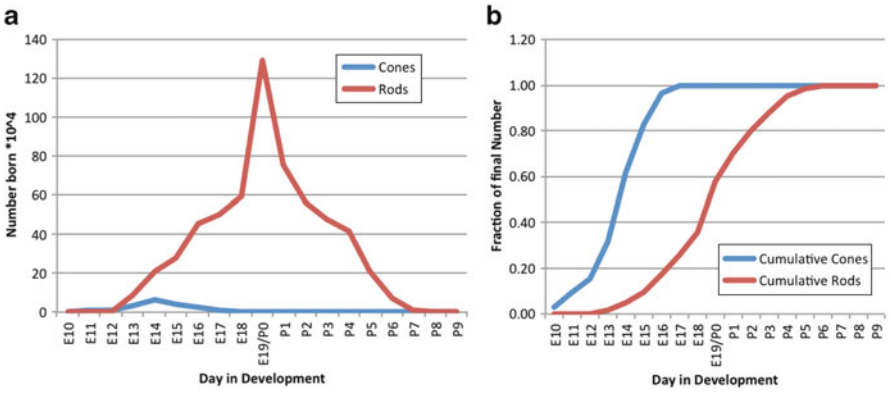

Fig. 9.1 Birthdates of rods and cones in the mouse retina. Classical ^{3}H-thymidine birthdating for rods and cones was carried out in the mouse (Carter-Dawson and LaVail 1979). A single injection of ^{3}H-thymidine was given on each day in development in different mice, and it was estimated that the label was available for approximately 30 min. Carter-Dawson and LaVail calculated that this would lead to heavily labeled nuclei for approximately 50 % of the cells born on a given day, as approximately 50 % of the cell cycle is the S-phase (Young 1985). At the termination of development, they quantified the percentage of all rods and all cones that were heavily labeled from the injection on a given day. Using the values of Young (Young 1985) for the fraction of all retinal cells that are rods and cones, 72.3 % and 2.2 %, respectively, the data of Carter-Dawson and LaVail were transformed into the number of rods (*red*) and cones (*blue*) born on a given day (**a**) and the cumulative fraction of rods (*red*) and cones (*blue*) born over time in development (**b**)

periphery at E18. Rod photoreceptors are born between E13 and postnatal day 7 (P7) and thereby have a period of genesis that overlaps that of cones but extends well beyond it. Rod and cone birthdays in the rat are similar (Rapaport et al. 2004). Carter-Dawson and LaVail quantified the birthdates of rods and cones in the mouse, showing that, within 2 days after the commencement of rod genesis, rods already outnumber cones (Fig. 9.1a). Thus, by E15, there are a greater number of cells fated to be rods in the embryonic mouse retina than there are those fated to be cones. By the end of development, rods comprise 72.3 % of all mouse retinal cells, whereas cones only are 2.2 % (Young 1985). In the chick, which is another useful model of retinal development, there are clearly more cones than rods (Morris and Shorey 1967; Bruhn and Cepko 1996). However, a precise accounting of the frequency of rods and cones is not available and may be difficult to obtain as there is significant variation across the retina in the frequency of rods (Bruhn and Cepko 1996). However, in one estimate cones comprise approximately 80 % of all photoreceptors (Morris and Shorey 1967). Most of the data discussed here are taken from studies of mice and chicks, although many of the observations made in these species have also been made in *Xenopus* and zebrafish, two other excellent models of retinal development.

9.2 Genes Required for the Genesis of Rods and Cones

The development of photoreceptors can be broken down into several stages, those of cell fate determination, differentiation, and survival. As discussed next, the determination event seems to occur at approximately the point of genesis, or birthday (day of birth), and can be thought of as the decision point to become a photoreceptor or a rod or a cone. Genes required for determination can be defined as those genes whose loss of function leads to a reduction in the number of photoreceptors, with a concomitant increase in another cell type(s), exemplified by loss of Otx2 (Nishida et al. 2003). This definition is meant to allow a distinction between those genes required for determination versus survival, as those required for survival may lead to loss of photoreceptors but they will not lead to an increase in another cell type, such as Neurod1 (Morrow et al. 1999). Differentiation can be defined as the elaboration of phenotype, such as the onset of markers, or morphological changes, that reflect the determination event. Genes that have a major role in the direct regulation of differentiation genes are also referred to as terminal selector genes (Hobert 2011). Loss of a terminal selector gene will lead to a reduction in the expression of photoreceptor markers, but will not lead to an absence of cells that express early markers or other features of photoreceptor cells, such as Crx (Furukawa et al. 1999). Complicating the interpretation of these roles is the possibility that a gene may have more than one role. Removal of gene function at different stages in the development process may enable the appreciation of multiple roles. However, genes with more than one role can present problems of interpretation, if, for example, a gene whose loss leads to a cell fate switch concomitantly leads to a change in proliferation and/or survival.

9.2.1 *Notch1*

Notch1 is a gene that acts at a very early, perhaps the earliest, point in photoreceptor determination (Jadhav et al. 2006; Yaron et al. 2006). The absence of Notch1 leads to an increase in the number of photoreceptors. Notch1 conditional knockout (CKO) mice have been examined following the introduction of Cre using different Cre strains, or by infection with a retrovirus carrying Cre. Overproduction of cone photoreceptors occurs if Notch1 is removed early, and other cell types are diminished accordingly. It is a bit difficult to do quantitative bookkeeping on this point, however, as there may also be a reduction in proliferation and survival. Nonetheless, one can see a preponderance of cells expressing cone markers, and a reduction in the expression of markers of other early-born cell types, by either microarray, in situ hybridization, or immunohistochemistry, if Notch1 is removed early in development. In the postnatal period, when a retrovirus was used to introduce Cre, there was an increase in rods (Jadhav et al. 2006; Mizeracka et al. 2013a). This experiment was done when proliferation was almost over, and almost every clone in the control was only a single cell, reducing the impact of a change in proliferation. Increases in photoreceptor number also were seen when a chemical inhibitor of gamma secretase, DAPT, which inhibits the enzyme needed to make functional Notch receptor, was added to cultures of the retina (Nelson et al. 2007). Furthermore, when Rbpj, which is a protein that acts in a complex with Notch to regulate transcription, was removed in mice, overproduction of photoreceptors was observed, as well as overproduction of ganglion cells (Riesenberg et al. 2009). It is possible that ganglion cell overproduction occurred because there are multiple Notch genes and all use Rbpj for transcriptional regulation. All of these observations demonstrate the importance of Notch1 in suppressing the photoreceptor fate.

Notch1 is expressed in both mitotic retinal progenitor cells (RPCs) and newly postmitotic cells (Nelson et al. 2006; Trimarchi et al. 2008b; Bao and Cepko 1997; Jadhav et al. 2006; Yaron et al. 2006). Its expression is then extinguished as neurons differentiate, whereas its expression is maintained in Müller glia, which also maintain the expression of many other RPC genes (Blackshaw et al. 2004). The aforementioned studies of the Notch1 CKO were conducted such that Notch1 was removed from mitotic cells. We were interested in whether Notch1 was needed in the newly postmitotic cells to regulate the number of rods. This question was of interest as we wish to understand how RPCs influence the fate of their progeny. It may be there are different types of RPCs and that they determine the fate of their progeny by passing down determinants, such as transcription factors (TFs), microRNAs, and/or chromatin state. These determinants might dictate the fate of progeny, reducing or eliminating the need for extrinsic cues or stochastic processes in the choice of fate within newly postmitotic cells. To this end, we used two methods to remove Notch1 from newly postmitotic cells (Mizeracka et al. 2013a). Almost every cell (~96 %) from which Notch1 was removed at this time became a rod, whereas about 30 % of wild-type cells became bipolar neurons and Müller glia.

This study demonstrated that the newborn retinal cells still need Notch1 to escape the rod fate. Interestingly, it is not just the signal from Notch 1 that is required, but new transcription and translation, because the gene itself is required in the postmitotic cells. It is not clear at this time if members of the delta or jagged families of ligands for Notch are required for this function of Notch1 in postmitotic cells. Several ligand genes are expressed at the right time and place to have this role (Bao and Cepko 1997; Nelson and Reh 2008; Rocha et al. 2009), but the role of these ligands in signaling newly postmitotic cells, versus signaling RPCs, has not been analyzed. An excellent candidate is Dll4, which has been shown to be regulated by Foxn4 (Luo et al. 2012), a TF that is required for the genesis of HCs and amacrine cell (ACs) (Li et al. 2004) versus photoreceptors. In addition to identification of the relevant ligand, it will be of interest to determine if the signaling is between siblings, to establish their asymmetry of photoreceptor and nonphotoreceptor fates.

9.2.2 Rax/Rx

The retinal and anterior homeobox genes identified in mouse as Rax (Furukawa et al. 1997a) and in *Xenopus* as Rx (Mathers et al. 1997) are the founding members of a group of genes with a paired-type homeobox, an octapeptide domain, and the OAR domain. Alignment of vertebrate and invertebrate genes has revealed a great deal of conservation among the sequences in these domains, and has led to a classification scheme comprising 3 groups (Wu et al. 2009). In the rodent lineage, there is a curious deletion such that they have only one Rax gene, the founding member of the group 1 Rax genes. Gain- and loss-of-function experiments in multiple species have shown multiple roles for Rax/Rx genes (Bailey et al. 2004; Muranishi et al. 2012). The group 2 Rax/Rx genes, which include some members referred to as Rax-L, Rax2, or Rx2 genes, are important in photoreceptor genesis and survival and differentiation. Interference with this type of Rax/Rx gene in chick (Chen and Cepko 2002), *Xenopus* (Wu et al. 2009), or zebrafish (Nelson et al. 2009) led to a reduction in photoreceptors. In the chick, this was at least in part caused by apoptosis as photoreceptors were being generated. In *Xenopus*, a clonal analysis following gain and loss of Rax-L indicated photoreceptor number increased or decreased, respectively (Wu et al. 2009), with changes also in amacrine and bipolar cells. The gain of function data from *Xenopus* is in contrast to gain of function using mouse Rax gene, overexpressed in rat, in which case there was a reduction in the formation of all types of neurons, and an increase in cells that resembled Müller glia/RPCs (Furukawa et al. 2000). Deletion of the sole Rax gene in midembryonic development in mouse using a CKO allele showed a reduction in photoreceptor cells, likely through its role in regulating Otx2 (Muranishi et al. 2011). Of interest was the lack of a requirement for the Rax gene for postnatal Otx2 expression, even though Rax was required for embryonic Otx2 expression (discussed further below). Given the data shown in Fig. 9.1 concerning rod and cone birthdays, it does not

appear that the embryonic versus postnatal dependence of Otx2 for Rax is a rod versus cone difference, but rather reflects a difference between embryonic and postnatal RPCs. Rax may also be involved in the direct regulation of some photoreceptor genes in mature photoreceptor cells, through binding to the PCE-1/Ret1 site (Kimura et al. 2000).

9.2.3 Otx2

Temporally downstream of Notch, commencing expression in some cells as they exit the cell cycle, is the Otx2 gene (Muranishi et al. 2012; Trimarchi et al. 2008a; Trimarchi et al. 2008b). Otx2 was found to be required for the production of rods and cones, as a CKO of Otx2 in the mouse was found to have no photoreceptors whereas there was an increase in the number of amacrine cells (Nishida et al. 2003). Although there was cell death in this model, the increase in amacrine fate was quite substantial and likely reflected a change in cell fate. Moreover, misexpression of Otx2 via a retroviral vector delivered to the postnatal mouse retina resulted in clones that contained only rods, whereas wild-type clones normally comprise rods, amacrine cells, bipolar cells, and Müller glia (Nishida et al. 2003). Subsequent to these early studies, it was reported that loss of Otx2 also led to loss of HCs, which do not express Otx2, and also to loss of bipolar cells, which express high levels of Otx2 (Sato et al. 2007).

Given its key role in photoreceptor fate determination, the regulation of Otx2 has been of interest. We used electroporation of chick and mouse retinas to assay for *cis*-regulatory modules (CRM) that show enhancer activity (Emerson and Cepko 2011). An Otx2 enhancer, Otx2ECR2, was found to drive expression in both chick and mouse retinas in the terminal cell cycle of RPCs that produce photoreceptors. Interestingly, when ECR2 was used to drive Cre, only photoreceptors showed a history of Otx2ECR2 expression, despite the fact that Otx2 is also expressed by bipolar cells, and, as discussed next, transiently in RPCs that produce HCs. Although many of the cells that express Otx2ECR2 are newly postmitotic cells fated to be rods or cones, Otx2ECR2 is expressed by a small number of RPCs in mouse, and these must be restricted to producing only photoreceptors. As clonal analyses have shown that some RPCs produce, for example, bipolar cells and rods in a terminal division (Turner and Cepko 1987; Turner et al. 1990), the restriction of ECR2 to some RPCs that only produce photoreceptors indicates that there are intrinsic differences among RPCs, at least in a terminal division.

Muranishi et al. used transgenic mice to assay for Otx2 enhancer activity (Muranishi et al. 2011). They report an enhancer that is different from Otx2ECR2, which they termed EELPOT. This approximately 500-bp enhancer is located further 5′ relative to ECR2, and had activity in a subset of embryonic cells, but interestingly did not have activity in the postnatal mouse retina. Sequence analysis revealed sites for several families of TFs, including E-boxes for neurogenic basic helix-loop-helix (bHLH genes), N-boxes for bHLH genes of the Hes/Hey type, and

binding sites for paired-type homeobox genes, which may include Otx2 or Crx. Rax was shown to transactivate this enhancer in a heterologous in vitro system. Similarly, bHLH genes were tested in this assay and some activity was detected for Ngn2. Hes1, Hes5, and Hey1, which are direct downstream targets of Notch and that bind to N-boxes, were able to reduce expression, even in the presence of Rax. The binding of Rax and Hes 1 to EELPOT in retinal extracts was demonstrated using ChIP. Binding was detected in embryonic, but not postnatal, extracts. Finally, a CKO of Rax was made and examined for Otx2 expression and photoreceptor cell numbers, as already discussed. Otx2 was quite reduced in the CKO and photoreceptor numbers were down. The activity of EELPOT in embryonic but not postnatal cells is reminiscent of the activity of Otx2ECR2 only in a subset of RPCs, and again points to intrinsic differences among the RPCs that produce photoreceptor cells, in this case, differences between embryonic and postnatal RPCs. The link to Notch, as perhaps the most upstream regulator of photoreceptor production, was suggested by these data. It is interesting, however, that Notch regulates rod production in the postnatal retina as well as the embryonic retina but apparently does not work through EELPOT in the postnatal retina.

9.2.4 Basic Helix-Loop-Helix

Multiple bHLH genes are expressed in the right time and place to have a role in photoreceptor development. These genes are heavily interconnected in a network (Kanekar et al. 1997; Hutcheson et al. 2005; Hernandez et al. 2007; Hatakeyama and Kageyama 2004), and are downstream of Notch signaling while also being upstream of the Notch ligands. Functional studies in multiple species have been carried out, showing that they play a role in photoreceptor development. However, it is difficult to assign roles precisely to any particular gene, given redundancy, compensation, and the lack of complementarity between some gain- and loss-of-function assays. However, a short summary of some examples of studies of expression and function is given here, with a focus on the mouse.

We carried out single-cell RNA profiling using microarrays in part to understand the complex patterns of expression of many types of TFs in RPCs, including the bHLH genes (Trimarchi et al. 2008b). A subset of the data regarding bHLH expression (shown in Fig. 9.2) illustrated that there are many patterns of expression of the bHLH genes, including those classified using the clade A, B, and E scheme (Skinner et al. 2010). They are expressed in overlapping patterns in mitotic cells, in newly postmitotic cells, and/or in photoreceptor cells. When double immunohistochemistry has been carried out for several of these proteins, a similar result has been found (Brzezinski et al. 2011). Akagi et al. performed a heroic series of experiments, examining single, double, and triple knockouts (KOs) in mice for effects on retinal development (Akagi et al. 2004). Ngn2, Ascl1 (Mash1), Neurod4 (Math3), and Neurod1, in different KO combinations, led to changes in the complement of retinal cell types. The triple KO for Ascl1, Neurod4, and Neurod1 led to an

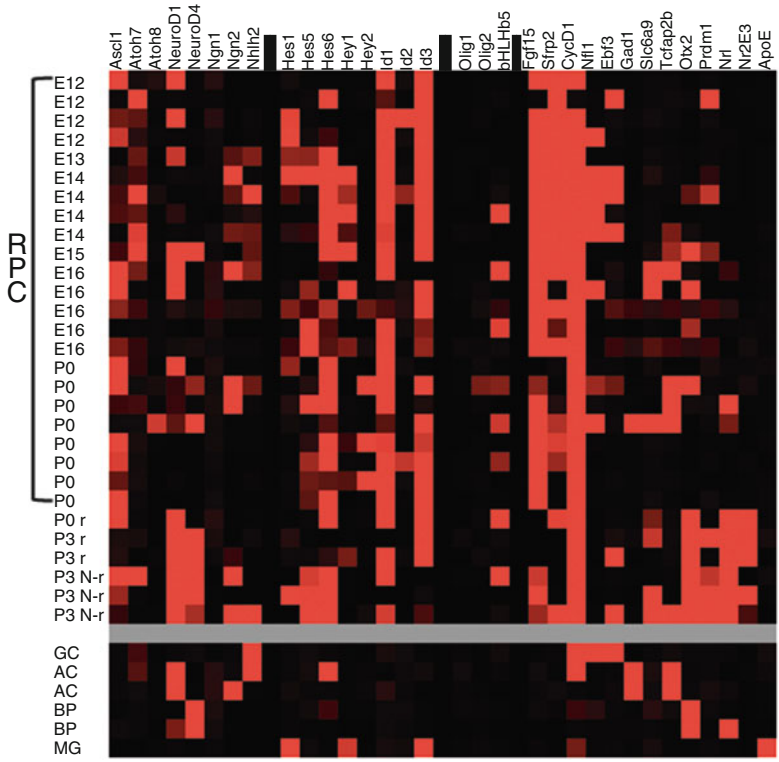

Fig. 9.2 Expression levels of basic helix-loop-helix (bHLH) genes. Individual single cells were used to prepare cDNA probes, which were amplified and applied to Affymetrix microarrays, with the resulting signal levels shown (Trimarchi et al. 2008b). Each cell is represented on a *row* and each bHLH gene in a *column*. The clade A bHLH genes are shown in the first group, clade B genes in the second group, and clade E genes in the third group, with groups demarcated by *black boxes* in the top row, and with the bHLH clade scheme of Skinner used (Skinner et al. 2010). Additional genes for reference cell types are shown on the right side of the heat map, after the clade E genes. The signal levels were scaled such that maximum red signal is 3,000. Retinal progenitor cells (*RPCs*) are shown with their corresponding ages, embryonic (*E*) through postnatal (*P*) stages. Cells demarcated by an *r* are immature rods; those with *N-r* are Notch-negative cells. (From Mizeracka et al. 2013b.) Several more mature cells are included for comparison. (From Cherry et al. 2009; Kim et al. 2008; Trimarchi et al. 2007.) *GC*, ganglion cell; *AC*, amacrine cell; *BP*, bipolar cell; *MG*, Müller glial cell. Genes that mark these cell types are Fgf15, SFRP2, and cycD1 for RPCs, Nfl1 and Ebf3 for GC, Gad1 and Slc6a9 for AC, Otx2 for rods and BP cells, Prdm1 for newly postmitotic cells fated to be rods, Nrl and Nr2E3 for rods, and ApoE for MG

approximately 66 % reduction in photoreceptors. Removal of only Neurod1 led to a defect only in rod survival (Morrow et al. 1999), whereas removal of Neurod4 along with Ascl1 led to a reduction in bipolar cells (Tomita et al. 2000). On the other hand, overexpression of a single bHLH gene, including either Ascl1 or Neurod4,

led to a significant increase in rods (Hatakeyama et al. 2001; Cherry et al. 2011) and a complete loss of Müller glia (Cai et al. 2000). Similarly, overexpression of Ngn1 in the chick led to overproduction of photoreceptor cells and resulted in reduced expression of other bHLH genes (Yan et al. 2009). Overexpression of bHLH genes in *Xenopus* (Wang and Harris 2005; Kanekar et al. 1997) and in zebrafish (Ochocinska and Hitchcock 2009) similarly led to excess photoreceptor cells.

Given the fact that at least some bHLH genes are negatively regulated by Hes/Hey factors, which are direct targets of Notch1 (Davis and Turner 2001), Notch1 signaling likely keeps the levels of neurogenic bHLHs in check and thus regulates the number of photoreceptors that are produced. Indeed, when we performed microarray analysis on single cells from a Notch1 CKO, one of the most noticeable changes was an increase in Neurod1 and Neurod4 (Mizeracka et al. 2013b). This increase was accompanied by a decrease in the Id factors, Id1 and Id3 (Mizeracka et al. 2013a; Mizeracka et al. 2013b). Ids also limit the activity of neurogenic bHLH factors by direct protein interaction (Benezra et al. 1990), and a functional analysis showed that Ids favor formation of Müeller glia (Mizeracka et al. 2013a). The overall chain of events downstream of Notch signaling, beginning in RPCs that are about to produce postmitotic daughter cells, needs to be elucidated to appreciate this network of TFs and their roles. It is likely that different RPCs, almost all of which can produce photoreceptors, use different bHLH genes, regulated by different upstream regulators, to produce photoreceptors and their distinctive siblings in terminal divisions, as is discussed further below.

9.2.5 Blimp1/PRDM1

Blimp1, or Prdm1, is a gene that is expressed in the period when cells fated to be photoreceptors are exiting mitosis (Hsiau et al. 2007) (Katoh et al. 2010; Brzezinski et al. 2010); this positions Blimp in that critical window where Notch, Otx2, and bHLH genes are expressed and are effecting fate decisions. Loss of Blimp1 was reported to lead to a reduction in rod and cone photoreceptors, with a concomitant gain in bipolar cells and abnormal cells with markers of proliferation (Katoh et al. 2010; Brzezinski et al. 2010). The excess bipolar cells were subsequently lost from cell death, perhaps because of their supernumerary status or perhaps being defective. As with bHLH genes, loss of Blimp1 creates a fate change only in a percentage of photoreceptors, as about 50 % of the normal number of rods remain in the Blimp1 CKO mouse; this may result from redundancy, as there are many members of this gene family and some are expressed in the mouse retina. Alternatively, there may be heterogeneity in the pool of RPCs and/or newly postmitotic daughters, and only some of these rely upon Blimp1 to become photoreceptors.

9.3 Genes That Regulate the Differentiation of Rod and Cone Photoreceptors

The network of genes involved in regulating rod- and cone-specific gene expression in differentiating cells define the specific properties of photoreceptor cells. As well, many of these genes are among the human disease genes that lead to blindness, and an understanding of their roles might lead to a greater understanding of these disease states (Blackshaw et al. 2001)(https://sph.uth.edu/retnet/). A short summary is given here of some of the genes that play a role in directing specific gene expression as photoreceptors differentiate.

9.3.1 Crx

Crx, a gene highly related to Otx2, is expressed in newborn rod and cone photoreceptors (Furukawa et al. 1997b; Chen et al. 1997). We examined the kinetics of expression of this gene and found that it could first be detected in cells that had exited the cell cycle, at about 24 h after the last S-phase (Trimarchi et al. 2008b); this would put Crx temporally later than Otx2, thyroid hormone receptor beta (Thrb) (discussed later), and the aforementioned bHLH genes, in mice and chicks. In zebrafish, Crx is detected in cells that are cycling (Riesenberg et al. 2009), perhaps reflecting the more rapid kinetics of development in the fish relative to mice and chicks. Crx is also detected at lower levels in bipolar cells in several species. The Crx KO mouse has photoreceptors that move to their proper position and turn on some markers of photoreceptor cells (Furukawa et al. 1999). However, the cells fail to express almost every photoreceptor gene and eventually degenerate (Furukawa et al. 1999; Livesey et al. 2000; Morrow et al. 2005), identifying Crx as a terminal selector gene for photoreceptors, necessary for differentiation and survival, but not for determination. In humans, there are also Crx mutations that lead to several forms of photoreceptor disease (Rivolta et al. 2001). Otx2 may regulate Crx directly (Nishida et al. 2003), and both Crx and Otx2 are able to bind to a similar sequence and directly regulate many photoreceptor genes (Chen et al. 1997; Furukawa et al. 1997b; White et al. 2013; Peng and Chen 2005). The combined loss of Otx2 and Crx leads to a phenotype of greater loss of photoreceptor gene expression (Koike et al. 2007).

9.3.2 Nuclear Hormone Receptors

Several members of the nuclear hormone receptor family of TFs play critical roles in photoreceptor differentiation, with some specific roles in opsin regulation clearly identified (Forrest and Swaroop 2012). Thrb has been characterized as the earliest

cone-specific marker (Sjoberg et al. 1992; Ng et al. 2001). It was studied in the early chick retina for its kinetics relative to Otx2 and NeuroD1 (Trimarchi et al. 2008a). These three genes are expressed with almost identical kinetics in overlapping patterns, with some cells expressing all three genes and other cells expressing one or two of these genes. They initiate expression in what appears to be the terminal S/G_2 phase of the cell cycle. Nr2b3 (RXRg) is also expressed in early cones, although it is not specific to cones, as it is also expressed in cells of the inner nuclear layer (INL) and ganglion cell layer (GCL) (Roberts et al. 2005). Thrb and Nr2b3 have been shown to be important in the regulation of cone opsin genes in mice (Ng et al. 2001). Loss of Thrb led to loss of expression of the medium-wavelength opsin, whereas the short-wavelength opsin was earlier in expression and was maintained in all cones. Nr2b3 is required for proper regulation of short-wavelength opsin (Roberts et al. 2005). Thrb and Nr2b3 regulate, and are themselves the target of, the E3 SUMO ligase, Pias3 (Onishi et al. 2009). Unliganded Thrb2 and liganded RXRγ positively regulate Pias3, which then carries out SUMOylation of Nr1f1, Nr2b3, and Thrb1. These SUMOylated TFs then repress S opsin in M cones and liganded Thrb2 and RXRγ activate M opsin. Interestingly, the Pias-3-mediated SUMOylation of the rod-specific transcription factor, Nr2E3, is required to inhibit S opsin in rods (Onishi et al. 2010). The use of Pias3 in both rods and cones for the inhibition of S opsin may reflect an ancient decision to make S opsin the default opsin type for both rods and cones. Thyroid hormone also plays an important role in opsin regulation within the retinas of salmonid fish. As these fish mature, they undergo a switch from UV to blue opsin-expressing cones. Although originally reported to be the result of a cell death and regeneration mechanism (Allison et al. 2006), it has now been established that this switch is a cone opsin expression switch within single cones (Cheng and Flamarique 2007; Cheng et al. 2009).

Nr2e3(PNR) encodes another nuclear hormone receptor that has been shown to be important in the differentiation of rods versus cones (Chen et al. 2005; Corbo and Cepko 2005; Haider et al. 2009). This gene is completely dependent upon the expression of Nrl, as there is almost no expression of Nr2e3 in the Nrl KO mouse. The rd7 mouse model of the Nr2e3 loss of function has a different phenotype, however, from the Nrl KO. In contrast to the Nrl KO mouse, rd7 rods express rod genes. As in the Nrl KO mouse, however, rd7 shows derepression of cone genes. However, there is a more limited number of cone genes that are derepressed in rods relative to the number seen in the Nrl KO. Nr2e3 is thus required for the repression of a subset of cone genes within rods and is not required for rod gene expression. There are also a small number of additional short-wavelength cones in rd7 (Akhmedov et al. 2000; Haider et al. 2001; Corbo and Cepko 2005), but not nearly the number that one observes in the Nrl KO mouse. These abnormalities likely underlie manifestations of Nr2E3 mutations in the human disease, the enhanced S-cone syndrome, wherein there is increased sensitivity to short wavelengths of light (Haider et al. 2000). As mentioned, the E3 SUMO ligase, Pias3, is involved in the regulation of rod versus cone genes, at least partially through Nr2e3 (Onishi et al. 2009). Nr2e3 works in the realm of differentiation rather than determination,

as KO or misexpression leads to misregulation of rod and cone genes, but not a complete transformation of rods or cones into other cell types.

Additional members of the nuclear hormone receptor family have roles to play in the proper regulation of rod and cone genes, as reviewed by Forrest and Swaroop (2012). Some examples include Nr1f1 (RORa) (Fujieda et al. 2009), which is important for the proper levels of cone opsin gene expression within cones. Loss of Nr3b2 (ERRb) leads to the degeneration of rods, and thus it is not a rod determination gene, but a gene important for rod differentiation and survival, in contrast to the role played by Nr1f2 (RORb) whose loss leads to a loss of rod determination (Jia et al. 2009; Srinivas et al. 2006; Montana et al. 2011), as is discussed further.

There is likely a network of nuclear hormone receptors, some of which may be linked through their use of coreceptors, the RXRs. A more in depth view of this network is required to properly interpret the roles of these receptors. However, at this point, all except Nr1f2 appear to be important for the proper regulation of rod and cone genes.

9.3.3 Nrl

In rats and mice, the genesis of cones precedes the genesis of rods, although there is a period of significant overlap in the mid- to late embryonic period (Fig. 9.1). The early-born cones are recognizable by expression of Thrb and Nr2b3. These early-born rods do not have a marker that has been recognized by in situ hybridization or immunohistochemistry, although polymerase chain reaction (PCR) of embryonic mouse tissue reveals expression of Nrl. In addition, an Nrl-GFP transgenic mouse shows green fluorescent protein (GFP) as early at E12 (Akimoto et al. 2006), but this early expression has not been shown to be specific to early-born rods. Nrl is a leucine zipper gene of the Maf family, discovered by the group of Anand Swaroop (Swaroop et al. 1992). They described Nrl as a rod-specific gene in the mouse and report expression of the protein in the cytoplasm of human cones (Swain et al. 2001). A null mutation of Nrl in the mouse showed the very interesting phenotype of loss of rods (Mears et al. 2001). Cells fated to be rods were not dead, however, but were transformed into short-wavelength cones. The cones are very close to bona fide cones, as judged by gene expression, morphology, and physiology (Daniele et al. 2005; Mears et al. 2001; Yoshida et al. 2004). This report led to the model that short-wavelength cones are the default type of photoreceptor; that is, when cells fated to be photoreceptors are first born, they enter the pathway of short-wavelength cones (Fig. 9.3a) (Swaroop et al. 2010). In normal development, many of the default cones are hypothesized to become rods when Nrl is expressed. As Nrl appears to be a critical node, one would like to know how Nrl is regulated. Two groups have examined this question, and report that there are conserved binding sites for Nr1f2 and Otx2/Crx upstream of Nrl (Montana et al. 2011; Kautzmann et al. 2011), in keeping with the loss of Nrl in the Nr1f2 KO mouse (Jia et al. 2009).

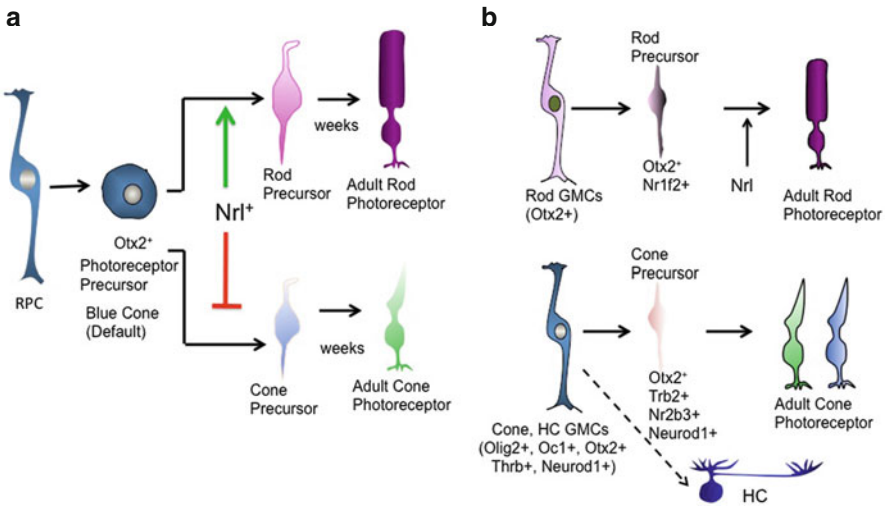

Fig. 9.3 Model of rod versus cone determination and the ganglion mother cells (GMCs) that make them (**a**). A model for photoreceptor development in which all photoreceptors originate from a common photoreceptor precursor, which follows a default pathway to differentiate into a blue cone unless instructed otherwise (Swaroop et al. 2010). Nrl, acting in combination with other transcription factors (TFs), is proposed to induce the rod fate, where it directly activates many rod genes and represses many cone genes. (**b**) A revised model, based upon lineage data (from Emerson et al. 2013; Emerson and Cepko 2011; Hafler et al. 2012; Suzuki et al. 2013) wherein distinct RPCs produce cones and rods, and there is not a common photoreceptor precursor that is, by default, a blue cone. In mice, some RPCs that produce a cone also produce a horizontal cell (HC) and express Olig2, Oc1, Otx2, Thrb, and Neurod1

As pointed out by Montana et al., these regulators cannot explain the specificity for Nrl expression in rods versus cones as both Otx2/Crx and Nr1f2 are expressed in both rods and cones.

The genes regulated by Crx and Nrl have been described in some detail, using expression profiling and CHIP-seq (Hao et al. 2012; Yoshida et al. 2004; Corbo et al. 2007, 2010; Hsiau et al. 2007). They act together at many loci in rods, and both can be considered to be terminal selector genes, directly regulating many of the genes required for rod function. Their importance is underscored by the observation that many of their target genes are human disease genes.

9.3.4 Sall3

S*palt* homologue 3 (Sall3), a transcription factor homologous to the *Drosophila Spalt*, which directly regulates *Drosophila* opsin expression, plays a role in cone differentiation in mice. It is a positive regulator of the short-wavelength opsin gene, along with additional cone genes, and is required for proper HC development

(de Melo et al. 2011). Misexpression studies show that it is sufficient to induce the short-wavelength cone opsin gene in rods, although it is unable to transfate rods or other cell types into cones. Similarly, it plays a role in the intermediate stages of HC differentiation.

9.4 RPCs That Produce Rods and Cones

Almost every clone derived from retroviral marking in the embryonic or postnatal retina or rats and mice contained a photoreceptor (Turner and Cepko 1987; Turner et al. 1990). In addition, many clones, even two-cell clones, contained a photoreceptor and another retinal cell type. These data indicated that almost every RPC has the ability to make a photoreceptor, and that many RPCs are multipotent. However, these data did not address whether all RPCs are equivalent. To probe the nature of RPCs, and to see if they differed from each other, we performed single-cell profiling using microarrays (Trimarchi et al. 2008b). These data showed many differences among RPCs across development as well as at a single time in development. One category of genes that varied was the bHLH category (Fig. 9.2). Olig2, a Clade E bHLH gene, showed variation in expression within RPCs across time. The single-cell microarray data regarding expression of Olig2 were corroborated by in situ hybridization (Hafler et al. 2012) and immunohistochemistry for Olig2 (Nakamura et al. 2006; Shibasaki et al. 2007). All these assays showed that Olig2 was expressed primarily in RPCs, but only in a subset of RPCs at each time in development. Two Cre knock-in lines for Olig2 in mice were available (Schuller et al. 2008; Dessaud et al. 2007). We thus were able to use these lines to analyze the descendants of Olig2+ RPCs using two methods. One method was the classic Cre fate mapping method. By crossing these two different Olig2-Cre strains to three different floxed reporter lines, we could see all the cells with an expression history for Olig2. These fate mapping experiments showed that cells with an Olig2 history comprised primarily rods, cones, HCs, and amacrine cells. Some bipolar cells showed history but Müller glia and RGCs almost never showed any Olig2 history. These results demonstrated that Olig2-expressing RPCs did not behave as totipotent RPCs.

A second method was developed to allow a determination of the types of cells descendant from Olig2-expressing RPCs, at clonal resolution, across time. The classic Cre fating experiment does not provide for temporal resolution, unless used in conjunction with a tamoxifen-regulated allele of Cre, which was not available in this case. The Cre fate mapping method also does not readily provide clonal resolution. Importantly, cells that do not derive from Olig2-expressing RPCs, but rather from expression of Olig2 in postmitotic cells, are mixed in with those cells produced by Olig2-expressing RPCs. As we wished to examine only those cells produced by Olig2-expressing RPCs, and to do so with temporal and clonal resolution, we developed a retroviral marking strategy to accomplish this goal

(Beier et al. 2011). This method relies upon expression of the avian retroviral receptor, TVA (Bates et al. 1998).

The TVA receptor is not normally expressed in mammals. However, one can direct expression of TVA to Cre-expressing cells by crossing a Cre line to a floxed TVA line (Beier et al. 2011). Alternatively, one can make a TVA knock-in or transgenic line. Schuller et al. had made a knock-in of both Cre and TVA into the Olig2 locus (Schuller et al. 2008) and had generously given this line to us for our experiments. Infection of TVA-expressing cells can be accomplished using retroviruses that carry on their surface the avian EnvA glycoprotein. By using gamma retroviruses for this analysis, only clones deriving from mitotic cells that express TVA would be produced, as gamma retroviruses are not able to integrate their DNA into postmitotic cells and thus are unable to initiate expression in such cells (Roe et al. 1993). If a gamma retrovirus integrates its genome into a host cell that then exits mitosis, a one-cell clone will be formed. If the integrated cell divides to make two postmitotic daughters, a two-cell clone is formed, etc. We thus infected the Olig2-Cre-IRES-TVA line of mice with a retroviral genome with an EnvA glycoprotein, or used the same virus with a promiscuous glycoprotein that allows infection of any type of RPC, as a control. The clone sizes and compositions from infection at several different ages were then compared.

The clonal analysis showed the same trends as the Cre fate mapping experiment but revealed a significant skew in terms of both the size and composition of clones from infection of Olig2-expressing RPCs (Hafler et al. 2012). First, the clones generated by Olig2-expressing RPCs were only 1 or 2 cells, indicating that these RPCs were terminally dividing RPCs. In contrast, the average clone size, for example, for the control group of clones infected by a retrovirus that could infect any RPC, was 32 cells/clone (range, 1–234 cells). Second, there was a striking specificity in the types of cells produced by infection at different times. When infections of Olig2-expressing RPCs were performed at E14.5, only cones and HCs were marked. They comprised single-cell clones of either cell type, or 2-cell clones of 2 cones, 2 HCs, or 1 cone and 1 HC. In contrast, when infections were performed at P0, almost every clone was a single rod, with a few clones comprising an amacrine cell, and a very few 2-cell clones comprising either 2 rods or a rod and an amacrine cell. If marking was initiated at P3, the clones were almost entirely a single rod, although a few single amacrine cells and single bipolar cells were marked as well. Statistical analyses indicated an extreme skew in these data from each infection time point, relative to the cells expected based upon birthdates and the cell types in the control set of clones. Similarly, there was an extreme skew toward small clone size. Results from tamoxifen-regulated Cre fate mapping experiments using history of Ngn2 or Ascl1 also showed an interesting skew in rods and cones (Brzezinski et al. 2011). When marking was initiated at E12.5–E13.5, small “clumps” of cells were labeled, with rods marked preferentially by Ascl1 history, and cones and HCs marked preferentially with Ngn2 history.

The clones produced by Olig2-expressing embryonic RPCs are reminiscent of the clones marked in a study of the chick retina using retroviruses (Rompani and Cepko 2008) and in a study using live imaging of the zebrafish

retina (Godinho et al. 2007). The chick has three types of HCs, referred to as HC1, HC2, and HC3, which have different patterns of connectivity to rods and cones. Large clones with many types of retinal cells, and all three types of HCs, were observed following marking near the beginning of chick retinal development. Later marking led to the production of clones with many types of cells, but only one or two HCs. Analysis of the combinations of the types of HCs within clones with two HCs revealed a nonrandom distribution in the types of HCs. Clones with only two HCs had homotypic pairs, either two H1 cells or two H3 cells. No pairs of only H2 cells were seen. Moreover, the clones that had only a single HC were skewed toward H2. When the numbers of each type of HC seen in clones with larger numbers of HC were analyzed, there was a skew toward even numbers of H1 or H3, but not H2. We interpret these data to mean that there is a specific and restricted RPC that divides once to make a pair of H1 cells, and a different RPC that divides once to make a pair of H3 cells. The preponderance toward even numbers suggests that clones contain multiple RPCs of these restricted types. Interestingly, the RPC that makes an H2 does not make a pair of H2 cells. In consideration of the data from the Olig2-expressing RPCs in mouse, we predict that the sibling of the chick H2 cell is a cone photoreceptor. In zebrafish, live imaging showed nonapical divisions of an RPC that produced only HCs (Godinho et al. 2007). The types of HCs produced were not ascertained in this study.

A recent lineage study used live imaging of zebrafish retinas and a fluorescent reporter based upon the Thrb gene (Suzuki et al. 2013). Suzuki et al. found that this reporter marked RPCs that made a terminal division that resulted in L cones, those that express the long-wavelength opsin. Alternatively, RPCs that express this reporter make a terminal division that produces a pair of HCs. A prior division also was seen in three cases where a retinal ganglion cells was produced. If they used a reporter based upon Crx, homotypic pairs of cones were observed, with live imaging in one case showing that the homotypic pairs of UV or M cones were made in terminal divisions.

The finding of restricted RPCs that make limited divisions and specific cell types, along with the observation of larger clones with many cell types, has suggested that the vertebrate retina uses the same strategy as the ventral nerve cord of *Drosophila* (Baumgardt et al. 2007; Pearson and Doe 2004; Zhong 2003) or the medulla of *Drosophila* (Li et al. 2013a, b). We have proposed that the terminally dividing RPCs, of the type identified using the Olig2-TVA line and the zebrafish Thrb reporter, are like the ganglion mother cells (GMCs) of the *Drosophila* ventral nerve cord and medulla. GMCs typically divide only once and make specific types of daughter cells. The types of daughters that are made are different in different segments and different at different times. The temporal order in the nerve cord is reminiscent of the temporal order seen during retinal development. The GMCs are produced by neuroblasts, which make large clones when they are marked, much as we see large clones when we mark with a virus that can mark any RPC. The neuroblasts in each segment rely on a temporal order of TFs to produce their temporal cohorts of GMCs, and even though the same temporal TFs are used in each segment, different types of cells are made in each segment. In the vertebrate

retina, there is also expression of paralogues of these temporal TFs, and some of these genes have similar roles in setting up temporal identity in the vertebrate retina (Blackshaw et al. 2004; Brzezinski et al. 2010; Elliott et al. 2008; Trimarchi et al. 2008b; Katoh et al. 2010).

The foregoing studies demonstrate that different RPCs make different daughter cell types in terminal divisions. In the mouse, the E13.5–E14.5 Olig2-expressing RPCs make cones and HCs whereas the postnatal Olig2-RPCs make rods and amacrine cells. The Thrb-expressing RPCs in zebrafish make HCs or L cones and the Crx-expressing RPCs make homotypic pairs of other cone types. The TFs that are responsible for the production of rods versus cones in such RPCs have recently been discovered, as described next.

9.5 Otx2 and Onecut Genes Direct Formation of Cones Versus Rods

The Thrb gene is an early marker of cones. We thus reasoned that the upstream regulators of Thrb would be informative regarding the genesis of cones. We used electroporation (Matsuda and Cepko 2004) to identify a noncoding region of Thrb ("ThrbCRM1") that directed expression to early cones in the chick and in the mouse. This conserved region was reduced to 40 bp and was found to label early cones, as well as two other cell types. Investigation of these other cell types showed that they were a subset of RPCs, as well as newborn HCs. Reversing the strategy described for the Olig2-RPCs, we used ThrbCRM1 to drive expression of the murine retrovirus receptor, CAT1 (Albritton et al. 1989), in the chick using electroporation. Infecting these electroporated retinas with a retrovirus with the murine glycoprotein gene, gp70, led to the identification of cells produced by the RPCs that expressed ThrbCRM1. The progeny were cones and HCs, providing evidence that, as in the mouse, there is a specific RPC in the chick that makes cones and HCs. The clones comprised almost entirely one cell. Larger clones were not seen, so these RPCs were making terminal divisions.

Investigation of the TFs that could bind and activate the 40-bp ThrbCRM1 led to the identification of Otx2 and Onecut1 (Oc1) as both necessary and sufficient, not only for activation of this enhancer, but also the endogenous Thrb gene in both chick and mouse. Chromatin immunoprecipitation experiments using retinal extracts confirmed that these two proteins bind ThrbCRM1. Otx2 and Oc1 were found to overlap in expression with Olig2, in a subset of RPC cells, as predicted by the activity of ThrbCRM1 in a subset of RPCs, and the previous observations from infection of Olig2-TVA in the mouse.

It was of interest to determine if Otx2 and Oc1 regulate the production of cones, HCs, and potentially, rod photoreceptors. To this end, gain and loss of function experiments were carried out in mice and chicks. Misexpression of Oc1 in the postnatal mouse retina, where Otx2 is expressed, could induce the formation of

immature cones. This induction was dependent on Otx2 as removal of a conditional allele of Otx2 prevented this induction. The induced cones did not progress to fully differentiated cones. We speculate that this might be caused by two factors. One is that expression of the Oc1 gene is normally reduced as cones mature, and the misexpression construct was constitutive. Second, a gene such as Sall3 might need to be upregulated at the proper time and proper level, and misexpression of Oc1 did not lead to this. Interestingly, in addition to induction of cones, cells with markers of HCs were produced from introduction of Oc1 into the postnatal mouse retina.

Two methods were used to examine the necessity of Oc1. Electroporation of the chick retina with a construct in which the Engrailed repressor domain was fused to Oc1 led to a reduction in Thrb reporter expression. This construct also led to an upregulation of MafA, the chick homologue of Nrl (Ochi et al. 2004), and to expression of a rhodopsin promoter construct. These findings suggest an induction of rod genesis. In embryonic Oc1 KO mice, a reduction in Thrb RNA and an upregulation in Nrl RNA were seen. These data all point to a role of the Oc1 gene in regulating the rod versus cone decision. These data suggest a revised model for rod and cone genesis (Fig. 9.3b).

9.6 Model of Rod Versus Cone Determination

In keeping with the notion of GMCs and neuroblasts in retinal development, we propose that there are specific GMCs that produce rods, cones, HCs, and likely other retinal cell types as well (Fig. 9.4). At least some of the types of GMCs that produce cones also produce HCs, and the GMCs that make cones are not the same ones that make rods. Rods are proposed to be produced by multiple types of GMCs. We propose multiple GMCs for rod production for two reasons. One reason is the nature of two-cell clones that are produced by viral infection in the postnatal period of rats and mice (Turner and Cepko 1987; Turner et al. 1990). Here, we see clones in which there are two rods, a rod and amacrine cell, a rod and a bipolar cell, or a rod and a Müller glial cell. Although one could model these clone types as deriving from a single type of rod GMC with competence to make all four cell types (Gomes et al. 2011), the Olig2 lineage data argue against this idea. Olig2-expressing RPCs in the postnatal period make either two rods or a rod and amacrine cell, whereas Olig2-negative RPCs make rod and bipolar and rod and Müller glial clones. Interestingly, the newly postmitotic daughter cells that would normally take on the non-rod fates still rely on Notch1 (Mizeracka et al. 2013a) and Numb (Kechad et al. 2012) to escape the rod fate. This finding argues against GMCs passing on irreversible fate decisions to their daughters. Rather, it is likely that determinants are passed to daughter cells from GMCs (Kechad et al. 2012), and although different determinants are passed from different GMCs, the newly postmitotic daughter cells must work out their fate using at least some other cue, such as the Notch signal, to adopt the proper fate. Because deletion of the Notch gene in the newly postmitotic cell prevented the acquisition of the non-rod fate, Notch

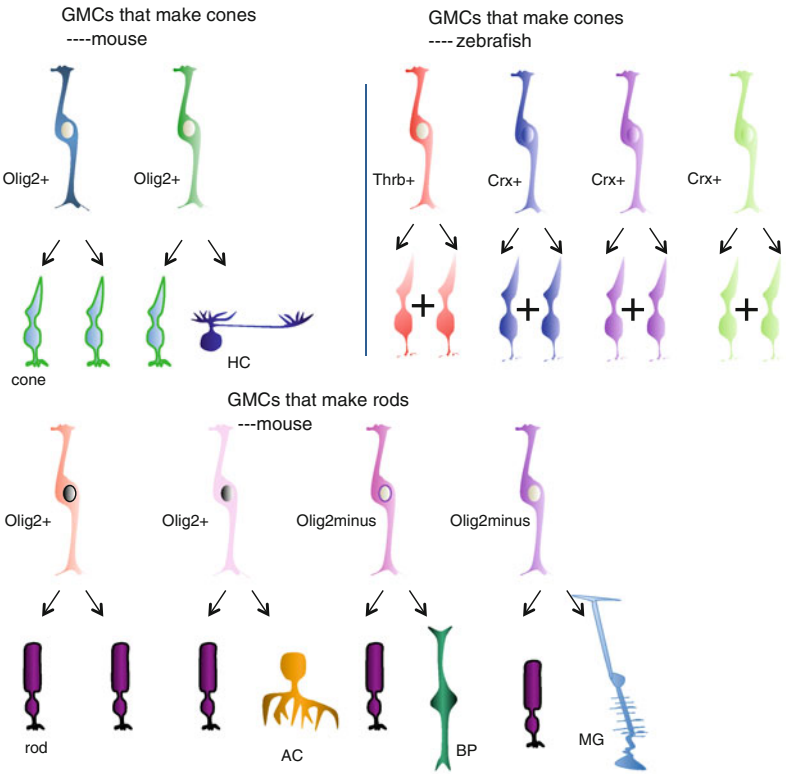

Fig. 9.4 Distinct RPCs that make terminal divisions to produce at least one rod or one cone, based upon marker expression and lineage tracing experiments, are shown (Emerson and Cepko 2011; Hafler et al. 2012; Rompani and Cepko 2008; Turner and Cepko 1987; Turner et al. 1990); (Suzuki et al. 2013). These are modeled to behave as GMCs similar to those seen in *Drosophila*. Three types of two cell clones are produced at E13.5–E14.5 in the mouse from Olig2-expressing GMCs. At least two of these clone types include a cone, as shown, and one type produces two HCs (not shown). The GMC that produces only cones likely uses the Otx2ECR2 to regulate Otx2 (Emerson and Cepko 2011). In zebrafish, a reporter based upon Thrb makes a pair of L cones from a terminal division (Suzuki et al. 2013). Other Thrb-expressing RPCs divide to make a pair of HCs (not shown). Crx-expressing RPCs produce homotypic pairs with respect to cone opsin type, with live imaging showing that these are likely the result of terminal divisions, as shown. GMCs that produce rods, but not cones, exist later in development in mice. They do not express Oc1 or Thrb and some of them express Olig2. At least some of those that make only rods express Otx2ECR2. *HC*, horizontal cell; *BP*, bipolar cell; *AC*, amacrine cell; *MG*, Müller glial cell

transcription and translation in the newly postmitotic cell are needed to generate Notch signals (Mizeracka et al. 2013a). This Notch dependence has also been seen in the *Drosophila* ventral nerve cord in the newly postmitotic daughters of the GMCs (Spana and Doe 1996) as well as in vertebrate neurons that are produced as asymmetrical pairs (Del Barrio et al. 2007). Regarding the rod versus cone fate, the

network operating in cone GMCs includes, at least in part, Otx2 and Oc1. The daughters of these GMCs then differentially regulate the Oc1 and Otx2 genes, as HCs upregulate Oc genes (Wu et al. 2012) and downregulate Otx2, and cones do the opposite. Additional levels of regulation downstream then can lead to photoreceptors with rod gene expression, as interference with Oc1 function leads to cells with Nrl or MafA, and thus likely will become rods. Because Nrl was upregulated following introduction of an Oc1-EnR allele, it is likely that Oc1 positively regulates a repressor of Nrl expression.

It is worth considering the phenotypes of the KO mice for Nrl, Nr1f2, and Nr2e3 in terms of the foregoing model. The fact that rods are proposed to be produced by RPCs that are distinct from the GMCs that make cones render the idea of a default blue cone fate unlikely. The lack of a blue cone default state is further supported by the lack of an upregulation of the early cone genes in the embryonic Nrl KO retina (Emerson et al. 2013). Rather than being a reflection of a normal developmental state, expression of blue cone genes in the aforementioned KO strains likely results from dysregulation of rod and cone genes in maturing photoreceptors. The blue cone gene expression program may be the program that is set up by expression of the common TFs in photoreceptors, including TFs such as Crx/Otx2 and Nr1f2. The differential expression of specific TFs, such as Nrl and Nr2e3, and their cofactors and regulators, such as Pias3, then defines the specific gene expression programs for rods and the different types of cones. The difference in these models is not merely a semantic one, as the blue cone default model invokes the blue cone fate as the state into which a newly postmitotic cell fated to be a photoreceptor enters, no matter which RPC produces it. Instead, we propose that the newly postmitotic cells fated to be photoreceptors are already instructed by their GMCs to be a cone or a rod, by virtue of differences within the GMCs that make these cells. We have defined the Oc1 gene as one of the critical differences between rod and cone GMCs. There are undoubtedly others, and the networks of which they are part need to be elucidated to understand the actual mechanisms that guide, and then lock, each type of photoreceptor into its final state. These networks must also be understood in terms of the spatial patterning across the retina that govern the frequency of the different types of photoreceptors, as exemplified by the fovea and similar specialized areas in different organisms. The final fate may also be dependent upon chromatin changes to make the changes irreversible.

Finally, it is interesting to note the similarities of the genes discussed herein to those used in the development of *Drosophila* photoreceptors. The discovery of the determination network that defines the eye field in disparate organisms has been well noted and discussed (Fernald 2006; Gehring 2011). In addition to these early genes, the *Drosophila* homologue of Otx/Crx, orthodenticle (Otd) (Vandendries et al. 1996), and the *Drosophila* homologue of Sall3, Spalt (Domingos et al. 2004), are required for *Drosophila* S cone regulation. The *Drosophila* Onecut gene likely also plays a role in photoreceptor differentiation, as it is able to bind to a conserved enhancer upstream of the Rh1 gene, and a dominant negative allele disrupts photoreceptor differentiation (Nguyen et al. 2000). These genes have some common expression patterns, as well as some shared functions in *Drosophila* and

mammals; for example, Crx and Otd are functionally similar in developing photoreceptors (Ranade et al. 2008; Tahayato et al. 2003). However, we are now proposing that the strategy used by the vertebrate retina for the production of the diverse set of cell types does not follow that of the *Drosophila* retina but rather follows that of the *Drosophila* ventral nerve cord and medulla (Pearson and Doe 2004). The terminally dividing RPCs have the same properties as GMCs, and there is support for both expression and function for the homologues of the *Drosophila* temporal TFs (Elliott et al. 2008). The strategy used for diversification of cells during development may be an overarching theme in many tissues, with specific cohorts of TFs acting to specify and differentiate the distinct cell types in tissues. Photoreceptors may have enough deep homology that they use some of the same TFs used by *Drosophila* photoreceptors for development, evolving different roles over time.

Finally, in keeping with the ventral nerve cord and medulla strategy, it may be that there are lineages in the vertebrate retina that are distinct; that is, specific and distinct RPCs may be upstream of specific GMCs. There is some evidence for this, in that RPCs that express Cad6 preferentially make RGCs that express Cad6, as well as other retinal cell types (De la Huerta et al. 2012). This observation could mean that there are some limited types of daughter cells produced by Cad6-expressing RPCs, or perhaps there is one division that produces a Cad6 RGC, with a sibling being a more generic type of RPC. Similarly, analysis of clones with many HCs in large chick clones shows biases toward the types of HCs that they contain, perhaps implying differences among very early RPCs toward one type of HC (Rompani and Cepko, unpublished data). Future lineage and molecular studies are needed to determine the full set of RPCs and GMCs and the mechanisms that reliably lead to the production of such a beautifully complex tissue.

References

Akagi T, Inoue T, Miyoshi G, Bessho Y, Takahashi M, Lee JE, Guillemot F, Kageyama R (2004) Requirement of multiple basic helix-loop-helix genes for retinal neuronal subtype specification. J Biol Chem 279(27):28492–28498. doi:10.1074/jbc.M400871200

Akhmedov NB, Piriev NI, Chang B, Rapoport AL, Hawes NL, Nishina PM, Nusinowitz S, Heckenlively JR, Roderick TH, Kozak CA, Danciger M, Davisson MT, Farber DB (2000) A deletion in a photoreceptor-specific nuclear receptor mRNA causes retinal degeneration in the rd7 mouse. Proc Natl Acad Sci USA 97(10):5551–5556

Akimoto M, Cheng H, Zhu D, Brzezinski JA, Khanna R, Filippova E, Oh EC, Jing Y, Linares JL, Brooks M, Zareparsi S, Mears AJ, Hero A, Glaser T, Swaroop A (2006) Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. Proc Natl Acad Sci USA 103(10):3890–3895. doi:10.1073/pnas.0508214103

Albritton LM, Tseng L, Scadden D, Cunningham JM (1989) A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell 57(4):659–666

Allison WT, Dann SG, Veldhoen KM, Hawryshyn CW (2006) Degeneration and regeneration of ultraviolet cone photoreceptors during development in rainbow trout. J Comp Neurol 499 (5):702–715. doi:10.1002/cne.21164

Altshuler D, Lo Turco JJ, Cepko C (1991) Specification of cell type in the vertebrate retina. In: Lam M-K, Shatz C (eds) Development of the visual system. MIT Press, Cambridge, pp 37–58

Bailey TJ, El-Hodiri H, Zhang L, Shah R, Mathers PH, Jamrich M (2004) Regulation of vertebrate eye development by Rx genes. Int J Dev Biol 48(8-9):761–770. doi:10.1387/ijdb.041878tb

Bao ZZ, Cepko CL (1997) The expression and function of Notch pathway genes in the developing rat eye. J Neurosci 17(4):1425–1434

Bates P, Rong L, Varmus HE, Young JA, Crittenden LB (1998) Genetic mapping of the cloned subgroup A avian sarcoma and leukosis virus receptor gene to the TVA locus. J Virol 72(3):2505–2508

Baumgardt M, Miguel-Aliaga I, Karlsson D, Ekman H, Thor S (2007) Specification of neuronal identities by feedforward combinatorial coding. PLoS Biol 5(2):e37. doi:10.1371/journal.pbio.0050037

Beier KT, Samson ME, Matsuda T, Cepko CL (2011) Conditional expression of the TVA receptor allows clonal analysis of descendents from Cre-expressing progenitor cells. Dev Biol 353 (2):309–320. doi:10.1016/j.ydbio.2011.03.004

Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H (1990) The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell 61(1):49–59

Blackshaw S, Fraioli RE, Furukawa T, Cepko CL (2001) Comprehensive analysis of photoreceptor gene expression and the identification of candidate retinal disease genes. Cell 107 (5):579–589

Blackshaw S, Harpavat S, Trimarchi J, Cai L, Huang H, Kuo WP, Weber G, Lee K, Fraioli RE, Cho SH, Yung R, Asch E, Ohno-Machado L, Wong WH, Cepko CL (2004) Genomic analysis of mouse retinal development. PLoS Biol 2(9):E247

Bruhn SL, Cepko CL (1996) Development of the pattern of photoreceptors in the chick retina. J Neurosci 16(4):1430–1439

Brzezinski JA, Lamba DA, Reh TA (2010) Blimp1 controls photoreceptor versus bipolar cell fate choice during retinal development. Development (Camb) 137(4):619–629. doi:10.1242/dev.043968

Brzezinski JA, Kim EJ, Johnson JE, Reh TA (2011) Ascl1 expression defines a subpopulation of lineage-restricted progenitors in the mammalian retina. Development (Camb) 138 (16):3519–3531. doi:10.1242/dev.064006

Cai L, Morrow EM, Cepko CL (2000) Misexpression of basic helix-loop-helix genes in the murine cerebral cortex affects cell fate choices and neuronal survival. Development (Camb) 127(14):3021–3030

Carter-Dawson LD, LaVail MM (1979) Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. J Comp Neurol 188(2):263–272. doi:10.1002/cne.901880205

Chen CM, Cepko CL (2002) The chicken RaxL gene plays a role in the initiation of photoreceptor differentiation. Development (Camb) 129(23):5363–5375

Chen S, Wang QL, Nie Z, Sun H, Lennon G, Copeland NG, Gilbert DJ, Jenkins NA, Zack DJ (1997) Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron 19(5):1017–1030

Chen J, Rattner A, Nathans J (2005) The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes. J Neurosci 25(1):118–129. doi:10.1523/JNEUROSCI.3571-04.2005

Cheng CL, Flamarique IN (2007) Chromatic organization of cone photoreceptors in the retina of rainbow trout: single cones irreversibly switch from UV (SWS1) to blue (SWS2) light sensitive opsin during natural development. J Exp Biol 210(pt 23):4123–4135. doi:10.1242/jeb.009217

Cheng CL, Gan KJ, Flamarique IN (2009) Thyroid hormone induces a time-dependent opsin switch in the retina of salmonid fishes. Invest Ophthalmol Vis Sci 50(6):3024–3032. doi:10.1167/iovs.08-2713

Cherry TJ, Trimarchi JM, Stadler MB, Cepko CL (2009) Development and diversification of retinal amacrine interneurons at single cell resolution. Proc Natl Acad Sci USA 106 (23):9495–9500. doi:10.1073/pnas.0903264106

Cherry TJ, Wang S, Bormuth I, Schwab M, Olson J, Cepko CL (2011) NeuroD factors regulate cell fate and neurite stratification in the developing retina. J Neurosci 31(20):7365–7379. doi:10.1523/JNEUROSCI.2555-10.2011

Corbo JC, Cepko CL (2005) A hybrid photoreceptor expressing both rod and cone genes in a mouse model of enhanced S-cone syndrome. PLoS Genet 1(2):e11. doi:10.1371/journal.pgen.0010011

Corbo JC, Myers CA, Lawrence KA, Jadhav AP, Cepko CL (2007) A typology of photoreceptor gene expression patterns in the mouse. Proc Natl Acad Sci USA 104(29):12069–12074. doi:10.1073/pnas.0705465104

Corbo JC, Lawrence KA, Karlstetter M, Myers CA, Abdelaziz M, Dirkes W, Weigelt K, Seifert M, Benes V, Fritsche LG, Weber BH, Langmann T (2010) CRX ChIP-seq reveals the cis-regulatory architecture of mouse photoreceptors. Genome Res 20(11):1512–1525. doi:10.1101/gr.109405.110

Daniele LL, Lillo C, Lyubarsky AL, Nikonov SS, Philp N, Mears AJ, Swaroop A, Williams DS, Pugh EN Jr (2005) Cone-like morphological, molecular, and electrophysiological features of the photoreceptors of the Nrl knockout mouse. Invest Ophthalmol Vis Sci 46(6):2156–2167. doi:10.1167/iovs.04-1427

Davis RL, Turner DL (2001) Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning. Oncogene 20(58):8342–8357. doi:10.1038/sj.onc.1205094

De la Huerta I, Kim IJ, Voinescu PE, Sanes JR (2012) Direction-selective retinal ganglion cells arise from molecularly specified multipotential progenitors. Proc Natl Acad Sci USA 109(43):17663–17668. doi:10.1073/pnas.1215806109

de Melo J, Peng GH, Chen S, Blackshaw S (2011) The Spalt family transcription factor Sall3 regulates the development of cone photoreceptors and retinal horizontal interneurons. Development (Camb) 138(11):2325–2336. doi:10.1242/dev.061846

Del Barrio MG, Taveira-Marques R, Muroyama Y, Yuk DI, Li S, Wines-Samuelson M, Shen J, Smith HK, Xiang M, Rowitch D, Richardson WD (2007) A regulatory network involving Foxn4, Mash1 and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool. Development (Camb) 134(19):3427–3436. doi:10.1242/dev.005868

Dessaud E, Yang LL, Hill K, Cox B, Ulloa F, Ribeiro A, Mynett A, Novitch BG, Briscoe J (2007) Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. Nature (Lond) 450(7170):717–720. doi:10.1038/nature06347

Domingos PM, Brown S, Barrio R, Ratnakumar K, Frankfort BJ, Mardon G, Steller H, Mollereau B (2004) Regulation of R7 and R8 differentiation by the spalt genes. Dev Biol 273(1):121–133. doi:10.1016/j.ydbio.2004.05.026

Elliott J, Jolicoeur C, Ramamurthy V, Cayouette M (2008) Ikaros confers early temporal competence to mouse retinal progenitor cells. Neuron 60(1):26–39. doi:10.1016/j.neuron.2008.08.008

Emerson MM, Cepko CL (2011) Identification of a retina-specific Otx2 enhancer element active in immature developing photoreceptors. Dev Biol 360(1):241–255. doi:10.1016/j.ydbio.2011.09.012

Emerson MM, Surzenko N, Goetz JJ, Trimarchi J, Cepko CL (2013) The Otx2 and Onecut factors promote cone photoreceptor and horizontal cell genesis over rod photoreceptors. Dev Cell 154(4):928–939

Fernald RD (2006) Casting a genetic light on the evolution of eyes. Science 313 (5795):1914–1918. doi:10.1126/science.1127889

Forrest D, Swaroop A (2012) Minireview: the role of nuclear receptors in photoreceptor differentiation and disease. Mol Endocrinol 26(6):905–915. doi:10.1210/me.2012-1010

Fujieda H, Bremner R, Mears AJ, Sasaki H (2009) Retinoic acid receptor-related orphan receptor alpha regulates a subset of cone genes during mouse retinal development. J Neurochem 108 (1):91–101. doi:10.1111/j.1471-4159.2008.05739.x

Furukawa T, Kozak CA, Cepko CL (1997a) rax, a novel paired-type homeobox gene, shows expression in the anterior neural fold and developing retina. Proc Natl Acad Sci USA 94(7):3088–3093

Furukawa T, Morrow EM, Cepko CL (1997b) Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell 91(4):531–541

Furukawa T, Morrow EM, Li T, Davis FC, Cepko CL (1999) Retinopathy and attenuated circadian entrainment in Crx-deficient mice. Nat Genet 23(4):466–470. doi:10.1038/70591

Furukawa T, Mukherjee S, Bao ZZ, Morrow EM, Cepko CL (2000) rax, Hes1, and notch1 promote the formation of Müller glia by postnatal retinal progenitor cells. Neuron 26(2):383–394

Gehring WJ (2011) Chance and necessity in eye evolution. Genome Biol Evol 3:1053–1066. doi:10.1093/gbe/evr061

Godinho L, Williams PR, Claassen Y, Provost E, Leach SD, Kamermans M, Wong RO (2007) Nonapical symmetric divisions underlie horizontal cell layer formation in the developing retina in vivo. Neuron 56(4):597–603. doi:10.1016/j.neuron.2007.09.036

Gomes FL, Zhang G, Carbonell F, Correa JA, Harris WA, Simons BD, Cayouette M (2011) Reconstruction of rat retinal progenitor cell lineages in vitro reveals a surprising degree of stochasticity in cell fate decisions. Development (Camb) 138(2):227–235. doi:10.1242/dev.059683

Hafler BP, Surzenko N, Beier KT, Punzo C, Trimarchi JM, Kong JH, Cepko CL (2012) Transcription factor Olig2 defines subpopulations of retinal progenitor cells biased toward specific cell fates. Proc Natl Acad Sci USA 109(20):7882–7887. doi:10.1073/pnas.1203138109

Haider NB, Jacobson SG, Cideciyan AV, Swiderski R, Streb LM, Searby C, Beck G, Hockey R, Hanna DB, Gorman S, Duhl D, Carmi R, Bennett J, Weleber RG, Fishman GA, Wright AF, Stone EM, Sheffield VC (2000) Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. Nat Genet 24(2):127–131. doi:10.1038/72777

Haider NB, Naggert JK, Nishina PM (2001) Excess cone cell proliferation due to lack of a functional NR2E3 causes retinal dysplasia and degeneration in rd7/rd7 mice. Hum Mol Genet 10(16):1619–1626

Haider NB, Mollema N, Gaule M, Yuan Y, Sachs AJ, Nystuen AM, Naggert JK, Nishina PM (2009) Nr2e3-directed transcriptional regulation of genes involved in photoreceptor development and cell-type specific phototransduction. Exp Eye Res 89(3):365–372. doi:10.1016/j.exer.2009.04.006

Hao H, Kim DS, Klocke B, Johnson KR, Cui K, Gotoh N, Zang C, Gregorski J, Gieser L, Peng W, Fann Y, Seifert M, Zhao K, Swaroop A (2012) Transcriptional regulation of rod photoreceptor homeostasis revealed by in vivo NRL targetome analysis. PLoS Genet 8(4):e1002649. doi:10.1371/journal.pgen.1002649

Hatakeyama J, Kageyama R (2004) Retinal cell fate determination and bHLH factors. Semin Cell Dev Biol 15(1):83–89. doi:10.1016/j.semcdb.2003.09.005

Hatakeyama J, Tomita K, Inoue T, Kageyama R (2001) Roles of homeobox and bHLH genes in specification of a retinal cell type. Development (Camb) 128(8):1313–1322

Hernandez J, Matter-Sadzinski L, Skowronska-Krawczyk D, Chiodini F, Alliod C, Ballivet M, Matter JM (2007) Highly conserved sequences mediate the dynamic interplay of basic helix-loop-helix proteins regulating retinogenesis. J Biol Chem 282(52):37894–37905. doi:10.1074/jbc.M703616200

Hobert O (2011) Regulation of terminal differentiation programs in the nervous system. Annu Rev Cell Dev Biol 27:681–696. doi:10.1146/annurev-cellbio-092910-154226

Holt CE, Bertsch TW, Ellis HM, Harris WA (1988) Cellular determination in the *Xenopus* retina is independent of lineage and birth date. Neuron 1(1):15–26

Hsiau TH, Diaconu C, Myers CA, Lee J, Cepko CL, Corbo JC (2007) The cis-regulatory logic of the mammalian photoreceptor transcriptional network. PloS One 2(7):e643. doi:10.1371/journal.pone.0000643

Hutcheson DA, Hanson MI, Moore KB, Le TT, Brown NL, Vetter ML (2005) bHLH-dependent and -independent modes of Ath5 gene regulation during retinal development. Development (Camb) 132(4):829–839. doi:10.1242/dev.01653

Jadhav AP, Mason HA, Cepko CL (2006) Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development (Camb) 133(5):913–923. doi:10.1242/dev.02245

Jia L, Oh EC, Ng L, Srinivas M, Brooks M, Swaroop A, Forrest D (2009) Retinoid-related orphan nuclear receptor RORbeta is an early-acting factor in rod photoreceptor development. Proc Natl Acad Sci USA 106(41):17534–17539. doi:10.1073/pnas.0902425106

Kanekar S, Perron M, Dorsky R, Harris WA, Jan LY, Jan YN, Vetter ML (1997) Xath5 participates in a network of bHLH genes in the developing *Xenopus* retina. Neuron 19 (5):981–994

Katoh K, Omori Y, Onishi A, Sato S, Kondo M, Furukawa T (2010) Blimp1 suppresses Chx10 expression in differentiating retinal photoreceptor precursors to ensure proper photoreceptor development. J Neurosci 30(19):6515–6526. doi:10.1523/JNEUROSCI.0771-10.2010

Kautzmann M-AI, Kim DS, Felder-Schmittbuhl M-P, Swaroop A (2011) Combinatorial regulation of photoreceptor differentiation factor, neural retina leucine zipper gene NRL, revealed by in vivo promoter analysis. J Biol Chem 286(32):28247–28255. doi:10.1074/jbc.M111.257246

Kechad A, Jolicoeur C, Tufford A, Mattar P, Chow RW, Harris WA, Cayouette M (2012) Numb is required for the production of terminal asymmetric cell divisions in the developing mouse retina. J Neurosci 32(48):17197–17210. doi:10.1523/JNEUROSCI.4127-12.2012

Kim DS, Ross SE, Trimarchi JM, Aach J, Greenberg ME, Cepko CL (2008) Identification of molecular markers of bipolar cells in the murine retina. J Comp Neurol 507(5):1795–1810. doi:10.1002/cne.21639

Kimura A, Singh D, Wawrousek EF, Kikuchi M, Nakamura M, Shinohara T (2000) Both PCE-1/RX and OTX/CRX interactions are necessary for photoreceptor-specific gene expression. J Biol Chem 275(2):1152–1160

Koike C, Nishida A, Ueno S, Saito H, Sanuki R, Sato S, Furukawa A, Aizawa S, Matsuo I, Suzuki N, Kondo M, Furukawa T (2007) Functional roles of Otx2 transcription factor in postnatal mouse retinal development. Mol Cell Biol 27(23):8318–8329. doi:10.1128/MCB.01209-07

Li S, Mo Z, Yang X, Price SM, Shen MM, Xiang M (2004) Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. Neuron 43(6):795–807. doi:10.1016/j.neuron.2004.08.041

Li X, Chen Z, Desplan C (2013a) Temporal patterning of neural progenitors in *Drosophila*. Curr Top Dev Biol 105:69–96. doi:10.1016/B978-0-12-396968-2.00003-8

Li X, Erclik T, Bertet C, Chen Z, Voutev R, Venkatesh S, Morante J, Celik A, Desplan C (2013b) Temporal patterning of *Drosophila* medulla neuroblasts controls neural fates. Nature (Lond) 498(7455):456–462. doi:10.1038/nature12319

Livesey FJ, Furukawa T, Steffen MA, Church GM, Cepko CL (2000) Microarray analysis of the transcriptional network controlled by the photoreceptor homeobox gene Crx. Curr Biol 10(6):301–310

Luo H, Jin K, Xie Z, Qiu F, Li S, Zou M, Cai L, Hozumi K, Shima DT, Xiang M (2012) Forkhead box N4 (Foxn4) activates Dll4-Notch signaling to suppress photoreceptor cell fates of early retinal progenitors. Proc Natl Acad Sci USA 109(9):E553–562. doi:10.1073/pnas.1115767109

Mathers PH, Grinberg A, Mahon KA, Jamrich M (1997) The Rx homeobox gene is essential for vertebrate eye development. Nature 387(6633):603–607. doi:10.1038/42475

Matsuda T, Cepko CL (2004) Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc Natl Acad Sci USA 101(1):16–22

Mears AJ, Kondo M, Swain PK, Takada Y, Bush RA, Saunders TL, Sieving PA, Swaroop A (2001) Nrl is required for rod photoreceptor development. Nat Genet 29(4):447–452. doi:10.1038/ng774

Mizeracka K, Demaso CR, Cepko CL (2013a) Notch1 is required in newly postmitotic cells to inhibit the rod photoreceptor fate. Development (Camb). doi:10.1242/dev.090696

Mizeracka K, Trimarchi JM, Stadler MB, Cepko CL (2013b) Analysis of gene expression in wild type and Notch1 mutant retinal cells by single cell profiling. Dev Dyn. doi:10.1002/dvdy.24006

Montana CL, Lawrence KA, Williams NL, Tran NM, Peng GH, Chen S, Corbo JC (2011) Transcriptional regulation of neural retina leucine zipper (Nrl), a photoreceptor cell fate determinant. J Biol Chem 286(42):36921–36931. doi:10.1074/jbc.M111.279026

Morris VB, Shorey CD (1967) An electron microscope study of types of receptor in the chick retina. J Comp Neurol 129(4):313–340. doi:10.1002/cne.901290404

Morrow EM, Furukawa T, Lee JE, Cepko CL (1999) NeuroD regulates multiple functions in the developing neural retina in rodent. Development (Camb) 126(1):23–36

Morrow EM, Furukawa T, Raviola E, Cepko CL (2005) Synaptogenesis and outer segment formation are perturbed in the neural retina of Crx mutant mice. BMC Neurosci 6:5. doi:10.1186/1471-2202-6-5

Muranishi Y, Terada K, Inoue T, Katoh K, Tsujii T, Sanuki R, Kurokawa D, Aizawa S, Tamaki Y, Furukawa T (2011) An essential role for RAX homeoprotein and NOTCH-HES signaling in Otx2 expression in embryonic retinal photoreceptor cell fate determination. J Neurosci 31(46):16792–16807. doi:10.1523/JNEUROSCI.3109-11.2011

Muranishi Y, Terada K, Furukawa T (2012) An essential role for Rax in retina and neuroendocrine system development. Dev Growth Differ 54(3):341–348. doi:10.1111/j.1440-169X.2012.01337.x

Nakamura K, Harada C, Namekata K, Harada T (2006) Expression of olig2 in retinal progenitor cells. Neuroreport 17(4):345–349. doi:10.1097/01.wnr.0000203352.44998.6b

Nelson BR, Reh TA (2008) Relationship between Delta-like and proneural bHLH genes during chick retinal development. Dev Dyn 237(6):1565–1580. doi:10.1002/dvdy.21550

Nelson BR, Gumuscu B, Hartman BH, Reh TA (2006) Notch activity is downregulated just prior to retinal ganglion cell differentiation. Dev Neurosci 28(1-2):128–141. doi:10.1159/000090759

Nelson BR, Hartman BH, Georgi SA, Lan MS, Reh TA (2007) Transient inactivation of Notch signaling synchronizes differentiation of neural progenitor cells. Dev Biol 304(2):479–498. doi:10.1016/j.ydbio.2007.01.001

Nelson SM, Park L, Stenkamp DL (2009) Retinal homeobox 1 is required for retinal neurogenesis and photoreceptor differentiation in embryonic zebrafish. Dev Biol 328(1):24–39. doi:10.1016/j.ydbio.2008.12.040

Ng L, Hurley JB, Dierks B, Srinivas M, Salto C, Vennstrom B, Reh TA, Forrest D (2001) A thyroid hormone receptor that is required for the development of green cone photoreceptors. Nat Genet 27(1):94–98. doi:10.1038/83829

Nguyen DN, Rohrbaugh M, Lai Z (2000) The *Drosophila* homolog of Onecut homeodomain proteins is a neural-specific transcriptional activator with a potential role in regulating neural differentiation. Mech Dev 97(1-2):57–72

Nishida A, Furukawa A, Koike C, Tano Y, Aizawa S, Matsuo I, Furukawa T (2003) Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. Nat Neurosci 6(12):1255–1263. doi:10.1038/nn1155

Ochi H, Sakagami K, Ishii A, Morita N, Nishiuchi M, Ogino H, Yasuda K (2004) Temporal expression of L-Maf and RaxL in developing chicken retina are arranged into mosaic pattern. Gene Expr Patterns 4(5):489–494. doi:10.1016/j.modgep.2004.03.005

Ochocinska MJ, Hitchcock PF (2009) NeuroD regulates proliferation of photoreceptor progenitors in the retina of the zebrafish. Mech Dev 126(3-4):128–141. doi:10.1016/j.mod.2008.11.009

Onishi A, Peng GH, Hsu C, Alexis U, Chen S, Blackshaw S (2009) Pias3-dependent SUMOylation directs rod photoreceptor development. Neuron 61(2):234–246. doi:10.1016/j.neuron.2008.12.006

Onishi A, Peng GH, Poth EM, Lee DA, Chen J, Alexis U, de Melo J, Chen S, Blackshaw S (2010) The orphan nuclear hormone receptor ERRbeta controls rod photoreceptor survival. Proc Natl Acad Sci USA 107(25):11579–11584. doi:10.1073/pnas.1000102107

Pearson BJ, Doe CQ (2004) Specification of temporal identity in the developing nervous system. Annu Rev Cell Dev Biol 20:619–647. doi:10.1146/annurev.cellbio.19.111301.115142

Peng GH, Chen S (2005) Chromatin immunoprecipitation identifies photoreceptor transcription factor targets in mouse models of retinal degeneration: new findings and challenges. Vis Neurosci 22(5):575–586. doi:10.1017/S0952523805225063

Ranade SS, Yang-Zhou D, Kong SW, McDonald EC, Cook TA, Pignoni F (2008) Analysis of the Otd-dependent transcriptome supports the evolutionary conservation of CRX/OTX/OTD functions in flies and vertebrates. Dev Biol 315(2):521–534. doi:10.1016/j.ydbio.2007.12.017

Rapaport DH, Wong LL, Wood ED, Yasumura D, LaVail MM (2004) Timing and topography of cell genesis in the rat retina. J Comp Neurol 474(2):304–324

Riesenberg AN, Liu Z, Kopan R, Brown NL (2009) Rbpj cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. J Neurosci 29 (41):12865–12877. doi:10.1523/JNEUROSCI.3382-09.2009

Rivolta C, Berson EL, Dryja TP (2001) Dominant Leber congenital amaurosis, cone-rod degeneration, and retinitis pigmentosa caused by mutant versions of the transcription factor CRX. Hum Mutat 18(6):488–498. doi:10.1002/humu.1226

Roberts MR, Hendrickson A, McGuire CR, Reh TA (2005) Retinoid X receptor (gamma) is necessary to establish the S-opsin gradient in cone photoreceptors of the developing mouse retina. Invest Ophthalmol Vis Sci 46(8):2897–2904. doi:10.1167/iovs.05-0093

Rocha SF, Lopes SS, Gossler A, Henrique D (2009) Dll1 and Dll4 function sequentially in the retina and pV2 domain of the spinal cord to regulate neurogenesis and create cell diversity. Dev Biol 328(1):54–65. doi:10.1016/j.ydbio.2009.01.011

Roe T, Reynolds TC, Yu G, Brown PO (1993) Integration of murine leukemia virus DNA depends on mitosis. EMBO J 12(5):2099–2108

Rompani SB, Cepko CL (2008) Retinal progenitor cells can produce restricted subsets of horizontal cells. Proc Natl Acad Sci USA 105(1):192–197. doi:10.1073/pnas.0709979104

Sato S, Inoue T, Terada K, Matsuo I, Aizawa S, Tano Y, Fujikado T, Furukawa T (2007) Dkk3-Cre BAC transgenic mouse line: a tool for highly efficient gene deletion in retinal progenitor cells. Genesis 45(8):502–507. doi:10.1002/dvg.20318

Schuller U, Heine VM, Mao J, Kho AT, Dillon AK, Han YG, Huillard E, Sun T, Ligon AH, Qian Y, Ma Q, Alvarez-Buylla A, McMahon AP, Rowitch DH, Ligon KL (2008) Acquisition of granule neuron precursor identity is a critical determinant of progenitor cell competence to form Shh-induced medulloblastoma. Cancer Cell 14(2):123–134. doi:10.1016/j.ccr.2008.07.005

Shibasaki K, Takebayashi H, Ikenaka K, Feng L, Gan L (2007) Expression of the basic helix-loop-factor Olig2 in the developing retina: Olig2 as a new marker for retinal progenitors and late-born cells. Gene Expr Patterns 7(1–2):57–65. doi:10.1016/j.modgep.2006.05.008

Sjoberg M, Vennstrom B, Forrest D (1992) Thyroid hormone receptors in chick retinal development: differential expression of mRNAs for alpha and N-terminal variant beta receptors. Development (Camb) 114(1):39–47

Skinner MK, Rawls A, Wilson-Rawls J, Roalson EH (2010) Basic helix-loop-helix transcription factor gene family phylogenetics and nomenclature. Differentiation 80(1):1–8. doi:10.1016/j.diff.2010.02.003

Spana EP, Doe CQ (1996) Numb antagonizes Notch signaling to specify sibling neuron cell fates. Neuron 17(1):21–26

Srinivas M, Ng L, Liu H, Jia L, Forrest D (2006) Activation of the blue opsin gene in cone photoreceptor development by retinoid-related orphan receptor beta. Mol Endocrinol 20(8):1728–1741. doi:10.1210/me.2005-0505

Suzuki SC, Bleckert A, Williams PR, Takechi M, Kawamura S, Wong RO (2013) Cone photoreceptor types in zebrafish are generated by symmetric terminal divisions of dedicated precursors. Proc Natl Acad Sci USA 110(37):15109–15114. doi:10.1073/pnas.1303551110

Swain PK, Hicks D, Mears AJ, Apel IJ, Smith JE, John SK, Hendrickson A, Milam AH, Swaroop A (2001) Multiple phosphorylated isoforms of NRL are expressed in rod photoreceptors. J Biol Chem 276(39):36824–36830. doi:10.1074/jbc.M105855200

Swaroop A, Xu JZ, Pawar H, Jackson A, Skolnick C, Agarwal N (1992) A conserved retina-specific gene encodes a basic motif/leucine zipper domain. Proc Natl Acad Sci USA 89(1):266–270

Swaroop A, Kim D, Forrest D (2010) Transcriptional regulation of photoreceptor development and homeostasis in the mammalian retina. Nat Rev Neurosci 11(8):563–576. doi:10.1038/nrn2880

Tahayato A, Sonneville R, Pichaud F, Wernet MF, Papatsenko D, Beaufils P, Cook T, Desplan C (2003) Otd/Crx, a dual regulator for the specification of ommatidia subtypes in the *Drosophila* retina. Dev Cell 5(3):391–402

Tomita K, Moriyoshi K, Nakanishi S, Guillemot F, Kageyama R (2000) Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. EMBO J 19(20):5460–5472. doi:10.1093/emboj/19.20.5460

Trimarchi JM, Stadler MB, Roska B, Billings N, Sun B, Bartch B, Cepko CL (2007) Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. J Comp Neurol 502(6):1047–1065. doi:10.1002/cne.21368

Trimarchi JM, Harpavat S, Billings NA, Cepko CL (2008a) Thyroid hormone components are expressed in three sequential waves during development of the chick retina. BMC Dev Biol 8:101. doi:10.1186/1471-213X-8-101

Trimarchi JM, Stadler MB, Cepko CL (2008b) Individual retinal progenitor cells display extensive heterogeneity of gene expression. PloS One 3(2):e1588. doi:10.1371/journal.pone.0001588

Turner DL, Cepko CL (1987) A common progenitor for neurons and glia persists in rat retina late in development. Nature (Lond) 328(6126):131–136

Turner DL, Snyder EY, Cepko CL (1990) Lineage-independent determination of cell type in the embryonic mouse retina. Neuron 4(6):833–845

Vandendries ER, Johnson D, Reinke R (1996) orthodenticle is required for photoreceptor cell development in the *Drosophila* eye. Dev Biol 173(1):243–255. doi:10.1006/dbio.1996.0020

Wang JC, Harris WA (2005) The role of combinational coding by homeodomain and bHLH transcription factors in retinal cell fate specification. Dev Biol 285(1):101–115. doi:10.1016/j.ydbio.2005.05.041

Wetts R, Fraser SE (1988) Multipotent precursors can give rise to all major cell types of the frog retina. Science 239(4844):1142–1145

White MA, Myers CA, Corbo JC, Cohen BA (2013) Massively parallel in vivo enhancer assay reveals that highly local features determine the cis-regulatory function of ChIP-seq peaks. Proc Natl Acad Sci USA. doi:10.1073/pnas.1307449110

Wu HY, Perron M, Hollemann T (2009) The role of *Xenopus* Rx-L in photoreceptor cell determination. Dev Biol 327(2):352–365. doi:10.1016/j.ydbio.2008.12.017

Wu F, Sapkota D, Li R, Mu X (2012) Onecut 1 and Onecut 2 are potential regulators of mouse retinal development. J Comp Neurol 520(5):952–969. doi:10.1002/cne.22741

Yan RT, He L, Wang SZ (2009) Pro-photoreceptor activity of chick neurogenin 1. Invest Ophthalmol Vis Sci 50(12):5567–5576. doi:10.1167/iovs.09-3647

Yaron O, Farhy C, Marquardt T, Applebury M, Ashery-Padan R (2006) Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. Development (Camb) 133(7):1367–1378. doi:10.1242/dev.02311

Yoshida S, Mears AJ, Friedman JS, Carter T, He S, Oh E, Jing Y, Farjo R, Fleury G, Barlow C, Hero AO, Swaroop A (2004) Expression profiling of the developing and mature Nrl-/- mouse retina: identification of retinal disease candidates and transcriptional regulatory targets of Nrl. Hum Mol Genet 13(14):1487–1503. doi:10.1093/hmg/ddh160

Young RW (1985) Cell differentiation in the retina of the mouse. Anat Rec 212(2):199–205. doi:10.1002/ar.1092120215

Zhong W (2003) Diversifying neural cells through order of birth and asymmetry of division. Neuron 37(1):11–14

Chapter 10
Cell Polarity in Differentiation and Patterning of Photoreceptors

Jarema J. Malicki

Abstract The vertebrate photoreceptor cell has a unique morphology. Its apical terminus differentiates into a bulky photosensitive structure, the outer segment, while its basal end forms synaptic connections. This highly polarized morphology is essential for photoreceptor function: the apical terminus of the cell collects information, while the basally located synaptic apparatus transmits it to the nervous system. Formation of photoreceptor polarity requires parallel differentiation of several features, such as subcompartments of the cell membrane and the cytoplasm, as well as the positioning of organelles, particularly the nucleus and mitochondria, in specific areas of the cell body. Another form of polarity, planar cell polarity, is seen in the orientation of photoreceptors in the plane of the photoreceptor cell layer. This chapter outlines several key molecular mechanisms that mediate the formation of cell polarity in photoreceptors and are essential for their differentiation, survival, and ultimately their function in visual perception.

Keywords Apicobasal polarity • Membrane compartment • Planar cell polarity

10.1 Introduction

Retinal photoreceptor cells are essential mediators of visual perception in vertebrates. Their cell bodies consist of a linear array of features: the photosensitive outer segment, the inner segment that harbors mitochondria and the bulk of protein synthesis machinery, the cell nucleus region, and the synaptic terminal (Rodieck 1973; Dowling 1987; Kennedy and Malicki 2009) (Fig. 10.1). The outer segment is a unique structure unlike any other found in eukaryotic cells. It is roughly as

J.J. Malicki (✉)
Department of Biomedical Science, MRC Centre for Developmental and Biomedical Genetics, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK
e-mail: j.malicki@sheffield.ac.uk

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_10, © Springer Japan 2014

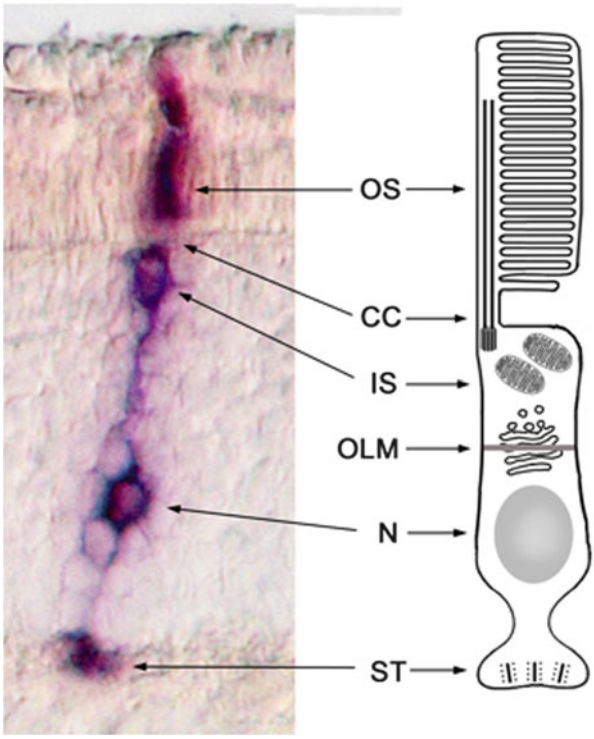

Fig. 10.1 Overview of photoreceptor morphology. *Left-hand panel* shows a section through the mouse retina at postnatal day 25. A single photoreceptor is infected with a retrovirus and visualized via alkaline phosphatase expression. (Image courtesy of Dr. R. Sanuki, Takahisa Furukawa Laboratory.) Schematized view of the photoreceptor cell (*right*). Position of the OLM relative to the nucleus varies for different photoreceptor types. *CC*, connecting cilium; *IS*, inner segment; *N*, cell nucleus region; *OLM*, outer limiting membrane; *OS*, outer segment; *ST*, synaptic terminal

voluminous as the rest of the cell body and consists of hundreds of membrane folds that harbor the visual pigment, opsin, and other components of the phototransduction apparatus (Steinberg et al. 1980; Townes-Anderson et al. 1985).

The photoreceptor outer segment is a wavelength-selective detector of photons that generates changes in membrane potential which convey information about the changes of light intensity. It connects to the rest of the cell body via a narrow constriction that forms around an array of microtubules typical of eukaryotic cilia. On closer inspection, the architecture of this connecting region, the connecting cilium (Fig. 10.1), contains all major structural features of the so-called transition zone, which is present at the base of most, if not all, eukaryotic cilia. The outer segment itself is a uniquely modified cilium that features an exceptionally extensive membrane surface area. Its membranes are constantly removed at the apical terminus and added at the base. The rate of outer segment replacement is remarkable: it is estimated that every day 10% of mouse rod outer segment length is replaced and about 4,500 μm^2 of ciliary membrane is added to *Xenopus* photoreceptors (LaVail 1973; Papermaster et al. 1985).

Because cilia do not contain ribosomes or any other constituents of protein synthesis machinery, all outer segment proteins, including approximately 10^8–10^9 of photopigment molecules are imported from the cell body, which requires that approximately 100–1,000 photopigment molecules are translocated from the cytoplasm every second (Malicki and Besharse 2012). For the photoreceptor to

function, this has to be accompanied by the transport of all other components of the phototransduction apparatus, including cytoplasmic proteins and membrane-embedded ion channels (Pugh and Lamb 2000; Wensel 2008). This torrential protein traffic into the outer segment is the most prominent defining feature of photoreceptor polarity.

In addition to outer segment-directed traffic, three other features define photoreceptor polarity: its epithelial characteristics, most visibly recognizable by the presence of a belt of cell junctions; a morphologically prominent synaptic terminal; and a defined distribution of organelles. The synaptic terminal is also fairly bulky although rather small in comparison to the outer segment. It maintains synaptic connections with a number of interneurons and features unique synapses characterized by the presence of ribbons, which tether vesicles and enable sustained neurotransmitter release (Hallermann and Silver 2013). Outside photoreceptor cells, these so-called ribbon synapses are present only in the inner retina and in auditory hair cells (Safieddine et al. 2012).

Whereas the outer segment, the synaptic terminal, and the membrane subdivision into apical and basolateral domains define apicobasal polarity of the photoreceptor cell, photoreceptors are also polarized within the plane of the cell layer that they form. This second form of polarity bears characteristics of planar cell polarity.

This chapter outlines mechanisms that contribute to several polarity features in vertebrate photoreceptors: the subdivision of cell membrane, the outer segment compartment, and the position of organelles. As planar cell polarity in vertebrate photoreceptors has not been studied in depth, we comment on it only briefly. Some other key mechanisms are not discussed in detail. Although studied in considerable depth, protein trafficking in the photoreceptor cytoplasm and intraflagellar transport are mentioned very briefly only as they are discussed in other chapters (Deretic 2014).

10.2 Apicobasal Polarity of the Photoreceptor Cell

Apicobasal polarity of photoreceptors is established and maintained by a combination of several processes. The most prominent among these are the formation of membrane subcompartments, directional trafficking of proteins and lipids, and the translocation of cellular organelles to specific areas in the cell cytoplasm. The latter process positions the nucleus in the middle of the cell and mitochondria apical to the nucleus and basal to the cilium (Townes-Anderson et al. 1985, 1988).

10.2.1 Membrane Compartments

The photoreceptor cell membrane is grossly subdivided into three compartments: the basolateral surface and two apical compartments, the inner and the outer segments. The first subdivision limits protein diffusion in the plane of the cell membrane; the second also affects cytoplasmic trafficking.

10.2.1.1 Apical and Basolateral Membrane Compartments

Vertebrate and invertebrate photoreceptor cells share an important characteristic with epithelial cells: their surface is subdivided into apical and basolateral domains by a belt of cell junctions (Miller and Dowling 1970; Uga and Smelser 1973; Longley and Ready 1995). In the vertebrate retina, presumably because of its appearance in microscopic preparations, this subdivision has been historically known as the outer limiting membrane (OLM) (Fig. 10.2). It contains cell junctions that connect photoreceptor cells to the apical processes of Müller glia and are easily visible in electron micrographs (Fig. 10.2c) (Miller and Dowling 1970; Uga and Smelser 1973; Kitambi and Malicki 2008). The OLM most likely has at least two functions: it provides structural support and creates a diffusion barrier in the outer retina.

The integrity of cell junctions that subdivide the cell surface and the size of the apical cell membrane domain are regulated by similar mechanisms in photoreceptors and epithelial cells of vertebrates and invertebrates. Genetic analysis performed so far revealed that key players in these processes belong to Crumbs/Stardust/Patj, Par-6/Par-3/aPKC/Cdc42, and Scribble/Dgl/Lgl complexes (St Johnston and Ahringer 2010). Proteins that contribute to these complexes are encoded by loci originally found in genetic screens for regulators of embryonic development in *Caenorhabditis elegans* and *Drosophila*. The *crumbs* and *stardust* genes were identified in the fly because their mutations affect embryonic epithelia and consequently lead to a loss of pattern in the embryonic cuticle (Jurgens et al. 1984; Wieschaus et al. 1984). The Par ("partition defective") genes were found based on their function in the early nematode embryo (Kemphues et al. 1988).

Subsequent vertebrate genetic screens conducted mostly in zebrafish, but also in medaka, identified some of the same genes as regulators of eye development, and thus homologues of fly *crumbs* and *stardust* were found in zebrafish genetic screens as mutants named *oko meduzy*(*ome*) and *nagie oko*(*nok*), respectively (Driever et al. 1996; Malicki et al. 1996). Another zebrafish mutant, *heart and soul* (*has*), was shown to encode atypical protein kinase C (aPKC) (Horne-Badovinac et al. 2001). A Par-6 mutant was also identified in zebrafish at a later time (Munson et al. 2008), and the function of par-3 was investigated in zebrafish using antisense morpholino knockdown (Wei et al. 2004). Remarkably, all mutants and morphants of apicobasal polarity genes feature severe disorganization of retinal neurons, and in all cases where this was investigated, the protein products of these genes localize to the vicinity of cell junctions in the outer limiting membrane of the photoreceptor cell layer (Wei and Malicki 2002; Wei et al. 2004; Omori and Malicki 2006).

How do apicobasal polarity genes function in photoreceptor cells? In fly photoreceptors, as one may expect based on the studies of embryonic tissues, the absence of apicobasal polarity determinants in mutant animals leads to a reduction of the apical membrane size (Izaddoost et al. 2002; Pellikka et al. 2002; Hong et al. 2003). In vertebrates, the analysis of this question is confounded by the fact that apicobasal polarity genes also function at the early stages of eye development in the neuroepithelium from which the retina, including photoreceptors, differentiates

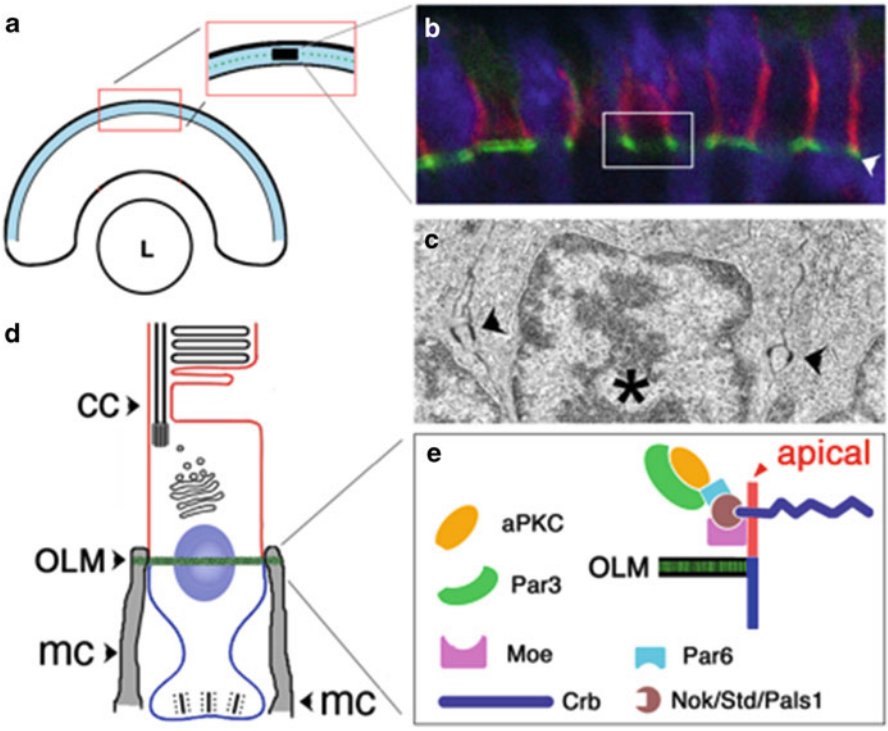

Fig. 10.2 Subdivision of the photoreceptor cell membrane into apical and basolateral compartments by cell junctions of the outer limiting membrane (OLM). (**a**) Schematic representation of the retina. Photoreceptor cell layer is indicated in *blue*. *L*, lens. (**b**) Confocal image of the outer limiting membrane in the retina of larval zebrafish. Cell junctions are visualized by phalloidin staining (*green*; *arrowhead*). The Crumbs polypeptide is detected via antibody staining (*red*). It localizes apical to cell junctions. (Reprinted with permission from Kennedy and Malicki 2009.) *White box* indicates approximate area shown in (**c**). (**c**) Electron micrograph of a section through the photoreceptor cell layer in larval zebrafish. *Arrowheads* indicate cell junctions between photoreceptors and apical termini of Muller glia. Nucleus indicated with *asterisk*. (Reprinted with permission from Kennedy and Malicki 2009.) (**d**) Schematic representation of the photoreceptor cell. Nucleus is highlighted in *blue*. A belt of cell junctions (highlighted in *green*), erroneously called the outer limiting membrane, subdivides the photoreceptor cell surface into the apical (*red*) and basolateral (*blue*) domains. *CC*, connecting cilium; *mc*, Müller glia; *OLM*, outer limiting membrane. (**e**) Schematic representation of the protein complex that regulates the formation of cell junctions in the outer limiting membrane (OLM). The formation of the apical cell membrane domain (*red*) of the photoreceptor cell, and the integrity of the junctional complexes in the OLM, require the function of Crumbs, a transmembrane protein that features a large extracellular domain and a short cytoplasmic tail. Crumbs cytoplasmic moiety binds a MAGUK protein Stardust/Nagie oko and a FERM-domain protein Mosaic eyes (Moe), which also bind each other. Par6, Par3, and aPKC are also thought to contribute to this protein complex. *Crb*, *Crumbs*; *Moe*, *Mosaic eyes*; *Nok*, *Nagie oko*; *Std*, *Stardust*. (Modified with permission from Kennedy and Malicki 2009)

(Malicki and Driever 1999; Yamaguchi et al. 2010). Mutations of genes that encode protein components of Crumbs and Par6 protein complexes compromise the polarity of neuroepithelial cells, leading to a dramatic disorganization of retinal neurons (Malicki and Driever 1999; Pujic and Malicki 2001; Wei and Malicki 2002). Photoreceptors of such mutant retinae no longer form a layer but instead are scattered throughout the retina. Although in most of these mutants photoreceptors are still polarized to some extent (as evidenced by an asymmetrical accumulation of opsin), the apical cell membrane domain is no longer delineated by a belt of cell junctions and thus its size cannot be easily evaluated (Omori and Malicki 2006).

The Role of the Crumbs Complex in Apical Membrane

An exception to the foregoing are *crumbs* genes, five of which are found in the zebrafish genome. It appears that only one of them, *crb2a*/*oko meduzy*, functions at early stages of retinal development. In the eye, its paralogue, *crb2b*, is expressed in photoreceptor cells only and its absence does not result in gross deformities of the neuronal organization, which makes it possible to study its function in the photoreceptor cell apical membrane. Similar to that which has been observed in the fly, *crb2b* knockdown results in a strong reduction of the apical membrane size (Omori and Malicki 2006). *crumbs* overexpression, on the other hand (*crb2a* was used in this experiment), has the opposite effect and produces expansion of the apical membrane domain (Hsu and Jensen 2010). These results recapitulate observations previously made in the fly and demonstrate that *crumbs* genes are essential determinants of the apical membrane size both in vertebrates and in the fly.

Somewhat unexpectedly for genes with such a broad role in cell polarity, in human population mutations in one of *crumbs* homologues, *CRB1*, lead to retinal disorders, including Leber congenital amaurosis (LCA), one of the most severe human inherited retinal dystrophies (den Hollander et al. 1999; Richard et al. 2006b). Phenotypic consequences of human CRB1 mutations are variable and, in the most severe cases, can involve a loss of neuronal lamination in the eye (Jacobson et al. 2003). Although mammalian genomes contain three *crumbs* genes, pathogenic mutations in human CRB2 and CRB3 have not been reported so far, which may be a consequence of early lethality of the mammalian *crumbs* mutations, which has been demonstrated for *Crb2b* in the mouse (Xiao et al. 2011).

The function of *crumbs* genes in vertebrate photoreceptors has been also studied in the mouse. Loss of *Crb1* in the *rd8* mutant mouse (presumptive null) causes limited developmental defects and progressive retinal degeneration (Mehalow et al. 2003). *Crb1*-targeted knockout produces an even weaker phenotype in which the photoreceptor cell layer is initially normal and develops structural aberrations, which may include formation of photoreceptor rosettes, only at around 3 months of age (van de Pavert et al. 2004). Light exposure exacerbates photoreceptor defects in *crumbs* mutant mice and flies alike (Johnson et al. 2002; van de Pavert et al. 2004). Knockout of mouse *Crb2*, on the other hand, affects gastrulation and causes embryonic lethality (Xiao et al. 2011). A conditional knockout of the

same gene in the retina results in an early defect (Alves et al. 2013). In this mutant, junctions of the apical retina are already disrupted during embryogenesis, and a severe disorganization of the outer retina is seen during early postnatal development. This phenotype is still milder than that of zebrafish mutants as the inner plexiform layer of the *Crb2* mutant retina remains fairly intact. The mutant phenotype of the mouse *Crb3* gene has not been reported so far.

The outer limiting membrane may not be the only site of *crumbs* function. Crumbs proteins have been shown to localize to cilia and play a role in ciliogenesis both in tissue culture conditions and in the zebrafish model, (Fan et al. 2004; Omori and Malicki 2006). Until recently, however, subcellular localization studies did not detect Crumbs proteins in photoreceptor connecting cilia (Hsu et al. 2006; Omori and Malicki 2006; van Rossum 2006; Hsu and Jensen 2010). A recent report suggests that Crb3 protein is expressed in the photoreceptor inner segment and colocalizes with acetylated tubulin in the connecting cilium (Herranz-Martín et al. 2012). If confirmed, this finding will provide evidence that *crumbs* contributes to photoreceptor differentiation via multiple mechanisms. One of these mechanisms operates at the junctions of the OLM to determine the apical surface area, whereas another one may function in the connecting cilium, perhaps to regulate ciliary transport. Interestingly, *crumbs* also contributes to photoreceptor morphogenesis by acting as an adhesion molecule: in zebrafish, extracellular domains of Crumbs proteins mediate adhesion between photoreceptors (Zou et al. 2012).

The C-terminal cytoplasmic region of Crumbs interacts directly with several other determinants of apicobasal polarity: Stardust/Nagie oko/Pals1, Par-6, and Mosaic Eyes/Yurt, some of which also bind each other (Fig. 10.2e) (Hong et al. 2001; Hurd et al. 2003; Lemmers et al. 2004). The loss of Stardust or its binding partner *DPATJ* in the fly causes a reduction of the apical membrane domain in photoreceptors (Hong et al. 2003; Richard et al. 2006a). Similarly, a conditional knockout of mouse *pals1*, a *stardust* homologue, causes photoreceptor defects, including disorganization of the photoreceptor cell layer and rosette formation (Park et al. 2011; Cho et al. 2012). Although this is difficult to evaluate in disorganized retinae, the inner and outer segments of photoreceptors appear to be shorter in *Pals1* mutants. Thus, it appears likely that the function of the Crb/Std/DPATJ complex in the determination of the apical membrane size is conserved in fly and vertebrate photoreceptors.

Moe/Yurt and *Medeka/ArhGEF18* Loci

In addition to mutations in the components of the core apical polarity complexes, Crb/Std/Patj, and Par6/Par3/aPKC, genetic screens revealed two additional mutant loci characterized by severe apicobasal polarity phenotypes in the eye: *mosaic eyes* (*moe*) in zebrafish and *medeka* (*med*) in medaka, a Japanese killifish (*Oryzias latipes*). Analysis of both loci provided interesting insights into apicobasal polarity: *moe* has been proposed to encode a negative regulator of *crumbs* function, whereas the protein product of the *med* gene appears to link the core apicobasal polarity

determinants to cytoskeletal rearrangements. Mutations in *moe* and *med* loci produce phenotypes typical for fish apicobasal polarity defects: loss of eye pigmentation caused by degeneration of the retinal pigmented epithelium, and a severe disorganization of the retinal lamination (Jensen et al. 2001; Herder et al. 2013).

The *mosaic eyes* (*moe*) locus encodes a FERM-domain protein that, as already mentioned, closely interacts with the Crb/Std/Patj complex by binding both Crumbs and Stardust/Nagie oko (Fig. 10.2e) (Hsu et al. 2006). In contrast to *crumbs*/*oko meduzy* and *stardust*/*nagie oko*, however, *mosaic eyes* loss of function appears to cause an expansion of the apical membrane in photoreceptors, particularly in the outer segments. A similar phenotype is found in the fly, where in contrast to *crumbs* mutations, defects in a *moe* homologue, *yurt*, cause expansion of the photoreceptor apical (stalk) membrane (Laprise et al. 2006). Moe and Crumbs proteins also display different localization patterns. Crumbs accumulates apically to the outer limiting membrane (OLM), whereas Moe localization in zebrafish photoreceptors is most pronounced basally to OLM, although a weaker Moe signal is also present in the apical region of the cell, especially at early stages of photoreceptor differentiation (Hsu et al. 2006). This pattern is also found in the fly, where Moe and Crumbs expression colocalize apically in the photoreceptor stalk membrane (Laprise et al. 2006). In zebrafish eye embryonic neuroepithelium, the localization of these two proteins is codependent; this is not true in photoreceptors, however, as Crumbs localizes correctly to the apical surface in *moe* mutants. Analysis of the *moe*; *crb* double-mutant phenotype would be informative but is difficult in zebrafish photoreceptors as it requires a laborious procedure of generating mosaic retinae containing doubly mutant cells, which has not been performed so far. Such an analysis in the fly revealed, however, that the *crumbs* phenotype is epistatic to the one seen in *yurt* mutants and suggested that *yurt* acts as a negative regulator of *crumbs* function (Laprise et al. 2006).

Yet another interesting contribution to the understanding of apicobasal polarity came from the studies of the *medeka* locus, which encodes ArhGEF18 (p114RhoGEF), a Rho GEF (guanine nucleotide exchange factor) (Herder et al. 2013). ArhGEF18 interacts physically with Lulu, a FERM-domain protein related to Mosaic eyes/Yurt (Nakajima and Tanoue 2011). It also appears to be phosphorylated by aPKCm, and requires PatJ and Par3 for its junctional localization. Finally, the catalytic activity of ArhGEF18 is upregulated by Lulu (Nakajima and Tanoue 2011). Downstream effects of ArhGEF18 function on the cytoskeleton in the retina appear to be mediated by RhoA and its effector Rock2 (Herder et al. 2013). Interference with RhoA and Rock2 via dominant constructs in the medaka retina causes a mislocalization of actin bundles and m-phase nuclei from the apical surface of retinal neuroepithelium, indicating defective apicobasal polarity (Herder et al. 2013).

The *ArhGEF18* photoreceptor phenotype has not been investigated for reasons of a disorganization of the mutant retina, which is similar to that seen in zebrafish polarity mutants. Although it has been observed that *medeka* (*med*) mutant photoreceptors retain grossly normal morphology in mosaic retinae (Herder et al. 2013), the contribution of *ArhGEF18* and its downstream effectors to the specification of

the apical surface in these cells remains to be investigated. It is quite likely that, similar to *crumbs* and *mosaic eyes*, *ArhGEF18* contributes to apical surface specification. Tissue culture experiments suggested that *ArhGEF18* spatially restricts RhoA activation to drive junction formation (Terry et al. 2011), and this may also be the case in photoreceptor cells. Whether the entire pathway plays a role in photoreceptors is less clear, however, as RhoA effectors appear to differ depending on the tissue context (Herzog et al. 2011). Regardless of the exact nature of its downstream effectors, ArhGEF18 provides an important connection between membrane-associated regulators of apicobasal polarity and the cytoskeleton.

Relationship to Müller Glia

Photoreceptor polarity forms and is maintained in the context of surrounding cells, such as glia and retinal pigment epithelium. Apical termini of Müller glial cells branch in the outer retina and contact photoreceptors (Fig. 10.2c,d). Each glial cell contacts several photoreceptors and forms electron-dense junctions with photoreceptor cell somata (Fig. 10.2c) (Miller and Dowling 1970; Uga and Smelser 1973; Kitambi and Malicki 2008). Additional junctions also form between glial processes and between photoreceptors (Miller and Dowling 1970; Uga and Smelser 1973; Zou et al. 2012). The latter type of junctions is structurally important, as evidenced by the studies of disorganized retinae of N-cadherin mutants. In these animals, photoreceptor cells adhere to each other in the absence of glial processes (Wei et al. 2006).

Although the apical termini of Müller glia express many of the same polarity determinants as photoreceptor cells, the protein composition of photoreceptor–glia junctions is asymmetrical. Crb1, for example, is expressed at a high level in glia and at a low level in photoreceptors of the mouse retina, and MPP4, a Pals1-related MAGUK, is present only at the photoreceptor side of the junctional complex (Kantardzhieva et al. 2005; van Rossum 2006). In contrast to Crb1 and MPP4, Crb2, Crb3, Pals1, and Patj are, however, expressed at approximately equal levels at both sides of photoreceptor–glia cell junctions (van Rossum 2006). As one would expect based on data from other tissues, silencing of *Pals1* in Müller glia results in a loss of Crb localization from the apical termini of Müller glia (van Rossum 2006). These observations suggest that the major determinants of apicobasal polarity function similarly in epithelia and retinal Müller glia. The asymmetry of some components at photoreceptor–glia junctions is, however, noteworthy as it may reflect very different morphological and mechanical characteristics of these cells. Müller glia span the entire thickness of the retina and thus may have to withstand unusually pronounced mechanical strains. Their apical surface is also smaller compared to that of photoreceptor cells, which, again, may increase mechanical strain at their apical cell junctions.

10.2.1.2 The Outer Segment Compartment

The outer segment, a highly modified cilium, is another major membrane compartment of the photoreceptor cell. The most conspicuous indication of its unique protein content is an extremely high concentration of the visual pigment, opsin. The outer segment is estimated to contain 10^8–10^9 of opsin molecules and yet opsin is very efficiently excluded from the rest of the photoreceptor cell membrane (Pugh and Lamb 2000; Calvert et al. 2001; Deretic 2006). It is, in fact, very well documented that ectopic opsin is toxic to photoreceptor cells, and opsin mislocalization results in a rapid photoreceptor loss in human and animal models alike (Sung et al. 1994; Bessant et al. 1999; Tsujikawa and Malicki 2004; Concepcion and Chen 2010). In addition to opsin, many phototransduction proteins are also confined to the outer segment and so are polypeptides that maintain the unique membrane architecture of the outer segment (Kennedy and Malicki 2009).

The Ciliary Transition Zone

Eukaryotic cilia are cell-surface protrusions supported by microtubule-based cytoskeleton (Rosenbaum and Witman 2002). As the outer segment is a uniquely differentiated cilium, its proximal portion displays a typical feature of ciliary architecture: an axoneme that consists of nine evenly spaced microtubule doublets (Fig. 10.3d). This region of the photoreceptor cell is referred to as the connecting cilium. Distal to the connecting cilium, ciliary microtubule doublets gradually spread apart and transform into singlets that run along the inner surface of the outer segment cell membrane (Cohen 1965; Wen et al. 1982). On the ultrastructural level, the diffusion barrier that separates the outer segment compartment from the rest of the photoreceptor cell membrane is very different from that which exists between the apical and basolateral domains. Whereas the latter involves a belt of cell junctions (references above), no such structure is seen at the outer segment base. Instead, the ultrastructural feature that is likely to function as a diffusion barrier is an array of Y-shaped links (also referred to as Y connectors; see arrowheads in Fig. 10.3d) that extend between ciliary microtubules and the cell membrane (Tokuyasu and Yamada 1959; Röhlich 1975; Besharse et al. 1985). These structures are not unique to photoreceptors and are found in the so-called transition zone of cilia in many eukaryotic phyla, including insects, nematodes, and algae (Thurm 1964; Ringo 1967; Gilula and Satir 1972; Röhlich 1975; Williams et al. 2011). They correlate spatially with membrane bead-like features seen at the base of cilia in freeze-fracture experiments and referred to as the ciliary necklace (Gilula and Satir 1972). A particularly prominent ciliary necklace has been described in vertebrate photoreceptors (Fig. 10.3e) (Röhlich 1975; Dentler 1981).

Until recently, the molecular composition of the transition zone was unknown. In the past several years, however, advances in human genetics combined with animal modeling revealed mutations that disrupt transition zone structure, including

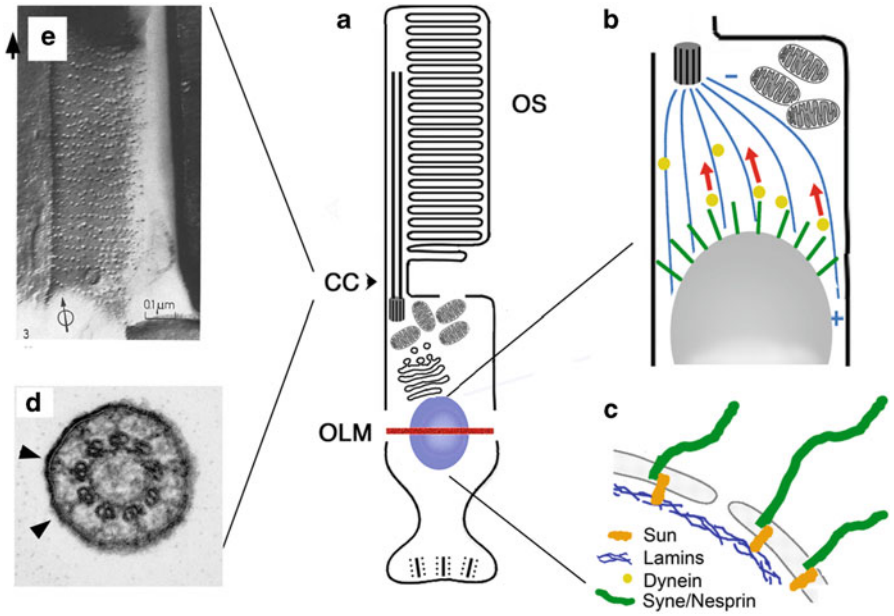

Fig. 10.3 Nuclear positioning and connecting cilium in vertebrate photoreceptor cell. (**a**) Schematic representation of photoreceptor cell. Nucleus is highlighted in *blue*. (**b**) The nucleus is by far the most voluminous organelle in the cytoplasm of the photoreceptor cell. Its position is affected by the activity of a microtubule dependant motor, dynein, and nuclear envelope components that feature C-terminal KASH domain (Syne/Nesprin family proteins in vertebrates). The KASH domain contains a lipophilic segment thought to span the outer membrane of the nuclear envelope. The cytoplasmic portion of many KASH-domain proteins is exceptionally long (close to 10,000 amino acids in some cases). (**c**) Schematic representation of the nuclear envelope. KASH-domain proteins such as Syne/Nesprins span its outer membrane and SUN proteins span the inner membrane. The C-termini of these proteins are thought to bind each other in the perinuclear space. Nuclear lamins line the inner surface of the nuclear envelope. (**d**) Transmission electron micrograph of transverse section through the cilium. Links (*arrowheads*) are present between microtubules of the ciliary axoneme and the membrane. (Image courtesy of Joe Besharse.) (**e**) Freeze-fracture experiments on rat photoreceptors reveal the presence of bead-shaped protrusions on the connecting cilium membrane termed the ciliary necklace: these are thought to correlate with Y-shaped links seen in transmission microscopy. (Reprinted with permission from Rohlich 1975)

the Y-links. Specific Y-link defects were first characterized by Craige and colleagues (2010), who modeled the function of Cep290, a human ciliopathy locus in *Chlamydomonas* (Craige et al. 2010). In this species of algae, Cep290 localizes to the transition zone and its mutations cause a loss or a collapse of transition zone Y-link structures: this is associated with an increased distance between transition zone microtubules and the membrane. The protein composition of flagella is also altered (Craige et al. 2010).

Subsequent analysis in *C. elegans* revealed the function of eight additional loci in the formation of the ciliary transition zone: *mks-1*, *-3*, *-5*, *-6*, *-9*, *-10*, as well as

nphp-1 and *-4* (Williams et al. 2011). (*mks-9* and *mks-10* are also known as *b9d1*/*mksr-1* and *b9d2*/*mksr-2* respectively, and *mks-5* and *mks-6* are also referred to as *rpgrip1lnphp8* and *cc2d2a*.) Defects in all eight loci are known to cause human syndromic ciliopathies. Most of them encode cytoplasmic proteins: two of them, *mks-5* and *mks-6*, feature a calcium-regulated membrane docking C2 module (Nalefski and Falke 1996; Williams et al. 2011), and three others, *mks-1*, *mks-9*, and *mks-10*, contain a related B9 domain and are the only polypeptides known to contain this motif. As the C2/B9 domains are thought to mediate membrane interactions, they may attach Y-links to the ciliary membrane (Williams et al. 2011).

Based on genetic analysis, transition zone loci described by Williams et al. form two partially redundant functional modules: one containing the nephrocystins, *nphp-1* and *nphp-4*, and the other the remaining loci with the exception of *mks-5*. The latter gene displays unique characteristics in that it is redundant with loci from both modules. The unique function of *mks-5* is corroborated by protein localization studies, which revealed a hierarchical organization of transition zone genes: *mks-5* appears to function upstream in the assembly of transition zone proteins. It is necessary to localize protein products of *mks-9* and *mks-10* while these two genes are, in turn, required to localize the transmembrane polypeptide encoded by *mks-3* and a C2-domain protein product of *mks-6* (Williams et al. 2011). *mks-5* also functions upstream of *nphp-1* and *nphp-4* to partially localize their protein products. The key role of this genetic cassette is evident in the transition zone phenotypes of double mutants: in the most severe cases of *nphp-6*/*nphp-4* mutants, for example, the transition zone membrane is no longer associated with the axoname and the Y-links are completely absent (Williams et al. 2011).

In parallel to genetic analysis in algae and nematodes, tandem affinity purification of protein complexes from mammalian cells identified related sets of interacting proteins that localize to the transition zone (Chih et al. 2011; Garcia-Gonzalo et al. 2011). These complexes included proteins that were not analyzed in *Chlamydomonas* or *C. elegans*: Tectonic (Tctn) 1, 2, and 3, Ahi1, Tmem231, and Tmem17. All these localize to the ciliary transition zone and at least some of them appear to function in the assembly of protein complexes that localize to this region: in mutant Tctn1 fibroblasts, MKS1 and MKS3 are mislocalized, and siRNA knockdown of Tmem231 and Tmem17 in IMCD3 cells causes a partial loss of MKS6 and MKS9 from the ciliary transition zone (Chih et al. 2011; Garcia-Gonzalo et al. 2011).

Transition Zone Barrier Function

Importantly, studies of transition zone protein complexes have revealed that they mediate the diffusion barrier function that has been known to exist at the base of cilia in most, if not all, cells, including photoreceptors. The function of this barrier is complex as it has to selectively regulate the movement of polypeptides both into and out of the cilium. In the case of photoreceptor opsins, it allows their entry into

the cilium but blocks diffusion back into the cell membrane. By contrast, certain other cell membrane proteins may have to be kept out of the cilium. This barrier mechanism has to be selective in both directions: it has to keep some proteins out but let others in and vice versa. Finally, in the case of some proteins, barrier permeability may change depending on physiological conditions, as may take place in hedgehog cascade components, which change ciliary localization in response to hedgehog signaling (Ocbina et al. 2011).

Experiments performed in the nematode model and in mammalian cells revealed that at least some diffusion barrier characteristics are compromised in mutants of transition zone proteins. A transmembrane protein TRAM-1a, which in *C. elegans* localizes to dendritic tips but not cilia, diffuses into the ciliary shaft in several mutants, including *mks-5*, *mks-6*, *nphp-1*, and *nphp-4* (Williams et al. 2011). Similarly, in cell culture conditions, GFP-GPI, an artificial membrane-associated protein, is more abundantly found in cilia following siRNA knockdowns of MKS6 and MKS9 (Chih et al. 2011). These experiments demonstrate a loss of barrier function for nonciliary membrane proteins. In other experiments, loss or downregulation of transition zone components resulted in decreased ciliary localization of proteins, including adenylyl cyclase III (AC3), polycystin 2 (Pkd2), somatostatin receptor 3 (SSTR3), serotonin receptor 6 (HTR6), and smoothened (Chih et al. 2011; Garcia-Gonzalo et al. 2011). This occurrence may reflect loss of barrier function causing unrestricted diffusion of these proteins from the ciliary shaft or, less likely, an abnormally high impermeability of the diffusion barrier, which hampers their entry into the cilium. Predictably, these defects appear to be selective, as at least in certain transition zone mutants, the ciliary localization of SSTR3 is not affected but the localizations of AC3 and Pkd2 are (Garcia-Gonzalo et al. 2011).

Transition Zone in Vertebrate Photoreceptors

Is the transition zone barrier mechanism revealed by genetic analysis in lower eukaryotes and cell culture studies relevant to photoreceptors? Multiple lines of evidence suggest that this is the case. First, many of the transition zone proteins localize to the photoreceptor cell connecting cilium. This includes NPHP1, MKS5/RPGRIP1L/NPHP8, NPHP6/Cep290, and JBTS3/AHI (Zhao et al. 2003; Fliegauf et al. 2006; Louie et al. 2010; Bachmann-Gagescu et al. 2011; Rachel et al. 2012). In addition, mutations in several genes, including NPHP1, 4, 6, NPHP8/MKS5, and AHI1 involve a varying frequency of blindness (reviewed in Hildebrandt et al. 2009; Coppieters et al. 2010; Mockel et al. 2011). The absence of reports on human inherited photoreceptor abnormalities caused by mutations in other loci, such as those that encode B9 domain proteins, is likely the result of early lethality of such mutations. Finally, as explained in more detail below, studies in animal models also support the conclusion that transition zone barrier function is necessary for vertebrate photoreceptor differentiation and survival.

Consistent with human abnormalities, mutants of genes encoding transition zone proteins in vertebrates reveal faulty photoreceptor differentiation. Particularly dramatic examples are the *Ahi1* mutant mouse and the *wpk* rat, which carries a mutation in the MKS3 locus (Smith et al. 2006; Louie et al. 2010). In both mutants, photoreceptors appear to lack outer segments (Tammachote et al. 2009; Louie et al. 2010). Outer segments do differentiate but are smaller and severely disorganized in the *rd16* mutant mouse, which contains a partial deletion of the Cep290 sequence, presumably a hypomorphic defect (Rachel et al. 2012). Finally, the zebrafish *sentinel*^{w38} mutant, thought to carry a null mutation in the MKS6 gene, features shorter or absent outer segment (Bachmann-Gagescu et al. 2011). This mutant also involves opsin mislocalization and a striking accumulation of vesicles in the apical inner segment. The latter phenotype is very rare but has been previously observed following interference with the function of Rab8, a small GTP-ase essential for cilia formation (Moritz et al. 2001). Phenotypic differences among transition zone mutants are noteworthy as they imply functional differences. Vesicle accumulation suggests an inability of opsin carrier vesicles to dock at the periciliary membrane, a phenomenon not seen in other mutants. The role of MKS6 in opsin transport may thus differ considerably from that of MKS3 and Ahi1.

Mutations that affect transition zone genes appear to cause phenotypes of severity comparable to that produced by defects in intraflagellar transport loci (IFT). Lesions in the zebrafish IFT genes *oval/ift88*, *elipsa/ift54*, and *fleer* cause a complete absence of the outer segment (Doerre and Malicki 2002; Tsujikawa and Malicki 2004). Comparably severe phenotypes are caused by defects in mouse Ahi1 and rat MKS3 genes (Tammachote et al. 2009; Louie et al. 2010; Tiwari et al. 2013). This finding is somewhat surprising as transition zone mutants, including double mutants, cause only moderate cilia morphology defects in other cells (Chih et al. 2011; Williams et al. 2011). Is the loss of outer segments in transition zone mutants then solely the result of compromised diffusion barrier function?

The outer segment is unique in that its main receptor protein, opsin, is not only a functional but also a structural component. This conclusion is based on the observation that deletion of the rod opsin gene in knockout mice blocks outer segment differentiation (Lem et al. 1999). It is thus possible that, in the absence of a transition zone-mediated diffusion barrier, the outer segment does not form simply because opsin fails to accumulate at a sufficiently high concentration in the ciliary membrane. One has to keep in mind, however, that in addition to barrier function, transition zone proteins may also play a role in interactions with the ciliary transport machinery (Zhao and Malicki 2011). Defects of these functions may also contribute to the severity of mutant phenotypes.

The dramatic consequences of transition zone defects are perhaps easier to understand in the light of microscopic observations showing that vertebrate photoreceptor transition zone is much more robust in comparison to these in other cilia: in most mammalian cilia, the ciliary necklace contains approximately 4 to 7 strands of bead-like protrusions, but in rat rod photoreceptors it contains 30 to 40 strands (Röhlich 1975; Dentler 1981). This, again, may be related to the exceptionally high

opsin concentration in outer segment membranes. It is thus perhaps not surprising that transition zone defects abrogate outer segment formation.

The functional complexity of the transition zone suggests similarly complex protein composition of this structure. It is thus not unexpected that in addition to proteins already discussed, many others appear to contribute to its structure in cilia, including TMEM237, Cep162, Septins, Nek4, Plk1, and nuclear pore components (Dishinger et al. 2010; Chih et al. 2011; Coene et al. 2011; Huang et al. 2011; Kee et al. 2012; Seeger-Nukpezah et al. 2012; Wang et al. 2013). As the functions of these polypeptides in the retina remain largely unknown, they are not discussed here in detail.

10.2.2 Organelle Positioning

In many cell types, organelles distribute nonrandomly in the cytoplasm, and this is also the case in photoreceptors. Mitochondria form a tight cluster in the apical region of the photoreceptor cell just basal to the connecting cilium (shown schematically in Fig. 10.3a), the photoreceptor nucleus is positioned roughly in the center of the cell body, and the Golgi apparatus localizes mainly between the nucleus and the mitochondrial cluster (Papermaster et al. 1985; Townes-Anderson et al. 1985, 1988). Although little is known about cellular machinery that localizes mitochondria or the Golgi apparatus in the photoreceptor cell, mechanisms which determine the position of photoreceptor cell nuclei are relatively well characterized (Fig. 10.3b, c).

10.2.2.1 The Nucleus

As in the case of apicobasal membrane subdivision, similarities exist between nucleus positioning in fly and vertebrate photoreceptors. Genetic analyses in *Drosophila*, zebrafish, and most recently the mouse revealed that microtubule-dependent motors and nuclear membrane-associated polypeptides determine the position of photoreceptor nuclei. The first insight into this mechanism came from observations that mutations in the fly *p150/glued* gene, which encodes a dynactin component, cause basal mispositioning of photoreceptor nuclei (Fan and Ready 1997; Whited et al. 2004). This phenotype is enhanced by second-site heterozygous defects in the dynein intermediate chain and suppressed by second-site kinesin mutations (Whited et al. 2004), supporting the idea that nucleus position is determined by antagonistic action of motors: dynein, which pulls nuclei in the apical direction, and kinesin, which does the opposite.

Analysis of zebrafish mutants revealed that vertebrate photoreceptors also require dynein to position their nuclei. This first became obvious following the analysis of the zebrafish mutant *mikre oko* (*mok*) ^{m632}, initially found in the same

large-scale screen that identified apicobasal polarity mutants (Malicki et al. 1996; Doerre and Malicki 2001). Subsequent cloning revealed that the *mikre oko* locus encodes the zebrafish homologue of fly *p150/glued*. Coincidently, both zebrafish and fly mutations, mok^{m632} and $glued^{1}$, respectively, involve similar C-terminal truncations of the p150 polypeptide (Swaroop et al. 1987; Tsujikawa et al. 2007). The role of the dynein motor in nucleus positioning in vertebrate photoreceptors was confirmed in several ways. First, the overexpression of another dynactin component, *p50*, known to disrupt the dynactin complex, causes a phenotype similar to that observed in mok^{m632} (Tsujikawa et al. 2007). Second, morpholino knockdown of *lis1*, a regulator of dynein motor function, also causes this phenotype in zebrafish (Tsujikawa et al. 2007). Finally, photoreceptor nucleus displacement is observed in the *ale oko* (*ako*) ^{jj50} mutant, which disrupts *p50* function (Jing and Malicki 2009). These studies led to the conclusion that cytoplasmic dynein pulls the vertebrate photoreceptor nucleus toward the apical terminus of the cell (Fig. 10.3b) (Tsujikawa et al. 2007). This model is also supported by observations that microtubules in photoreceptor cytoplasm direct their minus ends apically (Troutt and Burnside 1988).

Analysis in the fly also revealed that another group of proteins, functionally very different from cytoplasmic motors, plays a role in the positioning of photoreceptor nuclei. These proteins are associated with the nuclear envelope and were first identified via the analysis of the *Drosophila klarsicht* (*klar*) gene. Similar to observations in dynactin mutants, photoreceptor nuclei are mispositioned basally in *klar* mutant flies (Mosley-Bishop et al. 1999). The C-terminal region of the *klar* gene product was found homologous to the Syne/ANC-1 family of proteins and became known as the KASH (Klarsicht/ANC-1/Syne-1 Homology) domain (Starr and Han 2002). It consists of a transmembrane region followed by a short C-terminal sequence and is now known to localize KASH-domain proteins to the outer membrane of the nuclear envelope (Sosa et al. 2013). KASH-domain proteins frequently feature a long stretch of more than 8,000 amino acids thought to extend as much as 0.5 μm into the cytoplasm (Starr and Han 2002). As discussed below, this cytoplasmic region appears to interact with the cytoskeleton.

In agreement with the observation that Klarsicht localizes to the nuclear envelope, mutations in nuclear lamins, which localize to the inner membrane of the nuclear envelope, also misposition nuclei in fly photoreceptors (Patterson et al. 2004). Furthermore, studies in *C. elegans* revealed that the *unc-84* locus encodes a transmembrane protein that localizes to the nuclear envelope and is required for the migration and positioning of nuclei during nematode development (Malone et al. 1999). Its C-terminus features so-called SUN (sad1, unc-84) domain, also found in vertebrate proteins (Malone et al. 2003). In contrast to KASH-motif proteins, SUN-domain polypeptides localize to the inner membrane of the nuclear envelope. SUN and KASH domains are thought to bind each other in the perinuclear space (Hodzic et al. 2004; Padmakumar et al. 2005). This binding interaction has been recently characterized via X-ray crystallography (Sosa et al. 2012).

Nuclear envelope proteins also function in vertebrate photoreceptors to position the nucleus, as demonstrated by the interference with the zebrafish *Syne2a* using a dominant-negative construct. This treatment causes basal displacement of photoreceptor nuclei (Tsujikawa et al. 2007). Similarly, the overexpression of dominant-negative KASH-EGFP in mouse cone photoreceptors causes basal displacement of their nuclei, which may reflect a failure of nuclei to migrate to their proper position, faulty maintenance of nucleus position, or both (Razafsky et al. 2012). Consistent with that, knockouts of mouse *Syne2* and *Sun1* decrease photoreceptor survival, a phenotype accompanied by abnormalities in photoreceptor morphology suggestive of nucleus positioning defects (Yu et al. 2011; Razafsky et al. 2012).

Recent models of nucleus positioning postulate that cytoplasmic domains of KASH proteins interact with both microtubules and actin filaments (Tapley and Starr 2013). The N-terminal calponin-related region of Syne-1 and -2 proteins (also known as Nesprin-1 and -2) binds actin, and Nesprin-4 directly associates with kinesin (Starr and Han 2002; Zhen et al. 2002; Roux et al. 2009). Nesprin-3 also recruits intermediate filaments to the nuclear envelope (Wilhelmsen et al. 2005; Taranum et al. 2012). The severe photoreceptor nucleus mispositioning phenotypes observed in zebrafish dynactin mutants suggest that microtubule-dependent mechanisms play a particularly important role in this cell type. The contribution of nuclear envelope interactions with actin and intermediate filaments in photoreceptors remains to be evaluated. Finally, one has to note that defects in nuclear envelope-associated proteins, lamins in particular, but also KASH-domain polypeptides, are known to cause many rare human disorders, including muscular dystrophies, progeria, and cerebellar ataxia (De Sandre-Giovannoli et al. 2003; Gros-Louis et al. 2007; Meinke et al. 2011; Worman 2012). None of these diseases is, however, associated with photoreceptor loss.

10.2.2.2 Other Organelles

Whereas mechanisms that position the photoreceptor nucleus are relatively well investigated, very little is known about positioning of other organelles in the photoreceptor cell. The Golgi apparatus is frequently associated with centrosomes (Bornens 2008). Consistent with that, at least in some cells, microtubule-dependent motors affect its subcellular localization (Smith et al. 2000). It remains to be investigated, however, whether this is also the case in photoreceptors. A curious feature of photoreceptor polarity is a voluminous cluster of mitochondria that occupies nearly the entire apical region of the cell (Townes-Anderson et al. 1988; Knabe and Kuhn 1996; Kitambi and Malicki 2008). These apical mitochondria tightly adhere to each other and presumably provide metabolic support for outer segment formation and maintenance (Linton et al. 2010). They appear to colocalize with microtubules in at least some species (Knabe and Kuhn 1996). It is thus conceivable that microtubule-dependent motors position them in the cytoplasm and, given the orientation of the photoreceptor microtubules, minus end-directed motors are likely to play this role.

In the *Drosophila* eye and in vascular retinae of vertebrates, mitochondria also accumulate at synaptic terminals (Meinertzhagen and O'Neil 1991; Bentmann et al. 2005; Stone et al. 2008). The mechanisms that drive synaptic localization of mitochondria in fly photoreceptors are relatively well investigated. The loss of mitochondria from photoreceptor synaptic terminals in the fly mutant *milton* is associated with blindness (Stowers et al. 2002). Milton, a cytoplasmic protein, appears to physically associate with a kinesin heavy chain and a mitochondrial Rho GTPase, Miro (Guo et al. 2005; Glater et al. 2006). It remains to be investigated whether this or a similar mechanism also functions in the vertebrate photoreceptor synaptic terminal.

10.2.3 Polarization of Cytoplasmic Trafficking

The subcompartmentalization of the photoreceptor cell membrane and the unique protein content of each compartment require dedicated intracellular trafficking pathways, which deliver compartment-specific proteins to correct destinations. Given the remarkable volume and membrane surface area of the outer segment and its key role in photoreceptor function, protein transport into this compartment has been now investigated for decades (Young 1967; Young and Droz 1968; LaVail 1973; Papermaster et al. 1986). Opsin, the most abundant component of outer segment membranes, is naturally the best studied component of this process. It is initially transported from the Golgi to the base of the photoreceptor connecting cilium in cytoplasmic vesicles. From there it most likely translocates as an integral component of the ciliary membrane, although alternative hypotheses are being considered, and arguments can be produced against intramembrane transport models (Sung and Tai 2000; Gilliam et al. 2012).

On the molecular level, several proteins are now known to interact with opsin C-terminal sequences and most likely facilitate its translocation into the outer segment (Tai et al. 1999; Deretic et al. 2005; Chuang et al. 2007). Nevertheless, many steps of this process remain a mystery. If opsin does translocate in the plane of the ciliary membrane, it remains to be determined what mechanisms mediate the docking of opsin containing vesicles at the base of the cilium and how opsin molecules interact with ciliary transport mechanisms, such as IFT. In fact, even after decades of research, it remains uncertain whether opsin is transported via an IFT-dependent mechanism. Finally, opsin has to be deposited into rod photoreceptor discs. This is another area that requires further analysis, as at least two competing models exist to explain the incorporation of opsin into these structures (Steinberg et al. 1980; Chuang et al. 2007).

It has to be kept in mind that membrane compartmentalization as well as synapse formation and later function also require targeted delivery of polypeptides to specific membrane destinations. The importance of intracellular trafficking extends

then beyond opsin to many inner segment proteins such as Crumbs, transition zone proteins such as MKS2 and MKS3, and to synapse components. Because intracellular transport is the focus of another chapter in this volume (Deretic 2014), this important topic is mentioned here in passing only.

10.3 Planar Cell Polarity

In addition to apicobasal polarity, epithelial sheets also display another form of cell polarity, known as the planar polarity (Wang and Nathans 2007; Goodrich and Strutt 2011). It has been extensively studied in *Drosophila* wing and eye. In the fly wing, it manifests itself in the orientation of bristles that differentiate on the surface of cells. In the *Drosophila* eye, planar polarity is reflected in the orientation of ommatidia. In vertebrates, one of the most conspicuous and also best studied examples of planar polarity is the orientation of mechanosensory cells, so-called hair cells in the auditory and vestibular systems. The apical surface of these cells contains two asymmetrically distributed features: the kinocilium (true cilium) and a bundle of microvilli, known as stereocilia (Hudspeth and Jacobs 1979; Hudspeth 1989). The kinocilium itself is not positioned centrally on the apical surface but is instead displaced toward its periphery. Similarly, stereocilia, which differ in length, are also distributed asymmetrically so that the longest ones are the closest to the kinocilium. In hair cells of mechanosensory epithelia, the vector of polarity defined by the kinocilium and stereocilia consistently points in the same direction (Eaton 1997; Wang and Nathans 2007).

Although it is not so obvious as in hair cells, the apical surface of photoreceptors is also asymmetrical in that the photoreceptor connecting cilium is positioned at its periphery. It has been shown that this asymmetrical feature is oriented in the same way in a field of neighboring photoreceptors, a hallmark of planar cell polarity (Fig. 10.4). Analysis of flat mounted retinae from adult zebrafish revealed that cone photoreceptor basal bodies are consistently positioned on the side of the apical surface directed toward the optic nerve, indicating that these cells are polarized in the plane of the photoreceptor cell layer (Fig. 10.4d) (Ramsey and Perkins 2013). Planar polarity is present in blue and red/green double cones but not in rods and UV cones, which may be because red/green and blue cones closely adhere to each other and form linear pentameric arrays. These are separated from each other by UV-sensitive cones and rods (Fig. 10.4c) (Zou et al. 2012). This arrangement resembles to some extent the organization of mechanosensory epithelia, where polarized hair cells are separated from each other by supporting cells that do not display obvious polarity (Eaton 1997).

Several questions need to be answered regarding the planar polarity of the zebrafish photoreceptor cell layer. First, what mechanisms coordinate the polarity of pentameric photoreceptor units? This process may be under the control of core polarity genes, such as *flamingo*, *frizzled*, and *van Gogh/strabismus*, known to function in other polarized epithelia, including those in the mammalian cochlea

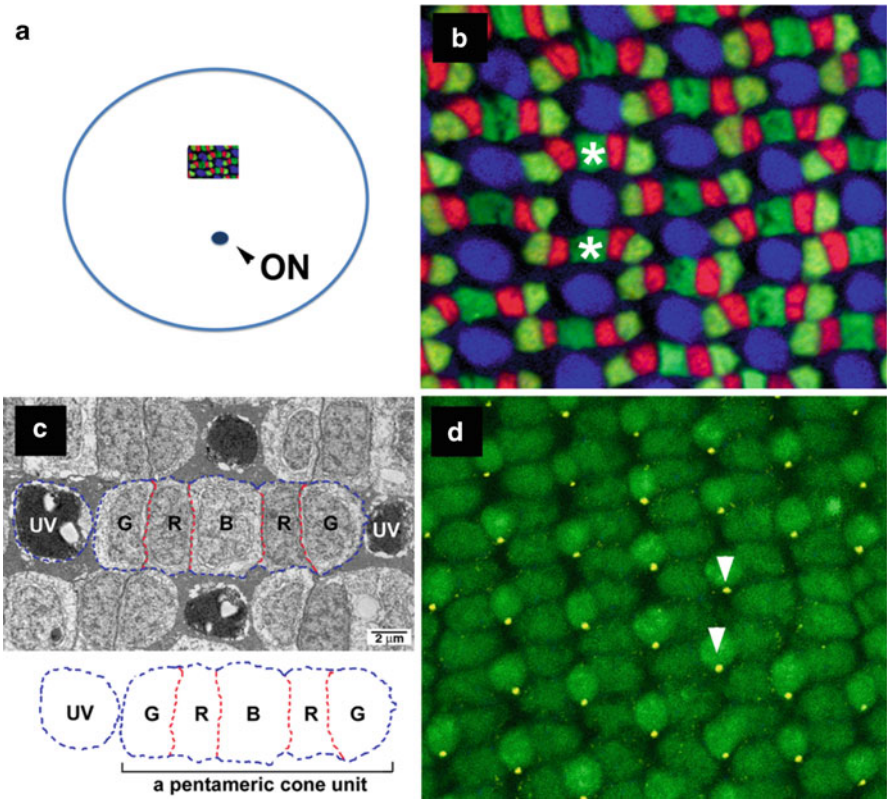

Fig. 10.4 Planar cell polarity in the photoreceptor cell layer. (**a**) Schematic representation of the retina shows optic nerve (*ON*) and a fragment of photoreceptor mosaic (not to scale). (**b**) Photoreceptor mosaic in the zebrafish retina. Note the remarkably regular arrangement of cones. UV cones, *blue*; blue cones are indicated with *asterisks*. (Image courtesy of Xiangyun Wei.) (**c**) Electron micrograph of tangential section through the retina. In the plane of this section, red/green (*R*, *G*) double cones and blue cones (*B*) closely adhere to each other, forming pentameric units. *UV*, ultraviolet-sensitive cones. (Reprinted with permission from Zou et al. 2012.) (**d**) Photoreceptor mosaic in the retina. Basal bodies of blue cones (*arrowheads*) are visualized with an anti-gamma tubulin antibody staining as *yellow dots*. In all cells shown in this image, they are displaced from the center of the apical surface in the same direction toward the optic nerve. (Image courtesy of Brian Perkins)

(Curtin et al. 2003; Wang et al. 2006). The second question to be answered is what drives peripheral positioning of the connecting cilium at the apical surface of the photoreceptor cell? Again in this case, potential mechanisms are suggested by research performed on hair cells. These studies revealed, for example, that apical surface asymmetry is generated via the remodeling of the apical cytoskeleton by LGN, mammalian inscuteable, and G-protein signaling (Ezan et al. 2013; Tarchini et al. 2013). Also to be addressed is the significance of planar polarity in the retina.

In the vertebrate ear or in the fly wing, planar polarity has an obvious functional importance. What would that be in the photoreceptor cell layer is not clear. Perhaps polarity is related to the formation of the regular photoreceptor mosaic pattern that characterizes teleost fish retinae (Larison and Bremiller 1990; Kitambi and Malicki 2008). If so, it may be worthwhile to investigate this form of polarity in the retinae of other teleosts.

10.4 Closing Remarks

Differentiation of photoreceptor morphology involves polarized intracellular trafficking coordinated with the formation, and subsequently the maintenance, of distinct membrane and cytoplasmic compartments. Some of the mechanisms that operate in the photoreceptor cell are remarkably conserved in evolution, and insights into their function came from such seemingly unlikely sources as research on the genetics of the unicellular green alga *Chlamydomonas*. Indeed, this very line of analysis produced advances in the understanding of Cep290, a major photoreceptor connecting cilium component, and a protein known to be defective in several forms of human blindness. Similarly, genetic analysis in *C. elegans* generated many insights into the function of the connecting cilium, whereas fruit fly genetics revealed mechanisms involved in the formation of photoreceptor membrane subdivisions: c*rumbs*, a key regulator of cell-surface compartmentalization and a gene involved in an early form of human blindness was initially identified in the fruit fly embryo.

Vertebrate photoreceptors are unique and fascinating in many ways. In addition to mechanisms shared with many tissues, photoreceptor morphogenesis likely involves genetic pathways uniquely tailored to the specialized functional features of these cells. The exceptionally bulky and physiologically active photoreceptor cilium requires particularly robust cytoplasmic and intraflagellar trafficking mechanisms. It also places an unusually high burden on photoreceptor metabolism. These exceptional features of the photoreceptor cell may also be particularly challenging to understand as they cannot be modeled in other cell types or in less complex model organisms. On the other hand, the size of the photoreceptor cilium and the abundance of its components, opsins, for example, open experimental opportunities that are not available elsewhere. Hence, research on vertebrate photoreceptors is a source of both unique challenges and equally unique opportunities.

Acknowledgments The author is thankful to Drs. Tomer Avidor-Reiss, Kai Erdmann, and Anton Nikolaev for critical reading of an earlier version of this manuscript and insightful comments. Dr. Yoshihiro Omori helped with figure preparation, and Gemma Stephens provided valuable editorial assistance. Jarema Malicki is funded in part by an R01 award from the National Eye Institute, USA.

References

Alves CH, Sanz AS, Park B, Pellissier LP, Tanimoto N, Beck SC, Huber G, Murtaza M, Richard F, Sridevi Gurubaran I et al (2013) Loss of CRB2 in the mouse retina mimics human retinitis pigmentosa due to mutations in the CRB1 gene. Hum Mol Genet 22(1):35–50

Bachmann-Gagescu R, Phelps IG, Stearns G, Link BA, Brockerhoff SE, Moens CB, Doherty D (2011) The ciliopathy gene cc2d2a controls zebrafish photoreceptor outer segment development through a role in Rab8-dependent vesicle trafficking. Hum Mol Genet 20(20):4041–4055

Bentmann A, Schmidt M, Reuss S, Wolfrum U, Hankeln T, Burmester T (2005) Divergent distribution in vascular and avascular mammalian retinae links neuroglobin to cellular respiration. J Biol Chem 280(21):20660–20665

Besharse JC, Forestner DM, Defoe DM (1985) Membrane assembly in retinal photoreceptors. III. Distinct membrane domains of the connecting cilium of developing rods. J Neurosci 5(4):1035–1048

Bessant DA, Khaliq S, Hameed A, Anwar K, Payne AM, Mehdi SQ, Bhattacharya SS (1999) Severe autosomal dominant retinitis pigmentosa caused by a novel rhodopsin mutation (Ter349Glu). Mutations in brief no. 208. Online. Hum Mutat 13(1):83

Bornens M (2008) Organelle positioning and cell polarity. Nat Rev Mol Cell Biol 9(11):874–886

Calvert PD, Govardovskii VI, Krasnoperova N, Anderson RE, Lem J, Makino CL (2001) Membrane protein diffusion sets the speed of rod phototransduction. Nature (Lond) 411 (6833):90–94

Chih B, Liu P, Chinn Y, Chalouni C, Komuves LG, Hass PE, Sandoval W, Peterson AS (2011) A ciliopathy complex at the transition zone protects the cilia as a privileged membrane domain. Nat Cell Biol 14(1):61–72

Cho S-H, Kim JY, Simons DL, Song JY, Le JH, Swindell EC, Jamrich M, Wu SM, Kim S (2012) Genetic ablation of Pals1 in retinal progenitor cells models the retinal pathology of Leber congenital amaurosis. Hum Mol Genet 21(12):2663–2676

Chuang JZ, Zhao Y, Sung CH (2007) SARA-regulated vesicular targeting underlies formation of the light-sensing organelle in mammalian rods. Cell 130(3):535–547

Coene KLM, Mans DA, Boldt K, Gloeckner CJ, van Reeuwijk J, Bolat E, Roosing S, Letteboer SJF, Peters TA, Cremers FPM et al (2011) The ciliopathy-associated protein homologs RPGRIP1 and RPGRIP1L are linked to cilium integrity through interaction with Nek4 serine/threonine kinase. Hum Mol Genet 20(18):3592–3605

Cohen AI (1965) New details of the ultrastructure of the outer segments and ciliary connectives of the rods of human and macaque retinas. Anat Rec 152:63–79

Concepcion F, Chen J (2010) Q344ter mutation causes mislocalization of rhodopsin molecules that are catalytically active: a mouse model of Q344ter-induced retinal degeneration. PloS ONE 5(6):e10904

Coppieters F, Lefever S, Leroy BP, De Baere E (2010) CEP290, a gene with many faces: mutation overview and presentation of CEP290base. Hum Mutat 31(10):1097–1108

Craige B, Tsao CC, Diener DR, Hou Y, Lechtreck KF, Rosenbaum JL, Witman GB (2010) CEP290 tethers flagellar transition zone microtubules to the membrane and regulates flagellar protein content. J Cell Biol 190(5):927–940

Curtin JA, Quint E, Tsipouri V, Arkell RM, Cattanach B, Copp AJ, Henderson DJ, Spurr N, Stanier P, Fisher EM et al (2003) Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse. Curr Biol 13(13):1129–1133

De Sandre-Giovannoli A, Bernard R, Cau P, Navarro C, Amiel J, Boccaccio I, Lyonnet S, Stewart CL, Munnich A, Le Merrer M et al (2003) Lamin: a truncation in Hutchinson-Gilford progeria. Science 300(5628):2055

den Hollander AI, ten Brink JB, de Kok YJ, van Soest S, van den Born LI, van Driel MA, van de Pol DJ, Payne AM, Bhattacharya SS, Kellner U et al (1999) Mutations in a human homologue of *Drosophila crumbs* cause retinitis pigmentosa (RP12). Nat Genet 23(2):217–221

Dentler WL (1981) Microtubule-membrane interactions in cilia and flagella. Int Rev Cytol 72:1–47

Deretic D (2006) A role for rhodopsin in a signal transduction cascade that regulates membrane trafficking and photoreceptor polarity. Vision Res 46(27):4427–4433

Deretic D (2014) Role and mechanism of ciliary transport. Furukawa T, Hurley JB, Kawamura S (eds) Vertebrate photoreceptors: Functional molecular bases. Springer, Tokyo, pp. 139–165

Deretic D, Williams AH, Ransom N, Morel V, Hargrave PA, Arendt A (2005) Rhodopsin C terminus, the site of mutations causing retinal disease, regulates trafficking by binding to ADP-ribosylation factor 4 (ARF4). Proc Natl Acad Sci USA 102(9):3301–3306

Dishinger JF, Kee HL, Jenkins PM, Fan S, Hurd TW, Hammond JW, Truong YN-T, Margolis B, Martens JR, Verhey KJ (2010) Ciliary entry of the kinesin-2 motor KIF17 is regulated by importin-β2 and RanGTP. Nat Cell Biol 12(7):703–710

Doerre G, Malicki J (2001) A mutation of early photoreceptor development, *mikre oko*, reveals cell–cell interactions involved in the survival and differentiation of zebrafish photoreceptors. J Neurosci 21(17):6745–6757

Doerre G, Malicki J (2002) Genetic analysis of photoreceptor cell development in the zebrafish retina. Mech Dev 110(1-2):125–138

Dowling J (1987) The retina. Harvard University Press, Cambridge

Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DY, Zwartkruis F, Abdelilah S, Rangini Z et al (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. Development (Camb) 123:37–46

Eaton S (1997) Planar polarization of *Drosophila* and vertebrate epithelia. Curr Opin Cell Biol 9 (6):860–866

Ezan J, Lasvaux L, Gezer A, Novakovic A, May-Simera H, Belotti E, Lhoumeau AC, Birnbaumer L, Beer-Hammer S, Borg JP et al (2013) Primary cilium migration depends on G-protein signalling control of subapical cytoskeleton. Nat Cell Biol 15(9):1107–1115

Fan SS, Ready DF (1997) Glued participates in distinct microtubule-based activities in *Drosophila* eye development. Development (Camb) 124(8):1497–1507

Fan S, Hurd TW, Liu CJ, Straight SW, Weimbs T, Hurd EA, Domino SE, Margolis B (2004) Polarity proteins control ciliogenesis via kinesin motor interactions. Curr Biol 14(16):1451–1461

Fliegauf M, Horvath J, von Schnakenburg C, Olbrich H, Muller D, Thumfart J, Schermer B, Pazour GJ, Neumann HP, Zentgraf H et al (2006) Nephrocystin specifically localizes to the transition zone of renal and respiratory cilia and photoreceptor connecting cilia. J Am Soc Nephrol 17(9):2424–2433

Garcia-Gonzalo FR, Corbit KC, Sirerol-Piquer MS, Ramaswami G, Otto EA, Noriega TR, Seol AD, Robinson JF, Bennett CL, Josifova DJ et al (2011) A transition zone complex regulates mammalian ciliogenesis and ciliary membrane composition. Nat Genet 43(8):776–784

Gilliam JC, Chang JT, Sandoval IM, Zhang Y, Li T, Pittler SJ, Chiu W, Wensel TG (2012) Three-dimensional architecture of the rod sensory cilium and its disruption in retinal neurodegeneration. Cell 151(5):1029–1041

Gilula NB, Satir P (1972) The ciliary necklace. A ciliary membrane specialization. J Cell Biol 53(2):494–509

Glater EE, Megeath LJ, Stowers RS, Schwarz TL (2006) Axonal transport of mitochondria requires milton to recruit kinesin heavy chain and is light chain independent. J Cell Biol 173(4):545–557

Goodrich LV, Strutt D (2011) Principles of planar polarity in animal development. Development (Camb) 138(10):1877–1892

Gros-Louis F, Dupré N, Dion P, Fox MA, Laurent S, Verreault S, Sanes JR, Bouchard J-P, Rouleau GA (2007) Mutations in SYNE1 lead to a newly discovered form of autosomal recessive cerebellar ataxia. Nat Genet 39(1):80–85

Guo X, Macleod GT, Wellington A, Hu F, Panchumarthi S, Schoenfield M, Marin L, Charlton MP, Atwood HL, Zinsmaier KE (2005) The GTPase dMiro is required for axonal transport of mitochondria to *Drosophila* synapses. Neuron 47(3):379–393

Hallermann S, Silver RA (2013) Sustaining rapid vesicular release at active zones: potential roles for vesicle tethering. Trends Neurosci 36(3):185–194

Herder C, Swiercz JM, Müller C, Peravali R, Quiring R, Offermanns S, Wittbrodt J, Loosli F (2013) ArhGEF18 regulates RhoA-Rock2 signaling to maintain neuro-epithelial apico-basal polarity and proliferation. Development (Camb) 140:2787–2797

Herranz-Martín S, Jimeno D, Paniagua AE, Velasco A, Lara JM, Aijón J, Lillo C (2012) Immunocytochemical evidence of the localization of the Crumbs homologue 3 protein (CRB3) in the developing and mature mouse retina. PLOS ONE 7(11):e50511

Herzog D, Loetscher P, van Hengel J, Knüsel S, Brakebusch C, Taylor V, Suter U, Relvas JB (2011) The small GTPase RhoA is required to maintain spinal cord neuroepithelium organization and the neural stem cell pool. J Neurosci 31(13):5120–5130

Hildebrandt F, Attanasio M, Otto E (2009) Nephronophthisis: disease mechanisms of a ciliopathy. J Am Soc Nephrol 20(1):23–35

Hodzic DM, Yeater DB, Bengtsson L, Otto H, Stahl PD (2004) Sun2 is a novel mammalian inner nuclear membrane protein. J Biol Chem 279(24):25805–25812

Hong Y, Stronach B, Perrimon N, Jan LY, Jan YN (2001) Drosophila Stardust interacts with Crumbs to control polarity of epithelia but not neuroblasts. Nature (Lond) 414(6864):634–638

Hong Y, Ackerman L, Jan LY, Jan YN (2003) Distinct roles of Bazooka and Stardust in the specification of *Drosophila* photoreceptor membrane architecture. Proc Natl Acad Sci USA 100(22):12712–12717

Horne-Badovinac S, Lin D, Waldron S, Schwarz M, Mbamalu G, Pawson T, Jan Y, Stainier DY, Abdelilah-Seyfried S (2001) Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. Curr Biol 11(19):1492–1502

Hsu Y-C, Jensen AM (2010) Multiple domains in the Crumbs Homolog 2a (Crb2a) protein are required for regulating rod photoreceptor size. BMC Cell Biol 11(1):60

Hsu YC, Willoughby JJ, Christensen AK, Jensen AM (2006) Mosaic Eyes is a novel component of the Crumbs complex and negatively regulates photoreceptor apical size. Development (Camb) 133(24):4849–4859

Huang L, Szymanska K, Jensen VL, Janecke AR, Innes AM, Davis EE, Frosk P, Li C, Willer JR, Chodirker BN et al (2011) TMEM237 is mutated in individuals with a Joubert syndrome related disorder and expands the role of the TMEM family at the ciliary transition zone. Am J Hum Genet 89(6):713–730

Hudspeth AJ (1989) How the ear's works work. Nature (Lond) 341(6241):397–404

Hudspeth AJ, Jacobs R (1979) Stereocilia mediate transduction in vertebrate hair cells (auditory system/cilium/vestibular system). Proc Natl Acad Sci USA 76(3):1506–1509

Hurd TW, Gao L, Roh MH, Macara IG, Margolis B (2003) Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. Nat Cell Biol 5(2):137–142

Izaddoost S, Nam SC, Bhat MA, Bellen HJ, Choi KW (2002) Drosophila Crumbs is a positional cue in photoreceptor adherens junctions and rhabdomeres. Nature (Lond) 416(6877):178–183

Jacobson SG, Cideciyan AV, Aleman TS, Pianta MJ, Sumaroka A, Schwartz SB, Smilko EE, Milam AH, Sheffield VC, Stone EM (2003) Crumbs homolog 1 (CRB1) mutations result in a thick human retina with abnormal lamination. Hum Mol Genet 12(9):1073–1078

Jensen AM, Walker C, Westerfield M (2001) Mosaic eyes: a zebrafish gene required in pigmented epithelium for apical localization of retinal cell division and lamination. Development (Camb) 128(1):95–105

Jing X, Malicki J (2009) Zebrafish ale oko, an essential determinant of sensory neuron survival and the polarity of retinal radial glia, encodes the p50 subunit of dynactin. Development (Camb) 136(17):2955–2964

Johnson K, Grawe F, Grzeschik N, Knust E (2002) Drosophila crumbs is required to inhibit light-induced photoreceptor degeneration. Curr Biol 12(19):1675–1680

Jurgens G, Wieschaus E, Nusslein-Volhard C, Kluding C (1984) Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. Roux Arch Dev Biol 193:283–295

Kantardzhieva A, Gosens I, Alexeeva S, Punte IM, Versteeg I, Krieger E, Neefjes-Mol CA, den Hollander AI, Letteboer SJ, Klooster J et al (2005) MPP5 recruits MPP4 to the CRB1 complex in photoreceptors. Invest Ophthalmol Vis Sci 46(6):2192–2201

Kee HL, Dishinger JF, Blasius TL, Liu CJ, Margolis B, Verhey KJ (2012) A size-exclusion permeability barrier and nucleoporins characterize a ciliary pore complex that regulates transport into cilia. Nat Cell Biol 14(4):431–437

Kemphues KJ, Priess JR, Morton DG, Cheng NS (1988) Identification of genes required for cytoplasmic localization in early *C. elegans* embryos. Cell 52(3):311–320

Kennedy B, Malicki J (2009) What drives cell morphogenesis: a look inside the vertebrate photoreceptor. Dev Dyn 238(9):2115–2138

Kitambi SS, Malicki JJ (2008) Spatiotemporal features of neurogenesis in the retina of medaka, *Oryzias latipes*. Dev Dyn 237(12):3870–3881

Knabe W, Kuhn HJ (1996) The role of microtubules and microtubule-organising centres during the migration of mitochondria. J Anat 189(pt 2):383–391

Laprise P, Beronja S, Silva-Gagliardi NF, Pellikka M, Jensen AM, McGlade CJ, Tepass U (2006) The FERM protein yurt is a negative regulatory component of the Crumbs complex that controls epithelial polarity and apical membrane size. Dev Cell 11(3):363–374

Larison K, Bremiller R (1990) Early onset of phenotype and cell patterning in the embryonic zebrafish retina. Development (Camb) 109:567–576

LaVail MM (1973) Kinetics of rod outer segment renewal in the developing mouse retina. J Cell Biol 58(3):650–661

Lem J, Krasnoperova NV, Calvert PD, Kosaras B, Cameron DA, Nicolo M, Makino CL, Sidman RL (1999) Morphological, physiological, and biochemical changes in rhodopsin knockout mice. Proc Natl Acad Sci USA 96(2):736–741

Lemmers C, Michel D, Lane-Guermonprez L, Delgrossi MH, Medina E, Arsanto JP, Le Bivic A (2004) CRB3 binds directly to Par6 and regulates the morphogenesis of the tight junctions in mammalian epithelial cells. Mol Biol Cell 15(3):1324–1333

Linton JD, Holzhausen LC, Babai N, Song H, Miyagishima KJ, Stearns GW, Lindsay K, Wei J, Chertov AO, Peters TA et al (2010) Flow of energy in the outer retina in darkness and in light. Proc Natl Acad Sci USA 107(19):8599–8604

Longley RL Jr, Ready DF (1995) Integrins and the development of three-dimensional structure in the *Drosophila* compound eye. Dev Biol 171(2):415–433

Louie CM, Caridi G, Lopes VS, Brancati F, Kispert A, Lancaster MA, Schlossman AM, Otto EA, Leitges M, Grone HJ et al (2010) AHI1 is required for photoreceptor outer segment development and is a modifier for retinal degeneration in nephronophthisis. Nat Genet 42(2):175–180

Malicki J, Besharse JC (2012) Kinesin-2 family motors in the unusual photoreceptor cilium. Vis Res 75:33–36

Malicki J, Driever W (1999) *oko meduzy* mutations affect neuronal patterning in the zebrafish retina and reveal cell-cell interactions of the retinal neuroepithelial sheet. Development (Camb) 126:1235–1246

Malicki J, Neuhauss SC, Schier AF, Solnica-Krezel L, Stemple DL, Stainier DY, Abdelilah S, Zwartkruis F, Rangini Z, Driever W (1996) Mutations affecting development of the zebrafish retina. Development (Camb) 123:263–273

Malone CJ, Fixsen WD, Horvitz HR, Han M (1999) UNC-84 localizes to the nuclear envelope and is required for nuclear migration and anchoring during *C. elegans* development. Development (Camb) 126(14):3171–3181

Malone CJ, Misner L, Le Bot N, Tsai M-C, Campbell JM, Ahringer J, White JG (2003) The *C. elegans* hook protein, ZYG-12, mediates the essential attachment between the centrosome and nucleus. Cell 115(7):825–836

Mehalow AK, Kameya S, Smith RS, Hawes NL, Denegre JM, Young JA, Bechtold L, Haider NB, Tepass U, Heckenlively JR et al (2003) CRB1 is essential for external limiting membrane

integrity and photoreceptor morphogenesis in the mammalian retina. Hum Mol Genet 12(17):2179–2189
Meinertzhagen IA, O'Neil SD (1991) Synaptic organization of columnar elements in the lamina of the wild type in *Drosophila melanogaster*. J Comp Neurol 305(2):232–263
Meinke P, Nguyen TD, Wehnert MS (2011) The LINC complex and human disease. Biochem Soc Trans 39(6):1693–1697
Miller RF, Dowling JE (1970) Intracellular responses of the Müller (glial) cells of mudpuppy retina: their relation to b-wave of the electroretinogram. J Neurophysiol 33(3):323–341
Mockel A, Perdomo Y, Stutzmann F, Letsch J, Marion V, Dollfus H (2011) Retinal dystrophy in Bardet-Biedl syndrome and related syndromic ciliopathies. Prog Retin Eye Res 30(4):258–274
Moritz OL, Tam BM, Hurd LL, Peranen J, Deretic D, Papermaster DS (2001) Mutant rab8 impairs docking and fusion of rhodopsin-bearing post-Golgi membranes and causes cell death of transgenic *Xenopus* rods. Mol Biol Cell 12(8):2341–2351
Mosley-Bishop KL, Li Q, Patterson L, Fischer JA (1999) Molecular analysis of the klarsicht gene and its role in nuclear migration within differentiating cells of the *Drosophila* eye. Curr Biol 9 (21):1211–1220
Munson C, Huisken J, Bit-Avragim N, Kuo T, Dong PD, Ober EA, Verkade H, Abdelilah-Seyfried S, Stainier DY (2008) Regulation of neurocoel morphogenesis by Pard6 gamma b. Dev Biol 324(1):41–54
Nakajima H, Tanoue T (2011) Lulu2 regulates the circumferential actomyosin tensile system in epithelial cells through p114RhoGEF. J Cell Biol 195(2):245–261
Nalefski EA, Falke JJ (1996) The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci 5(12):2375–2390
Ocbina PJR, Eggenschwiler JT, Moskowitz I, Anderson KV (2011) Complex interactions between genes controlling trafficking in primary cilia. Nat Genet 43(6)(6):547–553
Omori Y, Malicki J (2006) oko meduzy and related crumbs genes are determinants of apical cell features in the vertebrate embryo. Curr Biol 16(10):945–957
Padmakumar VC, Libotte T, Lu W, Zaim H, Abraham S, Noegel AA, Gotzmann J, Foisner R, Karakesisoglou I (2005) The inner nuclear membrane protein Sun1 mediates the anchorage of Nesprin-2 to the nuclear envelope. J Cell Sci 118(pt 15):3419–3430
Papermaster DS, Schneider BG, Besharse JC (1985) Vesicular transport of newly synthesized opsin from the Golgi apparatus toward the rod outer segment. Ultrastructural immunocytochemical and autoradiographic evidence in *Xenopus* retinas. Invest Ophthalmol Vis Sci 26 (10):1386–1404
Papermaster DS, Schneider BG, DeFoe D, Besharse JC (1986) Biosynthesis and vectorial transport of opsin on vesicles in retinal rod photoreceptors. J Histochem Cytochem 34(1):5–16
Park B, Alves CH, Lundvig DM, Tanimoto N, Beck SC, Huber G, Richard F, Klooster J, Andlauer TFM, Swindell EC et al (2011) PALS1 is essential for retinal pigment epithelium structure and neural retina stratification. J Neurosci 31(47):17230–17241
Patterson K, Molofsky AB, Robinson C, Acosta S, Cater C, Fischer JA (2004) The functions of Klarsicht and nuclear lamin in developmentally regulated nuclear migrations of photoreceptor cells in the *Drosophila* eye. Mol Biol Cell 15(2):600–610
Pellikka M, Tanentzapf G, Pinto M, Smith C, McGlade CJ, Ready DF, Tepass U (2002) Crumbs, the *Drosophila* homologue of human CRB1/RP12, is essential for photoreceptor morphogenesis. Nature (Lond) 416(6877):143–149
Pugh E, Lamb T (2000) Phototransduction in vertebrate rods and cones. In: Stavenga DG, de Grip WJ, Pugh Jr EN (eds) Handbook of biological physics, vol. 3. Elsevier, Amsterdam
Pujic Z, Malicki J (2001) Mutation of the zebrafish glass onion locus causes early cell-nonautonomous loss of neuroepithelial integrity followed by severe neuronal patterning defects in the retina. Dev Biol 234(2):454–469
Rachel RA, May-Simera HL, Veleri S, Gotoh N, Choi BY, Murga-Zamalloa C, McIntyre JC, Marek J, Lopez I, Hackett AN et al (2012) Combining Cep290 and Mkks ciliopathy alleles in mice rescues sensory defects and restores ciliogenesis. J Clin Invest 122(4):1233–1245

Ramsey M, Perkins BD (2013) Basal bodies exhibit polarized positioning in zebrafish cone photoreceptors. J Comp Neurol 521(8):1803–1816

Razafsky D, Blecher N, Markov A, Stewart-Hutchinson PJ, Hodzic D (2012) LINC complexes mediate the positioning of cone photoreceptor nuclei in mouse retina. PLOS ONE 7(10): e47180

Richard M, Grawe F, Knust E (2006a) DPATJ plays a role in retinal morphogenesis and protects against light-dependent degeneration of photoreceptor cells in the *Drosophila* eye. Dev Dyn 235(4):895–907

Richard M, Roepman R, Aartsen WM, van Rossum AG, den Hollander AI, Knust E, Wijnholds J, Cremers FP (2006b) Towards understanding CRUMBS function in retinal dystrophies. Hum Mol Genet 15(Spec No 2):R235–R243

Ringo DL (1967) Flagellar motion and fine structure of the flagellar apparatus in *Chlamydomonas*. J Cell Biol 33(3):543–571

Rodieck RW (1973) The vertebrate retina. Principles of structure and function. WH Freeman, San Francisco

Röhlich P (1975) The sensory cilium of retinal rods is analogous to the transitional zone of motile cilia. Cell Tissue Res 161(3):421–430

Rosenbaum JL, Witman GB (2002) Intraflagellar transport. Nat Rev Mol Cell Biol 3(11):813–825

Roux KJ, Crisp ML, Liu Q, Kim D, Kozlov S, Stewart CL, Burke B (2009) Nesprin 4 is an outer nuclear membrane protein that can induce kinesin-mediated cell polarization. Proc Natl Acad Sci USA 106(7):2194–2199

Safieddine S, El-Amraoui A, Petit C (2012) The auditory hair cell ribbon synapse: from assembly to function. Annu Rev Neurosci 35:509–528

Seeger-Nukpezah T, Liebau MC, Höpker K, Lamkemeyer T, Benzing T, Golemis EA, Schermer B (2012) The centrosomal kinase Plk1 localizes to the transition zone of primary cilia and induces phosphorylation of nephrocystin-1. PLOS ONE 7(6):e38838

Smith DS, Niethammer M, Ayala R, Zhou Y, Gambello MJ, Wynshaw-Boris A, Tsai LH (2000) Regulation of cytoplasmic dynein behaviour and microtubule organization by mammalian Lis1. Nat Cell Biol 2(11):767–775

Smith UM, Consugar M, Tee LJ, McKee BM, Maina EN, Whelan S, Morgan NV, Goranson E, Gissen P, Lilliquist S et al (2006) The transmembrane protein meckelin (MKS3) is mutated in Meckel-Gruber syndrome and the wpk rat. Nat Genet 38(2):191–196

Sosa BA, Rothballer A, Kutay U, Schwartz TU (2012) LINC complexes form by binding of three KASH peptides to domain interfaces of trimeric SUN proteins. Cell 149(5):1035–1047

Sosa BA, Kutay U, Schwartz TU (2013) Structural insights into LINC complexes. Curr Opin Struct Biol 23(2):285–291

St. Johnston D, Ahringer J (2010) Cell polarity in eggs and epithelia: parallels and diversity. Cell 141(5):757–774

Starr DA, Han M (2002) Role of ANC-1 in tethering nuclei to the actin cytoskeleton. Science 298 (5592):406–409

Steinberg RH, Fisher SK, Anderson DH (1980) Disc morphogenesis in vertebrate photoreceptors. J Comp Neurol 190(3):501–508

Stone J, van Driel D, Valter K, Rees S, Provis J (2008) The locations of mitochondria in mammalian photoreceptors: relation to retinal vasculature. Brain Res 1189:58–69

Stowers RS, Megeath LJ, Górska-Andrzejak J, Meinertzhagen IA, Schwarz TL (2002) Axonal transport of mitochondria to synapses depends on milton, a novel *Drosophila* protein. Neuron 36(6):1063–1077

Sung CH, Tai AW (2000) Rhodopsin trafficking and its role in retinal dystrophies. Int Rev Cytol 195:215–267

Sung CH, Makino C, Baylor D, Nathans J (1994) A rhodopsin gene mutation responsible for autosomal dominant retinitis pigmentosa results in a protein that is defective in localization to the photoreceptor outer segment. J Neurosci 14(10):5818–5833

Swaroop A, Swaroop M, Garen A (1987) Sequence analysis of the complete cDNA and encoded polypeptide for the Glued gene of *Drosophila melanogaster*. Proc Natl Acad Sci USA 84(18):6501–6505

Tai AW, Chuang JZ, Bode C, Wolfrum U, Sung CH (1999) Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. Cell 97(7):877–887

Tammachote R, Hommerding CJ, Sinders RM, Miller CA, Czarnecki PG, Leightner AC, Salisbury JL, Ward CJ, Torres VE, Gattone VH 2nd et al (2009) Ciliary and centrosomal defects associated with mutation and depletion of the Meckel syndrome genes MKS1 and MKS3. Hum Mol Genet 18(17):3311–3323

Tapley EC, Starr DA (2013) Connecting the nucleus to the cytoskeleton by SUN-KASH bridges across the nuclear envelope. Curr Opin Cell Biol 25(1):57–62

Taranum S, Sur I, Müller R, Lu W, Rashmi RN, Munck M, Neumann S, Karakesisoglou I, Noegel AA (2012) Cytoskeletal interactions at the nuclear envelope mediated by nesprins. Int J Cell Biol 2012:736524

Tarchini B, Jolicoeur C, Cayouette M (2013) A molecular blueprint at the apical surface establishes planar asymmetry in cochlear hair cells. Dev Cell 27(1):88–102

Terry SJ, Zihni C, Elbediwy A, Vitiello E, Leefa Chong San IV, Balda MS, Matter K (2011) Spatially restricted activation of RhoA signalling at epithelial junctions by p114RhoGEF drives junction formation and morphogenesis. Nat Cell Biol 13(2):159–166

Thurm U (1964) Mechanoreceptors in the cuticle of the honey bee: fine structure and stimulus mechanism. Science 145(3636):1063–1065

Tiwari S, Hudson S, Gattone VH, Miller C, Chernoff EAG, Belecky-Adams TL (2013) Meckelin 3 is necessary for photoreceptor outer segment development in rat Meckel syndrome. PLOS ONE 8(3):e59306

Tokuyasu K, Yamada E (1959) The fine structure of the retina studied with the electron microscope. IV. Morphogenesis of outer segments of retinal rods. J Biophys Biochem Cytol 6:225–230

Townes-Anderson E, MacLeish PR, Raviola E (1985) Rod cells dissociated from mature salamander retina: ultrastructure and uptake of horseradish peroxidase. J Cell Biol 100(1):175–188

Townes-Anderson E, Dacheux RF, Raviola E (1988) Rod photoreceptors dissociated from the adult rabbit retina. J Neurosci 8(1):320–331

Troutt LL, Burnside B (1988) Microtubule polarity and distribution in teleost photoreceptors. J Neurosci 8(7):2371–2380

Tsujikawa M, Malicki J (2004) Intraflagellar transport genes are essential for differentiation and survival of vertebrate sensory neurons. Neuron 42(5):703–716

Tsujikawa M, Omori Y, Biyanwila J, Malicki J (2007) Mechanism of positioning the cell nucleus in vertebrate photoreceptors. Proc Natl Acad Sci USA 104(37):14819–14824

Uga S, Smelser GK (1973) Comparative study of the fine structure of retinal Müller cells in various vertebrates. Invest Ophthalmol 12(6):434–448

van de Pavert SA, Kantardzhieva A, Malysheva A, Meuleman J, Versteeg I, Levelt C, Klooster J, Geiger S, Seeliger MW, Rashbass P et al (2004) Crumbs homologue 1 is required for maintenance of photoreceptor cell polarization and adhesion during light exposure. J Cell Sci 117(pt 18):4169–4177

van Rossum AGSH (2006) Pals1/Mpp5 is required for correct localization of Crb1 at the subapical region in polarized Muller glia cells. Hum Mol Genet 15(18):2659–2672

Wang Y, Nathans J (2007) Tissue/planar cell polarity in vertebrates: new insights and new questions. Development (Camb) 134(4):647–658

Wang Y, Guo N, Nathans J (2006) The role of Frizzled3 and Frizzled6 in neural tube closure and in the planar polarity of inner-ear sensory hair cells. J Neurosci 26(8):2147–2156

Wang W-J, Tay HG, Soni R, Perumal GS, Goll MG, Macaluso FP, Asara JM, Amack JD, Tsou M-FB (2013) CEP162 is an axoneme-recognition protein promoting ciliary transition zone assembly at the cilia base. Nat Cell Biol 15(6):591–601

Wei X, Malicki J (2002) nagie oko, encoding a MAGUK-family protein, is essential for cellular patterning of the retina. Nat Genet 31(2):150–157
Wei X, Cheng Y, Luo Y, Shi X, Nelson S, Hyde DR (2004) The zebrafish Pard3 ortholog is required for separation of the eye fields and retinal lamination. Dev Biol 269(1):286–301
Wei X, Zou J, Takechi M, Kawamura S, Li L (2006) Nok plays an essential role in maintaining the integrity of the outer nuclear layer in the zebrafish retina. Exp Eye Res 83(1):31–44
Wen GY, Soifer D, Wisniewski HM (1982) The doublet microtubules of rods of the rabbit retina. Anat Embryol (Berl) 165(3):315–328
Wensel TG (2008) Signal transducing membrane complexes of photoreceptor outer segments. Vis Res 48(20):2052–2061
Whited JL, Cassell A, Brouillette M, Garrity PA (2004) Dynactin is required to maintain nuclear position within postmitotic *Drosophila* photoreceptor neurons. Development (Camb) 131 (19):4677–4686
Wieschaus E, Ntisslein-Volhard C, Jiirgens G (1984) Mutations affecting the pattern of the larval cuticle in *D. melanogaster*. Roux Arch Dev Biol 193:296–307
Wilhelmsen K, Litjens SHM, Kuikman I, Tshimbalanga N, Janssen H, van den Bout I, Raymond K, Sonnenberg A (2005) Nesprin-3, a novel outer nuclear membrane protein, associates with the cytoskeletal linker protein plectin. J Cell Biol 171(5):799–810
Williams CL, Li C, Kida K, Inglis PN, Mohan S, Semenec L, Bialas NJ, Stupay RM, Chen N, Blacque OE et al (2011) MKS and NPHP modules cooperate to establish basal body/transition zone membrane associations and ciliary gate function during ciliogenesis. J Cell Biol 192 (6):1023–1041
Worman HJ (2012) Nuclear lamins and laminopathies. J Pathol 226(2):316–325
Xiao Z, Patrakka J, Nukui M, Chi L, Niu D, Betsholtz C, Pikkarainen T, Pikkarainan T, Vainio S, Tryggvason K (2011) Deficiency in Crumbs homolog 2 (Crb2) affects gastrulation and results in embryonic lethality in mice. Dev Dyn 240(12):2646–2656
Yamaguchi M, Imai F, Tonou-Fujimori N, Masai I (2010) Mutations in N-cadherin and a Stardust homolog, Nagie oko, affect cell-cycle exit in zebrafish retina. Mech Dev 127(5-6):247–264
Young RW (1967) The renewal of photoreceptor cell outer segments. J Cell Biol 33(1):61–72
Young RW, Droz B (1968) The renewal of protein in retinal rods and cones. J Cell Biol 39(1):169–184
Yu J, Lei K, Zhou M, Craft CM, Xu G, Xu T, Zhuang Y, Xu R, Han M (2011) KASH protein Syne-2/Nesprin-2 and SUN proteins SUN1/2 mediate nuclear migration during mammalian retinal development. Hum Mol Genet 20(6):1061–1073
Zhao C, Malicki J (2011) Nephrocystins and MKS proteins interact with IFT particle and facilitate transport of selected ciliary cargos. EMBO J 30(13):2532–2544
Zhao Y, Hong DH, Pawlyk B, Yue G, Adamian M, Grynberg M, Godzik A, Li T (2003) The retinitis pigmentosa GTPase regulator (RPGR)-interacting protein: subserving RPGR function and participating in disk morphogenesis. Proc Natl Acad Sci USA 100(7):3965–3970
Zhen Y-Y, Libotte T, Munck M, Noegel AA, Korenbaum E (2002) NUANCE, a giant protein connecting the nucleus and actin cytoskeleton. J Cell Sci 115(pt 15):3207–3222
Zou J, Wang X, Wei X (2012) Crb apical polarity proteins maintain zebrafish retinal cone mosaics via intercellular binding of their extracellular domains. Dev Cell 22(6):1261–1274

Chapter 11
Photoreceptor Degeneration: Molecular Mechanisms of Photoreceptor Degeneration

Jerome E. Roger and Anand Swaroop

Abstract Rod and cone photoreceptors initiate the visual process by capturing photons and transducing the information into chemical and electrical signals. These functionally specialized neurons have high metabolic activity and oxygen consumption, making them vulnerable to genetic insults and changes in microenvironment. Inherited retinal degenerative diseases are clinically and genetically heterogeneous, with more than 200 genes identified so far. In a majority of retinal degenerations, the genetic defects affect diverse functions in photoreceptors, such as phototransduction, gene regulation, splicing, or intracellular transport. To develop efficient therapies, it is critical to elucidate how genetic defects affect cellular functions and activate death pathways. Caspase-dependent and -independent apoptosis appear to be the major route for photoreceptor cell death in retinal diseases, although the importance of necrosis and autophagy has been demonstrated. Distinct molecules associated with oxidative or endoplasmic reticulum stress can activate these interconnected cell death pathways. Notably, in most inherited diseases, the first signs of photoreceptor dysfunction or loss are observed years after birth and late in life, indicating adaptive mechanisms that protect the cells and suggesting their breakdown may lead to cell death. Mitochondria are predicted to play a critical role in integrating cellular homeostasis to stress and initiation of death pathways. Investigations of adaptive behavior and pre-death molecules that modulate the response to genetic factors should provide attractive targets for the development of better therapeutic approaches.

Keywords Cell death • Dystrophy • Retina • Neuro degeneration

J.E. Roger • A. Swaroop (✉)
Neurobiology-Neurodegeneration & Repair Laboratory, National Eye Institute, National Institutes of Health, MSC0610, 6 Center Drive, Bethesda, MD 20892, USA
e-mail: jeromeeroger@gmail.com; swaroopa@nei.nih.gov

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_11, © Springer Japan (outside the USA) 2014

11.1 Introduction

Vision is probably the most important of all senses in humans, allowing us to integrate the information from the surrounding world. From a functional perspective, the eye is sometimes compared to a camera with the ability to focus images with an optimal amount of light on the retina, which acts as the film. However, the light-sensitive retina is much more complicated and versatile than a camera, functioning as an extension of the central nervous system to detect, integrate, and process visual information before sending it to the cortex. Retinal photoreceptors capture light photons and initiate visual transduction cascade. The vertebrate retina contains two types of photoreceptors for light detection: rods optimally function under dim light conditions, whereas cones mediate color vision and high resolution in daylight conditions. The rod and cone photoreceptors are specialized neurons with distinct compartments to carry out distinct functions, as elaborated here.

- *The outer segment* is made of membranous discs containing the phototransduction machinery (Hubbell and Bownds 1979), including visual pigment proteins, called opsins, which vary between rod and different types of cone photoreceptors. The outer segment discs are in contact with the retinal pigment epithelium (RPE), which serves as a selective barrier for transport of macromolecules between choroidal capillaries and photoreceptors and participates in the retinoid cycle necessary for phototransduction.
- *The inner segment* encloses the metabolic machinery, including mitochondria, ribosomes, and other organelles associated with protein synthesis and transport to the outer segment and synaptic region. Massive amounts of protein are transported from the inner to the outer segment through a microtubule-based connecting cilium.
- *The nucleus* is the control center localized in the outer nuclear layer, with discrete chromatin architecture depending on the type of the photoreceptor (Carter-Dawson and LaVail 1979; Solovei et al. 2009).
- *The synaptic terminal*, called the "spherule" for rods and the "pedicle" for cone cells, is located in the outer plexiform layer and is connected to different types of interneurons, called bipolar cells, for signal transmission. Photoreceptors also make synapses with horizontal cells.

The photoreceptors have a high metabolic rate and carry out a complex phototransduction process (Fain et al. 2010; Hubbell and Bownds 1979; McBee et al. 2001). The unique characteristics of photoreceptors contribute to their high vulnerability to genetic defects and to changes in metabolism or microenvironment. Thus, photoreceptor dysfunction or death is commonly observed in many blinding diseases, including retinal and macular degeneration. Retinal degenerative diseases constitute a genetically and clinically heterogeneous group with almost 250 human genetic loci associated with Mendelian forms (such as retinitis pigmentosa and cone dystrophies) and more than 200 genes identified so far (*Retnet; https://sph.uth.edu/retnet/sym-dis.htm*) (Ratnapriya and Swaroop 2013). In addition, dysfunction and

death of retinal photoreceptors are observed in complex multifactorial diseases including age-related macular degeneration (AMD) (Jackson et al. 2002; Swaroop et al. 2009).

Genetic defects associated with photoreceptor degeneration can lead to different outcomes in terms of visual dysfunction:

- Functional abnormalities, consequent to developmental defects or death of some/all of the photoreceptors.
- Functional abnormalities associated with early cell death.
- No or minimal functional abnormalities in early to mid-life, but late disease onset (including cell death).

Rod and cone photoreceptor function and survival can be differentially impacted in retinal diseases. Consequently, a clearer understanding of molecular mechanisms underlying photoreceptor cell death is crucial for developing effective treatments or cure to prevent vision loss or to preserve/restore vision. However, the clinical and genetic diversity associated with distinct forms of photoreceptor degeneration has complicated the elucidation of molecular mechanisms and hampered disease management (Goetz et al. 2012; Swaroop et al. 2007). Recent advances in gene therapy of Leber congenital amaurosis (LCA) caused by *RPE65* mutations demonstrate the invaluable contribution of understanding fundamental cellular processes in designing therapies for blinding retinal diseases and other neurodegenerative disorders (Jacobson et al. 2012). Several excellent reviews have discussed the clinical and genetic aspects of retinal and macular degeneration (Ambati and Fowler 2012; Bramall et al. 2010; Fletcher 2010; Lim and Wong 2012; Ratnapriya and Swaroop 2013; Wright et al. 2010). In this chapter, we therefore focus on the discussion of cell death mechanisms involved in photoreceptor death. We also examine how the knowledge of affected cellular pathways can contribute to the development of therapeutics.

11.2 Retinal Diseases and Photoreceptor Degeneration

11.2.1 Retinitis Pigmentosa (RP)

RP is a genetically heterogeneous form of inherited blindness with prevalence ranging from 1 in 3,000 to 1 in 5,000 individuals worldwide. More than 50 genes have been identified for RP with autosomal dominant, autosomal recessive, or X-linked modes of inheritance. In almost half the patients it is difficult to assign the inheritance; these are called simplex RP cases. One can observe characteristic abnormal pigmentary deposits predominantly in the peripheral retina of RP patients. In some instances, nonocular phenotypes can be associated with RP in syndromic diseases.

In one form of RP, referred to as rod-cone dystrophy, patients initially exhibit progressive loss of night and peripheral vision because of the dysfunction or death of rod photoreceptors. Eventually, at later stages of the disease cone photoreceptors also die, resulting in loss of central vision and a severe restriction of the visual field.

Cone-rod dystrophy (CRD) has a prevalence of 1 in 40,000, with more than 15 genes identified so far. Even though CRD and RP overlap genetically and clinically, CRD often exhibits an initial loss of cone photoreceptors with subsequent or concomitant loss of rods.

11.2.2 Macular Degeneration (MD)

Macular degeneration initially affects the central part of the retina, called the macula, associated with high visual acuity. MD can be inherited in a Mendelian manner or manifest as a complex late-onset disease; the latter is termed age-related macular degeneration (AMD).

Mutations in several genes are associated with MD (Stone 2007). Among the monogenic disorders affecting the macula, Stargardt disease is the most common form of inherited juvenile MD with a prevalence of 1 in 8,000 to 1 in 10,000. In most cases, the disease is caused by mutations in the *ABCA4* gene (Allikmets 1997), but *ELOVL4* mutations have also been associated (Zhang et al. 2001). Stargardt patients display accumulation of yellowish-white deposits in the macular region and eventually exhibit photoreceptor and RPE cell death. Histological examination of donor eyes demonstrates the presence of oxidized cholesterol and insoluble aggregates in these deposits from incomplete degradation of shed photoreceptor outer segments (Molday and Zhang 2010). Another inherited MD, called Best disease or vitelliform macular dystrophy, is caused by mutations in the *VDM2* gene that encodes the RPE protein Bestrophin. Best disease is also characterized by the accumulation of yellow, insoluble deposits within the subretinal space and in the RPE (Sun et al. 2002). Mutations in *RDS*, *TIMP-3*, and *EFEMP1* (also known as *Fibulin-3*) genes also cause different forms of MD (Stone 2007).

In contrast to monogenic disease, AMD is a late-onset multifactorial disorder caused by interaction of environmental and genetic components (Priya et al. 2012; Swaroop et al. 2007; Chamberlain et al. 2006). In developed countries, AMD is a leading cause of blindness in the elderly. In the United States, almost 2 million people past the age of 50 years have some form of AMD, and the prevalence is more than 13% for people older than 85 years (Smith et al. 2001; Friedman et al. 2004); http://www.visionproblemsus.org/index.html). AMD is characterized by decreased central vision because of the loss of photoreceptors and RPE in the macular region of the retina. The Age-Related Eye Diseases Study (AREDS) classified AMD into four categories based on clinical features (Davis et al. 2005). Geographic atrophy is the major advanced form, characterized by the accumulation of large deposits, termed drusen, between the RPE and the Bruch's membrane, and scattered areas of RPE atrophy and photoreceptor degeneration in the macula (Davis et al. 2005).

Drusen are composed of numerous proteins and lipids, but their origin is still unclear (Rudolf et al. 2008). A more severe form of late AMD, called choroidal neovascularization (CNV), accounts for only about 10 % of cases and is characterized by the presence of new blood vessels in the macula. The nascent expanding blood vessels are leaky and lead to photoreceptor cell death. The patients usually experience sudden loss of central vision with distortion of straight lines and/or a dark spot in their central visual field (Lim et al. 2012).

Genome-wide association studies (GWAS) have identified a number of genetic risk variants linked to advanced stages of AMD (Priya et al. 2012). More recently, meta-analysis of GWAS from a large consortium of investigators has established as many as 19 AMD risk loci (Fritsche et al. 2013).

11.2.3 Cone Dystrophy

Cone dystrophies include a group of rare disorders that affect the function and survival of cone photoreceptors, resulting in high sensitivity to bright light, poor color vision, and low visual acuity (Simunovic and Moore 1998). Most cone dystrophy patients have better vision at dusk. Achromatopsia are usually autosomal recessive and represent a majority of stationary cone dystrophy. Individuals have impaired color discrimination, caused by mutations in one of the following five genes: *CNGB3*, *CNGA3*, *GNAT2*, *PDE6C*, *PDE6H* (Hamel 2007). In contrast, progressive cone dystrophy is inherited as autosomal dominant and overlaps with cone-rod dystrophy. For instance, mutations in *GUCA1A* encoding Guanylate Cyclase Activating Protein 1 (GUCAP1) are associated with autosomal dominant cone dystrophy, CRD or MD (Michaelides et al. 2005). Cone opsin gene mutations lead to X-linked cone dystrophy with varying severity from color blindness to progressive cone dystrophy (Gardner et al. 2010).

11.2.4 Leber Congenital Amaurosis (LCA)

LCA is a clinically and genetically heterogeneous congenital or early onset retinal disease (den Hollander et al. 2008) that is characterized by vision loss associated with other clinical symptoms including nystagmus and nonrecordable rod and cone electroretinogram (ERG) (Traboulsi 2010). LCA accounts for as much as 5% of all inherited retinopathies but 20% of the children attending schools for the blind and visually impaired (Koenekoop 2004). Seventeen genes have so far been associated with autosomal recessive LCA, and two, *CRX* and *IMPDH1*, can cause autosomal dominant disease (den Hollander et al. 2008; Freund et al. 1998; Bowne et al. 2002). LCA is the first neurodegenerative disease with effective gene-replacement therapy for patients with *RPE65* mutations (Cideciyan et al. 2008).

11.3 Interdependence of Rod and Cone Photoreceptors for Survival

An interesting feature of retinal degeneration is that rod-specific mutations do not lead only to rod cell death. One would expect that mutations in genes exclusively expressed in rods would affect only night vision and have no major effect on daylight vision. However, patients with mutations in rod-specific genes also experience loss of cone photoreceptors. The kinetics of cone death is variable, depending on the affected gene (LaVail 1981; Punzo et al. 2009; Wright et al. 2004). Thus, in addition to cell-autonomous mechanisms mediating death pathways in rods, non-cell-autonomous factors appear to contribute to the death of normal and otherwise healthy cones. Chimera generated from a mixture of wild-type cells and those expressing mutant rhodopsin show homogenous retinal degeneration independent of the patch of normal or mutant cells (Huang et al. 1993). Similarly, female hemizygous-transgenic *rds* mutant mice also show uniform cell death in the outer nuclear layer, including those of genetically normal photoreceptors (Kedzierski et al. 1998). These studies reveal that photoreceptor death involves not only genetic defects but also cell interactions.

Even mutations that affect other cell types, such as those in RPE-specific genes, can cause non-cell-autonomous photoreceptor death. For instance, in mutant mice as well as human patients, mutations in *RPE65*, an enzyme expressed specifically in RPE, lead to absence of visual response and slow degeneration of rods (Redmond et al. 1998; Gu et al. 1997; Marlhens et al. 1997). Interestingly, rod photoreceptors are generally unaffected in cone dystrophies caused by cone-specific gene mutations.

11.4 Pathways of Photoreceptor Cell Death

Cell death is a part of normal development and can occur in different modes, such as apoptosis, necrosis and autophagy. Multiple interdependent pathways may participate sequentially or overlap to mediate photoreceptor cell death (Kunchithapautham and Rohrer 2007a, b; Metrailler et al. 2012; Lo et al. 2011; Lohr et al. 2006; Doonan et al. 2003).

11.4.1 Apoptosis

Apoptosis is the major cell death pathway (Portera-Cailliau et al. 1994), at times referred as programmed cell death (PCD). Apoptosis is a complex process leading to DNA fragmentation and generation of apoptotic bodies that can be phagocytized and quickly removed to prevent the surrounding cells from further damage by extrusion of cellular contents. Prompt removal of cellular debris avoids

inflammation. The TUNEL assay that labels the fragmented DNA is extensively used to detect apoptosis, but other cell death pathways, such as necrosis and autolysis of the cells, can also exhibit DNA fragmentation (Grasl-Kraupp et al. 1995).

Apoptosis is a critical part of normal retinal development especially for photoreceptors that may be produced in excess (Vecino et al. 2004). In addition, apoptosis is a major cause of cell death under disease conditions, such as RP, retinal detachment, or light-induced apoptosis (Portera-Cailliau et al. 1994; Gregory and Bird 1995; Hafezi et al. 1997; Chang et al. 1995; Cook et al. 1995). Apoptosis is an active process involving several steps controlled by the cell itself (Fig. 11.1). The first step, called induction phase, triggers apoptosis by a wide variety of stimuli such as tumor necrosis factor (TNF), Fas-ligand, and other extracellular factors. During this phase, production of necessary effectors, called pro-apototic factors, is increased. Among them, c-Fos and c-Jun form a heterodimer complex, termed activator protein complex 1 (AP1) (Hafezi et al. 1997; Rich et al. 1997; Vuong et al. 2013; Wenzel et al. 2000). The subsequent executive phase leads to morphological changes including chromatin condensation, DNA fragmentation, cell shrinkage, and ultimately cell fragmentation into apoptotic bodies, in which organelles remain intact to prevent inflammation and damages to the surrounding cells. During the degradation phase, cleavage of proteins, further DNA fragmentation, and cellular shrinkage allow apoptotic bodies to be removed without inflammation by macrophages and activated microglia (Langmann 2007; Ng and Streilein 2001; Joly et al. 2009). Apoptosis can occur in a caspase-dependent or -independent manner.

11.4.1.1 Caspase-Dependent Apoptosis

Caspases, a family of highly conserved cysteine proteases, play a central role in the executive phase. The initiator caspases (2, 9, 8, 10) activate the effector caspases (3, 6, 7) by cleavage. The role of activated caspases is to conduct proteolytic degradation of intracellular targets leading to the cell death program. During this phase, mitochondria play a critical role by releasing cytochrome c by permeabilization of the mitochondrial outer membrane (Green and Kroemer 2004). B-cell lymphoma 2 (Bcl-2) family members participate in the release of cytochrome c; some of these members are pro-apoptotic [e.g., Bcl-2 homologous antagonist/killer (Bak) and Bcl-2-associated X protein (Bax)], whereas others are anti-apoptotic (such as Bcl-2 and Bcl-xL). Cytochrome c activates the caspase cascade by binding to apoptotic peptidase activating factor 1 (Apaf1) and triggering the cleavage of initiator caspases, such as caspase-9, in an ATP-dependent manner (Brenner and Mak 2009; Liu et al. 1996). The cleavage of procaspase-9 to caspase-9 allows the cleavage and activation of the downstream effector caspases, such as caspase-3, which initiate the degradation phase by activating DNase. Caspase-dependent apoptosis has been demonstrated in *rd1* (calcium overload) and *rds* (structural defects) mutant mice and in a light damage model (oxidative stress) (Jomary et al. 2001; Lohr et al. 2006; Perche et al. 2007).

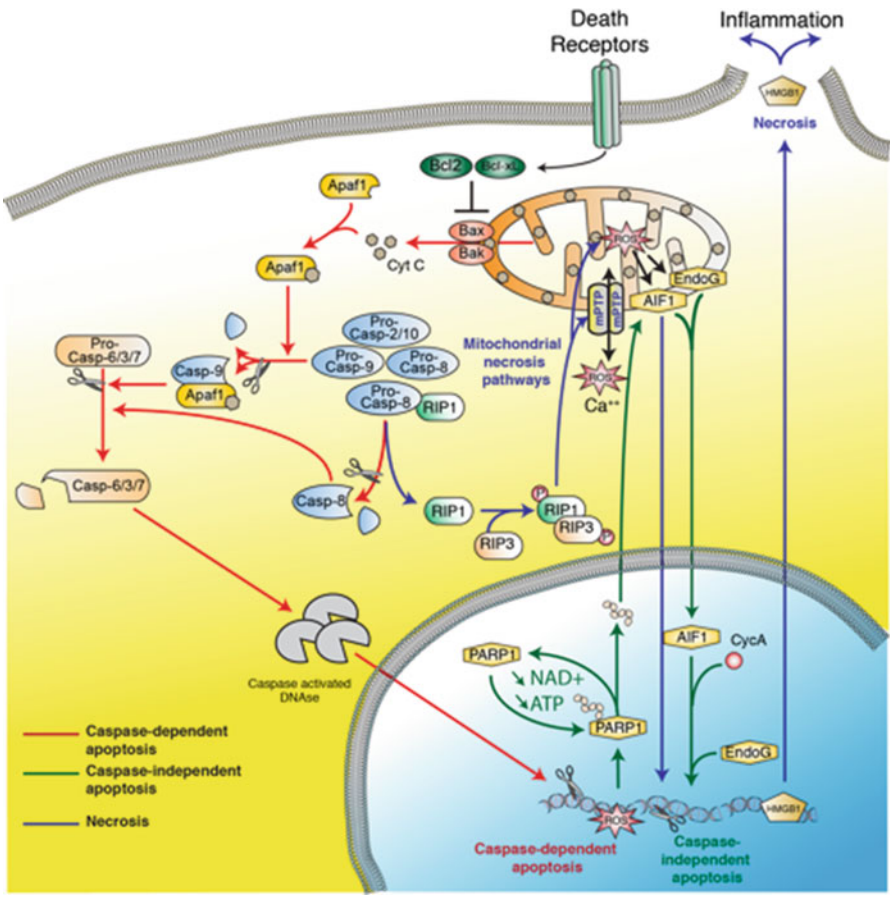

Fig. 11.1 Crosstalk between apoptotic cell death pathway and necrosis. Upon activation of death receptors, caspase-dependent apoptosis involves mitochondria and the release of cytochrome c, which activates APAF1 to cleave procaspases (2, 10, 9,8). The complex APAF1 and Caspase-9 then activate effector caspases such as caspase-3, -6, and -7, allowing the activation of caspase-activated DNAses that cleave genomic DNA and participate at the formation of apoptotic bodies. Caspase-independent apoptosis is also dependent on mitochondria and induced by cell death effectors such as AIF1, EndoG, and PARP1. AIF1 and EndoG are released from mitochondria under oxidative stress condition and translocate into the nucleus. PARP detects DNA strand breaks and transfers polymers of ADP-ribose to a wide range of nuclear proteins. High PARP activity leads to a high ATP consumption and accumulation of poly(ADP-ribosyl) polymers, which leads to the release of AIF1 from the mitochondria to trigger caspase-independent apoptosis. Caspase-dependent apoptosis and necrosis activation can overlap after cleavage of procaspase-8 by the release of RIP1 protein, the key protein to trigger necrosis. Phosphorylation of RIP1 and RIP3 leads to the formation of a pronecrotic complex initiating necrosis. This complex opens the mitochondrial permeability transition pores (mPTP) leading to an increase of intracellular Ca^{2+}, reactive oxygen species (ROS), and the translocation of AIF1 into the nucleus. Necrosis activation ultimately causes the disruption of the plasma membrane and the release of HMGB1 into the extracellular environment to mediate inflammation

11.4.1.2 Caspase-Independent Apoptosis

Although caspase-dependent apoptosis occurs in several retinal degenerative diseases, increasing evidence using a wide range of caspase inhibitors indicates that apoptosis can occur in a caspase-independent manner (Borner and Monney 1999; Bouchier-Hayes et al. 2005) in both in vitro and in vivo models of photoreceptor cell death (Carmody and Cotter 2000; Donovan and Cotter 2002) (Fig. 11.1). Caspase-independent apoptosis is also dependent on mitochondria and induced by cell death effectors, such as apoptosis-inducing factor 1 (AIF1), endonuclease G (EndoG), or poly(ADP-ribose) polymerase I (PARP1).

AIF1 is an oxido-reductase normally localized in the mitochondrial intermembrane space (Cande et al. 2002), and its activity is directly correlated to oxidative stress (Klein et al. 2002). After induction by appropriate apoptotic stimuli, AIF1 translocates to the nucleus and induces chromatin condensation and DNA fragmentation in cooperation with cyclophilin A to elicit apoptosis (Susin et al. 1999). In purified mitochondria, AIF1 triggers cytochrome C and caspase-9 release. AIF1 is involved in apoptosis triggered by retinal detachment in rodent models (Hisatomi et al. 2001), and its inhibition has a protective effect on photoreceptor degeneration (Hisatomi et al. 2008).

EndoG, similar to AIF1, is confined to the mitochondria under normal conditions but translocates to the nucleus upon apoptotic stimulation (Lemarie et al. 2004). In a mouse model of achromatopsia, these two factors are shown to participate in cone cell death caused by endoplasmic reticulum stress (Thapa et al. 2012).

PARP proteins also play a key role in mediating caspase-independent apoptosis. These factors detect DNA strand breaks and are involved in DNA repair (Barth et al. 2006). After activation, PARP enzymes use NAD^+ as a substrate to transfer polymers of ADP-ribose onto itself as well as to many nuclear protein targets. Excessive DNA damage leads to enhanced activation of PARP1, with consequent accumulation of poly(ADP-ribosyl) polymers, which play a critical role in the release of AIF from mitochondria to trigger caspase-independent pathways (Yu et al. 2002; Yu et al. 2006). PARP activation has been shown in several models of retinal degeneration (Kaur et al. 2011; Paquet-Durand et al. 2007). Notably, the lack of PARP1 in the mouse retina does not alter retinal function and protects photoreceptors from cell death (Sahaboglu et al. 2010). Several studies suggest a possible clinical relevance of the pharmacological inhibition of PARP proteins (Jagtap and Szabo 2005; Paquet-Durand et al. 2007).

Photoreceptor cell death is shown to be caspase independent in a number of models of retinal degeneration, such as in *rd* mouse, *N*-methyl-*N*-nitrosourea (MNU)-treated mice, or in light-induced apoptosis (Donovan and Cotter 2002; Doonan et al. 2003; Donovan et al. 2001; Chahory et al. 2010). In these models, cytochrome c release from the mitochondria or activation of the caspase cascade could not be detected (Doonan et al. 2003).

It is widely accepted that caspase-dependent and -independent mechanisms often act in cooperation during apoptosis (Lohr et al. 2006; Kroemer et al. 2009). Hence, the success of therapeutics based on apoptosis inhibition would depend on targeting both caspase-dependent and caspase-independent mechanisms.

11.4.2 Autophagy

Autophagy is an alternative and equally critical death pathway that plays an important role during development and homeostasis of mature tissues. Growing evidence demonstrates that autophagy might be even more important under disease conditions (Kim et al. 2013; Mizushima et al. 2008). The primary function of this catabolic process is to maintain cellular integrity by targeting proteins (e.g., aggregates) and dysfunctional organelles (e.g., mitochondria) to the lysosome for degradation (Marino et al. 2011). A stringent regulation of autophagy is critical for cell homeostasis under normal conditions, and loss of control of degradation can be lethal. During starvation, the degradation of cellular components by autophagy provides resources for survival. Autophagy is an adaptive response under stress and disease conditions to promote cell survival; however, under other scenarios, it may promote cell death. The main autophagy pathway, called macroautophagy, involves organelle and protein degradation by engulfment in a double membrane and formation of an autophagosome, which is then targeted to and fuses with the lysosome for degradation of the enclosed material by lysosomal proteases. In contrast, microautophagy is characterized by direct invagination of the lysosomal membrane to completely enclose the cytoplasmic material. Chaperone-mediated autophagy (CMA) is a more complex and specific pathway in which cytosolic soluble proteins containing a recognition motif for chaperones are selectively transported to the lysosome. The translocation to the lysosome is triggered after binding of the chaperone to a lysosomal receptor, named lysosome-associated membrane protein type 2A (LAMP2A) (Cuervo 2010).

Macroautophagy is a nonspecific process often activated in response to nutrient deprivation and stress conditions. In contrast, CMA is usually activated after long periods of starvation leading to an increase of LAMP2A synthesis (Cuervo and Dice 2000; Marino et al. 2011; Massey et al. 2004). Crosstalk among different autophagic pathways can compensate for each other (Kaushik et al. 2008; Massey et al. 2008). Recent work on photoreceptor aging indicates that dysfunction of macroautophagy is correlated with significant increase of CMA markers in aged animals (Rodriguez-Muela et al. 2013). Such compensatory mechanisms can explain the delay between the start of expression of macroautophagy markers and apparent cell death occurring much later. Interestingly, inhibition of CMA in the retina does not lead to an increase in macroautophagy; instead, cells become more sensitive to stress.

In aging, AMD, and a majority of retinal dystrophies, rod photoreceptors die first, followed later by cones, which have a much slower rate of cell death (Kolesnikov et al. 2010; Punzo et al. 2009). Interestingly, macroautophagy is not activated in dying cones. On the contrary, it is inhibited with a concomitant increase of LAMP2A expression specifically in the lysosomal membrane of cones, potentially reflecting starvation of these cells. Microarray analysis of dying cone photoreceptors in mouse models of RP has identified several genes associated with insulin metabolism, suggesting that the level of intracellular glucose in cones may

be altered (Punzo et al. 2009). Recently, glucose starvation of cones in vitro has also been shown to trigger autophagy (Balmer et al. 2013).

It is often difficult to determine whether autophagy is the cause or the consequence of photoreceptor stress and death. For instance, RPE accumulates proteins, lipofuscin, and damaged mitochondria during aging and in AMD (Kaarniranta et al. 2010; Kaarniranta et al. 2013; Krohne et al. 2010; Wang et al. 2009a, b). Accumulation of lipofuscin in the RPE can alter the fusion between the autophagosome and the lysosome. In addition, accumulation of A2E, the major lipofuscin fluorophore reportedly inhibits ATP-driven proton pump in the RPE cells, leading to increase of lysosomal pH. Increase in the autophagy flux and accumulation of toxic products may thus contribute to drusen formation, which are hallmarks of AMD (Bergmann et al. 2004). Autophagy is an important pathway for photoreceptor maintenance and homeostasis, especially during disc outer segment shedding (Kim et al. 2013; Long et al. 1986). Autophagy may also regulate opsin protein levels to adjust to lighting environment (Reme et al. 1999).

The ubiquitin proteasome system (UPS) is a major protein degradation pathway. Several lines of evidence indicate a crosstalk between autophagy and proteasomal degradation, especially in selective autophagy (Kraft et al. 2010). The UPS controls protein stability and degradation of the target proteins by posttranslational modifications, including covalent addition of several monomers of a small protein ubiquitin, transferred by sequential action of E1-, E2-, and E3-ligases. The proteasome complex then degrades poly-ubiquitinated proteins. This complex regulates a number of intracellular proteins associated with cell signaling, cell-cycle regulation, and synaptic plasticity. UPS also plays a critical role in regulating cell death (Tai and Schuman 2008; Vucic et al. 2011). Protein aggregation is a characteristic feature of a wide variety of neurological diseases. Parkinson's disease, Alzheimer's disease, and prion disease are among the most common neurodegenerative diseases, which reveal protein aggregation and dysfunction of the UPS-triggered apoptosis (Bence et al. 2001; Sherman and Goldberg 2001; Snyder et al. 2003). Several mutations associated with inherited retinal degeneration, such as those in rhodopsin, cause protein misfolding, mistargeting, or aggregation (Comitato et al. 2007; Surgucheva et al. 2005; Illing et al. 2002). In the case of formation of the transducin βγ-subunit complex, critical for phototransduction, lack of transducin γ-subunit (Gγ1) in mice leads to increase in production of its partner, transducin β1 (Gβ1), which in the absence of Gγ1 cannot be folded properly and is targeted to the proteasome (Lobanova et al. 2013). Consequently, large increases in Gβ1 overload the UPS, causing cellular stress and photoreceptor cell death. Defects in degrading misfolded proteins have also been observed in rodent models, including *rds* (PRPH2), $Rho^{-/-}$, and Rho^{P23H}. Such mechanisms are also described for extended polyglutamine proteins, such as SCA7 and Huntingtin, leading to neuronal cell death (Lobanova et al. 2013; Yvert et al. 2000; Abe et al. 2000; Helmlinger et al. 2002; Schipper-Krom et al. 2012). Mutations in components of the UPS have also been identified in autosomal dominant RP patients (Friedman et al. 2009).

11.4.3 Necrosis

Necrosis, also called necroptosis, is characterized by an increase in cell volume, swelling of the organelles, and rupture of the plasma membrane causing the release of intracellular components in the surrounding microenvironment and initiating an inflammatory response (Vandenabeele et al. 2010). If apoptosis is a regulated cellular process that can be beneficial and necessary for the organism, necrosis almost always has a negative impact. At first, necrosis was thought to be a nonspecific, uncontrolled cell death mechanism, but recent evidence indicates some level of regulation of this pathway (McCall 2010). Several regulators of necrosis have now been identified, including receptor-interacting protein (RIP) kinases. RIP1 is essential to trigger necrosis in response to tumor necrosis factor (TNF)-α, and FasL stimulation and promotes activation of nuclear factor κB (NF-κB) (Ea et al. 2006; Moquin and Chan 2010; Christofferson and Yuan 2010). In case of failure to activate caspase-dependant apoptosis, RIP1 interacts with RIP3 to form a pronecrotic complex, stabilized by phosphorylation that leads to necrosis pathway activation. Often, apoptosis and necrosis pathways overlap and crosstalk (Fig. 11.1) (Brenner and Mak 2009; Zhang et al. 2009). In necrosis, high-mobility group box 1 (HMGB1), a non-histone chromatin-binding protein, is usually released into the extracellular matrix and acts as a pro-inflammatory factor (Fang et al. 2012). However, little is known about the downstream effectors of necrosis, and additional studies are required.

The importance of RIP and necrotic pathway activation in the secondary death of cone photoreceptors has recently been demonstrated in models of rod-cone dystrophy (Murakami et al. 2012). In the *rd10* mouse, a model of rod-cone dystrophy caused by a mutation in the rod-specific gene, cGMP phosphodiesterase β-subunit (Pde6β), an increase in RIP proteins has been observed in the late phase of retinal degeneration during the period of cone death but not in earlier stages, when most of the rods die. A detailed morphological analysis of dying photoreceptors shows that necrosis is an important cell death pathway for cones in contrast to rods, where apoptosis appears to be the preferred cell death pathway. In concordance, lack of RIP3 expression does not prevent rod cell death but leads to preservation of cones. The importance of RIP proteins in mediating necrosis is also shown recently in a mouse model of retinal degeneration induced by subretinal injection of dsRNA, a component of the drusen, causing RPE cell death and inflammation in AMD patients (Murakami et al. 2013). In such a model, lack of RIP3 expression has a protective effect by preventing necrosis and inflammation and suppressing the release of HMGB1 (Fang et al. 2012; Murakami et al. 2013). Recent studies have highlighted the contribution of necrosis in several models of photoreceptor degeneration, such as retinal detachment, light damage, and retinal ischemia (Shahinfar et al. 1991; Trichonas et al. 2010; Rosenbaum et al. 2010).

11.5 Mediators of Cell Death Pathways

11.5.1 Calcium Signaling and Photoreceptor Cell Death

Under dark conditions, rod photoreceptors are depolarized because of an inward flow of sodium and calcium through cyclic nucleotide-gated channels (cGMP-dependent) located in the outer segment. Upon light stimulation, 11-*cis*-retinal bound to rhodopsin is isomerized into all-*trans*-retinal, triggering the release of α-subunit of the G-protein transducin, which in turn activates a photoreceptor-specific cyclic nucleotide phosphodiesterase (PDE). Activated PDE hydrolyzes cGMP to GMP, reducing its intracellular concentration, and thereby closing cyclic nucleotide-gated channels and triggering an outward flux of potassium leading to hyperpolarization of the photoreceptor membrane. Channel closure also leads to a decrease in calcium influx and a decrease of glutamate release at the synaptic terminal. cGMP concentration is restored by activation of guanylate cyclase upon reduction in intracellular calcium. Enzyme dysregulation, altered energy utilization, and changes in signaling molecule concentration are predicted to cause photoreceptor cell death when the visual transduction cascade is continuously active. Thus, calcium may play a crucial role in cell death.

Overload of intracellular calcium is a major contributor to cell death, including in photoreceptors (Wenzel et al. 2005; Zhivotovsky and Orrenius 2011; Fox et al. 1999; Takano et al. 2004; Read et al. 2002; Fox et al. 2003; Sharma and Rohrer 2004). However, it is still unclear whether the additional Ca^{2+} enters from the extracellular space or is released from intracellular storage in mitochondria or endoplasmic reticulum (Orrenius et al. 2003). The importance of Ca^{2+} in photoreceptor cell death has been investigated in the *rd1* mouse retina, where a strong activation of Ca^{2+}-dependent enzymes occurs (Paquet-Durand et al. 2006; Hauck et al. 2006). Here, photoreceptors go under severe and rapid degeneration caused by a mutation in PDE-β, which, when mutated, also causes photoreceptor death in humans (Bowes et al. 1990; McLaughlin et al. 1993). The mutated PDE6β is not functional, potentially leading to cGMP accumulation and constitutive opening of cGMP-gated channels, resulting in an influx of Ca^{2+} into the cells. The deleterious effect of Ca^{2+} has been further demonstrated using *rd1* mice that also lack a major L-type voltage-dependent Ca^{2+} channel at the photoreceptor synapse. In the double mutant mice, photoreceptor cell death is slower compared to the *rd1* mice (Read et al. 2002). Calpains are a group of 15 Ca^{2+}-activated cysteine proteases that have been implicated in degeneration of neuronal tissues (Paquet-Durand et al. 2006; Smith and Schnellmann 2012; Nakazawa 2011). Association of calpains with calpastatins in the endoplasmic reticulum specifically inhibits their activity, and overexpression of calpastatins has neuroprotective effects (Suzuki et al. 2004; Schoch et al. 2013; Wingrave et al. 2004; Cao et al. 2007). In the retina, calpains also play a critical role in photoreceptor cell death, and their inhibition can delay photoreceptor demise (Paquet-Durand et al. 2010; Mahajan et al. 2012; Ozaki et al. 2013). In the *rd1* mutant, calpains are strongly activated during photoreceptor

cell death with a concomitant downregulation of calpastatin and cyclic AMP response element-binding protein (CREB)-1 (Paquet-Durand et al. 2006). CREB1, a transcription factor critical for neuronal survival (Finkbeiner 2000; Mantamadiotis et al. 2002), is critical for integrating Ca^{2+} and cAMP signals. It can be activated by the AC/cAMP/PKA pathway, triggered by high intracellular Ca^{2+}, or by Ca^{2+}/calmodulin-dependent protein kinases (CAMK).

11.5.2 Oxidative Stress and Photoreceptor Cell Death

Oxidative stress is closely linked to a number of neurodegenerative diseases (Andersen 2004; Komeima et al. 2006; Shen et al. 2005; Anderson et al. 1999) and is produced by increase in free radicals that cause damage to proteins, lipids, and DNA. Reactive oxygen species (ROS) are highly reactive molecules produced by normal oxygen metabolism from the mitochondria or by exogenous sources such as ionizing radiation. Varying rates of ROS production have been proposed to underlie differential rates of cell death in neurodegenerative diseases sharing the same mutation (Wright et al. 2004). Neuronal cells have very high metabolism and their membranes are rich in lipids and unsaturated fatty acids, which are ideal targets of lipid peroxidation, thus making them highly susceptible to damage by ROS.

During aging, the scavenging activities of endogenous antioxidants decrease and trigger oxidative stress (Klein and Ackerman 2003). However, it is still difficult to discriminate whether oxidative stress is the cause or the consequence of neuronal cell death (Andersen 2004). Additional investigations are required to identify signaling pathways activated by ROS and to define their role in death pathways.

Similar to other neuronal cells, photoreceptors are under constant environmental and intrinsic stress. Their function and location in the retina and the presence of high concentration of lipids in the outer segments make photoreceptors specially vulnerable as they are exposed to light, and ultraviolet radiations in particular, which can lead to the production of free radicals (Kagan et al. 1973; Oguni et al. 1996; Yang et al. 2003). These deleterious effects are amplified by high oxygen tension caused by proximity of the choroidal blood vessels that also participate in the production of ROS (Nickla and Wallman 2010). Interestingly, impairment of choroidal blood circulation in light-exposed albino rats is shown to delay photoreceptor cell death (Tanito et al. 2007b).

Mitochondrial metabolism is a major source of ROS. In photoreceptors, mitochondria are located in the inner segments. Mouse cone photoreceptors contain twofold more mitochondria than rods, and the ratio reaches tenfold in humans and primates (Hoang et al. 2002; Perkins et al. 2003). If rod and cone mitochondrial membranes have the same width, the total membrane surface is greater in cones compared to rods, suggesting high-energy demand and higher cytochrome c oxidase activity in cones (Hoang et al. 2002; Perkins et al. 2003). Oxidative stress and photoreceptor degeneration are tightly linked (Costa et al. 2008;

Shen et al. 2005), as has been described in models of retinal degeneration (Cao and Phillis 1995; Costa et al. 2008; Komeima et al. 2006; Kowluru and Odenbach 2004). As a by-product of energy production, mitochondria produce free radicals, including superoxide (O_2^-) radicals. Mitochondria are also equipped with antioxidant mechanisms to preserve cell homeostasis. Among them are superoxide dismutase (SOD), in charge of converting O_2^- to hydrogen peroxide (H_2O_2), and glutathione peroxidase and related enzymes, which convert H_2O_2 to H_2O, making glutathione an important cellular antioxidant. Transgenic mice with gain-of-function mutation in *SOD1*, associated with familial amyotrophic lateral sclerosis in human, show specific light-induced photoreceptor cell death (Mittag et al. 1999). In addition, decreased expression of SOD2 leads to photoreceptor cell death (Justilien et al. 2007). Such alterations in antioxidant properties of mitochondria may also be linked to AMD (Khandhadia and Lotery 2010). Because rod photoreceptors outnumber cones, it has been proposed that rod death leads to a great increase of oxygen tension on the remaining cone photoreceptors, thereby increasing their oxidative stress (Yu et al. 2000). Such mechanisms might participate in the non-cell-autonomous death occurring in rod-specific inherited retinal degeneration and explain the initially slow death rate in cones. Oxidative damage is reported in a transgenic pig model with rhodopsin mutation, in which cone photoreceptors progressively accumulate oxidative damage over time in parallel to their death (Shen et al. 2005). A similar pattern of oxidative damage and cell death is observed in *rd1* mice (Komeima et al. 2006). In contrast, reduction of superoxide radical production can reduce cone cell death (Komeima et al. 2008). Thus, loss of rod photoreceptors in RP may lead to hyperoxia in the outer nuclear layer, causing oxidative damage and slow death of cone photoreceptors.

The presence of free radicals in photoreceptor outer segments can also result in peroxidation of lipids, which account for about 15% of the total weight of photoreceptors compared to about 1% in most other cells (Bramall et al. 2010). Photoreceptors have a high amount of decosahexaenoic acid (DHA), a very long chain polyunsaturated fatty acid, which can be oxidized to highly reactive molecules such as molondialdehde (MDA) and 4-hydroxy-2-neoenal (4-HNE), two by-products that indicate the level of oxidative stress in cells (Moreira et al. 2010). Furthermore, oxidized phosphatidylcholine increases in human photoreceptors and RPE located in the macula during aging and at even higher levels in AMD patients (Suzuki et al. 2007). Oxidative stress is also shown to damage mitochondrial DNA in human RPE (Liang and Godley 2003). The decreased antioxidant capacity during aging, together with a concomitant damage to mitochondrial DNA, can contribute to AMD pathophysiology. Our recent studies in cone-only $Nrl^{-/-}$ mouse retina have shown an association between rapid and transient death of cones and transient increase of oxidative stress that are followed by long-term preservation of the remaining cone photoreceptors after lipid peroxidation suppresses (Roger et al. 2012).

Daily shedding and renewal of photoreceptor outer segments by the RPE can also be considered an antioxidant mechanism, designed to remove oxidized lipids and their by-products (Winkler 2008). Additional antioxidant mechanisms can

protect photoreceptors; for example, thioredoxins are small proteins playing an important role in the redox signaling cascade (Tanaka et al. 2000). Their expression is upregulated in response to oxidative stress and shown to have neuroprotective effects, especially in the retina (Tanito et al. 2002a, b, 2005, 2007a; Wang et al. 2008; Kong et al. 2010). Thioredoxin-like protein rod-derived cone survival factor (RdCVF) has been identified based on its property to sustain the viability of cone photoreceptors (Leveillard et al. 2004). RdCVF proteins are localized in the cone extracellular matrix, and lack of their expression in mice affects photoreceptor function and increases oxidative stress, suggesting a relationship between RdCVF and redox signaling (Cronin et al. 2010).

11.5.3 Purine and Photoreceptor Death

Intracellular ATP is a major source of cellular energy. When released into the extracellular space, it can also be used as a purinergic neurotransmitter in the central and peripheral nervous system. If the intracellular concentration of ATP is in the millimolar range, the extracellular concentration ranging from nanomolar to micromolar must result from the balance between release and degradation (Schwiebert 2000). Enzymes located at the cellular membrane, called ectonucleotidases, which produce ADP or AMP, control ATP concentration in the extracellular space. ATP can be released by damaged membranes of dying cells, by ATP transporters, channels, or osmotic transporters, or by exocytosis from synaptic vesicles. Two families of receptors, called P2X (seven subtypes) and P2Y receptors (eight subtypes), mediate ATP signaling. P2X receptors assemble into ATP-activated cation channels, selectively permeable to sodium and calcium ions, leading to an increase of intracellular calcium concentration and depolarization of the cell membrane (Abbracchio et al. 2009). P2X receptors have low affinity for ATP and have a fast response because they do not require the diffusion of a second messenger (Volonte et al. 2003). In contrast, P2Y receptors are metabotropic receptors belonging to the family of seven-transmembrane domain G protein-coupled receptors that generate a slower response because they have a higher affinity for their ligand and are coupled to potassium or calcium channels (Weisman et al. 2012).

ATP is demonstrated to regulate retinal function, especially as a neuromodulator released by Müller glia cells (Ward et al. 2010; Neal and Cunningham 1994; Newman 2003). $P2X_7$ and $P2Y_6$ receptors are expressed in the plexiform layers of rodent retinas, especially in the photoreceptor synaptic terminals (Puthussery and Fletcher 2004; Puthussery et al. 2006; Zhang et al. 2012). In addition to their role in modulating neurotransmission, P2 receptors are shown to be involved in cell homeostasis in the central nervous system (Franke and Illes 2006). The release of ATP into the extracellular environment can also participate in apoptosis-induced cell death after injury (Wang et al. 2004). In the retina, intravitreal injections of ATP, but not its breakdown products (i.e., ADP or AMP), induce extensive and

selective photoreceptor apoptosis through activation of P2 purinoreceptors in a dose-dependent manner and lead to severe photoreceptor degeneration and impaired ERG (Puthussery and Fletcher 2009). Notably, injection of the purinergic antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) prevents ATP-induced apoptosis and reduces photoreceptor cell death in *rd1* mouse retina. The critical role of $P2RX_7$ in ATP-mediated apoptosis has been reported in cells under physiological stress such as nutrient starvation (Notomi et al. 2011). Injection of a $P2RX_7$ agonist triggered photoreceptor cell death in wild-type animals but has no effect in mice lacking $P2X_7$ receptor. Apoptosis can be mediated by activation of caspase-8 and -9, leading to the translocation of AIF1 from mitochondria to the nuclei (Notomi et al. 2011). Such a mechanism has also been identified in AMD patients with subretinal hemorrhage caused by choroidal neovascularization (CNV), in which increased levels of ATP are observed (Notomi et al. 2013). In vitro, extravascular blood initiates apoptotic pathways after binding to $P2X_7$ receptors, suggesting an important role for ATP in exacerbating photoreceptor cell apoptosis in AMD patients with CNV. The preceding discussion suggests that dying photoreceptors may release ATP into the extracellular space, and released ATP can bind to P2 receptors on neighboring photoreceptors and contribute to cell death by excessive stimulation.

11.6 Complexities Associated with Photoreceptor Degeneration

In addition to the large number of genes associated with photoreceptor degeneration, different mutations in the same gene can have diverse outcomes in terms of disease type, progression, and severity. For instance, mutations in *CRX*, a critical transcription factor for photoreceptor development, can cause dominant LCA, cone-rod dystrophy, or RP (Huang et al. 2012). In addition to allelic differences, environmental factors and genetic modifiers can also cause phenotypic variability. Modifier genes can protect or have additional deleterious effects, as described for *RPGR*/*RPGRIP1L*, *RPE65*, or *CEP290* (Cideciyan et al. 2013; Coppieters et al. 2010; Fahim et al. 2011; Khanna et al. 2009). Furthermore, clear heterogeneity exists in disease progression and cell death location within the retina. Nonuniform photoreceptor cell death illustrates the importance and differential sensitivity of photoreceptors expressing the exact same mutation (Jacobson et al. 2000). In mice, photoreceptor degeneration can follow in either way around a central/peripheral axis, suggesting that a gradient of molecules makes the photoreceptor cells more or less sensitive to cellular stress. One can also hypothesize the existence of distinct rod subtypes in the retina with differential sensitivity to cell death. Consequently, gene-profiling analysis from macula versus peripheral retina may reveal factors influencing photoreceptor homeostasis (Sharon et al. 2002). The heterogeneity of retinal photoreceptors is illustrated with different distribution of cones among

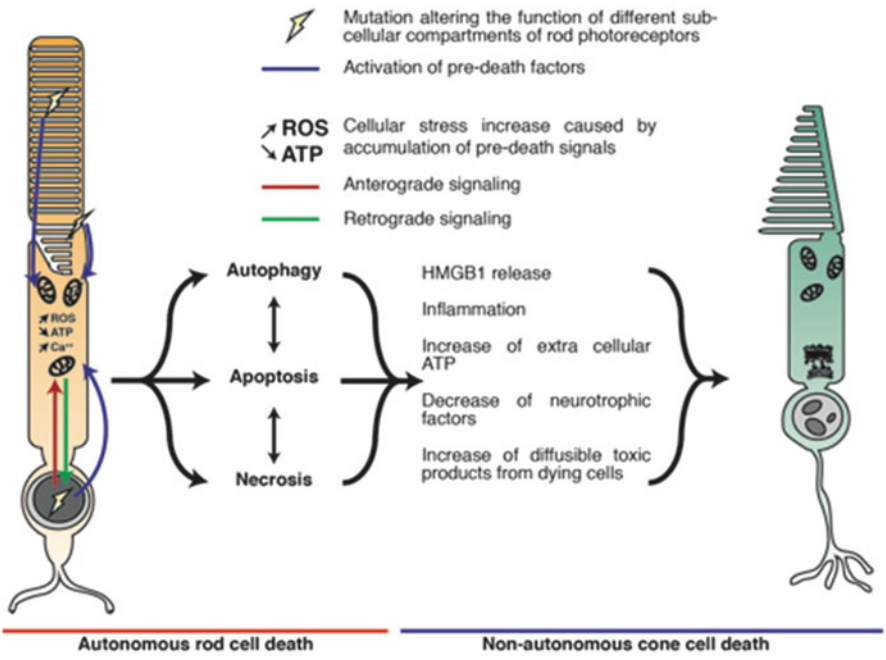

Fig. 11.2 Convergence of cell death signals to trigger autonomous and nonautonomous cell death during photoreceptor degeneration. The large number of mutations in genes causing photoreceptor degeneration can alter any subcellular compartments of the photoreceptor cells. Mitochondria play an important role in the integration of pre-death factors generated by the cellular stress caused by mutated genes over time. Exchange of signals from the nucleus to the mitochondria (anterograde signaling) and vice versa (retrograde signaling) is an important part of this integration of life and death signal by the mitochondria to maintain cell homeostasis. Accumulation above a certain threshold of pre-death signal and cellular stress, such as ROS, activates photoreceptor cell death. In rod-cone dystrophies, rod photoreceptor cell death by apoptosis, necrosis, or autophagy leads to nonautonomous cone cell death by the release of toxic signals or a decrease of neurotrophic factors produced

vertebrates and especially by a dorsal–ventral gradient of short and medium/long wavelength opsins in the mouse retina (Applebury et al. 2000; Ng et al. 2001). However, very few molecules displaying gradient have so far been identified in the retina. Alternatively, a gradient of neurotrophic factors may create variability in the neuroprotective effects, generating resistance of photoreceptors to cellular stress (Cornish et al. 2005).

A large number of mutations leading to photoreceptor cell death converge to common death pathways such as apoptosis, necrosis, and autophagy (Fig. 11.2). Consequently, all these mutations must share common death effectors (Swaroop et al. 2010). Many genes associated with photoreceptor degeneration are ubiquitously expressed and exhibit extensive functional diversity. In addition, mutations in disease-causing genes do not always lead directly to nonfunctional photoreceptors. Slow progression of disease in many mutations supports a model involving the

accumulation of toxic molecules and damage before cell death (Clarke et al. 2001). LCA and AMD represent two extremes, with LCA-causing genes leading to early childhood blindness, whereas in AMD the first signs of the disease appear after many decades.

It is critical to identify pre-cell death molecules shared by different types of mutations because it is unlikely that each mutated gene triggers its own pre-apoptotic pathway (Swaroop et al. 2010). In such a model, the pre-death signal must reach a certain level to trigger cell death. The pre-death signal may also participate in death of neighboring photoreceptors because of the accumulation of toxic products extruded by dying cells into the microenvironment or the depletion of pro-survival factors [such as rod-derived cone viability factor (Rdcvf) and ciliary neurotrophic factor (Cntf)] (Fig. 11.2). The presence of diffusible toxic factors released by dying photoreceptors is supported by studies using chimeric animals in which the retina contains both normal and mutant photoreceptors and the cell death rate is proportional to the number of mutant cells (Burns et al. 2002; Huang et al. 1993). In such a model, the pre-death signal may converge and be integrated by an organelle in the cell to trigger cell death. One organelle where such integration takes place is the mitochondria, as the production of ROS appears to be correlated with the rate of photoreceptor cell death (Perez-Campo et al. 1998; Sastre et al. 2000; Wright et al. 2004). Overexpression of antioxidant defenses, such as SOD, which has neuroprotective effects, can extend cellular lifespan (Sampayo et al. 2003). However, the correlation between oxidative stress and neurodegeneration is still unclear, because most genes do not alter or directly induce ROS production. Mitochondrial DNA is more vulnerable to mutations compared to nuclear DNA, and such accumulation of genomic abnormalities would occur independently in each photoreceptor (Wright et al. 2009). The consequence could be altered mitochondrial bioenergetic functions, which then act as redox damage sensors. Mitochondria and the nucleus are suggested to have anterograde (nucleus to mitochondria) and retrograde signaling to maintain homeostasis and prevent the triggering of cell death pathways (Galganska et al. 2010). Oversaturation of ROS can modify proteins involved in cell death pathways and damage key mitochondrial proteins (Luce et al. 2010). The other consequence of ROS would be the modification of lipids in the mitochondria, which may influence the release of cytochrome c (Ott et al. 2002). In the retina, P53 and HDAC4 are two important components regulating photoreceptor cell death partly because of their sensitivity to oxidative stress (Ali et al. 1998; Chen and Cepko 2009).

11.7 Concluding Remarks and Therapeutic Perspective

Photoreceptor degeneration has often been associated with classical caspase-dependent apoptotic pathways. However, as discussed here, caspase-independent apoptosis and nonapoptotic pathways, such as necrosis or autophagy, can also play important roles. In addition, crosstalk among different death pathways would make

therapeutic approaches for photoreceptor degeneration challenging (Doonan et al. 2005; Lohr et al. 2006). One target can be mitochondria, which are one of the main hubs to integrate cellular stress signal and activate downstream cell death pathways (Lezi and Swerdlow 2012; Newmeyer and Ferguson-Miller 2003).

Retinal dystrophies offer great therapeutic potential because of easy anatomical access of the eye, an immunologically confined environment, and availability of noninvasive tools for assessing treatment outcomes in clinical trials. Such tools include direct vision assessment, the electroretinogram to test retinal function, and optical coherence tomography, which provides live imaging of the retina.

To prevent loss of vision in individuals with predisposing mutations, several approaches must be attempted concurrently: gene therapy, cell therapy, prosthesis implantation, and more recently optogenetic methods. Neuroprotection can also be used to preserve photoreceptors by blocking cell death or to strengthen endogenous pro-survival pathways. Such methods present the advantage of being mutation independent. However, studies using neurotrophic factors to delay degeneration showed significant variation in efficiency depending on the type of animal model studied, reflecting the complexity of cell death pathways (Trifunovic et al. 2012). The heterogeneous effects observed with neurotrophic factors suggest that multiple and combined pharmacological approaches may be required to thwart photoreceptor cell death.

Neurotrophic factors (i.e., bFGF, NGF, BDNF, PEDF, CNTF) have shown different degrees of neuroprotection in mouse models. Among these neurotrophic factors, ciliary neurotrophic factor (CNTF) has shown efficiency in more than ten different animal models (Wen et al. 2012; MacDonald et al. 2007). The use of encapsulated CNTF-expressing cells implanted in the eye of RP patients has shown promising results, especially for cone preservation, although the protective mechanism is still unclear (Sieving et al. 2006; Talcott et al. 2011). However, sustained CNTF expression leads to morphological and functional alterations (Bok et al. 2002; Rhee et al. 2007). It is still unclear if CNTF directly protects photoreceptors or indirectly by activating secretion from Müller glia cells of extrinsic factors having neuroprotective effects on photoreceptors.

As discussed here, oxidative stress and photoreceptor cell death are linked, and endogenous antioxidants play critical roles in redox homeostasis and photoreceptor survival (i.e., RdCVF, glutathione-*S*-transferase), which have been tested for different type of retinal dystrophies. For instance, in a clinical trial involving patients with RP, the combination of vitamin E, diltiazem, and taurin showed protective effects by delaying the progressive visual field reduction (Pasantes-Morales et al. 2002). Still, therapeutic success appears to be dependent on the genetic background and the nature of the mutation, as described for neurotrophic factors. In regard to the great variety of genetic modifications leading to photoreceptor cell death, it is critical to identify downstream death effectors commonly used in most mutant photoreceptors. The identification of a drug inhibiting such common death effectors will be of interest for further pharmaceutical development as it could have broader neuroprotective effects in a large number of retinal diseases. All these approaches require a detailed understanding of death pathways

activated during photoreceptor degeneration and of the intracellular effectors mediating neuroprotection. The technological advances made during the past few years in high-throughput genomic sequencing and in proteomics will certainly further our understanding of cellular mechanisms mediating photoreceptor homeostasis and assist in the development of therapeutics.

References

Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H (2009) Purinergic signalling in the nervous system: an overview. Trends Neurosci 32(1):19–29. doi:10.1016/j.tins.2008.10.001

Abe T, Tsuda T, Yoshida M, Wada Y, Kano T, Itoyama Y, Tamai M (2000) Macular degeneration associated with aberrant expansion of trinucleotide repeat of the SCA7 gene in 2 Japanese families. Arch Ophthalmol 118(10):1415–1421

Ali RR, Reichel MB, Kanuga N, Munro PM, Alexander RA, Clarke AR, Luthert PJ, Bhattacharya SS, Hunt DM (1998) Absence of p53 delays apoptotic photoreceptor cell death in the rds mouse. Curr Eye Res 17(9):917–923

Allikmets R (1997) A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. Nat Gen 17(1):122. doi:10.1038/ng0997-122a

Ambati J, Fowler BJ (2012) Mechanisms of age-related macular degeneration. Neuron 75(1):26–39. doi:10.1016/j.neuron.2012.06.018

Andersen JK (2004) Oxidative stress in neurodegeneration: cause or consequence? Nat Med 10(Suppl):S18–S25. doi:10.1038/nrn1434

Anderson KM, Seed T, Ou D, Harris JE (1999) Free radicals and reactive oxygen species in programmed cell death. Med Hypotheses 52(5):451–463. doi:10.1054/mehy.1997.0521

Applebury ML, Antoch MP, Baxter LC, Chun LL, Falk JD, Farhangfar F, Kage K, Krzystolik MG, Lyass LA, Robbins JT (2000) The murine cone photoreceptor: a single cone type expresses both S and M opsins with retinal spatial patterning. Neuron 27(3):513–523

Balmer D, Emery M, Andreux P, Auwerx J, Ginet V, Puyal J, Schorderet DF, Roduit R (2013) Autophagy defect is associated with low glucose-induced apoptosis in 661W photoreceptor Cells. PloS One 8(9):e74162. doi:10.1371/journal.pone.0074162

Barth E, Radermacher P, Szabo C (2006) The world according to poly(ADP-ribose) polymerase (PARP)–update 2006. Intensive Care Med 32(10):1470–1474. doi:10.1007/s00134-006-0336-x

Bence NF, Sampat RM, Kopito RR (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. Science 292(5521):1552–1555. doi:10.1126/science.292.5521.1552

Bergmann M, Schutt F, Holz FG, Kopitz J (2004) Inhibition of the ATP-driven proton pump in RPE lysosomes by the major lipofuscin fluorophore A2-E may contribute to the pathogenesis of age-related macular degeneration. FASEB J 18(3):562–564. doi:10.1096/fj.03-0289fje

Bok D, Yasumura D, Matthes MT, Ruiz A, Duncan JL, Chappelow AV, Zolutukhin S, Hauswirth W, LaVail MM (2002) Effects of adeno-associated virus-vectored ciliary neurotrophic factor on retinal structure and function in mice with a P216L rds/peripherin mutation. Exp Eye Res 74(6):719–735

Borner C, Monney L (1999) Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Differ 6(6):497–507. doi:10.1038/sj.cdd.4400525

Bouchier-Hayes L, Lartigue L, Newmeyer DD (2005) Mitochondria: pharmacological manipulation of cell death. J Clin Invest 115(10):2640–2647. doi:10.1172/JCI26274

Bowes C, Li T, Danciger M, Baxter LC, Applebury ML, Farber DB (1990) Retinal degeneration in the rd mouse is caused by a defect in the beta subunit of rod cGMP-phosphodiesterase. Nature (Lond) 347(6294):677–680. doi:10.1038/347677a0

Bowne SJ, Sullivan LS, Blanton SH, Cepko CL, Blackshaw S, Birch DG, Hughbanks-Wheaton D, Heckenlively JR, Daiger SP (2002) Mutations in the inosine monophosphate dehydrogenase 1 gene (IMPDH1) cause the RP10 form of autosomal dominant retinitis pigmentosa. Hum Mol Genet 11(5):559–568

Bramall AN, Wright AF, Jacobson SG, McInnes RR (2010) The genomic, biochemical, and cellular responses of the retina in inherited photoreceptor degenerations and prospects for the treatment of these disorders. Annu Rev Neurosci 33:441–472. doi:10.1146/annurev-neuro-060909-153227

Brenner D, Mak TW (2009) Mitochondrial cell death effectors. Curr Opin Cell Biol 21(6):871–877. doi:10.1016/j.ceb.2009.09.004

Burns J, Clarke G, Lumsden CJ (2002) Photoreceptor death: spatiotemporal patterns arising from one-hit death kinetics and a diffusible cell death factor. Bull Math Biol 64(6):1117–1145. doi:10.1006/bulm.2002.0320

Cande C, Cecconi F, Dessen P, Kroemer G (2002) Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? J Cell Sci 115(pt 24):4727–4734

Cao G, Xing J, Xiao X, Liou AK, Gao Y, Yin XM, Clark RS, Graham SH, Chen J (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. J Neurosci 27(35):9278–9293. doi:10.1523/JNEUROSCI.2826-07.2007

Cao X, Phillis JW (1995) The free radical scavenger, alpha-lipoic acid, protects against cerebral ischemia-reperfusion injury in gerbils. Free Radic Res 23(4):365–370

Carmody RJ, Cotter TG (2000) Oxidative stress induces caspase-independent retinal apoptosis in vitro. Cell Death Differ 7(3):282–291. doi:10.1038/sj.cdd.4400646

Carter-Dawson LD, LaVail MM (1979) Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. J Comp Neurol 188(2):245–262. doi:10.1002/cne.901880204

Chahory S, Keller N, Martin E, Omri B, Crisanti P, Torriglia A (2010) Light induced retinal degeneration activates a caspase-independent pathway involving cathepsin D. Neurochem Int 57(3):278–287. doi:10.1016/j.neuint.2010.06.006

Chamberlain M, Baird P, Dirani M, Guymer R (2006) Unraveling a complex genetic disease: age-related macular degeneration. Surv Ophthalmol 51(6):576–586. doi:10.1016/j.survophthal.2006.08.003

Chang CJ, Lai WW, Edward DP, Tso MO (1995) Apoptotic photoreceptor cell death after traumatic retinal detachment in humans. Arch Ophthalmol 113(7):880–886

Chen B, Cepko CL (2009) HDAC4 regulates neuronal survival in normal and diseased retinas. Science 323(5911):256–259. doi:10.1126/science.1166226

Christofferson DE, Yuan J (2010) Cyclophilin A release as a biomarker of necrotic cell death. Cell Death Differ 17(12):1942–1943. doi:10.1038/cdd.2010.123

Cideciyan AV, Aleman TS, Boye SL, Schwartz SB, Kaushal S, Roman AJ, Pang JJ, Sumaroka A, Windsor EA, Wilson JM, Flotte TR, Fishman GA, Heon E, Stone EM, Byrne BJ, Jacobson SG, Hauswirth WW (2008) Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. Proc Natl Acad Sci USA 105(39):15112–15117. doi:10.1073/pnas.0807027105

Cideciyan AV, Jacobson SG, Beltran WA, Hauswirth WW, Aguirre GD (2013) Reply to Townes-Anderson: RPE65 gene therapy does not alter the natural history of retinal degeneration. Proc Natl Acad Sci USA 110(19):E1706

Clarke G, Lumsden CJ, McInnes RR (2001) Inherited neurodegenerative diseases: the one-hit model of neurodegeneration. Hum Mol Gen 10(20):2269–2275

Comitato A, Spampanato C, Chakarova C, Sanges D, Bhattacharya SS, Marigo V (2007) Mutations in splicing factor PRPF3, causing retinal degeneration, form detrimental aggregates in photoreceptor cells. Hum Mol Genet 16(14):1699–1707. doi:10.1093/hmg/ddm118

Cook B, Lewis GP, Fisher SK, Adler R (1995) Apoptotic photoreceptor degeneration in experimental retinal detachment. Invest Ophthalmol Vis Sci 36(6):990–996

Coppieters F, Casteels I, Meire F, De Jaegere S, Hooghe S, van Regemorter N, Van Esch H, Matuleviciene A, Nunes L, Meersschaut V, Walraedt S, Standaert L, Coucke P, Hoeben H, Kroes HY, Vande Walle J, de Ravel T, Leroy BP, De Baere E (2010) Genetic screening of LCA in Belgium: predominance of CEP290 and identification of potential modifier alleles in AHI1 of CEP290-related phenotypes. Hum Mut 31(10):E1709–E1766. doi:10.1002/humu.21336

Cornish EE, Madigan MC, Natoli R, Hales A, Hendrickson AE, Provis JM (2005) Gradients of cone differentiation and FGF expression during development of the foveal depression in macaque retina. Vis Neurosci 22(4):447–459. doi:10.1017/S0952523805224069

Costa BL, Fawcett R, Li GY, Safa R, Osborne NN (2008) Orally administered epigallocatechin gallate attenuates light-induced photoreceptor damage. Brain Res Bull 76(4):412–423. doi:10.1016/j.brainresbull.2008.01.022

Cronin T, Raffelsberger W, Lee-Rivera I, Jaillard C, Niepon ML, Kinzel B, Clerin E, Petrosian A, Picaud S, Poch O, Sahel JA, Leveillard T (2010) The disruption of the rod-derived cone viability gene leads to photoreceptor dysfunction and susceptibility to oxidative stress. Cell Death Differ 17(7):1199–1210. doi:10.1038/cdd.2010.2

Cuervo AM (2010) Chaperone-mediated autophagy: selectivity pays off. Trends Endocrinol Metab 21(3):142–150. doi:10.1016/j.tem.2009.10.003

Cuervo AM, Dice JF (2000) Regulation of lamp2a levels in the lysosomal membrane. Traffic 1(7):570–583

Davis MD, Gangnon RE, Lee LY, Hubbard LD, Klein BE, Klein R, Ferris FL, Bressler SB, Milton RC, Age-Related Eye Disease Study Group (2005) The Age-Related Eye Disease Study severity scale for age-related macular degeneration: AREDS Report No. 17. Arch Ophthalmol 123(11):1484–1498. doi:10.1001/archopht.123.11.1484

den Hollander AI, Roepman R, Koenekoop RK, Cremers FP (2008) Leber congenital amaurosis: genes, proteins and disease mechanisms. Prog Retin Eye Res 27(4):391–419. doi:10.1016/j.preteyeres.2008.05.003

Donovan M, Carmody RJ, Cotter TG (2001) Light-induced photoreceptor apoptosis in vivo requires neuronal nitric-oxide synthase and guanylate cyclase activity and is caspase-3-independent. J Biol Chem 276(25):23000–23008. doi:10.1074/jbc.M005359200

Donovan M, Cotter TG (2002) Caspase-independent photoreceptor apoptosis in vivo and differential expression of apoptotic protease activating factor-1 and caspase-3 during retinal development. Cell Death Differ 9(11):1220–1231. doi:10.1038/sj.cdd.4401105

Doonan F, Donovan M, Cotter TG (2003) Caspase-independent photoreceptor apoptosis in mouse models of retinal degeneration. J Neurosci 23(13):5723–5731

Doonan F, Donovan M, Cotter TG (2005) Activation of multiple pathways during photoreceptor apoptosis in the rd mouse. Invest Ophthalmol Vis Sci 46(10):3530–3538. doi:10.1167/iovs.05-0248

Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ (2006) Activation of IKK by TNF-alpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell 22(2):245–257. doi:10.1016/j.molcel.2006.03.026

Fahim AT, Bowne SJ, Sullivan LS, Webb KD, Williams JT, Wheaton DK, Birch DG, Daiger SP (2011) Allelic heterogeneity and genetic modifier loci contribute to clinical variation in males with X-linked retinitis pigmentosa due to RPGR mutations. PloS One 6(8):e23021. doi:10.1371/journal.pone.0023021

Fain GL, Hardie R, Laughlin SB (2010) Phototransduction and the evolution of photoreceptors. Curr Biol 20(3):R114–R124. doi:10.1016/j.cub.2009.12.006

Fang P, Schachner M, Shen YQ (2012) HMGB1 in development and diseases of the central nervous system. Mol Neurobiol 45(3):499–506. doi:10.1007/s12035-012-8264-y

Finkbeiner S (2000) CREB couples neurotrophin signals to survival messages. Neuron 25(1):11–14

Fletcher EL (2010) Mechanisms of photoreceptor death during retinal degeneration. Optom Vis Sci 87(4):269–275. doi:10.1097/OPX.0b013e3181c9132b

Fox DA, Poblenz AT, He L (1999) Calcium overload triggers rod photoreceptor apoptotic cell death in chemical-induced and inherited retinal degenerations. Ann NY Acad Sci 893:282–285

Fox DA, Poblenz AT, He L, Harris JB, Medrano CJ (2003) Pharmacological strategies to block rod photoreceptor apoptosis caused by calcium overload: a mechanistic target-site approach to neuroprotection. Eur J Ophthalmol 13(suppl 3):S44–S56

Franke H, Illes P (2006) Involvement of P2 receptors in the growth and survival of neurons in the CNS. Pharmacol Ther 109(3):297–324. doi:10.1016/j.pharmthera.2005.06.002

Freund CL, Wang QL, Chen S, Muskat BL, Wiles CD, Sheffield VC, Jacobson SG, McInnes RR, Zack DJ, Stone EM (1998) De novo mutations in the CRX homeobox gene associated with Leber congenital amaurosis. Nat Gen 18(4):311–312. doi:10.1038/ng0498-311

Friedman DS, O'Colmain BJ, Munoz B, Tomany SC, McCarty C, de Jong PT, Nemesure B, Mitchell P, Kempen J, Eye Diseases Prevalence Research Group (2004) Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol 122(4):564–572. doi:10.1001/archopht.122.4.564

Friedman JS, Ray JW, Waseem N, Johnson K, Brooks MJ, Hugosson T, Breuer D, Branham KE, Krauth DS, Bowne SJ, Sullivan LS, Ponjavic V, Granse L, Khanna R, Trager EH, Gieser LM, Hughbanks-Wheaton D, Cojocaru RI, Ghiasvand NM, Chakarova CF, Abrahamson M, Goring HH, Webster AR, Birch DG, Abecasis GR, Fann Y, Bhattacharya SS, Daiger SP, Heckenlively JR, Andreasson S, Swaroop A (2009) Mutations in a BTB-Kelch protein, KLHL7, cause autosomal-dominant retinitis pigmentosa. Am J Hum Genet 84(6):792–800. doi:10.1016/j.ajhg.2009.05.007

Fritsche LG, Chen W, Schu M, Yaspan BL, Yu Y, Thorleifsson G, Zack DJ, Arakawa S, Cipriani V, Ripke S, Igo RP, Jr., Buitendijk GH, Sim X, Weeks DE, Guymer RH, Merriam JE, Francis PJ, Hannum G, Agarwal A, Armbrecht AM, Audo I, Aung T, Barile GR, Benchaboune M, Bird AC, Bishop PN, Branham KE, Brooks M, Brucker AJ, Cade WH, Cain MS, Campochiaro PA, Chan CC, Cheng CY, Chew EY, Chin KA, Chowers I, Clayton DG, Cojocaru R, Conley YP, Cornes BK, Daly MJ, Dhillon B, Edwards AO, Evangelou E, Fagerness J, Ferreyra HA, Friedman JS, Geirsdottir A, George RJ, Gieger C, Gupta N, Hagstrom SA, Harding SP, Haritoglou C, Heckenlively JR, Holz FG, Hughes G, Ioannidis JP, Ishibashi T, Joseph P, Jun G, Kamatani Y, Katsanis N, C NK, Khan JC, Kim IK, Kiyohara Y, Klein BE, Klein R, Kovach JL, Kozak I, Lee CJ, Lee KE, Lichtner P, Lotery AJ, Meitinger T, Mitchell P, Mohand-Said S, Moore AT, Morgan DJ, Morrison MA, Myers CE, Naj AC, Nakamura Y, Okada Y, Orlin A, Ortube MC, Othman MI, Pappas C, Park KH, Pauer GJ, Peachey NS, Poch O, Priya RR, Reynolds R, Richardson AJ, Ripp R, Rudolph G, Ryu E, Sahel JA, Schaumberg DA, Scholl HP, Schwartz SG, Scott WK, Shahid H, Sigurdsson H, Silvestri G, Sivakumaran TA, Smith RT, Sobrin L, Souied EH, Stambolian DE, Stefansson H, Sturgill-Short GM, Takahashi A, Tosakulwong N, Truitt BJ, Tsironi EE, Uitterlinden AG, van Duijn CM, Vijaya L, Vingerling JR, Vithana EN, Webster AR, Wichmann HE, Winkler TW, Wong TY, Wright AF, Zelenika D, Zhang M, Zhao L, Zhang K, Klein ML, Hageman GS, Lathrop GM, Stefansson K, Allikmets R, Baird PN, Gorin MB, Wang JJ, Klaver CC, Seddon JM, Pericak-Vance MA, Iyengar SK, Yates JR, Swaroop A, Weber BH, Kubo M, Deangelis MM, Leveillard T, Thorsteinsdottir U, Haines JL, Farrer LA, Heid IM, Abecasis GR, Consortium AMDG (2013) Seven new loci associated with age-related macular degeneration. Nat Genet 45(4):433–439, 439e, 431–432. doi:10.1038/ng.2578

Galganska H, Karachitos A, Wojtkowska M, Stobienia O, Budzinska M, Kmita H (2010) Communication between mitochondria and nucleus: putative role for VDAC in reduction/oxidation mechanism. Biochim Biophys Acta 1797(6-7):1276–1280. doi:10.1016/j.bbabio.2010.02.004

Gardner JC, Webb TR, Kanuga N, Robson AG, Holder GE, Stockman A, Ripamonti C, Ebenezer ND, Ogun O, Devery S, Wright GA, Maher ER, Cheetham ME, Moore AT, Michaelides M, Hardcastle AJ (2010) X-linked cone dystrophy caused by mutation of the red and green cone opsins. Am J Hum Genet 87(1):26–39. doi:10.1016/j.ajhg.2010.05.019

Goetz KE, Reeves MJ, Tumminia SJ, Brooks BP (2012) eyeGENE(R): a novel approach to combine clinical testing and researching genetic ocular disease. Curr Opin Ophthalmol 23(5):355–363. doi:10.1097/ICU.0b013e32835715c9

Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R (1995) In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 21(5):1465–1468

Green DR, Kroemer G (2004) The pathophysiology of mitochondrial cell death. Science 305(5684):626–629. doi:10.1126/science.1099320

Gregory CY, Bird AC (1995) Cell loss in retinal dystrophies by apoptosis–death by informed consent! Br J Ophthalmol 79(2):186–190

Gu SM, Thompson DA, Srikumari CR, Lorenz B, Finckh U, Nicoletti A, Murthy KR, Rathmann M, Kumaramanickavel G, Denton MJ, Gal A (1997) Mutations in RPE65 cause autosomal recessive childhood-onset severe retinal dystrophy. Nat Genet 17(2):194–197. doi:10.1038/ng1097-194

Hafezi F, Steinbach JP, Marti A, Munz K, Wang ZQ, Wagner EF, Aguzzi A, Reme CE (1997) The absence of c-fos prevents light-induced apoptotic cell death of photoreceptors in retinal degeneration in vivo. Nat Med 3(3):346–349

Hamel CP (2007) Cone rod dystrophies. Orphanet J Rare Dis 2:7. doi:10.1186/1750-1172-2-7

Hauck SM, Ekstrom PA, Ahuja-Jensen P, Suppmann S, Paquet-Durand F, van Veen T, Ueffing M (2006) Differential modification of phosducin protein in degenerating rd1 retina is associated with constitutively active Ca^{2+}/calmodulin kinase II in rod outer segments. Mol Cell Proteomics 5(2):324–336. doi:10.1074/mcp.M500217-MCP200

Helmlinger D, Yvert G, Picaud S, Merienne K, Sahel J, Mandel JL, Devys D (2002) Progressive retinal degeneration and dysfunction in R6 Huntington's disease mice. Hum Mol Gen 11(26):3351–3359

Hisatomi T, Nakazawa T, Noda K, Almulki L, Miyahara S, Nakao S, Ito Y, She H, Kohno R, Michaud N, Ishibashi T, Hafezi-Moghadam A, Badley AD, Kroemer G, Miller JW (2008) HIV protease inhibitors provide neuroprotection through inhibition of mitochondrial apoptosis in mice. J Clin Invest 118(6):2025–2038. doi:10.1172/JCI34267

Hisatomi T, Sakamoto T, Murata T, Yamanaka I, Oshima Y, Hata Y, Ishibashi T, Inomata H, Susin SA, Kroemer G (2001) Relocalization of apoptosis-inducing factor in photoreceptor apoptosis induced by retinal detachment in vivo. Am J Pathol 158(4):1271–1278. doi:10.1016/S0002-9440(10)64078-3

Hoang QV, Linsenmeier RA, Chung CK, Curcio CA (2002) Photoreceptor inner segments in monkey and human retina: mitochondrial density, optics, and regional variation. Vis Neurosci 19(4):395–407

Huang L, Xiao X, Li S, Jia X, Wang P, Guo X, Zhang Q (2012) CRX variants in cone-rod dystrophy and mutation overview. Biochem Biophys Res Commun 426(4):498–503. doi:10.1016/j.bbrc.2012.08.110

Huang PC, Gaitan AE, Hao Y, Petters RM, Wong F (1993) Cellular interactions implicated in the mechanism of photoreceptor degeneration in transgenic mice expressing a mutant rhodopsin gene. Proc Natl Acad Sci USA 90(18):8484–8488

Hubbell WL, Bownds MD (1979) Visual transduction in vertebrate photoreceptors. Annu Rev Neurosci 2:17–34. doi:10.1146/annurev.ne.02.030179.000313

Illing ME, Rajan RS, Bence NF, Kopito RR (2002) A rhodopsin mutant linked to autosomal dominant retinitis pigmentosa is prone to aggregate and interacts with the ubiquitin proteasome system. J Biol Chem 277(37):34150–34160. doi:10.1074/jbc.M204955200

Jackson GR, Owsley C, Curcio CA (2002) Photoreceptor degeneration and dysfunction in aging and age-related maculopathy. Ageing Res Rev 1(3):381–396

Jacobson SG, Cideciyan AV, Iannaccone A, Weleber RG, Fishman GA, Maguire AM, Affatigato LM, Bennett J, Pierce EA, Danciger M, Farber DB, Stone EM (2000) Disease expression of RP1 mutations causing autosomal dominant retinitis pigmentosa. Invest Ophthalmol Vis Sci 41(7):1898–1908

Jacobson SG, Cideciyan AV, Ratnakaram R, Heon E, Schwartz SB, Roman AJ, Peden MC, Aleman TS, Boye SL, Sumaroka A, Conlon TJ, Calcedo R, Pang JJ, Erger KE, Olivares MB, Mullins CL, Swider M, Kaushal S, Feuer WJ, Iannaccone A, Fishman GA, Stone EM,

Byrne BJ, Hauswirth WW (2012) Gene therapy for leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years. Arch Ophthalmol 130(1):9–24. doi:10.1001/archophthalmol.2011.298

Jagtap P, Szabo C (2005) Poly(ADP-ribose) polymerase and the therapeutic effects of its inhibitors. Nat Rev Drug Discov 4(5):421–440. doi:10.1038/nrd1718

Joly S, Francke M, Ulbricht E, Beck S, Seeliger M, Hirrlinger P, Hirrlinger J, Lang KS, Zinkernagel M, Odermatt B, Samardzija M, Reichenbach A, Grimm C, Reme CE (2009) Cooperative phagocytes: resident microglia and bone marrow immigrants remove dead photoreceptors in retinal lesions. Am J Pathol 174(6):2310–2323. doi:10.2353/ajpath.2009.090023

Jomary C, Neal MJ, Jones SE (2001) Characterization of cell death pathways in murine retinal neurodegeneration implicates cytochrome c release, caspase activation, and bid cleavage. Mol Cell Neurosci 18(4):335–346. doi:10.1006/mcne.2001.1036

Justilien V, Pang JJ, Renganathan K, Zhan X, Crabb JW, Kim SR, Sparrow JR, Hauswirth WW, Lewin AS (2007) SOD2 knockdown mouse model of early AMD. Invest Ophthalmol Vis Sci 48(10):4407–4420. doi:10.1167/iovs.07-0432

Kaarniranta K, Hyttinen J, Ryhanen T, Viiri J, Paimela T, Toropainen E, Sorri I, Salminen A (2010) Mechanisms of protein aggregation in the retinal pigment epithelial cells. Front Biosci (Elite Ed) 2:1374–1384

Kaarniranta K, Sinha D, Blasiak J, Kauppinen A, Vereb Z, Salminen A, Boulton ME, Petrovski G (2013) Autophagy and heterophagy dysregulation leads to retinal pigment epithelium dysfunction and development of age-related macular degeneration. Autophagy 9(7):973–984

Kagan VE, Shvedova AA, Novikov KN, Kozlov YP (1973) Light-induced free radical oxidation of membrane lipids in photoreceptors of frog retina. Biochim Biophys Acta 330(1):76–79

Kaur J, Mencl S, Sahaboglu A, Farinelli P, van Veen T, Zrenner E, Ekstrom P, Paquet-Durand F, Arango-Gonzalez B (2011) Calpain and PARP activation during photoreceptor cell death in P23H and S334ter rhodopsin mutant rats. PloS One 6(7):e22181. doi:10.1371/journal.pone.0022181

Kaushik S, Massey AC, Mizushima N, Cuervo AM (2008) Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy. Mol Biol Cell 19(5):2179–2192. doi:10.1091/mbc.E07-11-1155

Kedzierski W, Bok D, Travis GH (1998) Non-cell-autonomous photoreceptor degeneration in rds mutant mice mosaic for expression of a rescue transgene. J Neurosci 18(11):4076–4082

Khandhadia S, Lotery A (2010) Oxidation and age-related macular degeneration: insights from molecular biology. Expert Rev Mol Med 12:e34. doi:10.1017/S146239941000164X

Khanna H, Davis EE, Murga-Zamalloa CA, Estrada-Cuzcano A, Lopez I, den Hollander AI, Zonneveld MN, Othman MI, Waseem N, Chakarova CF, Maubaret C, Diaz-Font A, MacDonald I, Muzny DM, Wheeler DA, Morgan M, Lewis LR, Logan CV, Tan PL, Beer MA, Inglehearn CF, Lewis RA, Jacobson SG, Bergmann C, Beales PL, Attie-Bitach T, Johnson CA, Otto EA, Bhattacharya SS, Hildebrandt F, Gibbs RA, Koenekoop RK, Swaroop A, Katsanis N (2009) A common allele in RPGRIP1L is a modifier of retinal degeneration in ciliopathies. Nat Genet 41(6):739–745. doi:10.1038/ng.366

Kim JY, Zhao H, Martinez J, Doggett TA, Kolesnikov AV, Tang PH, Ablonczy Z, Chan CC, Zhou Z, Green DR, Ferguson TA (2013) Noncanonical autophagy promotes the visual cycle. Cell 154(2):365–376. doi:10.1016/j.cell.2013.06.012

Klein JA, Ackerman SL (2003) Oxidative stress, cell cycle, and neurodegeneration. J Clin Invest 111(6):785–793. doi:10.1172/JCI18182

Klein JA, Longo-Guess CM, Rossmann MP, Seburn KL, Hurd RE, Frankel WN, Bronson RT, Ackerman SL (2002) The harlequin mouse mutation downregulates apoptosis-inducing factor. Nature (Lond) 419(6905):367–374. doi:10.1038/nature01034

Koenekoop RK (2004) An overview of Leber congenital amaurosis: a model to understand human retinal development. Surv Ophthalmol 49(4):379–398. doi:10.1016/j.survophthal.2004.04.003

Kolesnikov AV, Fan J, Crouch RK, Kefalov VJ (2010) Age-related deterioration of rod vision in mice. J Neurosci 30(33):11222–11231. doi:10.1523/JNEUROSCI.4239-09.2010

Komeima K, Rogers BS, Lu L, Campochiaro PA (2006) Antioxidants reduce cone cell death in a model of retinitis pigmentosa. Proc Natl Acad Sci USA 103(30):11300–11305. doi:10.1073/pnas.0604056103

Komeima K, Usui S, Shen J, Rogers BS, Campochiaro PA (2008) Blockade of neuronal nitric oxide synthase reduces cone cell death in a model of retinitis pigmentosa. Free Radic Biol Med 45(6):905–912. doi:10.1016/j.freeradbiomed.2008.06.020

Kong L, Zhou X, Li F, Yodoi J, McGinnis J, Cao W (2010) Neuroprotective effect of overexpression of thioredoxin on photoreceptor degeneration in Tubby mice. Neurobiol Dis 38(3):446–455. doi:10.1016/j.nbd.2010.03.005

Kowluru RA, Odenbach S (2004) Role of interleukin-1beta in the development of retinopathy in rats: effect of antioxidants. Invest Ophthalmol Vis Sci 45(11):4161–4166. doi:10.1167/iovs.04-0633

Kraft C, Peter M, Hofmann K (2010) Selective autophagy: ubiquitin-mediated recognition and beyond. Nat Cell Biol 12(9):836–841. doi:10.1038/ncb0910-836

Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G, Nomenclature Committee on Cell Death (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16(1):3–11. doi:10.1038/cdd.2008.150

Krohne TU, Stratmann NK, Kopitz J, Holz FG (2010) Effects of lipid peroxidation products on lipofuscinogenesis and autophagy in human retinal pigment epithelial cells. Exp Eye Res 90(3):465–471. doi:10.1016/j.exer.2009.12.011

Kunchithapautham K, Rohrer B (2007a) Apoptosis and autophagy in photoreceptors exposed to oxidative stress. Autophagy 3(5):433–441

Kunchithapautham K, Rohrer B (2007b) Autophagy is one of the multiple mechanisms active in photoreceptor degeneration. Autophagy 3(1):65–66

Langmann T (2007) Microglia activation in retinal degeneration. J Leukoc Biol 81(6):1345–1351. doi:10.1189/jlb.0207114

LaVail MM (1981) Analysis of neurological mutants with inherited retinal degeneration. Friedenwald Lecture. Invest Ophthalmol Vis Sci 21(5):638–657

Lemarie A, Lagadic-Gossmann D, Morzadec C, Allain N, Fardel O, Vernhet L (2004) Cadmium induces caspase-independent apoptosis in liver Hep3B cells: role for calcium in signaling oxidative stress-related impairment of mitochondria and relocation of endonuclease G and apoptosis-inducing factor. Free Radic Biol Med 36(12):1517–1531. doi:10.1016/j.freeradbiomed.2004.03.020

Leveillard T, Mohand-Said S, Lorentz O, Hicks D, Fintz AC, Clerin E, Simonutti M, Forster V, Cavusoglu N, Chalmel F, Dolle P, Poch O, Lambrou G, Sahel JA (2004) Identification and characterization of rod-derived cone viability factor. Nat Genet 36(7):755–759. doi:10.1038/ng1386

Lezi E, Swerdlow RH (2012) Mitochondria in neurodegeneration. Adv Exp Med Biol 942:269–286. doi:10.1007/978-94-007-2869-1_12

Liang FQ, Godley BF (2003) Oxidative stress-induced mitochondrial DNA damage in human retinal pigment epithelial cells: a possible mechanism for RPE aging and age-related macular degeneration. Exp Eye Res 76(4):397–403

Lim JH, Wickremasinghe SS, Xie J, Chauhan DS, Baird PN, Robman LD, Hageman G, Guymer RH (2012) Delay to treatment and visual outcomes in patients treated with anti-vascular endothelial growth factor for age-related macular degeneration. Am J Ophthalmol 153(4):678–686. doi:10.1016/j.ajo.2011.09.013

Lim LS, Wong TY (2012) Lipids and diabetic retinopathy. Expert Opin Biol Ther 12(1):93–105. doi:10.1517/14712598.2012.641531

Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86(1):147–157

Lo AC, Woo TT, Wong RL, Wong D (2011) Apoptosis and other cell death mechanisms after retinal detachment: implications for photoreceptor rescue. Ophthalmologica 226(suppl 1):10–17. doi:10.1159/000328206

Lobanova ES, Finkelstein S, Skiba NP, Arshavsky VY (2013) Proteasome overload is a common stress factor in multiple forms of inherited retinal degeneration. Proc Natl Acad Sci USA 110(24):9986–9991. doi:10.1073/pnas.1305521110

Lohr HR, Kuntchithapautham K, Sharma AK, Rohrer B (2006) Multiple, parallel cellular suicide mechanisms participate in photoreceptor cell death. Exp Eye Res 83(2):380–389. doi:10.1016/j.exer.2006.01.014

Long KO, Fisher SK, Fariss RN, Anderson DH (1986) Disc shedding and autophagy in the cone-dominant ground squirrel retina. Exp Eye Res 43(2):193–205

Luce K, Weil AC, Osiewacz HD (2010) Mitochondrial protein quality control systems in aging and disease. Adv Exp Med Biol 694:108–125

MacDonald IM, Sauve Y, Sieving PA (2007) Preventing blindness in retinal disease: ciliary neurotrophic factor intraocular implants. Can J Ophthalmol 42(3):399–402. doi:10.3129/can j ophthalmol.i07-039

Mahajan VB, Skeie JM, Bassuk AG, Fingert JH, Braun TA, Daggett HT, Folk JC, Sheffield VC, Stone EM (2012) Calpain-5 mutations cause autoimmune uveitis, retinal neovascularization, and photoreceptor degeneration. PLoS Genet 8(10):e1003001. doi:10.1371/journal.pgen.1003001

Mantamadiotis T, Lemberger T, Bleckmann SC, Kern H, Kretz O, Martin Villalba A, Tronche F, Kellendonk C, Gau D, Kapfhammer J, Otto C, Schmid W, Schutz G (2002) Disruption of CREB function in brain leads to neurodegeneration. Nat Genet 31(1):47–54. doi:10.1038/ng882

Marino G, Madeo F, Kroemer G (2011) Autophagy for tissue homeostasis and neuroprotection. Curr Opin Cell Biol 23(2):198–206. doi:10.1016/j.ceb.2010.10.001

Marlhens F, Bareil C, Griffoin JM, Zrenner E, Amalric P, Eliaou C, Liu SY, Harris E, Redmond TM, Arnaud B, Claustres M, Hamel CP (1997) Mutations in RPE65 cause Leber's congenital amaurosis. Nat Genet 17(2):139–141. doi:10.1038/ng1097-139

Massey A, Kiffin R, Cuervo AM (2004) Pathophysiology of chaperone-mediated autophagy. Int J Biochem Cell Biol 36(12):2420–2434. doi:10.1016/j.biocel.2004.04.010

Massey AC, Follenzi A, Kiffin R, Zhang C, Cuervo AM (2008) Early cellular changes after blockage of chaperone-mediated autophagy. Autophagy 4(4):442–456

McBee JK, Palczewski K, Baehr W, Pepperberg DR (2001) Confronting complexity: the interlink of phototransduction and retinoid metabolism in the vertebrate retina. Prog Retin Eye Res 20(4):469–529

McCall K (2010) Genetic control of necrosis: another type of programmed cell death. Curr Opin Cell Biol 22(6):882–888. doi:10.1016/j.ceb.2010.09.002

McLaughlin ME, Sandberg MA, Berson EL, Dryja TP (1993) Recessive mutations in the gene encoding the beta-subunit of rod phosphodiesterase in patients with retinitis pigmentosa. Nat Genet 4(2):130–134. doi:10.1038/ng0693-130

Metrailler S, Schorderet DF, Cottet S (2012) Early apoptosis of rod photoreceptors in Rpe65(−/−) mice is associated with the upregulated expression of lysosomal-mediated autophagic genes. Exp Eye Res 96(1):70–81. doi:10.1016/j.exer.2011.12.019

Michaelides M, Wilkie SE, Jenkins S, Holder GE, Hunt DM, Moore AT, Webster AR (2005) Mutation in the gene GUCA1A, encoding guanylate cyclase-activating protein 1, causes cone, cone-rod, and macular dystrophy. Ophthalmology 112(8):1442–1447. doi:10.1016/j.ophtha.2005.02.024

Mittag TW, Bayer AU, La VM (1999) Light-induced retinal damage in mice carrying a mutated SOD I gene. Exp Eye Res 69(6):677–683. doi:10.1006/exer.1999.0748

Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature (Lond) 451(7182):1069–1075. doi:10.1038/nature06639

Molday RS, Zhang K (2010) Defective lipid transport and biosynthesis in recessive and dominant Stargardt macular degeneration. Prog Lipid Res 49(4):476–492. doi:10.1016/j.plipres.2010.07.002

Moquin D, Chan FK (2010) The molecular regulation of programmed necrotic cell injury. Trends Biochem Sci 35(8):434–441. doi:10.1016/j.tibs.2010.03.001

Moreira PI, Sayre LM, Zhu X, Nunomura A, Smith MA, Perry G (2010) Detection and localization of markers of oxidative stress by in situ methods: application in the study of Alzheimer disease. Methods Mol Biol 610:419–434. doi:10.1007/978-1-60327-029-8_25

Murakami Y, Matsumoto H, Roh M, Giani A, Kataoka K, Morizane Y, Kayama M, Thanos A, Nakatake S, Notomi S, Hisatomi T, Ikeda Y, Ishibashi T, Connor KM, Miller JW, Vavvas DG (2013) Programmed necrosis, not apoptosis, is a key mediator of cell loss and DAMP-mediated inflammation in dsRNA-induced retinal degeneration. Cell Death Differ. doi:10.1038/cdd.2013.109

Murakami Y, Matsumoto H, Roh M, Suzuki J, Hisatomi T, Ikeda Y, Miller JW, Vavvas DG (2012) Receptor interacting protein kinase mediates necrotic cone but not rod cell death in a mouse model of inherited degeneration. Proc Natl Acad Sci USA 109(36):14598–14603. doi:10.1073/pnas.1206937109

Nakazawa M (2011) Effects of calcium ion, calpains, and calcium channel blockers on retinitis pigmentosa. J Ophthalmol 2011:292040. doi:10.1155/2011/292040

Neal M, Cunningham J (1994) Modulation by endogenous ATP of the light-evoked release of ACh from retinal cholinergic neurones. Br J Pharmacol 113(4):1085–1087

Newman EA (2003) Glial cell inhibition of neurons by release of ATP. J Neurosci 23(5):1659–1666

Newmeyer DD, Ferguson-Miller S (2003) Mitochondria: releasing power for life and unleashing the machineries of death. Cell 112(4):481–490

Ng L, Hurley JB, Dierks B, Srinivas M, Salto C, Vennstrom B, Reh TA, Forrest D (2001) A thyroid hormone receptor that is required for the development of green cone photoreceptors. Nat Genet 27(1):94–98. doi:10.1038/83829

Ng TF, Streilein JW (2001) Light-induced migration of retinal microglia into the subretinal space. Invest Ophthalmol Vis Sci 42(13):3301–3310

Nickla DL, Wallman J (2010) The multifunctional choroid. Prog Retin Eye Res 29(2):144–168. doi:10.1016/j.preteyeres.2009.12.002

Notomi S, Hisatomi T, Kanemaru T, Takeda A, Ikeda Y, Enaida H, Kroemer G, Ishibashi T (2011) Critical involvement of extracellular ATP acting on P2RX7 purinergic receptors in photoreceptor cell death. Am J Pathol 179(6):2798–2809. doi:10.1016/j.ajpath.2011.08.035

Notomi S, Hisatomi T, Murakami Y, Terasaki H, Sonoda S, Asato R, Takeda A, Ikeda Y, Enaida H, Sakamoto T, Ishibashi T (2013) Dynamic increase in extracellular ATP accelerates photoreceptor cell apoptosis via ligation of P2RX7 in subretinal hemorrhage. PloS One 8(1):e53338. doi:10.1371/journal.pone.0053338

Oguni M, Tamura H, Kato K, Setogawa T (1996) Chronic retinal effects by ultraviolet irradiation, with special reference to superoxide dismutases. Histol Histopathol 11(3):695–702

Orrenius S, Zhivotovsky B, Nicotera P (2003) Regulation of cell death: the calcium–apoptosis link. Nat Rev Mol Cell Biol 4(7):552–565. doi:10.1038/nrm1150

Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S (2002) Cytochrome c release from mitochondria proceeds by a two-step process. Proc Natl Acad Sci USA 99(3):1259–1263. doi:10.1073/pnas.241655498

Ozaki T, Ishiguro S, Hirano S, Baba A, Yamashita T, Tomita H, Nakazawa M (2013) Inhibitory peptide of mitochondrial mu-calpain protects against photoreceptor degeneration in rhodopsin transgenic S334ter and P23H rats. PloS One 8(8):e71650. doi:10.1371/journal.pone.0071650

Paquet-Durand F, Azadi S, Hauck SM, Ueffing M, van Veen T, Ekstrom P (2006) Calpain is activated in degenerating photoreceptors in the rd1 mouse. J Neurochem 96(3):802–814. doi:10.1111/j.1471-4159.2005.03628.x

Paquet-Durand F, Sanges D, McCall J, Silva J, van Veen T, Marigo V, Ekstrom P (2010) Photoreceptor rescue and toxicity induced by different calpain inhibitors. J Neurochem 115(4):930–940. doi:10.1111/j.1471-4159.2010.06983.x

Paquet-Durand F, Silva J, Talukdar T, Johnson LE, Azadi S, van Veen T, Ueffing M, Hauck SM, Ekstrom PA (2007) Excessive activation of poly(ADP-ribose) polymerase contributes to inherited photoreceptor degeneration in the retinal degeneration 1 mouse. J Neurosci 27(38):10311–10319. doi:10.1523/JNEUROSCI.1514-07.2007

Pasantes-Morales H, Quiroz H, Quesada O (2002) Treatment with taurine, diltiazem, and vitamin E retards the progressive visual field reduction in retinitis pigmentosa: a 3-year follow-up study. Metab Brain Dis 17(3):183–197

Perche O, Doly M, Ranchon-Cole I (2007) Caspase-dependent apoptosis in light-induced retinal degeneration. Invest Ophthalmol Vis Sci 48(6):2753–2759. doi:10.1167/iovs.06-1258

Perez-Campo R, Lopez-Torres M, Cadenas S, Rojas C, Barja G (1998) The rate of free radical production as a determinant of the rate of aging: evidence from the comparative approach. J Comp Physiol B Biochem System Environ Physiol 168(3):149–158

Perkins GA, Ellisman MH, Fox DA (2003) Three-dimensional analysis of mouse rod and cone mitochondrial cristae architecture: bioenergetic and functional implications. Mol Vis 9:60–73

Portera-Cailliau C, Sung CH, Nathans J, Adler R (1994) Apoptotic photoreceptor cell death in mouse models of retinitis pigmentosa. Proc Natl Acad Sci USA 91(3):974–978

Priya RR, Chew EY, Swaroop A (2012) Genetic studies of age-related macular degeneration: lessons, challenges, and opportunities for disease management. Ophthalmology 119 (12):2526–2536. doi:10.1016/j.ophtha.2012.06.042

Punzo C, Kornacker K, Cepko CL (2009) Stimulation of the insulin/mTOR pathway delays cone death in a mouse model of retinitis pigmentosa. Nat Neurosci 12(1):44–52. doi:10.1038/nn.2234

Puthussery T, Fletcher E (2009) Extracellular ATP induces retinal photoreceptor apoptosis through activation of purinoceptors in rodents. J Comp Neurol 513(4):430–440. doi:10.1002/cne.21964

Puthussery T, Fletcher EL (2004) Synaptic localization of P2X7 receptors in the rat retina. J Comp Neurol 472(1):13–23. doi:10.1002/cne.20045

Puthussery T, Yee P, Vingrys AJ, Fletcher EL (2006) Evidence for the involvement of purinergic P2X receptors in outer retinal processing. Eur J Neurosci 24(1):7–19. doi:10.1111/j.1460-9568.2006.04895.x

Ratnapriya R, Swaroop A (2013) Genetic architecture of retinal and macular degenerative diseases: the promise and challenges of next-generation sequencing. Genome Med 5(9):84. doi:10.1186/gm488

Read DS, McCall MA, Gregg RG (2002) Absence of voltage-dependent calcium channels delays photoreceptor degeneration in rd mice. Exp Eye Res 75(4):415–420

Redmond TM, Yu S, Lee E, Bok D, Hamasaki D, Chen N, Goletz P, Ma JX, Crouch RK, Pfeifer K (1998) Rpe65 is necessary for production of 11-*cis*-vitamin A in the retinal visual cycle. Nat Genet 20(4):344–351. doi:10.1038/3813

Reme CE, Wolfrum U, Imsand C, Hafezi F, Williams TP (1999) Photoreceptor autophagy: effects of light history on number and opsin content of degradative vacuoles. Invest Ophthalmol Vis Sci 40(10):2398–2404

Rhee KD, Ruiz A, Duncan JL, Hauswirth WW, Lavail MM, Bok D, Yang XJ (2007) Molecular and cellular alterations induced by sustained expression of ciliary neurotrophic factor in a mouse model of retinitis pigmentosa. Invest Ophthalmol Vis Sci 48(3):1389–1400. doi:10.1167/iovs.06-0677

Rich KA, Zhan Y, Blanks JC (1997) Aberrant expression of c-Fos accompanies photoreceptor cell death in the rd mouse. J Neurobiol 32(6):593–612

Rodriguez-Muela N, Koga H, Garcia-Ledo L, de la Villa P, de la Rosa EJ, Cuervo AM, Boya P (2013) Balance between autophagic pathways preserves retinal homeostasis. Aging Cell 12(3):478–488. doi:10.1111/acel.12072

Roger JE, Ranganath K, Zhao L, Cojocaru RI, Brooks M, Gotoh N, Veleri S, Hiriyanna A, Rachel RA, Campos MM, Fariss RN, Wong WT, Swaroop A (2012) Preservation of cone photoreceptors after a rapid yet transient degeneration and remodeling in cone-only Nrl−/− mouse retina. J Neurosci 32(2):528–541. doi:10.1523/JNEUROSCI.3591-11.2012

Rosenbaum DM, Degterev A, David J, Rosenbaum PS, Roth S, Grotta JC, Cuny GD, Yuan J, Savitz SI (2010) Necroptosis, a novel form of caspase-independent cell death, contributes to neuronal damage in a retinal ischemia-reperfusion injury model. J Neurosci Res 88(7):1569–1576. doi:10.1002/jnr.22314

Rudolf M, Malek G, Messinger JD, Clark ME, Wang L, Curcio CA (2008) Sub-retinal drusenoid deposits in human retina: organization and composition. Exp Eye Res 87(5):402–408. doi:10.1016/j.exer.2008.07.010

Sahaboglu A, Tanimoto N, Kaur J, Sancho-Pelluz J, Huber G, Fahl E, Arango-Gonzalez B, Zrenner E, Ekstrom P, Lowenheim H, Seeliger M, Paquet-Durand F (2010) PARP1 gene knock-out increases resistance to retinal degeneration without affecting retinal function. PloS One 5(11):e15495. doi:10.1371/journal.pone.0015495

Sampayo JN, Olsen A, Lithgow GJ (2003) Oxidative stress in *Caenorhabditis elegans*: protective effects of superoxide dismutase/catalase mimetics. Aging Cell 2(6):319–326

Sastre J, Pallardo FV, Vina J (2000) Mitochondrial oxidative stress plays a key role in aging and apoptosis. IUBMB Life 49(5):427–435. doi:10.1080/152165400410281

Schipper-Krom S, Juenemann K, Reits EA (2012) The ubiquitin-proteasome system in Huntington's disease: are proteasomes impaired, initiators of disease, or coming to the rescue? Biochem Res Int 2012:837015. doi:10.1155/2012/837015

Schoch KM, von Reyn CR, Bian J, Telling GC, Meaney DF, Saatman KE (2013) Brain injury-induced proteolysis is reduced in a novel calpastatin-overexpressing transgenic mouse. J Neurochem 125(6):909–920. doi:10.1111/jnc.12144

Schwiebert EM (2000) Extracellular ATP-mediated propagation of Ca(2+) waves. Focus on mechanical strain-induced Ca(2+) waves are propagated via ATP release and purinergic receptor activation. Am J Physiol Cell Physiol 279(2):C281–283

Shahinfar S, Edward DP, Tso MO (1991) A pathologic study of photoreceptor cell death in retinal photic injury. Curr Eye Res 10(1):47–59

Sharma AK, Rohrer B (2004) Calcium-induced calpain mediates apoptosis via caspase-3 in a mouse photoreceptor cell line. J Biol Chem 279(34):35564–35572. doi:10.1074/jbc.M401037200

Sharon D, Blackshaw S, Cepko CL, Dryja TP (2002) Profile of the genes expressed in the human peripheral retina, macula, and retinal pigment epithelium determined through serial analysis of gene expression (SAGE). Proc Natl Acad Sci USA 99(1):315–320. doi:10.1073/pnas.012582799

Shen J, Yang X, Dong A, Petters RM, Peng YW, Wong F, Campochiaro PA (2005) Oxidative damage is a potential cause of cone cell death in retinitis pigmentosa. J Cell Physiol 203(3):457–464. doi:10.1002/jcp.20346

Sherman MY, Goldberg AL (2001) Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron 29(1):15–32

Sieving PA, Caruso RC, Tao W, Coleman HR, Thompson DJ, Fullmer KR, Bush RA (2006) Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants. Proc Natl Acad Sci USA 103(10):3896–3901. doi:10.1073/pnas.0600236103

Simunovic MP, Moore AT (1998) The cone dystrophies. Eye 12(pt 3b):553–565. doi:10.1038/eye.1998.145

Smith MA, Schnellmann RG (2012) Calpains, mitochondria, and apoptosis. Cardiovasc Res 96(1):32–37. doi:10.1093/cvr/cvs163

Smith W, Assink J, Klein R, Mitchell P, Klaver CC, Klein BE, Hofman A, Jensen S, Wang JJ, de Jong PT (2001) Risk factors for age-related macular degeneration: pooled findings from three continents. Ophthalmology 108(4):697–704

Snyder H, Mensah K, Theisler C, Lee J, Matouschek A, Wolozin B (2003) Aggregated and monomeric alpha-synuclein bind to the S6' proteasomal protein and inhibit proteasomal function. J Biol Chem 278(14):11753–11759. doi:10.1074/jbc.M208641200

Solovei I, Kreysing M, Lanctot C, Kosem S, Peichl L, Cremer T, Guck J, Joffe B (2009) Nuclear architecture of rod photoreceptor cells adapts to vision in mammalian evolution. Cell 137(2):356–368. doi:10.1016/j.cell.2009.01.052

Stone EM (2007) Macular degeneration. Annu Rev Med 58:477–490. doi:10.1146/annurev.med.58.111405.133335

Sun H, Tsunenari T, Yau KW, Nathans J (2002) The vitelliform macular dystrophy protein defines a new family of chloride channels. Proc Natl Acad Sci USA 99(6):4008–4013. doi:10.1073/pnas.052692999

Surgucheva I, Ninkina N, Buchman VL, Grasing K, Surguchov A (2005) Protein aggregation in retinal cells and approaches to cell protection. Cell Mol Neurobiol 25(6):1051–1066. doi:10.1007/s10571-005-8474-1

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature (Lond) 397(6718):441–446. doi:10.1038/17135

Suzuki K, Hata S, Kawabata Y, Sorimachi H (2004) Structure, activation, and biology of calpain. Diabetes 53(suppl 1):S12–S18

Suzuki M, Kamei M, Itabe H, Yoneda K, Bando H, Kume N, Tano Y (2007) Oxidized phospholipids in the macula increase with age and in eyes with age-related macular degeneration. Mol Vis 13:772–778

Swaroop A, Branham KE, Chen W, Abecasis G (2007) Genetic susceptibility to age-related macular degeneration: a paradigm for dissecting complex disease traits. Human Mol Genet 16(Spec No. 2):R174–R182. doi:10.1093/hmg/ddm212

Swaroop A, Chew EY, Rickman CB, Abecasis GR (2009) Unraveling a multifactorial late-onset disease: from genetic susceptibility to disease mechanisms for age-related macular degeneration. Annu Rev Genomics Hum Genet 10:19–43. doi:10.1146/annurev.genom.9.081307.164350

Swaroop A, Kim D, Forrest D (2010) Transcriptional regulation of photoreceptor development and homeostasis in the mammalian retina. Nat Rev Neurosci 11(8):563–576. doi:10.1038/nrn2880

Tai HC, Schuman EM (2008) Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. Nat Rev Neurosci 9(11):826–838. doi:10.1038/nrn2499

Takano Y, Ohguro H, Dezawa M, Ishikawa H, Yamazaki H, Ohguro I, Mamiya K, Metoki T, Ishikawa F, Nakazawa M (2004) Study of drug effects of calcium channel blockers on retinal degeneration of rd mouse. Biochem Biophys Res Commun 313(4):1015–1022

Talcott KE, Ratnam K, Sundquist SM, Lucero AS, Lujan BJ, Tao W, Porco TC, Roorda A, Duncan JL (2011) Longitudinal study of cone photoreceptors during retinal degeneration and in response to ciliary neurotrophic factor treatment. Invest Ophthalmol Vis Sci 52(5):2219–2226. doi:10.1167/iovs.10-6479

Tanaka T, Nakamura H, Nishiyama A, Hosoi F, Masutani H, Wada H, Yodoi J (2000) Redox regulation by thioredoxin superfamily: protection against oxidative stress and aging. Free Radic Res 33(6):851–855

Tanito M, Agbaga MP, Anderson RE (2007a) Upregulation of thioredoxin system via Nrf2-antioxidant responsive element pathway in adaptive-retinal neuroprotection in vivo and in vitro. Free Radic Biol Med 42(12):1838–1850. doi:10.1016/j.freeradbiomed.2007.03.018

Tanito M, Kaidzu S, Anderson RE (2007b) Delayed loss of cone and remaining rod photoreceptor cells due to impairment of choroidal circulation after acute light exposure in rats. Invest Ophthalmol Vis Sci 48(4):1864–1872. doi:10.1167/iovs.06-1065

Tanito M, Masutani H, Kim YC, Nishikawa M, Ohira A, Yodoi J (2005) Sulforaphane induces thioredoxin through the antioxidant-responsive element and attenuates retinal light damage in mice. Invest Ophthalmol Vis Sci 46(3):979–987. doi:10.1167/iovs.04-1120

Tanito M, Masutani H, Nakamura H, Ohira A, Yodoi J (2002a) Cytoprotective effect of thioredoxin against retinal photic injury in mice. Invest Ophthalmol Vis Sci 43(4):1162–1167

Tanito M, Masutani H, Nakamura H, Oka S, Ohira A, Yodoi J (2002b) Attenuation of retinal photooxidative damage in thioredoxin transgenic mice. Neurosci Lett 326(2):142–146

Thapa A, Morris L, Xu J, Ma H, Michalakis S, Biel M, Ding XQ (2012) Endoplasmic reticulum stress-associated cone photoreceptor degeneration in cyclic nucleotide-gated channel deficiency. J Biol Chem 287(22):18018–18029. doi:10.1074/jbc.M112.342220

Traboulsi EI (2010) The Marshall M. Parks Memorial Lecture. Making sense of early-onset childhood retinal dystrophies: the clinical phenotype of Leber congenital amaurosis. Br J Ophthalmol 94(10):1281–1287. doi:10.1136/bjo.2009.165654

Trichonas G, Murakami Y, Thanos A, Morizane Y, Kayama M, Debouck CM, Hisatomi T, Miller JW, Vavvas DG (2010) Receptor interacting protein kinases mediate retinal detachment-induced photoreceptor necrosis and compensate for inhibition of apoptosis. Proc Natl Acad Sci USA 107(50):21695–21700. doi:10.1073/pnas.1009179107

Trifunovic D, Sahaboglu A, Kaur J, Mencl S, Zrenner E, Ueffing M, Arango-Gonzalez B, Paquet-Durand F (2012) Neuroprotective strategies for the treatment of inherited photoreceptor degeneration. Curr Mol Med 12(5):598–612

Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 11(10):700–714. doi:10.1038/nrm2970

Vecino E, Hernandez M, Garcia M (2004) Cell death in the developing vertebrate retina. Int J Dev Biol 48(8-9):965–974. doi:10.1387/ijdb.041891ev

Volonte C, Amadio S, Cavaliere F, D'Ambrosi N, Vacca F, Bernardi G (2003) Extracellular ATP and neurodegeneration. Curr Drug Targets CNS Neurol Disord 2(6):403–412

Vucic D, Dixit VM, Wertz IE (2011) Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. Nat Rev Mol Cell Biol 12(7):439–452. doi:10.1038/nrm3143

Vuong L, Brobst DE, Ivanovic I, Sherry DM, Al-Ubaidi MR (2013) p53 selectively regulates developmental apoptosis of rod photoreceptors. PloS One 8(6):e67381. doi:10.1371/journal.pone.0067381

Wang AL, Lukas TJ, Yuan M, Du N, Tso MO, Neufeld AH (2009a) Autophagy and exosomes in the aged retinal pigment epithelium: possible relevance to drusen formation and age-related macular degeneration. PloS One 4(1):e4160. doi:10.1371/journal.pone.0004160

Wang AL, Lukas TJ, Yuan M, Du N, Tso MO, Neufeld AH (2009b) Autophagy, exosomes and drusen formation in age-related macular degeneration. Autophagy 5(4):563–564

Wang X, Arcuino G, Takano T, Lin J, Peng WG, Wan P, Li P, Xu Q, Liu QS, Goldman SA, Nedergaard M (2004) P2X7 receptor inhibition improves recovery after spinal cord injury. Nat Med 10(8):821–827. doi:10.1038/nm1082

Wang XW, Tan BZ, Sun M, Ho B, Ding JL (2008) Thioredoxin-like 6 protects retinal cell line from photooxidative damage by upregulating NF-kappaB activity. Free Radic Biol Med 45(3):336–344. doi:10.1016/j.freeradbiomed.2008.04.028

Ward MM, Puthussery T, Vessey KA, Fletcher EL (2010) The role of purinergic receptors in retinal function and disease. Adv Exp Med Biol 664:385–391. doi:10.1007/978-1-4419-1399-9_44

Weisman GA, Woods LT, Erb L, Seye CI (2012) P2Y receptors in the mammalian nervous system: pharmacology, ligands and therapeutic potential. CNS Neurol Disord Drug Targets 11(6):722–738

Wen R, Tao W, Li Y, Sieving PA (2012) CNTF and retina. Prog Retin Eye Res 31(2):136–151. doi:10.1016/j.preteyeres.2011.11.005

Wenzel A, Grimm C, Marti A, Kueng-Hitz N, Hafezi F, Niemeyer G, Reme CE (2000) c-fos controls the "private pathway" of light-induced apoptosis of retinal photoreceptors. J Neurosci 20(1):81–88

Wenzel A, Grimm C, Samardzija M, Reme CE (2005) Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. Prog Retin Eye Res 24(2):275–306. doi:10.1016/j.preteyeres.2004.08.002

Wingrave JM, Sribnick EA, Wilford GG, Matzelle DD, Mou JA, Ray SK, Hogan EL, Banik NL (2004) Higher calpastatin levels correlate with resistance to calpain-mediated proteolysis and neuronal apoptosis in juvenile rats after spinal cord injury. J Neurotrauma 21(9):1240–1254. doi:10.1089/neu.2004.21.1240

Winkler BS (2008) An hypothesis to account for the renewal of outer segments in rod and cone photoreceptor cells: renewal as a surrogate antioxidant. Invest Ophthalmol Vis Sci 49 (8):3259–3261. doi:10.1167/iovs.08-1785

Wright AF, Chakarova CF, Abd El-Aziz MM, Bhattacharya SS (2010) Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. Nat Rev Genet 11(4):273–284. doi:10.1038/nrg2717

Wright AF, Jacobson SG, Cideciyan AV, Roman AJ, Shu X, Vlachantoni D, McInnes RR, Riemersma RA (2004) Lifespan and mitochondrial control of neurodegeneration. Nat Genet 36(11):1153–1158. doi:10.1038/ng1448

Wright AF, Murphy MP, Turnbull DM (2009) Do organellar genomes function as long-term redox damage sensors? Trends Genet 25(6):253–261. doi:10.1016/j.tig.2009.04.006

Yang JH, Basinger SF, Gross RL, Wu SM (2003) Blue light-induced generation of reactive oxygen species in photoreceptor ellipsoids requires mitochondrial electron transport. Invest Ophthalmol Vis Sci 44(3):1312–1319

Yu DY, Cringle SJ, Su EN, Yu PK (2000) Intraretinal oxygen levels before and after photoreceptor loss in the RCS rat. Invest Ophthalmol Vis Sci 41(12):3999–4006

Yu SW, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL (2006) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. Proc Natl Acad Sci USA 103(48):18314–18319. doi:10.1073/pnas.0606528103

Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297(5579):259–263. doi:10.1126/science.1072221

Yvert G, Lindenberg KS, Picaud S, Landwehrmeyer GB, Sahel JA, Mandel JL (2000) Expanded polyglutamines induce neurodegeneration and trans-neuronal alterations in cerebellum and retina of SCA7 transgenic mice. Hum Mol Genet 9(17):2491–2506

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325(5938):332–336. doi:10.1126/science.1172308

Zhang K, Kniazeva M, Han M, Li W, Yu Z, Yang Z, Li Y, Metzker ML, Allikmets R, Zack DJ, Kakuk LE, Lagali PS, Wong PW, MacDonald IM, Sieving PA, Figueroa DJ, Austin CP, Gould RJ, Ayyagari R, Petrukhin K (2001) A 5-bp deletion in ELOVL4 is associated with two related forms of autosomal dominant macular dystrophy. Nat Genet 27(1):89–93. doi:10.1038/83817

Zhang PP, Yang XL, Zhong YM (2012) Cellular localization of P2Y(6) receptor in rat retina. Neuroscience 220:62–69. doi:10.1016/j.neuroscience.2012.06.032

Zhivotovsky B, Orrenius S (2011) Calcium and cell death mechanisms: a perspective from the cell death community. Cell Calcium 50(3):211–221. doi:10.1016/j.ceca.2011.03.003

Chapter 12
Photoreceptor Transplantation and Regeneration

Valeria Marigo and Simona Casarosa

Abstract A recent study showed that an electronic chip implanted under the human retina restored some extent of vision to a blind patient. Because the device was implanted where the light-sensitive cells, the photoreceptors, should have been, this study demonstrated that it is possible to take advantage of the internal circuitry of the retina even in the absence of photoreceptors and in the presence of extensive glial and neuronal reorganization. This result strongly supports the development of cell replacement therapies for the cure of photoreceptor degeneration, provided that the cells are implanted in the same anatomical location. First, similar to other sensory neurons but in contrast to neurons lost in most degenerative diseases, photoreceptors are the first neurons of the circuit and only have to make efferent connections. Second, photoreceptors are histologically located in a restricted region of the organ. These features make them the most immediately transplantable type of neuron and interesting candidates for clinical trials involving cell transplantation.

In cell replacement therapies, the identification of the source of cells able to integrate and connect to the host tissue must be defined. For the retina, cells showing the best survival and integration rates are postmitotic rod precursors, rather than immature retinal progenitors. Given the difficulty of obtaining human fetal cells, many studies are undertaking differentiation of cells with such features starting from stem cells. Three main classes of stem cells are under investigation to be sources for in vitro photoreceptor generation: embryonic stem cells, induced pluripotent stem cells, and adult retinal stem cells. This chapter describes the current preclinical studies for in vitro generation and subsequent transplantation of photoreceptor precursors.

V. Marigo (✉)
Department of Life Sciences, University of Modena and Reggio Emilia,
Via G. Campi 287, 41125 Modena, Italy
e-mail: valeria.marigo@unimore.it

S. Casarosa
Centre for Integrative Biology (CIBIO), Via delle Regole 101, 38123 Mattarello (TN), Italy
e-mail: casarosa@science.unitn.it

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_12, © Springer Japan 2014

Keywords ES cells • iPS cells • Müller glia • Photoreceptor precursors • Retinal stem cells • Transplantation

12.1 Rod and Cone Precursors from Immature Retina

After many attempts at retinal transplantation, a question that remained open until few years ago concerned the molecular features characterizing cells with the ability to integrate and generate functional rods in degenerating retinae. A thorough study of murine rod progenitors and precursors determined that integration of donor cells requires cells derived from retinae at developmental times coincident with the peak of rod genesis (Bartsch et al. 2008; MacLaren et al. 2006). The developmental time window for donor cells that successfully integrated in recipient adult retinae was defined by transplantation of cells taken from murine retinae at E11.5 (embryonic day 11.5), E16.5, P1 (postnatal day 1) to P15, and adult. Undifferentiated progenitors from E11.5 retinae as well as cells from adult retinae failed to integrate after transplantation. The highest integration efficiency was obtained with cells dissociated from P3–P5 retinae, a stage in the mouse in which rod precursors are committed but not yet fully differentiated. Cells capable of integrating in a host retina were immature, postmitotic, and expressed the transcription factor Nrl. Proliferating progenitors otherwise showed no transplantation success (MacLaren et al. 2006). Integrated cells expressed rod-specific proteins, formed synapses with bipolar cells, and were functional. Expression of rod-specific markers was also shown not to derive from fusion of donor cells with recipient photoreceptors, demonstrating that the host environment supported rod maturation as well as integration. This was the first study demonstrating that integration in the retinal tissue requires donor cells with specific molecular characteristics and that donor cells should be derived from retinae that have not reached complete maturation. We need to assume that these molecular features are sufficient for the cells to complete their differentiation in situ after transplantation, because to restore visual function the immature transplanted cells will have to resume their developmental program, form a light-sensitive outer segment, and synaptically connect with the recipient tissue.

Although transplanted rods were shown to be able to complete differentiation in situ acquiring morphological and molecular features of adult rods, the low number of integrated cells, about 1,000 cells per retina, limited the assessment of functionality and of vision improvement. The protocol was improved by subretinal transplantation of higher number of cells with two injections in the superior and the inferior retina, resulting in a 20- to 30-fold increase in the number of integrated cells. These injections were performed in $Gnat^{-/-}$ mice lacking α-transducin (generated by J. Lem), unable to activate a phototransduction response and therefore facilitating the functional analysis of wild-type transplanted cells. Correlation of cell transplantation and improved vision was defined by in situ patch-clamp recording, optomotor head tracking responses to a rotating grating, and visually guided water-maze tests.

Although the sensitivity of scotopic ERG recording was not sufficient to discriminate between sham-injected and transplanted eyes, probably because the number of cells was still low, vision sensitivity of treated animals suggested that even low numbers of photoreceptors could be clinically valuable to blind patients (Pearson et al. 2012).

The environment in a degenerating retina can be very different from the normal retina and can differ from one type of retinal degeneration to another. To assess possible differential integration efficiency in different forms of the disease, the transplantation protocol described above was applied to six murine models of photoreceptor degeneration. Differences in integration number, outer segment formation and synapse connectivity were observed in the mutant murine retinae among the different models and when compared to the wild type (Barber et al. 2013). Transplantation success in different types of retinal degeneration was strongly influenced by glial scarring and, to a lesser extent, by changes of integrity in the outer limiting membrane. Disruption of the these barriers by reversible opening of the outer limiting membrane together with reduction of gliosis by chondroitinase ABC digestion of chondroitin sulfate proteoglycans could improve integration of donor cells (Barber et al. 2013). These treatments may affect the immune privilege of the eye. Researchers in fact observed a significant loss of integrated photoreceptors with time after transplantation, and no transplanted cells could be found after 6 months (West et al. 2010).

Outer nuclear layer thickness appeared not to affect the success of integration, and efficient integration could be achieved even in advanced stages of degeneration. This finding has important implication for clinical applications because patients are often diagnosed when many photoreceptors are already lost, and optical coherence tomography (OCT) analysis shows a reduced outer nuclear layer. Further studies on advanced forms of the disease will be very important because we can expect that a greater number of integrated cells will be required to restore vision in vastly degenerated retinae. Also, photoreceptor precursors transplanted in advanced degenerated retinae are expected not to find rods in the recipient organ, although their presence, even if they are not functional, could help the molecular and morphological maturation of immature transplanted cells. One study that addressed this important question showed that transplantation of photoreceptor precursors in fully degenerated retinae could give rise to polarized cells elongated toward the retina pigment epithelium (RPE). These cells were also able to form synaptic connections with bipolar cells. Although the morphology of the outer segment was very poor, appropriate localization of the phototransduction molecules could be observed (Singh et al. 2013).

Most investigators focused their research on replacement therapy for rods, the cell type affected in retinitis pigmentosa. This task may be challenging because of the high number of rods present in the human retina and the limited number of cells that could integrate in the animal models tested so far. Patients are also strongly affected by the loss of cone photoreceptors, which are present in lower numbers in the human retina but are very important for our daylight and color vision as well as our high visual acuity.

Because the highest efficiency of rod transplantation requires cells at the peak of rod genesis, we can expect that transplantation of cones may require cells at a different retinal developmental stage corresponding to the peak of cone genesis. Postnatal murine retinal precursors efficiently integrated and differentiated into rod photoreceptors but never into cones. Researchers thus decided to test whether E14.5–E17.5 embryonic murine retinal cells could integrate into an adult retina and differentiate into cones. The efficiency was low, with less than 1 % of the integrated cells expressing molecular and morphological features of cone photoreceptors (Lakowski et al. 2010). We need to understand the differences between the two experiments here presented. MacLaren and colleagues in rod transplantations were selecting cells based on expression of the Nrl transcription factor, so they were selecting rod precursors and excluding cone precursors (MacLaren et al. 2006). Lakowski and colleagues selected cells to be transplanted based on expression of the Crx transcription factor, so they subretinally injected both cone and rod photoreceptor precursors (Furukawa et al. 1997). This selection allowed the assessment also of cone transplantation potential, which had not been evaluated by MacLaren and colleagues. Consistent with the developmental biology studies calculating that at E15.5 more than 75 % of the photoreceptor progenitors (Crx^+) are cone precursors (Carter-Dawson and LaVail 1979), transplantation of E15.5 Crx-expressing cells into an adult retina showed better success of cone integration. Furthermore, the use of Crx as marker for the selection of donor cells, even at later developmental stages, had the advantage of choosing a transcription factor acting as a critical determinant of the entire photoreceptor lineage.

These two studies inferred that success in transplantation requires cells having recently undergone terminal mitosis but committed to the photoreceptor lineage. In fact, the low percentage of integrating cones quite nicely reflects the 1:35 cone-to-rod ratio of photoreceptors in the murine retina. It is known that postmitotic photoreceptor precursors retain some plasticity before full commitment. The recipient environment may affect final differentiation, as suggested by higher percentages of cones when transplants were performed on a P14 not fully developed retina or on retinae bearing a cone dystrophy compared to transplants on adult wild-type retina (Lakowski et al. 2010).

12.2 Photoreceptors from Embryonic Stem Cells and Induced Pluripotent Stem Cells

Embryonic stem cells (ESCs) originate from the inner cell mass of the mammalian blastocyst, a specific developmental stage that is reached at E4 in the mouse and at 5 days postfertilization in humans. By formal definition, stem cells are pluripotent cells, able to proliferate indefinitely and to differentiate into any cell type from the three germ layers (ectoderm, mesoderm, endoderm) given the appropriate environment and stimuli. After these premises, it is clear that in the past years

ESCs were envisaged as an important tool to obtain photoreceptor progenitors at the appropriate stage and in sufficient number for efficient transplantation purposes. A number of differentiation protocols have been published in the past 5 to 10 years, for both mouse and human ESCs. Although somewhat different, all these protocols recapitulated, at least to some extent, the stepwise development of retinal cells in vivo. ESCs appear to respond to secreted signaling molecules similarly to what happens during vertebrate neural and retinal development. More specifically, BMP and Wnt signaling blockade was demonstrated to be important for neural tissue patterning and for the formation of anterior structures, and most retinal differentiation protocols contained inhibitors of these pathways, such as Noggin and Dkk-1, in their culture medium (Ikeda et al. 2005; Lamba et al. 2006; Osakada et al. 2008; Mellough et al. 2012; Garita-Hernández et al. 2013). Noggin and Dkk-1 could also be substituted by small compounds, as shown by Sasai and colleagues (Osakada et al. 2009), leading to the generation of differentiation protocols that are more appropriate for translation to therapy. Those protocols that did not use these inhibitors otherwise demonstrated the presence of endogenous Dkk and Noggin, particularly in human ESCs (Meyer et al. 2009). Some of the protocols also included insulin-like growth factor 1 (IGF-1) (Lamba et al. 2006; Mellough et al. 2012), important for anterior neural/eye field specification (Pera et al. 2001) as well as Notch pathway inhibition, important in photoreceptor development (Jadhav et al. 2006; Osakada et al. 2008; Meyer et al. 2009). The initial differentiation steps generated $Pax6^+/Rax^+$ retinal precursors; in fact, coexpression of Rax and Pax6 is considered the hallmark of eye field/retinal precursor cells. The efficiency of generation of retinal precursors was quite variable among the different protocols, but, at least in the mouse, these cells could be purified by using a Rax-GFP knock-in ESC line (generated by Y. Sasai), thus allowing the differentiation cultures to continue with an enriched population of cells (Osakada et al. 2008; West et al. 2012). One of these studies also demonstrated that the purification of the Rax^+ progenitors led to more efficient terminal differentiation, suggesting that, as in vivo, a retinal progenitor cell niche was required for in vitro differentiation of photoreceptors (West et al. 2012). Differentiation protocols then continued with the formation, from the $Rax^+/Pax6^+$ precursors, of more specific photoreceptor progenitors, which expressed the transcription factor Crx (Furukawa et al. 1997). Most protocols then contemplated a final step of differentiation into more mature postmitotic photoreceptors that, in addition to Crx, also expressed terminal differentiation markers such as recoverin and rhodopsin. The efficiency of this final step could be ameliorated by the use of molecules such as taurine, retinoic acid, and sonic hedgehog (Shh) (Osakada et al. 2008; Mellough et al. 2012; West et al. 2012). A recently published study argued for the necessity of hypoxic conditions to improve the differentiation efficiency of mouse ESCs toward photoreceptors (Garita-Hernández et al. 2013), based on the low-oxygen conditions of mammalian embryogenesis (Dunwoodie 2009). Nevertheless, none of the discussed protocols was able to generate, in vitro, the morphological characteristics of terminally differentiated photoreceptors, such as the outer segment, although explant cultures of mammalian retinae previously showed that cells could acquire high levels of

morphological differentiation provided that they were maintained in an organized state (Feigenspan et al. 1993; Ogilvie et al. 1999). To overcome this problem, more recent studies investigated the use of biomaterials as scaffolds for three-dimensional cultures with some success (McUsic et al. 2012).

Integration capabilities of the in vitro differentiated cells were also tested by subretinal injections into adult wild-type mice, or into retinal degeneration mouse models (Lamba et al. 2009; Hambright et al. 2012; West et al. 2012). All these studies assessed terminal differentiation and integration of ESC-derived photoreceptors and, when possible, functionality showing alternative results. Lamba et al. (2009) described that transplantation of human ESC-derived photoreceptor progenitors into the subretinal space of adult mice achieved integration into the outer nuclear layer, expression of photoreceptor markers, and formation of morphologically identifiable outer segments. When transplanted into the subretinal space of adult $Crx^{-/-}$ mice (a model for Leber congenital amaurosis), the cells integrated into the retina and restored a light response to otherwise unresponsive animals. In contrast, other two studies described very limited terminal differentiation and integration of the subretinally injected cells, and no functional recovery at all, when assessed (Hambright et al. 2012; West et al. 2012).

Given the origin of ESCs, it is clear that the prospective application of their derivatives to cell transplantation in patients raised important ethical concerns. In more recent years, a new source of pluripotent cells was developed. In 2006, Shinya Yamanaka (Nobel Prize Laureate for Physiology and Medicine 2012 together with Sir John Gurdon "for the discovery that mature cells can be reprogrammed to become pluripotent"; http://www.nobelprize.org/nobel_prizes/medicine/laureates/2012/) showed that adult murine somatic cells such as fibroblasts could be reprogrammed to a pluripotent state by the misexpression of a surprisingly small number of transcription factors (Takahashi and Yamanaka 2006). This step was successfully achieved the subsequent year also for human cells, again by Yamanaka and independently by the Thomson laboratory (Takahashi et al. 2007; Yu et al. 2007). Induced pluripotent stem cells (iPSCs) share all the desirable self-renewal and pluripotency characteristics of ESCs, without carrying the ethical burden associated with the use of human embryos. Furthermore, iPSCs can be derived from patients, thus allowing for the development of personalized cell therapies and "custom" disease models (Yamanaka 2012). Protocols for retinal differentiation of ESCs were also applied with success to mouse and human iPSCs (Lamba et al. 2010; Tucker et al. 2011; Meyer et al. 2011; Boucherie et al. 2013), showing their equivalence to ESCs, at least for in vitro differentiation into photoreceptors. Lamba et al. (2010) and Tucker et al. (2011) also showed the capability of iPSC-derived photoreceptors to terminally differentiate and integrate into the adult mouse retina.

12.3 Shaping the Eye from Embryonic Stem Cells: In Vitro Morphogenesis of Optic Cups and Stratified Neural Retina

A recent breakthrough for in vitro retinal differentiation came from the seminal studies published by Sasai and collaborators (Eiraku et al. 2011; Nakano et al. 2012). They described the incredible self-organization of murine and human ES cells into three-dimensional structures that paralleled a retina. These studies started in 2010 by showing that, by adding extracellular matrix components (matrigel) to an already established retinal differentiation culture system (Osakada et al. 2008), mouse ESCs were able to spontaneously aggregate into polarized neuroepithelial structures with retinal characteristics. This phenomenon was observed after 6–7 days of culture with the budding of vesicles (expressing Rax) that subsequently invaginated, giving rise to optic cup-like structures patterned along the proximal–distal axis. These structures then proceeded to terminal differentiation, generating stratified mature retinal tissue (Eiraku et al. 2011) Remarkably, the whole process occurred outside the normal embryonic environment, and thus in the absence of both lens and surface ectoderm, whose necessity for the formation of the optic cups had been extensively debated (Ashery-Padan et al. 2000). This finding showed that, at least in this in vitro context, the lens was dispensable for optic cup invagination. In 2011 Meyer and colleagues, using modifications of their retinal-inducing culture conditions, showed the formation of optic vesicle-like aggregates harboring the molecular features of retinal progenitor cells, starting from human ESCs and iPSCs (Meyer et al. 2009). Cells in the optic vesicle-like structures further differentiated, showed a segregation that was reminiscent of retinal lamination, and gave rise to electrophysiologically active photoreceptors (Meyer et al. 2011). Despite these characteristics, the optic vesicles-like structures did not invaginate to form optic cups, and a RPE was never observed. Again, Sasai and colleagues in 2012 described the formation of complete optic cup-like structures from human ESCs (Nakano et al. 2012). As for their mouse counterparts, these cells showed self-organization into optic vesicle-like aggregates that subsequently invaginated to form optic cups. Most importantly, the tissue acquired a laminated structure with cells displaying some terminal differentiation characteristics. These findings not only represent a major advance in basic research but also have profound implications for the future of regenerative medicine applications. In fact, iPSC-derived optic cups may be a promising source of photoreceptor precursors for transplantation.

12.4 Retinal Stem Cells from Adult Eyes

The ability of newts to regenerate their eye has been known for a long time, having been discovered in the eighteenth century by Charles Bonnet. Subsequently, the retina became an experimental system for regeneration studies in lower vertebrates

during the twentieth century. These studies demonstrated that the eye has an intrinsic ability to regenerate the damaged tissue. In evolution, this competence declines and is eventually lost in mammals, that upon a genetic or physical insult are unable to substitute for damaged cells. During retinal development, differentiation proceeds from the central part to the periphery of the retina, but regenerating cells in the mature eye of cold-blooded vertebrates derive from a mitotically active zone at the periphery of the retina, the ciliary marginal zone (CMZ). The CMZ and its ability to proliferate persist throughout the lifetime, and CMZ cells can generate all types of retinal neurons and glia and have characteristics of neural stem cells (Locker et al. 2009). There is no evidence of proliferation in the marginal zone of the mammalian retina, but the characterization of the CMZ in lower vertebrates prompted research work to assess if cells in the mammalian ciliary body had the ability to reactivate proliferation when cultured in vitro in the presence of growth factors. Dissection of the ciliary epithelium followed by dissociation of the cells and culture at very low density generated floating clones of pigmented cells called retina neurospheres (Ahmad et al. 2000; Tropepe et al. 2000). These cells in several mammals appeared to be quiescent in the ciliary epithelium in situ, but separation of the cells from other ocular tissues allowed their in vitro reactivation and expansion. A deep analysis of these cells showed that they derived from large ciliary epithelial pigmented cells expressing low levels of P-cadherin (Ballios et al. 2012). Ciliary epithelial cells could be derived from different species such as man, rat, mouse, pig, and rabbit (Coles et al. 2004; Gu et al. 2007; Inoue et al. 2005; Ahmad et al. 2000) and could be expanded in vitro in DMEM-F12 serum-free medium in the presence of hormones and defined doses of growth factors. Pigmented neurospheres were visible within 1 week, and when exposed to differentiation protocols they expressed makers of retinal pigmented epithelial cells as well as Müller glia, rod photoreceptors, and bipolar and horizontal cells. Optimization of the differentiation protocol by plating neurospheres on extracellular matrix from Engelbreth-Holm-Swarm murine sarcoma (ECM) and by changing the culture medium to DMEM (Dulbecco's modified Eagle's medium) and serum allowed specific differentiation into a monolayer of RPE (Aruta et al. 2011).

The stem cell nature of these cells has been challenged by two reports suggesting that neurospheres were actually a clone of ciliary epithelial or RPE cells (Cicero et al. 2009; Gualdoni et al. 2010). The stronger criticism was the inability to induce photoreceptor differentiation by simple treatment with serum, which led the authors to hypothesize transdifferentiation and not differentiation in the production of mature retinal cell types. Specific studies better characterized the stem cell nature of retinal neurospheres by assessing expression of *Nanog* and *Nestin*. These two genes were found at higher levels in retinal neurospheres when compared to the ciliary epithelium. The hypothesis that retinal stem cells could derive from retinal or RPE cells could be discarded based on the observation that three to eight times more retinal neurospheres could be obtained from Chx10-null or Mitf-null eyes, characterized by reduction of neural retina and RPE progenitors, respectively (Coles et al. 2006). In vitro expansion of retinal stem cells was limited because only a small proportion of the cells in neurospheres had self-renewal capability,

but this property could be increased by exposure to pigment epithelium-derived factor (PEDF) (De Marzo et al. 2010). Interestingly, retinal neurospheres expressed much higher levels of Pax6, Chx10, and Rax when compared to ESCs, suggesting that retinal neurospheres contained cells with characteristics of retinal progenitors and a small population of retinal stem cells. Retinal neurospheres from adult ciliary epithelium are therefore clones with a mixed population of cells exhibiting retinal stem and progenitor nature.

Differentiation efficiency into photoreceptors was ameliorated by optimization of the differentiation protocol. To this purpose, similar results were obtained by plating retinal neurospheres on either laminin- or ECM-coated plates and by differentiating the cells in the presence of retinoic acid (RA), taurine, and basic fibroblast growth factor (FGF-2). Expression of photoreceptor markers such as rhodopsin, PDE6b, transducin, Crx, Nrl, peripherin, and recoverin could be detected at 20 days of differentiation and increased to 80 % of cells after 40 days of differentiation. At this same time, most of the cells had lost pigmentation and epithelial characteristics such as ciliation (Ballios et al. 2012; Demontis et al. 2012). The molecular analysis of gene and protein expressions was supported by a functional characterization of cells expressing rhodopsin. Patch-clamp recording defined expression and function of cGMP-gated channels regulated by endogenous cGMP as well as voltage-gated channels necessary for rod maturation (Demontis et al. 2012). In this same study a response to light, although weak, could be measured in rods derived from retinal neurospheres.

Altogether these studies indicate the ciliary epithelium as a promising source of rods for allogeneic transplantation because this tissue can be easily obtained from postmortem human donors, thus avoiding the ethical concerns regarding the use of ESCs or embryonic tissue. A good recovery of human retinal neurospheres was demonstrated until the seventh decade of life (Coles et al. 2004). Otherwise, these cells showed several limits so far: (1) they could be scarcely expanded in vitro; (2) a protocol for cryopreservation of retinal neurospheres has not been developed yet; and (3) it is unclear if these cells followed a proper developmental program in vitro to generate photoreceptor precursors apt to integrate in a damaged retina (see 12.1). The limited attempts in transplantations of cells derived from retinal neurospheres, although yielding not completely characterized outcomes, did not rule out the possibility of using retinal stem cells from adult eyes. Human cells dissociated from retinal neurospheres were able to integrate in neonatal retinae of immunodeficient mice, but they poorly differentiated into photoreceptors and functionality was not assessed (Coles et al. 2004). To favor cell integration and survival, subretinal transplantations were performed using biocompatible and biodegradable biomaterials such as HAMC. HAMC, composed of hyaluronan and methylcellulose, is fluid at room temperature and exhibits rapid gelation at body temperature, making this biomaterial scaffold a minimally invasive and injectable vehicle for cell delivery in the subretinal space. Neurospheres were dissociated into a single-cell suspension and mixed with HAMC. HAMC could increase cell survival after the injection of about 10,000 cells in the subretinal space, but cell integration in the outer nuclear layer could not be observed (Ballios et al. 2010). It is possible that,

as for ESCs, young progenitors and naïve cells were less prone to integration compared to committed photoreceptor precursors. Further studies are necessary to define whether retinal stem cells derived from the adult ciliary epithelium can be differentiated in vitro to the developmental stage that will allow sorting, transplantation, and integration in a degenerating retina.

12.5 Müller Glia Cells

Studies in the brain revealed that glia cells perform several functions such as maintaining homeostasis, providing myelin, and serving as a source of stem cells. The Müller cell is the sole glial cell generated from multipotential progenitors during retinal development. In fish and birds, retina regeneration is mediated by Müller glia cells spontaneously reentering the cell cycle in response to damage (Lamba et al. 2008). This discovery derived from the observation that intrinsic retinal progenitors from the inner nuclear layer sustained regeneration in fish. Upon injury in fish, Müller glia cells activated *Pax6* expression and divided asymmetrically to generate retinal progenitors. These progenitors then moved along the processes of Müller cells, exited the cell cycle, and differentiated into rods in the outer nuclear layer (Ahmad et al. 2011). The neurogenic potential of Müller glia cells was also demonstrated in birds but was more restricted and only a few types of neurons were replaced (Lamba et al. 2008). In mammals, Müller glia cells could re-enter cell cycle after chemical damage, suggesting neurogenic potential, but their differentiation into retinal neurons was very poor. Several studies were undertaken to discover differences between Müller glia cells in lower vertebrates and in mammals and to define whether Müller glia cells could have characteristics of stem cells. The first study directly addressing this question was the demonstration that Müller glia cells could form neurospheres in vitro and showed multipotentiality and self-renewal properties (Das et al. 2006). This study also suggested the neurogenic potential of Müller glia cells derived from the murine retina. Neurogenic potential was probably constrained in the mammalian retina, and in vitro culture conditions could reactivate Müller glia cells. The neurogenic potential could be enhanced when cells were removed from their natural environment, as also shown for human cells (Giannelli et al. 2011). In vitro culture induced a change in gene expression of human Müller glia cells with activation of the cell cycle, and retinal progenitor genes such as *GFAP*, *SOX2*, *CyclinD3*, and *Nestin* and downregulation of Müller glia makers such as glutamine synthetase (*GS*). Although murine Müller glia cells could be induced to differentiate in vitro into photoreceptors by treatment with γ-secretase, an inhibitor of the Notch pathway, differentiation of human Müller glia cells required coculture on a PA6 feeder layer and exposure to taurine that allowed 44 % of cells to be positive to photoreceptor markers. No benefit could be observed by exposure to Retinoic acid, Noggin, and to transforming growth factor (TGF)-β inhibitors. Differentiation was assessed by expression of Rhodopsin,

Recoverin, CNGA1, GNAT1, and PDE6B as well as by patch-clamp recording showing electrical resistance and capacitance similar to rods (Giannelli et al. 2011).

The neurogenic potential of Müller glia cells was further tested by transplantation into neonatal rat eyes of a side population of cells derived from activated Müller glia. The side population represents stem cells and progenitors that are characterized by expression of the ABCG2 transporter that enables cells to preferentially exclude a fluorescent DNA intercalating dye and can be sorted by FACS (fluorescence-activated cell sorting) (Goodell et al. 1996). Transplantation of sorted side population Müller cells demonstrated the ability of these cells to migrate and integrate into the outer nuclear layer (Das et al. 2006).

These studies suggested that Müller glia cells could be a source for cell transplantation but their integration in host retina has proven not to be very efficient so far. The interest in Müller glia cells for photoreceptor replacement otherwise resides in their potentialities for regeneration and comes from studies in lower vertebrates in which endogenous cells could reactivate and substitute damaged photoreceptors. In the adult mammalian retina, an acute in vivo neurotoxic injury was demonstrated to induce Müller glia dedifferentiation and production of photoreceptors. The number of newly generated retinal neurons was, however, very limited. The differences between cold-blooded vertebrates and mammals implied that Müller glia cells in evolution had lost molecular aspects that allowed them to be reactivated during retina regeneration. The characterization of molecular features in fish Müller glia cells absent in mammalian Müller glia cells might open new perspectives for molecular and/or pharmacological treatments to activate endogenous Müller glia cells in retinal degeneration. *Ascl1*, a gene expressed in retinal progenitors, but normally not in Müller glia, was shown to have a fundamental function in fish retinal regeneration because it was rapidly induced upon retinal damage (Fausett et al. 2008). *Ascl1a* induced expression of the pluripotency gene *lin-28*, a RNA-binding protein, that was crucial for Müller glia dedifferentiation and cell-cycle progression and which acted through downregulation of *let-7* miRNA (Ramachandran et al. 2010). Müller cell dedifferentiation was required before cell-cycle reentry, and the proneural basic helix-loop-helix (bHLH) transcription factor Ascl1 was necessary for regeneration of all retinal cell types. This function was mediated by decreasing *let-7* miRNA levels that repressed regeneration-associated mRNAs. Forced expression of Ascl1 in murine Müller glia cells could reprogram the cells to a neurogenic state, induce expression of pan-neuronal markers, and upregulate many retinal-specific neuronal markers (Pollak et al. 2013). Unfortunately, only bipolar neurons could be generated by Ascl1 viral reprogramming in the intact retina. Further studies are required to direct differentiation of reactivated Müller glia cells into photoreceptors.

New studies are trying to address whether Müller glia cells can be reactivated in situ by targeting specific signaling pathways such as Fgf2, Notch, Wnt, and Shh (Del Debbio et al. 2010; Fischer et al. 2002; Osakada et al. 2007; Wan et al. 2007). Intraocular injections of either Wnt or Shh were shown to cause reactivation of Müller glia in wild-type retinae. Reactivated Müller glia started to express retinal progenitor markers such as Rax, Pax6, and Chx10 and migrated out of the inner

nuclear layer to the injured area. During reactivation, cells that reentered the cell cycle could coexpress Müller glia markers together with opsin, and expression changed during migration to regenerate the damaged cells in the retina. Several problems that need to be addressed were highlighted by these studies: to achieve therapeutic regeneration a sufficient number of cells have to be reactivated and to fully differentiate into photoreceptor cells. Nevertheless, positive preliminary studies showed a weak recovery of functionality in S334ter rats after activation of the Wnt and Notch pathways (Del Debbio et al. 2010). Regeneration potentialities of Müller glia cells has not been confirmed by these studies yet, because the molecular features of regenerated cells were not well characterized. It is still unclear if regenerated opsin-expressing cells maintained expression of some Müller-specific marker, if they reached full differentiation, and if functional recovery derived either from newly regenerated rod cells or from neural protection on host photoreceptors. Nonetheless, the neurogenic potential of Müller glia cells could open important perspectives to sustain regeneration.

12.6 Transplantation and Regeneration Perspectives

During the 1980s and 1990s, retinal sheet transplantation was attempted, both with embryonic retinae that survived up to 10 months, and with vibratome sections of the photoreceptor layer (Ghosh and Ehinger 2000). One difficulty was to correctly position the embryonic tissue with photoreceptors facing the host RPE. Transplants often formed rosettes and integrated poorly into the host retina. Transplantation of single cells could overcome some of the limitations observed with embryonic tissue grafts. For more than 10 years researchers tried to identify sources of cells for photoreceptor cell transplantation. The information gained from these studies is very important for development of new protocols to obtain cells able to integrate in a degenerating retina. An accepted requirement to achieve integration and synaptic connection with bipolar cells is that the transplanted cells need to be postmitotic precursors expressing transcription factors involved in photoreceptor identity. Several sources of photoreceptor precursors have been proposed, but at the moment further studies are required to define the most effective ones (Fig. 12.1). None of the sources discussed here have overcome all the different obstacles to photoreceptor transplantation. ESCs, iPSCs, and adult retinal stem cells require extremely long differentiation procedures associated with a low efficiency in photoreceptor generation unsuited to therapeutic applications. Müller glia cells offer a system with a much faster differentiation protocol but so far only poor integration in the outer nuclear layer could be demonstrated.

Drawbacks for the use of ESCs and photoreceptor precursors are ethical and legal debates because the sources are human embryos. iPSCs elude these ethical and legal controversies but, as for ESCs, pluripotent cells are endowed with a not well characterized tumorigenic potential that needs to be completely excluded before any therapeutic exploitation. Differentiation through in vitro generation of

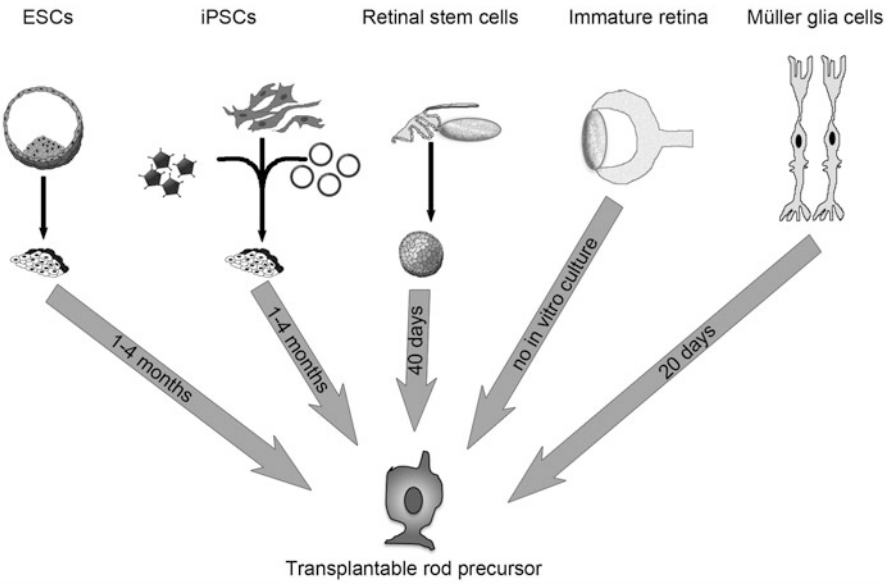

Fig. 12.1 Schematic representation of the different sources of cells for photoreceptor transplantation and the time in culture to derive photoreceptor precursors. We show embryonic stem cell (*ESC*) derivation from the inner cell mass, induced pluripotent stem cell (*iPSC*) derivation from fibroblasts after retroviral infection to express *Oct-3/4*, *Sox2*, *c-Myc*, and *Klf4*, or expression of these factors by using plasmids and retinal stem cells derived from the adult ciliary body

an eye cup may provide a promising source because the primary source can be iPSCs and differentiation to safe postmitotic precursors can be guided in vitro.

Once the proper source of retinal precursors has been identified, an efficient protocol to select cells for transplantation will be imperative for clinical translation. In the experiments just discussed, millions of cells were injected to achieve thousands of integrated cells, implying that many cells either remained in the subretinal space or died. Second, genetic modifications were applied to select cells expressing photoreceptor transcription factors, but these techniques are not desirable for therapy. A recent study addressed this issue and found that CD73/CD24-positive cells could be selected to gain high efficiency of photoreceptor integration (Lakowski et al. 2011).

Finally, allogeneic transplantation may result ineffective if immune rejection is evoked. However, the eye is frequently described as an immune-privileged organ. The subretinal space has been shown to elicit immune deviation although damage to the RPE or disruption of the inner blood–retinal barrier may affect the ocular immune privilege. Upon transplantation of photoreceptor precursors, an acute inflammatory reaction was observed posttransplantation in a limited number of eyes. Otherwise, an adaptive immune response caused cell loss at 4 months posttransplantation in the majority of eyes. These results suggested that photoreceptors from nonautologous sources could survive in the adult host retina, provided

immune responses were modulated by immune suppression with cyclosporine A (West et al. 2010). iPSCs offer a system to overcome the problems associated with immune rejection because they can be derived from patient biopsies once the genetic defect is corrected in vitro before transplantation.

A second, but not as well studied, opportunity for retinal degeneration therapy is in situ activation of regeneration. This field is rapidly evolving, but needs to be supported by gene therapy, because the regenerated endogenous photoreceptors will bear the same genetic mutation that is causing retinal degeneration in the patient.

References

Ahmad I, Tang L, Pham H (2000) Identification of neural progenitors in the adult mammalian eye. Biochem Biophys Res Commun 270:517–521. doi:10.1006/bbrc.2000.2473

Ahmad I, De Debbio CB, Das AV, Parameswaran S (2011) Müller glia: a promising target for therapeutic regeneration. Invest Ophthalmol Vis Sci 52:5758–5764. doi:10.1167/iovs.11-7308

Aruta C, Giordano F, De Marzo A et al (2011) In vitro differentiation of retinal pigment epithelium from adult retinal stem cells. Pigment Cell Melanoma Res 24:233–240. doi:10.1111/j.1755-148X.2010.00793.x

Ashery-Padan R, Marquardt T, Zhou X, Gruss P (2000) Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. Genes Dev 14:2701–11

Ballios BG, Cooke MJ, Van der Kooy D, Shoichet MS (2010) A hydrogel-based stem cell delivery system to treat retinal degenerative diseases. Biomaterials 31:2555–2564. doi:10.1016/j.biomaterials.2009.12.004

Ballios BG, Clarke L, Coles BLK et al (2012) The adult retinal stem cell is a rare cell in the ciliary epithelium whose progeny can differentiate into photoreceptors. Biol Open 1:237–246. doi:10.1242/bio.2012027

Barber AC, Hippert C, Duran Y et al (2013) Repair of the degenerate retina by photoreceptor transplantation. Proc Natl Acad Sci USA 110:354–359. doi:10.1073/pnas.1212677110/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1212677110

Bartsch U, Oriyakhel W, Kenna PF et al (2008) Retinal cells integrate into the outer nuclear layer and differentiate into mature photoreceptors after subretinal transplantation into adult mice. Exp Eye Res 86:691–700. doi:10.1016/j.exer.2008.01.018

Boucherie C, Mukherjee S, Henckaerts E, Thrasher AJ, Sowden JC, Ali RR (2013) Brief report: self-organizing neuroepithelium from human pluripotent stem cells facilitates derivation of photoreceptors. Stem Cells 31:408–414. doi:10.1002/stem.1268

Carter-Dawson LD, LaVail MM (1979) Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. J Comp Neurol 188:263–272. doi:10.1002/cne.901880205

Cicero SA, Johnson D, Reyntjens S et al (2009) Cells previously identified as retinal stem cells are pigmented ciliary epithelial cells. Proc Natl Acad Sci USA 106:6685–6690

Coles BLK, Angenieux B, Inoue T et al (2004) Facile isolation and the characterization of human retinal stem cells. Proc Natl Acad Sci USA 101:15772–15777

Coles BLK, Horsford DJ, McInnes RR, Van der Kooy D (2006) Loss of retinal progenitor cells leads to an increase in the retinal stem cell population in vivo. Eur J Neurosci 23:75–82. doi:10.1111/j.1460-9568.2005.04537.x

Das AV, Mallya KB, Zhao X et al (2006) Neural stem cell properties of Müller glia in the mammalian retina: regulation by Notch and Wnt signaling. Dev Biol 299:283–302. doi:10.1016/j.ydbio.2006.07.029

De Marzo A, Aruta C, Marigo V (2010) PEDF promotes retinal neurosphere formation and expansion in vitro. Adv Exp Med Biol 664:621–630

Del Debbio CB, Balasubramanian S, Parameswaran S et al (2010) Notch and Wnt signaling mediated rod photoreceptor regeneration by Müller cells in adult mammalian retina. PLoS One 5:e12425. doi:10.1371/journal.pone.0012425

Demontis GC, Aruta C, Comitato A, et al (2012) Functional and molecular characterization of rod-like cells from retinal stem cells derived from the adult ciliary epithelium. PLoS One 7: e33338. doi:10.1371/journal.pone.0033338 PONE-D-11-14163 [pii]

Dunwoodie SL (2009) The role of hypoxia in development of the mammalian embryo. Dev Cell 17:755–773. doi:10.1016/j.devcel.2009.11.008

Eiraku M, Takata N, Ishibashi H, Kawada M, Sakakura E, Okuda S, Sekiguchi K, Adachi T, Sasai Y (2011) Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature (Lond) 472:51–56. doi:10.1038/nature09941

Fausett BV, Gumerson JD, Goldman D (2008) The proneural basic helix-loop-helix gene Ascl1a is required for retina regeneration. J Neurosci 28:1109–1117. doi:10.1523/JNEUROSCI.4853-07.2008

Feigenspan A, Bormann J, Wassle H (1993) Organotypic slice culture of the mammalian retina. Vis Neurosci 10:203e17

Fischer AJ, McGuire CR, Dierks BD, Reh TA (2002) Insulin and fibroblast growth factor 2 activate a neurogenic program in Müller glia of the chicken retina. J Neurosci 22:9387–9398

Furukawa T, Morrow EM, Cepko CL (1997) Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell 91:531–541

Garita-Hernández M, Diaz-Corrales F, Lukovic D et al (2013) Hypoxia increases the yield of photoreceptors differentiating from mouse embryonic stem cells and improves the modeling of retinogenesis in vitro. Stem Cells 31:966–978. doi:10.1002/stem.1339

Ghosh F, Ehinger B (2000) Full-thickness retinal transplants: a review. Ophthalmologica 214:54–69

Giannelli SG, Demontis GC, Pertile G et al (2011) Adult human Müller glia cells are a highly efficient source of rod photoreceptors. Stem Cells 29:344–356. doi:10.1002/stem.579. Photoreceptor

Goodell MA, Brose K, Paradis G et al (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806

Gu P, Harwood LJ, Zhang X et al (2007) Isolation of retinal progenitor and stem cells from the porcine eye. Mol Vis 13:1045–1057

Gualdoni S, Baron M, Lakowski J et al (2010) Adult ciliary epithelial cells, previously identified as retinal stem cells with potential for retinal repair, fail to differentiate into new rod photoreceptors. Stem Cells 28:1048–1059

Hambright D, Park KY, Brooks M et al (2012) Long-term survival and differentiation of retinal neurons derived from human embryonic stem cell lines in un-immunosuppressed mouse retina. Mol Vis 18:920–936

Ikeda H, Osakada F, Watanabe K et al (2005) Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. Proc Natl Acad Sci USA 102:11331–11336. doi:10.1073/pnas.0500010102

Inoue Y, Yanagi Y, Tamaki Y et al (2005) Clonogenic analysis of ciliary epithelial derived retinal progenitor cells in rabbits. Exp Eye Res 81:437–445. doi:10.1016/j.exer.2005.03.003

Jadhav AP, Mason HA, Cepko CL (2006) Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development (Camb) 133:913–923

Lakowski J, Baron M, Bainbridge J et al (2010) Cone and rod photoreceptor transplantation in models of the childhood retinopathy Leber congenital amaurosis using flow-sorted Crx-positive donor cells. Hum Mol Genet 19:4545–4559. doi:10.1093/hmg/ddq378

Lakowski J, Han Y-T, Pearson RA et al (2011) Effective transplantation of photoreceptor precursor cells selected via cell surface antigen expression. Stem Cells 29:1391–1404. doi:10.1002/stem.694

Lamba DA, Karl MO, Ware CB, Reh TA (2006) Efficient generation of retinal progenitor cells from human embryonic stem cells. Proc Natl Acad Sci USA 103:12769–12774. doi:10.1073/pnas.0601990103

Lamba D, Karl M, Reh T (2008) Neural regeneration and cell replacement: a view from the eye. Cell Stem Cell 2:538–549. doi:10.1016/j.stem.2008.05.002

Lamba DA, Gust J, Reh TA (2009) Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. Cell Stem Cell 4:73–79. doi:10.1016/j.stem.2008.10.015

Lamba DA, McUsic A, Hirata RK et al (2010) Generation, purification and transplantation of photoreceptors derived from human induced pluripotent stem cells. PLoS One 5:e8763. doi:10.1371/journal.pone.0008763

Locker M, Borday C, Perron M (2009) Stemness or not stemness? Current status and perspectives of adult retinal stem cells. Curr Stem Cell Res Ther 4:118–130

MacLaren RE, Pearson RA, MacNeil A et al (2006) Retinal repair by transplantation of photoreceptor precursors. Nature (Lond) 444:203–207. doi:10.1038/nature05161

McUsic AC, Lamba DA, Reh TA (2012) Guiding the morphogenesis of dissociated newborn mouse retinal cells and hES cell-derived retinal cells by soft lithography-patterned microchannel PLGA scaffolds. Biomaterials 33:1396–1405. doi:10.1016/j.biomaterials.2011.10.083

Mellough CB, Sernagor E, Moreno-Gimeno I et al (2012) Efficient stage-specific differentiation of human pluripotent stem cells toward retinal photoreceptor cells. Stem Cells 30:673–686. doi:10.1002/stem.1037

Meyer JS, Shearer RL, Capowski EE et al (2009) Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc Natl Acad Sci USA 106:16698–16703. doi:10.1073/pnas.0905245106

Meyer JS, Howden SE, Wallace KA et al (2011) Optic vesicle-like structures derived from human pluripotent stem cells facilitate a customized approach to retinal disease treatment. Stem Cells 29:1206–1218. doi:10.1002/stem.674

Nakano T, Ando S, Takata N, Kawada M, Muguruma K, Sekiguchi K, Saito K, Yonemura S, Eiraku M, Sasai Y (2012) Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 10:771–785. doi:10.1016/j.stem.2012.05.009

Ogilvie JM, Speck JD, Lett JM, Fleming TT (1999) A reliable method for organ culture of neonatal mouse retina with long-term survival. J Neurosci Methods 87:57–65

Osakada F, Ooto S, Akagi T et al (2007) Wnt signaling promotes regeneration in the retina of adult mammals. J Neurosci 27:4210–4219. doi:10.1523/JNEUROSCI.4193-06.2007

Osakada F, Ikeda H, Mandai M et al (2008) Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nat Biotechnol 26:215–224. doi:10.1038/nbt1384

Osakada F, Jin Z-B, Hirami Y et al (2009) In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. J Cell Sci 122:3169–3179. doi:10.1242/jcs.050393

Pearson RA, Barber AC, Rizzi M et al (2012) Restoration of vision after transplantation of photoreceptors. Nature (Lond) 485:99–103. doi:10.1038/nature10997

Pera EM, Wessely O, Li SY, De Robertis EM (2001) Neural and head induction by insulin-like growth factor signals. Dev Cell 1:655–665

Pollak J, Wilken MS, Ueki Y et al (2013) Ascl1 reprograms mouse Müller glia into neurogenic retinal progenitors. Development (Camb) 140:2619–2631. doi:10.1242/dev.091355

Ramachandran R, Fausett BV, Goldman D (2010) Ascl1a regulates Müller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. Nat Cell Biol 12:1101–1107. doi:10.1038/ncb2115

Singh MS, Charbel Issa P, Butler R et al (2013) Reversal of end-stage retinal degeneration and restoration of visual function by photoreceptor transplantation. Proc Natl Acad Sci USA 110:1101–1106. doi:10.1073/pnas.1119416110

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676. http://dx.doi.org/10.1016/j.cell.2006.07.024

Takahashi K, Tanabe K, Ohnuki M et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Tropepe V, Coles BLK, Chiasson BJ et al (2000) Retinal stem cells in the adult mammalian eye. Science 287:2032–2036

Tucker BA, Park IH, Qi SD et al (2011) Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice. PLoS One 6:e18992. doi:10.1371/journal.pone.0018992

Wan J, Zheng H, Xiao H-L et al (2007) Sonic hedgehog promotes stem-cell potential of Müller glia in the mammalian retina. Biochem Biophys Res Commun 363:347–354. doi:10.1016/j.bbrc.2007.08.178

West EL, Pearson RA, Barker SE et al (2010) Long-term survival of photoreceptors transplanted into the adult murine neural retina requires immune modulation. Stem Cells 28:1997–2007. doi:10.1002/stem.520

West EL, Gonzalez-Cordero A, Hippert C et al (2012) Defining the integration capacity of embryonic stem cell-derived photoreceptor precursors. Stem Cells 30:1424–1435. doi:10.1002/stem.1123

Yamanaka S (2012) Induced pluripotent stem cells: past, present, and future. Cell Stem Cell 10:678–684. doi:10.1016/j.stem.2012.05.005

Yu J, Vodyanik MA, Smuga-Otto K, et al (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920. Epub 2007 Nov 20

Chapter 13
Molecular Mechanisms of the Function of Pineal Organs

Daisuke Kojima and Yoshitaka Fukada

Abstract The pineal organ in nonmammalian species is a light-sensitive brain structure mediating photosensory and photoendocrine functions. This chapter reviews the photopigments and the phototransduction pathways in the pineal organs of chicken, teleosts, and lamprey and those in the pineal-related organ, the parietal eye, of lizard. Chicken pinealocytes contain a rhodopsin-like molecules, pinopsin, which activates a G protein, transducin, in a light-dependent manner resulting in acute suppression of melatonin synthesis within the cells. Pinopsin is dominantly expressed in the avian and reptilian pineal organs, whereas teleost pineal organs have another rhodopsin-like molecule, exo-rhodopsin, instead of pinopsin. The pineal organs of lampreys exhibit antagonistic responses to green and UV light at the interneuron level: This UV response is mediated by parapinopsin in the photoreceptor cells. In lizards, the parietal eye photoreceptor cells show antagonistic responses to green and blue light at the photoreceptor cell level: parietopsin and pinopsin are likely to antagonistically regulate a cGMP pathway to elicit the responses. This chapter also introduces a more recent topic on a new pineal function as producing a neurosteroid, 7α-hydroxypregnenolone, that regulates behavioral activities in some species.

Keywords 7α-Hydroxypregnenolone • Exo-rhodopsin • Parapinopsin • Parietopsin • Pinopsin

D. Kojima (✉)
Department of Biological Sciences, Graduate School of Science,
The University of Tokyo, Bunkyo-Ku, Tokyo, Japan

Japan Science and Technology Agency (JST), Precursory Research for Embryonic Science and Technology (PRESTO), Kawaguchi, Saitama, Japan
e-mail: sdkojima@mail.ecc.u-tokyo.ac.jp

Y. Fukada (✉)
Department of Biological Sciences, Graduate School of Science,
The University of Tokyo, Bunkyo-Ku, Tokyo, Japan
e-mail: sfukada@mail.ecc.u-tokyo.ac.jp

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5_13,

13.1 Introduction

The pineal organ is an isolated brain structure and, in most non-mammalian vertebrate species, it is present in the dorsal brain just under the skull (Oksche 1965). The pineal organ of many nonmammalian vertebrates is sensitive to light because the pineal organ contains photoreceptor cells that respond to changes in ambient light condition, sending light information via neural connections to other neurons and secreting melatonin into circulating blood. In avian and mammalian species, the pineal organ functions as a neuroendocrine and/or photoendocrine gland. The mammalian pineal gland is not sensitive to light directly but its activity is indirectly regulated by the ambient light–dark information, which is transmitted from the retina via several neural connections (Klein 1985; Simonneaux and Ribelayga 2003).

The pineal organs of nonmammalian vertebrates generally consist of three basic cell types: photoreceptor cells (pinealocytes), projection neurons, and glial cells (Ekstrom and Meissl 2003). In lamprey, teleosts, and amphibians, the pineal photoreceptor cells have a well-developed lamellar outer segment that closely resembles those of retinal rod and cone photoreceptor cells. The photoreceptor cells transmit the light signals to the secondary afferent neurons (projection neurons) through synaptic contacts within the pineal organ. The projection neurons innervate several areas of the central brain, and in this sense they are similar to the retinal ganglion cells. The pinealocytes of reptiles and birds have a regressed outer segment with degenerated lammellar structure, and they are thus refered as "modified" photoreceptor cells. Most of the modified photoreceptor cells act as neuroendocrine cells specialized for secreting melatonin, rather than primary sensory cells. Note that mammalian pinealocytes, having no direct light sensitivity, lack any pronounced outer segment-like structure and instead function as specialized neuroendocrine cells (Ekstrom and Meissl 2003).

Physiological functions for the pineal organs have been reported mainly in circadian biologies. For example, surgical removal of the pineal gland from oscine passerine birds, such as the house sparrow (*Passer domestics*), abolishes the expression of circadian locomotor rhythms when birds are placed in constant darkness (Gaston and Menaker 1968), although the effects of pinealectomy are variable in other avians (Cassone et al. 2009). The pineal gland also plays a role in keeping a circadian rhythm of body temperature (Refinetti and Menaker 1992).

In some vertebrate species, such as lampreys and teleosts, the pineal organ constitutes a structural unit of a pineal complex with an accessory organ called the parapineal organ, which is also photosensitive. Some lizards have an extracranial photosensory organ, called the parietal eye or parietal organ, which is considered as a parapineal organ homologue (Ekstrom and Meissl 2003). Another example of extracranial photosensory organs is the frontal organ in frogs, but in this case it is likely to to be a specialization of the distal part of the pineal organ (Ekstrom and Meissl 2003).

In this chapter, we review the photopigments and the phototransduction pathways of chicken and teleost pineal organs. We then discuss the photopigments

in the lamprey pineal complex, in which light signals sent from multiple photoreceptor cells having different spectral sensitivities are likely to be converged at the level of interneurons exhibiting "chromatic" photoresponses. We also review the antagonistic phototransduction pathway of lizard parietal eye photoreceptor cells, where light signals from multiple photopigments are converged within the photoreceptor cells. Finally, a more recent topic is introduced on a new pineal function as producing a neurosteroid, 7α-hydroxypregnenolone, that regulates behavioral activities in some species.

13.2 Photopigments and Phototransduction Pathways in Pineal and Related Organs

13.2.1 Avian Pineal Gland

The intrinsic photosensitivity in the chicken pineal gland was reported in the late 1970s (Binkley et al. 1978; Deguchi 1979a,b,c; Kasal et al. 1979), and the presence of rhodopsin-like molecule(s) in the pineal gland was predicted by immunohistochemical and physiological studies in the 1980s. In 1994, a rhodopsin-like photoreceptive molecule, pinopsin, was identified in the chicken pineal gland (Okano et al. 1994) as the first example of a "nonvisual" opsin expressed in a tissue outside the retina. Pinopsin is closely related to vertebrate visual pigments in the primary structure (Fig. 13.1), and binds 11-*cis*-retinal as the chromophore to form a blue-sensitive pigment having the absorption maximum at 468 nm (Okano et al. 1994; Nakamura et al. 1999).

The absorption maximum of pinopsin is ~30 nm blue-shifted from the peak of the physiological action spectrum (500 nm) for light-induced inhibition of chicken pineal arylalkylamine *N*-acetyltransferase (Deguchi 1981), a rate-determining enzyme in the melatonin synthesis pathway. This difference suggested that the chicken pineal gland contains at least one additional photoreceptive molecule having longer wavelength sensitivity, such as red cone opsin, and it is indeed expressed in the pineal gland (Okano et al. 1994).

Chicken pinopsin is expressed only in the pineal gland (Okano et al. 1994; Max et al. 1995). Pinopsin protein is localized in the outer segment of pinealocytes (Hirunagi et al. 1997; Matsushita et al. 2000), which is considered as the primary photosensitive structure and likely to contain phototransduction proteins just as in the outer segments of retinal rods and cones (see following).

The chicken pineal gland has intermediate properties between a neuroendocrine and photoendocrine organ, producing and secreting melatonin in a manner dependent on both the efferent input signal and the light signal captured by intrinsic photoreceptors. Melatonin is a nighttime hormone involved in a variety of physiological aspects such as sleep–wake regulation. Melatonin production shows a

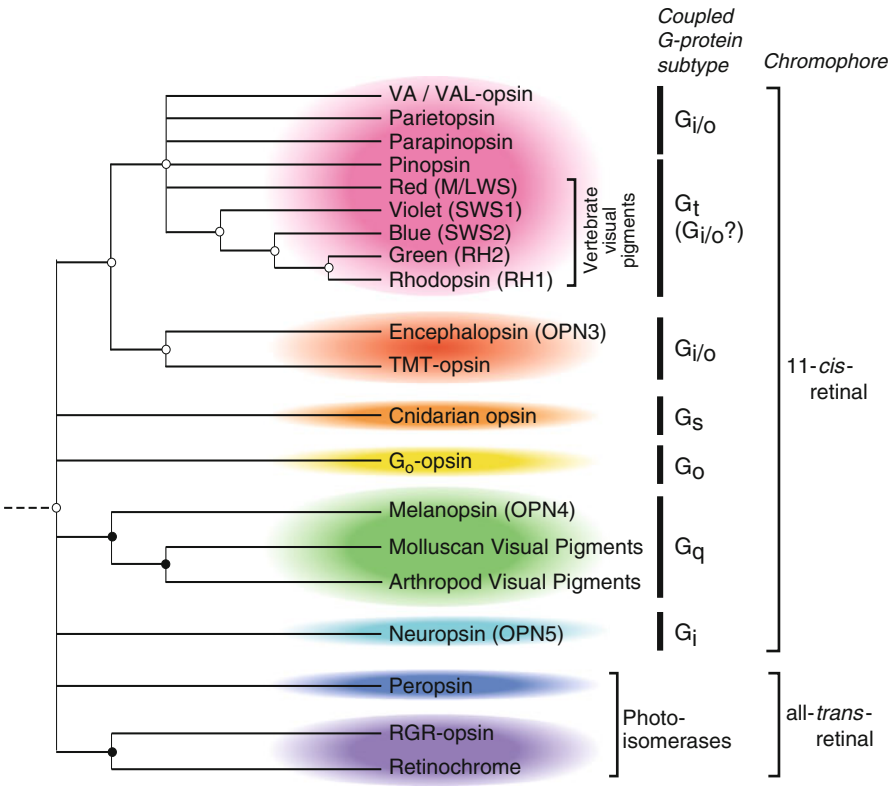

Fig. 13.1 Phylogenetic relationship among subfamilies in the opsin family. Nomemclatures for vertebrate visual pigments (M/LWS, SWS1, SWS2, RH2 and RH1) are indicated in parentheses according to Ebrey and Koutalos (2001)). Each node with a *closed circle* represents species divergence; *open circle* represents gene duplication. (Modified from Kojima et al. 2008)

nocturnal rhythm, which is controlled by the endogenous circadian clock in individual cells (pinealocytes) of the pineal gland. The phase of the circadian rhythm is shifted by light given at subjective night (phase-shifting effect; see Fig. 13.2). On the other hand, the chicken pinealocytes contain another phototransduction pathway mediating acute suppression of melatonin production by light (acute suppression effect; see Fig. 13.2). These two effects of light on chicken pineal cells can be discriminated by an administration of pertussis toxin (PTX). The PTX treatment does not influence the phase-shifting effect of cellular clocks but blocks the acute suppression effect on melatonin production (Zatz and Mullen 1988), indicating the the latter effect is mediated by PTX-sensitive molecules, most probably by a PTX-sensitive heterotrimeric GTP-binding protein (G protein). Consistently, the chicken pineal gland shows mRNA expression of α-subunits of PTX-sensitive G proteins, such as $G\alpha_{t1}$ (rod transducin-α), $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{o}$

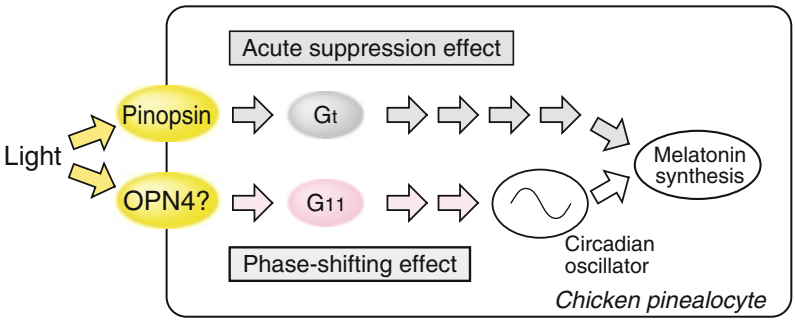

Fig. 13.2 Light signaling pathways in chicken pineal photoreceptor cells

(Okano et al. 1997; Matsushita et al. 2000; Kasahara et al. 2000). The rod-type transducin, G_{t1}, is activated by chicken pinopsin in a light-dependent manner in in vitro reconstitution assays as well as in the pineal homogenate (Max et al. 1998; Nakamura et al. 1999; Kasahara et al. 2000) and is colocalized with pinopsin in the "rudimentary" outer segment of pinealocytes (Matsushita et al. 2000; Kasahara et al. 2000). Taken together, the pinopsin-transducin (G_t) pathway is likely to be involved in the pineal phototransduction cascade and to account for the acute suppression effect of light in the melatonin production of the pinealocytes (Fig. 13.2).

The PTX-sensitive acute effect of light might be mediated by another photopigment, OPN5 (OPN5m), which has been recently reported to be present in chicken pineal glands (Yamashita et al. 2010). OPN5 is an ultraviolet light (UV)-sensitive photopigment and can activate G_i-type G protein in a UV-dependent manner (Yamashita et al. 2010; Kojima et al. 2011). $G_{i/o}$-type G proteins, being susceptible to PTX, are expressed in the pineal gland (Okano et al. 1997), although a direct effect of ultraviolet light on melatonin production in the chicken pineal gland has not been reported.

As just described, the circadian rhythm of melatonin production in the chicken pinealocytes is phase shifted by light in a PTX-"insensitive" manner (Zatz and Mullen 1988). Several classes of heterotrimeric G proteins, such as $G_{q/11}$ and G_s, are insensitive to PTX treatment as they lack the (PTX-catalyzed) ADP-ribosylated amino acid in the C-terminal region. Among these members, the α-subunit of G_{11} ($G\alpha_{11}$) is expressed in the chicken pineal gland (Matsushita et al. 2000; Kasahara et al. 2002). In a gain-of-function experiment using cultured chicken pinealocytes, selective activation of G_{11} caused phase shift of the circadian rhythm of melatonin production in a manner very similar to a light-triggered phase shift (Kasahara et al. 2002). G_{11} is thus likely to mediate the circadian phase-shifting effect in chicken pinealocytes (Fig. 13.2). Now an important question is the identities of photopigments triggering this pathway. There has been no report on activation of $G_{q/11}$-type G protein mediated by pinopsin, red cone opsin, or Opn5, although chicken rhodopsin can interact with $G\alpha_{11}$ in a light-dependent manner (Kasahara et al. 2002). It should be noted that vitamin A depletion in the culture medium only

reduces the acute melatonin supression but does not affect the circadian phase shift (Zatz 1994). Therefore, the two phototransduction pathways (Fig. 13.2) causing acute melatonin supression and the circadian phase shift in the chicken pineolocytes are likely to couple with different photopigments from each other. A potential candidate for the phase-shifting photopigment is OPN4, or melanopsin (Bailey and Cassone 2005; Chaurasia et al. 2005; Holthues et al. 2005; Bellingham et al. 2006; Torii et al. 2007), which is closely related in sequence to invertebrate G_q-coupled rhodopsins (Fig. 13.1). Invertebrate rhodopsins as well as the cepholochordate melanopsin homologue have been shown to activate G_q-type G protein in a light-dependent manner (Koyanagi et al. 2005; Terakita et al. 2008). The photoproduct (active state) of OPN4 is stable in the dark, and it is photo-convertible to the original state without further supply of its chromophore 11-*cis*-retinal. Such a bistable nature of OPN4 is consistent with the fact that the circadian phase-shifting pathway does not require a vitamin A supply in the pineal cell culture (Zatz 1994). The chicken pineal gland expresses two kinds of melanopsin genes, OPN4-1 (OPN4x) and OPN4-2 (OPN4m) (Bailey and Cassone 2005; Chaurasia et al. 2005; Holthues et al. 2005; Bellingham et al. 2006; Torii et al. 2007). The OPN4-1 and OPN4-2 genes encode blue-sensitive photopigments with absorption maxima at 476 and 484 nm, respectively (Torii et al. 2007). It is not known whether any of these OPN4 proteins contributes to the phase shift in the chicken pineal gland.

13.2.2 Teleost Pineal Organ

In contrast to pinopsin expression in the pineal gland of birds (Okano et al. 1994; Max et al. 1995; Kawamura et al. 1999) and reptiles (Kawamura and Yokoyama 1997; Taniguchi et al. 2001), the pineal organ of teleosts does not express pinopsin but it has, instead, a novel rhodopsin-like molecule, exo-rhodopsin (named after extraocular rhodopsin). The exo-rhodopsin was first identified in the zebrafish pineal gland (Mano et al. 1999), and later in the pineal gland of salmon (Philp et al. 2000a) and other teleosts. In zebrafish, exo-rhodopsin is specifically expressed in the pineal gland (but not in the retina), whereas rhodopsin is specifically present in the retinal rod photoreceptor cells but not in the pineal gland (Mano et al. 1999). Such mutually exclusive expression patterns between exo-rhodopsin (pineal gland) and rhodopsin (retina) were also reported in salmon (Philp et al. 2000a). A detailed mutational analysis of the zebrafish *exo-rhodopsin* promoter led to identification of a 12-bp *cis*-acting element, PIPE (pineal expression-promoting element), that is required for pineal specific gene expression (Asaoka et al. 2002).

Molecular phylogenetic analyses clearly indicate that the *exo-rhodopsin* gene emerged from the *rhodopsin* gene by gene duplication in the ray-finned fish lineage (Mano et al. 1999; Bellingham et al. 2003; Rennison et al. 2012). Interestingly, the teleost *exo-rhodopsin* genes retain the exon-intron structure that is conserved among the tetrapod *rhodopsin* genes, whereas the teleost *rhodopsin* genes are intronless.

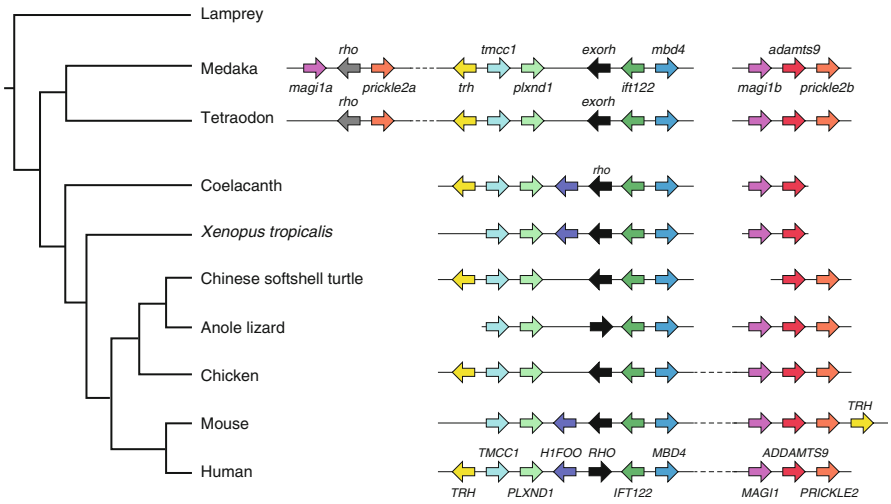

Fig. 13.3 Genome synteny of chromosomal regions containing vertebrate *rhodopsin*/*exo-rhodopsin* genes. In the genomes of teleosts (medaka and tetraodon), the *exo-rhodopsin* gene (*exorh*) is located between *plxnd1* and *ift122* loci. The *plxnd1-exorh-ift122* arrangement shows a synteny to the chromosomal region containing the *rhodopsin* gene in the coelacanth and tetrapods including humans (*PLXND1-H1FOO-RHO-IFT122*). Note that the teleost *rhodopsin* gene locus (*rho*) is located adjacent to *prickle2a* and *magi1a* loci, which appear to have emerged by a whole-genome duplication event in the teleost lineage. (From Taylor et al. 2003)

These observations suggest that a retroposition occurred in the teleost "*rhodopsin*" gene lineage just after the *exo-rhodopsin* gene had arisen (Bellingham et al. 2003). In this sense, *exo-rhodopsin* may be the true orthologue of tetrapod *rhodopsin* (Bellingham et al. 2003), and this idea is supported by a synteny analysis of *rhodopsin*/*exo-rhodopsin* genes (Fig. 13.3).

The zebrafish pineal gland also expresses the red cone opsin gene in a smaller subset of the cells (Robinson et al. 1995; Mano et al. 1999), whereas most of the zebrafish pineal photoreceptor cells express exo-rhodopsin (Mano et al. 1999). As are most of the vertebrate rhodopsins, exo-rhodopsin is a green-sensitive photoreceptive molecule having its absorption maximum at 498 nm (Tarttelin et al. 2011). The zebrafish red cone opsin, LWS1 or LWS2, has its absorption maximum at 558 nm (LWS1) or 548 nm (LWS2) (Chinen et al. 2003). Consistently, light-induced suppression of melatonin release measured in cultured zebrafish pineal glands has a spectral sensitivity peaking at ~500 nm with a shoulder at ~570 nm, suggesting that multiple photopigments including exo-rhodopsin and red cone opsin are involved in this response (Ziv et al. 2007). On the other hand, the pineal gland of rainbow trout showed an action spectrum of melatonin suppression, peaking at ~500 nm without any obvious shoulder in the longer-wavelength region (Max and Menaker 1992), and it also showed maximal sensitivity between 500 and 530 nm in electrophysiological recordings of photoresponses of projection neurons and photoreceptor cells (Dodt 1963; Meissl and Ekstrom 1988). The difference in

spectral sensitivity at a longer-wavelength region between zebrafish and trout pineal glands may reflect species variation in the composition of photoreceptive molecules whose identities have not yet been reported in the trout pineal gland. As an example of species variation, VA-opsin gene expression was detected in the 'salmon' pineal gland (Philp et al. 2000b) but not in the 'zebrafish' pineal gland (Kojima et al. 2000; Ziv et al. 2007).

With light illumination, exo-rhodopsin, similar to rhodopsin, is converted into its active state, meta II, which was shown to activate G-protein transducin in vitro (Tarttelin et al. 2011). As in the chicken pineal photoreceptor cells, transducin is likely to mediate the light-induced melatonin suppression in the pineal gland of teleosts, where immunoreactivity to the α-subunit of transducin was detected (van Veen et al. 1986). The meta II state of exo-rhodopsin has a lifetime more than tenfold shorter than that of meta II of rhodopsin (Tarttelin et al. 2011), which is a characteristic rather similar to cone opsins (Shichida and Imai 1998). This molecular behavior of exo-rhodopsin was in contrast to the prediction, because exo-rhodopsin is more similar to rhodopsin than to cone opsins in its primary structure (Mano et al. 1999), including the determinant amino acid residues for meta II lifetime of rhodopsin, Glu122, and Ile189 (Imai et al. 1997; Kuwayama et al. 2002). The structural basis for the shorter meta II lifetime for exo-rhodopsin appears quite different from the cone opsins but remains unsolved (Tarttelin et al. 2011).

13.2.3 Lamprey Pineal Complex

The lamprey has a pineal complex consisting of the pineal body in the dorsal part and parapineal body in the ventral part. The pineal body is located just under the pineal window, which is a less-pigmented part of the skull. The ventral portion of the pineal body shows immunoreactivities to rhodopsin antibody (Tamotsu et al. 1990) and rhodopsin mRNA expression (Koyanagi et al. 2004). Expression of the rhodopsin gene is consistent with the fact that the ventral pineal body has achromatic (luminosity-type) interneurons, which are maximally sensitive to green light (Morita and Dodt 1973; Uchida and Morita 1994). These observations suggest potential signal inputs from the rhodopsin-expressing photoreceptor cells to the luminosity-type interneurons. On the other hand, the dorsal and peripheral portion of the lamprey pineal body expresses a UV-sensitive opsin, parapinopsin (Koyanagi et al. 2004), which was originally identified in the catfish parapineal organ (Blackshaw and Snyder 1997). The lamprey parapinosin shows a bistable nature such that it is converted with UV light illumination to a thermally stable, green-sensitive photoproduct, which can be converted with green light illumination again to the original, UV-sensitive state (Koyanagi et al. 2004). The existence of the UV-sensitive pigment is consistent with the fact that the lamprey pineal body

contains "chromatic"-type interneurons, whose activity is inhibited maximally by 380-nm UV light but enhanced by 540-nm green light (Morita and Dodt 1973; Uchida and Morita 1994). The inhibitory action of the UV light on the chromatic-type interneurons is likely to be mediated by the parapinopsin-expressing cells, whereas the excitatory action of green light is unlikely to originate from rhodopsin-expressing cells in the ventral pineal body but could be mediated by unidentified photopigments, which must be present in the dorsal and peripheral portion of the lamprey pineal gland.

The parapineal body of the lamprey, as does the pineal body, has rhodopsin expression in the ventral part and parapinopsin expression in the dorsal part (Kawano-Yamashita et al. 2007; Koyanagi et al. 2004). In addition to rhodopsin and parapinopsin, red cone opsin immunoreactivities are observed in the pineal and parapineal bodies of lamprey (Tamotsu et al. 1994; Koyanagi et al. 2004). Some of the red cone opsin-immunoreactive signals were observed in the serotonin-containing pineal neurons (Tamotsu et al. 1994).

13.2.4 Reptile Parietal Organ

The parietal eye in reptiles is an extracranial photoreceptive organ that is considered to be a parapineal organ homologue (Ekstrom and Meissl 2003). Similar to vertebrate lateral eyes, the parietal eye has a retina-like layered structure, which consists of two cellular layers: the inner layer has photoreceptor cells and the outer has ganglion cells. In contrast to lateral eyes, the parietal eye has no interneurons, but the ganglion cells exhibit antagonistic chromatic responses (Dodt and Scherer 1968; Miller and Wolbarsht 1962): Green light induces an excitatory response in the parietal ganglion cells, whereas blue light causes an inhibitory response. The spectral sensitivities of these responses peak at ~520 nm (excitatory) and at ~450 nm (inhibitory) in the European lizard *Lacerta sicula campestris* (Dodt and Scherer 1968). The antagonistic chromatic response was found to originate from the individual photoreceptor cells in the parietal eyes of the desert night lizard *Xantusia vigilis* and the side-blotched lizard *Uta stansburiana* (Solessio and Engbretson 1993). In the parietal photoreceptor cells, green light depolarizes the membrane potential by increasing Na^+ conductance, and blue light on the background green illumination hyperpolarizes it by decreasing Na^+ conductance (Solessio and Engbretson 1993). It was proposed that the antagonistic nature of these photo-responses may provide lizards with a mechanism for enhanced detection of dawn and dusk (Solessio and Engbretson 1993).

The green light-induced depolarization in the parietal eye photoreceptor cells arises from openings of the cGMP-gated (CNG) cation channels located in their outer segments, and they are similar in properties to those found in retinal rod photoreceptor cells (Finn et al. 1997). The CNG channel openings in the parietal

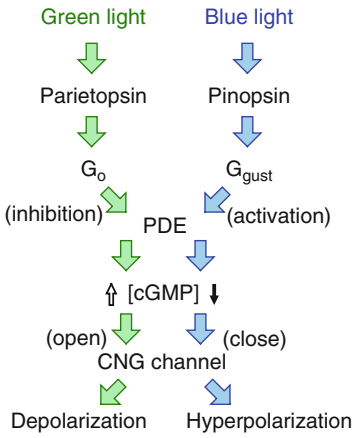

Fig. 13.4 Antagonistic phototransduction pathway in the parietal eye photoreceptor cells of side-blotched lizards. *CNG channel*, cGMP-gated cation channel

eye photoreceptor cells result from a rise in intracellular cGMP caused by a decrease in activity of phosphodiesterase (PDE), which hydrolyzes cGMP, rather than an increase in activity of guanylyl cyclase synthesizing cGMP (Xiong et al. 1998). It is proposed that PDE activity is regulated by a G protein (putatively termed G_1, active in the dark) and is antagonistically inhibited by another G protein (G_2) activated by light (Xiong et al. 1998). It remains unknown whether the G_1 is constitutively active or is activated by an upstream signal in the dark.

Consistent with the presence of two antagonistic light signaling pathways (Fig. 13.4), the parietal eye photoreceptor cells of side-blotched lizards were found to have blue- and green-sensitive photoreceptive molecules (opsins) in the same cells (Su et al. 2006): The blue one was pinopsin, originally found in the chicken pineal gland (Okano et al. 1994), and the green one was named parietopsin, having an absorption maximum at 522 nm with 11-*cis*-retinal bound. Interestingly, the parietal eye photoreceptor cells have two kinds of G-protein α-subunits, $G\alpha_{gust}$ and $G\alpha_o$, but do not have transducin-α (Su et al. 2006). Electrophysiological and pharmacological examination suggested that the $G\alpha_o$ inhibits the PDE to generate the depolarizing response to green light, probably via activation by parietopsin (Su et al. 2006) (Fig. 13.4). On the other hand, the structural similarity of pinopsin to rod/cone opsins together with the close similarity among $G\alpha_{gust}$ and $G\alpha_{t1/2}$ (transducin-α) suggested the hypothesis that the blue-light induced hyperpolarization in parietal eye photoreceptor cells could be mediated by a pinopsin-$G\alpha_{gust}$ pathway via PDE activation (Su et al. 2006) (Fig. 13.4). It should be noted that the parietal eye photoreceptor cells in another lizard, the green iguana (*Iguana iguana*), contain a combination of parietopsin and UV-sensitive opsin, parapinopsin, instead of pinopsin (Wada et al. 2012), indicating species variation in the opsin repertoire of the parietal eye photoreceptor cells among reptiles.

13.3 Light-Dependent Synthesis of 7α-Hydroxypregnenolone in Chicken Pineal Gland

The chicken pineal gland has been recently found to actively produce a neurosteroid hormone, 7α-hydroxypregnenolone (Hatori et al. 2011). This finding came from our pineal transcriptome analysis that aimed at understanding molecular mechanisms underlying light-dependent regulation of the circadian clock. The pineal transcripts prepared from dark-reared chicks or those exposed to light at various times of the day were subjected to a differential microarray analysis to search for the genes important for the light-dependent phase shift of the clock. This global transcriptome analysis revealed light-induced transcriptional activation of a set of genes involved in cholesterol biosynthesis (Hatori et al. 2011) along with upregulation of a transcript for *E4bp4*, a clock transcription factor gene that is known to repress *Per2* gene expression and hence associated with the phase delay of the chick pineal clock (Doi et al. 2001; Doi et al. 2004) (Fig. 13.5). The light induction of these genes turned out to be regulated by light activation of the transcription factor, sterol regulatory element-binding protein SREBP (Hatori et al. 2011) (Fig. 13.5). From these observations, a possibility emerged that pineal cholesterol biosynthesis is activated by light. In fact, we found that the chick pineal gland produces and secretes 7α-hydroxypregnenolone (Fig. 13.5) in organ culture in a light-stimulated manner (Hatori et al. 2011). It should be noted that the light stimulation of 7α-hydroxypregnenolone production occurs only at a specific time of the day: that is, this steroid production was not activated by light at late night nor during daytime but by light given at early night (Hatori et al. 2011), which is the time when the circadian clock is phase delayed by light exposure (Okano and Fukada 2003). Interestingly, locomotor activities of dark-reared chicks are stimulated far more strikingly by light exposure at early night when compared to light/dark ratios at late night and in daytime (Hatori et al. 2011) (Fig. 13.5). It is well

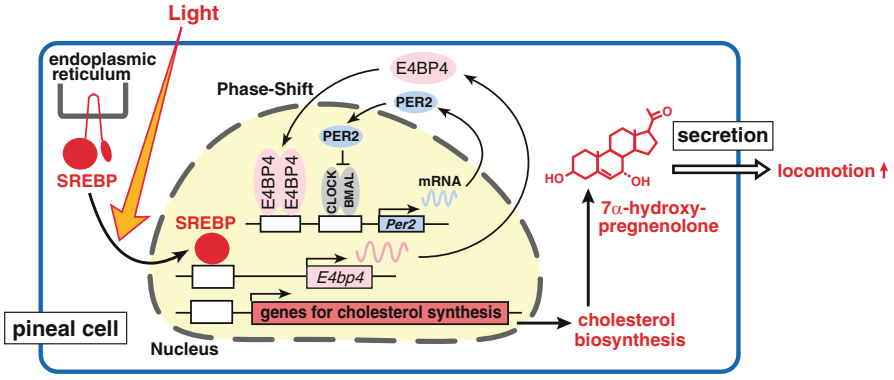

Fig. 13.5 Light-stimulated production of pineal cholesterol biosynthetic genes is associated with light-stimulated production of 7α-hydroxypregnenolone. (Modified from Hatori et al. 2011)

established that the canonical pineal hormone melatonin regulates sleep rhythms (Arendt and Skene 2005). The pineal gland appears to participate in the regulation of the sleep–wake state, not only by circadian production of melatonin in the dark but also by synthesis and secretion of 7α-hydroxypregnenolone in the light.

References

Arendt J, Skene DJ (2005) Melatonin as a chronobiotic. Sleep Med Rev 9(1):25–39. doi:10.1016/j.smrv.2004.05.002

Asaoka Y, Mano H, Kojima D, Fukada Y (2002) Pineal expression-promoting element (PIPE), a cis-acting element, directs pineal-specific gene expression in zebrafish. Proc Natl Acad Sci USA 99(24):15456–15461. doi:10.1073/pnas.232444199

Bailey MJ, Cassone VM (2005) Melanopsin expression in the chick retina and pineal gland. Brain Res Mol Brain Res 134(2):345–348. doi:10.1016/j.molbrainres.2004.11.003

Bellingham J, Tarttelin EE, Foster RG, Wells DJ (2003) Structure and evolution of the teleost extraretinal rod-like opsin (errlo) and ocular rod opsin (rho) genes: is teleost rho a retrogene? J Exp Zool B Mol Dev Evol 297(1):1–10. doi:10.1002/jez.b.18

Bellingham J, Chaurasia SS, Melyan Z, Liu C, Cameron MA, Tarttelin EE, Iuvone PM, Hankins MW, Tosini G, Lucas RJ (2006) Evolution of melanopsin photoreceptors: discovery and characterization of a new melanopsin in nonmammalian vertebrates. PLoS Biol 4(8):e254. doi:10.1371/journal.pbio.0040254

Binkley SA, Riebman JB, Reilly KB (1978) The pineal gland: a biological clock in vitro. Science 202(4373):1198–1120

Blackshaw S, Snyder SH (1997) Parapinopsin, a novel catfish opsin localized to the parapineal organ, defines a new gene family. J Neurosci 17(21):8083–8092

Cassone VM, Paulose JK, Whitfield-Rucker MG, Peters JL (2009) Time's arrow flies like a bird: two paradoxes for avian circadian biology. Gen Comp Endocrinol 163(1-2):109–116. doi:10.1016/j.ygcen.2009.01.003

Chaurasia SS, Rollag MD, Jiang G, Hayes WP, Haque R, Natesan A, Zatz M, Tosini G, Liu C, Korf HW, Iuvone PM, Provencio I (2005) Molecular cloning, localization and circadian expression of chicken melanopsin (Opn4): differential regulation of expression in pineal and retinal cell types. J Neurochem 92(1):158–170. doi:10.1111/j.1471-4159.2004.02874.x

Chinen A, Hamaoka T, Yamada Y, Kawamura S (2003) Gene duplication and spectral diversification of cone visual pigments of zebrafish. Genetics 163(2):663–675

Deguchi T (1979a) A circadian oscillator in cultured cells of chicken pineal gland. Nature (Lond) 282(5734):94–96

Deguchi T (1979b) Circadian rhythm of serotonin *N*-acetyltransferase activity in organ culture of chicken pineal gland. Science 203(4386):1245–1247

Deguchi T (1979c) Role of adenosine 3',5'-monophosphate in the regulation of circadian oscillation of serotonin *N*-acetyltransferase activity in cultured chicken pineal gland. J Neurochem 33(1):45–51

Deguchi T (1981) Rhodopsin-like photosensitivity of isolated chicken pineal gland. Nature (Lond) 290(5808):706–707

Dodt E (1963) Photosensitivity of the pineal organ in the teleost, *Salmo irideus* (Gibbons). Experientia (Basel) 19:642–643

Dodt E, Scherer E (1968) Photic responses from the parietal eye of the lizard *Lacerta sicula campestris* (De Betta). Vision Res 8(1):61–72. doi:10.1016/0042-6989(68)90064-3

Doi M, Nakajima Y, Okano T, Fukada Y (2001) Light-induced phase-delay of the chicken pineal circadian clock is associated with the induction of cE4bp4, a potential transcriptional repressor of cPer2 gene. Proc Natl Acad Sci USA 98(14):8089–8094. doi:10.1073/pnas.141090998

Doi M, Okano T, Yujnovsky I, Sassone-Corsi P, Fukada Y (2004) Negative control of circadian clock regulator E4BP4 by casein kinase Iepsilon-mediated phosphorylation. Curr Biol 14(11):975–980. doi:10.1016/j.cub.2004.05.043

Ebrey T, Koutalos Y (2001) Vertebrate photoreceptors. Prog Retin Eye Res 20(1):49–94

Ekstrom P, Meissl H (2003) Evolution of photosensory pineal organs in new light: the fate of neuroendocrine photoreceptors. Philos Trans R Soc Lond B Biol Sci 358(1438):1679–1700. doi:10.1098/rstb.2003.1303

Finn JT, Solessio EC, Yau KW (1997) A cGMP-gated cation channel in depolarizing photoreceptors of the lizard parietal eye. Nature (Lond) 385(6619):815–819. doi:10.1038/385815a0

Gaston S, Menaker M (1968) Pineal function: the biological clock in the sparrow? Science 160 (3832):1125–1127

Hatori M, Hirota T, Iitsuka M, Kurabayashi N, Haraguchi S, Kokame K, Sato R, Nakai A, Miyata T, Tsutsui K, Fukada Y (2011) Light-dependent and circadian clock-regulated activation of sterol regulatory element-binding protein, X-box-binding protein 1, and heat shock factor pathways. Proc Natl Acad Sci USA 108(12):4864–4869. doi:10.1073/pnas.1015959108

Hirunagi K, Ebihara S, Okano T, Takanaka Y, Fukada Y (1997) Immunoelectron-microscopic investigation of the subcellular localization of pinopsin in the pineal organ of the chicken. Cell Tissue Res 289(2):235–241

Holthues H, Engel L, Spessert R, Vollrath L (2005) Circadian gene expression patterns of melanopsin and pinopsin in the chick pineal gland. Biochem Biophys Res Commun 326 (1):160–165. doi:10.1016/j.bbrc.2004.11.022

Imai H, Kojima D, Oura T, Tachibanaki S, Terakita A, Shichida Y (1997) Single amino acid residue as a functional determinant of rod and cone visual pigments. Proc Natl Acad Sci USA 94(6):2322–2326. doi:10.1073/pnas.94.6.2322

Kasahara T, Okano T, Yoshikawa T, Yamazaki K, Fukada Y (2000) Rod-type transducin alpha-subunit mediates a phototransduction pathway in the chicken pineal gland. J Neurochem 75(1):217–224

Kasahara T, Okano T, Haga T, Fukada Y (2002) Opsin-G_{11}-mediated signaling pathway for photic entrainment of the chicken pineal circadian clock. J Neurosci 22(17):7321–7325

Kasal CA, Menaker M, Perez-Polo JR (1979) Circadian clock in culture: *N*-acetyltransferase activity of chick pineal glands oscillates in vitro. Science 203(4381):656–658

Kawamura S, Yokoyama S (1997) Expression of visual and nonvisual opsins in American chameleon. Vision Res 37(14):1867–1871

Kawamura S, Blow NS, Yokoyama S (1999) Genetic analyses of visual pigments of the pigeon (*Columba livia*). Genetics 153(4):1839–1850

Kawano-Yamashita E, Terakita A, Koyanagi M, Shichida Y, Oishi T, Tamotsu S (2007) Immunohistochemical characterization of a parapinopsin-containing photoreceptor cell involved in the ultraviolet/green discrimination in the pineal organ of the river lamprey *Lethenteron japonicum*. J Exp Biol 210(pt 21):3821–3829. doi:10.1242/jeb.007161

Klein DC (1985) Photoneural regulation of the mammalian pineal gland. Ciba Found Symp 117:38–56

Kojima D, Mano H, Fukada Y (2000) Vertebrate ancient-long opsin: a green-sensitive photoreceptive molecule present in zebrafish deep brain and retinal horizontal cells. J Neurosci 20(8):2845–2851

Kojima D, Torii M, Fukada Y, Dowling JE (2008) Differential expression of duplicated VAL-opsin genes in the developing zebrafish. J Neurochem 104(5):1364–1371. doi:10.1111/j.1471-4159.2007.05093.x

Kojima D, Mori S, Torii M, Wada A, Morishita R, Fukada Y (2011) UV-sensitive photoreceptor protein OPN5 in humans and mice. PloS One 6(10):e26388. doi:10.1371/journal.pone.0026388

Koyanagi M, Kawano E, Kinugawa Y, Oishi T, Shichida Y, Tamotsu S, Terakita A (2004) Bistable UV pigment in the lamprey pineal. Proc Natl Acad Sci USA 101(17):6687–6691. doi:10.1073/pnas.0400819101

Koyanagi M, Kubokawa K, Tsukamoto H, Shichida Y, Terakita A (2005) Cephalochordate melanopsin: evolutionary linkage between invertebrate visual cells and vertebrate photosensitive retinal ganglion cells. Curr Biol 15(11):1065–1069. doi:10.1016/j.cub.2005.04.063

Kuwayama S, Imai H, Hirano T, Terakita A, Shichida Y (2002) Conserved proline residue at position 189 in cone visual pigments as a determinant of molecular properties different from rhodopsins. Biochemistry 41(51):15245–15252. doi:10.1021/Bi026444k

Mano H, Kojima D, Fukada Y (1999) Exo-rhodopsin: a novel rhodopsin expressed in the zebrafish pineal gland. Brain Res Mol Brain Res 73(1-2):110–118

Matsushita A, Yoshikawa T, Okano T, Kasahara T, Fukada Y (2000) Colocalization of pinopsin with two types of G-protein alpha-subunits in the chicken pineal gland. Cell Tissue Res 299 (2):245–251

Max M, Menaker M (1992) Regulation of melatonin production by light, darkness, and temperature in the trout pineal. J Comp Physiol A 170(4):479–489

Max M, McKinnon PJ, Seidenman KJ, Barrett RK, Applebury ML, Takahashi JS, Margolskee RF (1995) Pineal opsin: a nonvisual opsin expressed in chick pineal. Science 267(5203):1502–1506

Max M, Surya A, Takahashi JS, Margolskee RF, Knox BE (1998) Light-dependent activation of rod transducin by pineal opsin. J Biol Chem 273(41):26820–26826. doi:10.1074/jbc.273.41.26820

Meissl H, Ekstrom P (1988) Photoreceptor responses to light in the isolated pineal organ of the trout, *Salmo gairdneri*. Neuroscience 25(3):1071–1076

Miller WH, Wolbarsht ML (1962) Neural activity in the parietal eye of a lizard. Science 135 (3500):316–317

Morita Y, Dodt E (1973) Slow photic responses of the isolated pineal organ of lamprey. Nova Acta Leopold 38:331–339

Nakamura A, Kojima D, Imai H, Terakita A, Okano T, Shichida Y, Fukada Y (1999) Chimeric nature of pinopsin between rod and cone visual pigments. Biochemistry 38(45):14738–14745. doi:10.1021/bi9913496

Okano T, Fukada Y (2003) Chicktacking pineal clock. J Biochem (Tokyo) 134(6):791–797. doi:10.1093/jb/mvg221

Okano T, Yoshizawa T, Fukada Y (1994) Pinopsin is a chicken pineal photoreceptive molecule. Nature (Lond) 372(6501):94–97. doi:10.1038/372094a0

Okano T, Yamazaki K, Kasahara T, Fukada Y (1997) Molecular cloning of heterotrimeric G-protein alpha-subunits in chicken pineal gland. J Mol Evol 44(suppl 1):S91–S97

Oksche A (1965) Survey of the development and comparative morphology of the pineal organ. Prog Brain Res 10:3–29

Philp AR, Bellingham J, Garcia-Fernandez J, Foster RG (2000a) A novel rod-like opsin isolated from the extra-retinal photoreceptors of teleost fish. FEBS Lett 468(2-3):181–188

Philp AR, Garcia-Fernandez JM, Soni BG, Lucas RJ, Bellingham J, Foster RG (2000b) Vertebrate ancient (VA) opsin and extraretinal photoreception in the Atlantic salmon (*Salmo salar*). J Exp Biol 203(pt 12):1925–1936

Refinetti R, Menaker M (1992) The circadian rhythm of body temperature. Physiol Behav 51 (3):613–637

Rennison DJ, Owens GL, Taylor JS (2012) Opsin gene duplication and divergence in ray-finned fish. Mol Phylogenet Evol 62(3):986–1008. doi:10.1016/j.ympev.2011.11.030

Robinson J, Schmitt EA, Dowling JE (1995) Temporal and spatial patterns of opsin gene expression in zebrafish (*Danio rerio*). Vis Neurosci 12(5):895–906

Shichida Y, Imai H (1998) Visual pigment: G-protein-coupled receptor for light signals. Cell Mol Life Sci 54(12):1299–1315

Simonneaux V, Ribelayga C (2003) Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. Pharmacol Rev 55(2):325–395. doi:10.1124/pr.55.2.2

Solessio E, Engbretson GA (1993) Antagonistic chromatic mechanisms in photoreceptors of the parietal eye of lizards. Nature (Lond) 364(6436):442–445. doi:10.1038/364442a0

Su CY, Luo DG, Terakita A, Shichida Y, Liao HW, Kazmi MA, Sakmar TP, Yau KW (2006) Parietal-eye phototransduction components and their potential evolutionary implications. Science 311(5767):1617–1621. doi:10.1126/science.1123802

Tamotsu S, Korf HW, Morita Y, Oksche A (1990) Immunocytochemical localization of serotonin and photoreceptor-specific proteins (rod-opsin, S-antigen) in the pineal complex of the river lamprey, *Lampetra japonica*, with special reference to photoneuroendocrine cells. Cell Tissue Res 262(2):205–216

Tamotsu S, Oishi T, Nakao K, Fukada Y, Shichida Y, Yoshizawa T, Morita Y (1994) Localization of iodopsin and rod-opsin immunoreactivity in the retina and pineal complex of the river lamprey, *Lampetra japonica*. Cell Tissue Res 278(1):1–10. doi:10.1007/s004410050188

Taniguchi Y, Hisatomi O, Yoshida M, Tokunaga F (2001) Pinopsin expressed in the retinal photoreceptors of a diurnal gecko. FEBS Lett 496(2-3):69–74

Tarttelin EE, Fransen MP, Edwards PC, Hankins MW, Schertler GF, Vogel R, Lucas RJ, Bellingham J (2011) Adaptation of pineal expressed teleost exo-rod opsin to non-image forming photoreception through enhanced meta II decay. Cell Mol Life Sci 68(22):3713–3723. doi:10.1007/s00018-011-0665-y

Taylor JS, Braasch I, Frickey T, Meyer A, Van de Peer Y (2003) Genome duplication, a trait shared by 22000 species of ray-finned fish. Genome Res 13(3):382–390. doi:10.1101/gr.640303

Terakita A, Tsukamoto H, Koyanagi M, Sugahara M, Yamashita T, Shichida Y (2008) Expression and comparative characterization of Gq-coupled invertebrate visual pigments and melanopsin. J Neurochem 105(3):883–890. doi:10.1111/j.1471-4159.2007.05184.x

Torii M, Kojima D, Okano T, Nakamura A, Terakita A, Shichida Y, Wada A, Fukada Y (2007) Two isoforms of chicken melanopsins show blue light sensitivity. FEBS Lett 581(27):5327–5331. doi:10.1016/j.febslet.2007.10.019

Uchida K, Morita Y (1994) Spectral sensitivity and mechanism of interaction between inhibitory and excitatory responses of photosensory pineal neurons. Pflugers Arch 427(3-4):373–377. doi:10.1007/BF00374547

van Veen T, Ostholm T, Gierschik P, Spiegel A, Somers R, Korf HW, Klein DC (1986) alpha-Transducin immunoreactivity in retinae and sensory pineal organs of adult vertebrates. Proc Natl Acad Sci USA 83(4):912–916

Wada S, Kawano-Yamashita E, Koyanagi M, Terakita A (2012) Expression of UV-sensitive parapinopsin in the iguana parietal eyes and its implication in UV-sensitivity in vertebrate pineal-related organs. PloS One 7(6):e39003. doi:10.1371/journal.pone.0039003

Xiong WH, Solessio EC, Yau KW (1998) An unusual cGMP pathway underlying depolarizing light response of the vertebrate parietal-eye photoreceptor. Nat Neurosci 1(5):359–365. doi:10.1038/1570

Yamashita T, Ohuchi H, Tomonari S, Ikeda K, Sakai K, Shichida Y (2010) Opn5 is a UV-sensitive bistable pigment that couples with Gi subtype of G protein. Proc Natl Acad Sci USA 107(51):22084–22089. doi:10.1073/pnas.1012498107

Zatz M (1994) Photoendocrine transduction in cultured chick pineal cells: IV. What do vitamin A depletion and retinaldehyde addition do to the effects of light on the melatonin rhythm? J Neurochem 62(5):2001–2011

Zatz M, Mullen DA (1988) Two mechanisms of photoendocrine transduction in cultured chick pineal cells: pertussis toxin blocks the acute but not the phase-shifting effects of light on the melatonin rhythm. Brain Res 453(1-2):63–71

Ziv L, Tovin A, Strasser D, Gothilf Y (2007) Spectral sensitivity of melatonin suppression in the zebrafish pineal gland. Exp Eye Res 84(1):92–99. doi:10.1016/j.exer.2006.09.004

Index

T. Furukawa et al. (eds.), *Vertebrate Photoreceptors: Functional Molecular Bases*,
DOI 10.1007/978-4-431-54880-5, © Springer Japan 2014