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Vertebrate vision: about physical determinants of photoreceptor sensitivity and kinetics

Doctoral Dissertation

Hanna Heikkinen

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Abstract

Rod and cone photoreceptors transform information about incoming light into neural signals with broadly similar molecular mechanisms. Yet their sensitivity, response kinetics and adaptation properties are quite different as rods mediate dim-light vision and cones function mainly under daylight. This thesis 1. addresses the functional differences between rods and cones as well as mammalian and non-mammalian photoreceptors and 2. provides novel findings regarding the existence and regulation of rod-cone interactions at the photoreceptor level.

Rod and cone photoresponses to brief flashes of light were recorded with electroretinogram (ERG) from isolated rodent and amphibian retinas. Various phototransduction models were used to compare their relevant parameters over a range of adapting conditions. The study focused on how the following physical factors shape and limit photoreceptor function: operating temperature, thermal stability of the amphibian long wavelength sensitive (A1-) visual pigment, outer segment dimensions, morphology and electrical connections between adjacent rods and cones.

Mammalian rod photoreceptors generate faster photoresponses but light-adapt less efficiently than amphibian rods. In the rodent and anuran rods studied in this thesis, the main differences could be accounted for by the higher operating temperature and smaller outer segment size of the rodent photoreceptors. Additionally, the slender outer segments of the mammalian rods enabled sufficient quantal responses and high quantum catch despite the observed desensitizing effect of warming.

Long wavelength -sensitive cone photoreceptors have been hypothesized to be desensitized by thermal excitation of their visual pigment molecules. However, it has been shown experimentally only in amphibian cones that utilize the A2-chromophore. The relative stability of the A1-based cone pigments – used by all terrestrial vertebrates – has remained unclear, as well as its role in limiting cone function. In this study, thermal isomerization rate of the long wavelength sensitive (A1)-visual pigment was estimated to play at most a minor role in regulating cone sensitivity of the frog Rana temporaria.

Finally, ERG light responses originating in mouse cone photoreceptors were found to be suppressed in the dark-adapted retina, apparently through direct electrical coupling between rods and cones. The results indicated this coupling is weakened by moderate background light, explaining a long known phenomenon of unknown origin: light-induced growth of cone flash responses in mammalian ERG. This is indicative of a previously unknown mechanism of retinal adaptation.

Tiivistelmä

Verkkokalvon valoherkät solut, sauvat ja tapit, muuntavat tietoa silmään saapuvasta valosta sähköisiksi signaaleiksi samankaltaisilla biokemiallisilla mekanismeilla. Sauvojen ja tappien tuottamat sähköiset valovasteet ovat kuitenkin hyvin erilaisia herkkyydeltään ja aikakäyttäytymiseltään. Myös niiden kyky muokata signaalivahvistustaan keskimääräisen valaistustason muuttuessa on hyvin erilainen. Tämä väitöskirja 1. käsittelee sauva- ja tappisolujen sekä nisäkkään ja vaihtolämpöisen eläimen näköaistinsolujen välisiä toiminnallisia eroja ja 2. esittää uutta tietoa sauvojen ja tappien välisestä vuorovaikutuksesta ja sen säätelystä.

Sauva- ja tappisolujen tuottamia sähköisiä valovasteita rekisteröitiin elektroretinogrammi-tekniikalla (ERG) jyrsijöiden ja sammakkoeläinten eristetyistä verkkokalvoista. Valovasteiden herkkyys- ja kinetiikkaparametreja vertailtiin keskenään eri adaptaatiotiloissa. Analyysissä keskityttiin selvittämään seuraavien fysikaalisten tekijöiden vaikutusta näköaistinsolujen toimintaan: toimintalämpötila, pitkän aallonpituuden (A1-)näköpigmentin terminen stabiilius, sauvan ja tapin ulkojäsenen koko ja mittasuhteet, morfologia sekä sauva- ja tappisoluja yhdistävät sähköiset kytkennät.

Nisäkkään sauvasolut tuottavat nopeampia valovasteita kuin vaihtolämpöisten eläinten vastaavat solut, mutta eivät kykene mukautumaan yhtä tehokkaasti keskimääräisen valaistustason muutoksiin. Näiden toiminnallisten erojen havaittiin selittyvän puhtaasti korkeamman toimintalämpötilan ja solujen valoherkkien ulkojäsenten mittasuhteiden avulla. Lisäksi nisäkkään sauvasolun pitkät ja kapeat ulkojäsenet mahdollistavat riittävän suuren impulssivasteen sekä tehokkaan fotonien keruun huolimatta korkeamman lämpötilan valovasteiden kokonaisvahvistusta pienentävästä vaikutuksesta.

Tappisolujen suhteellisen matalan herkkyyden on esitetty johtuvan osaksi tappisolujen näköpigmenttimolekyylien termisen (spontaanin) aktivoitumisen tuottamasta taustakohinasta. Tällaisen ”pimeävalon” olemassaolo on kuitenkin osoitettu kokeellisesti ainoastaan joidenkin sammakkoeläinten A2-kromoforia käyttävissä punaherkissä tapeissa. Sen merkitystä maaeläinten A1-pohjaista pigmenttiä hyödyntävissä tappisoluissa ei yleisesti tunneta. Tutkittaessa A1-pohjaisen tappipigmentin termisten isomerisaatioiden vaikutusta sammakon punaherkkien tappien toimintaan niillä todettiin olevan korkeintaan hyvin pieni vaikutus näiden solujen herkkyyden säätelyssä.

Sauvojen ja tappien välisestä sähköisen kytkennän olemassaolosta ja merkityksestä hiiren verkkokalvossa löytyi fysiologisia todisteita, ja sen näytettiin rajoittavan tappisolujen signalointia pimeässä. Tulokset viittaavat sauvojen ja tappien välisen sähköisiä synapseja hyödyntävän kytkennän säätelyyn taustavalossa. Samalla ne selittävät kauan tunnetun ilmiön, jonka aiheuttajaa ei tiedetä: tappilähtöisten ERG-komponenttien voimakkaan kasvun taustavalossa. Kyseessä on verkkokalvon hermoverkossa aiemmin tuntematon adaptaatiomekanismi.

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Preface

The research for this thesis was carried out at the Department of Biomedical Engineering and Computational Science at Aalto University School of Science and Technology (former Helsinki University of Technology) and at the Faculty of Biological and Environmental Sciences at University of Helsinki. It was financially supported by the Finnish Academy of Science, Finnish Cultural Foundation and International Graduate School in Biomedical Engineering and Medical Physics.

I was fortunate to enter a research group with great tradition and experience on the field, based on the work of generations of wonderful researchers. I need to thank Professor Ari Koskelainen for providing the conditions for the experimental work, endless ideas and always taking the time to discuss recent results and next experiments. Professor Kristian Donner has been a wonderful mentor in scientific writing and thinking, and thanks to him, writing this thesis went as smoothly as it did. I wish to thank both my advisors for their support and encouragement as well as numerous discussions concerning research and life.

I wish to thank both Professor Risto Ilmoniemi and emeritus Professor Toivo Katila for the research environment and the excellent facilities at the department. Special thanks go also to the rest of the staff at BECS for making this a pleasant place to work at.

During the course of this work, I have had wonderful colleagues. Large body of the work was planned, performed and completed together with Dr Soile Nymark, who has also become a dear friend during these years. We have shared the highs and lows of numerous projects together, with endless discussions mixing fluently professional and private matters. Thank you for all this, Soile! Also most special thanks go to Frans Vinberg. His calm and practical attitude, accompanied with remarkable expertise in the lab, really enabled me to finish this thesis in the middle of the twists and turns of the last subproject – even with a reasonable amount of sanity left. Teemu Turunen and Marja Pitkänen deserve warmest thanks for both their cheerful presence at the research group and their expert help with numerous experiments and tasks during this last year. The many colleagues and students who have worked in the cellular biophysics group during these years each have left some happy memories, and deserve warmest thanks for the many good days. Especially, Dr Petri Ala-Laurila and Rauli Albert never failed to brighten the day with their good humor, while the matters concerning research were taken ever so seriously and much was learned. Also Petri has been a great support to me lately through our discussions regarding science and other matters. I want to thank Liisa-Ida Sorsa, Hanna Mäki and Elina Sahala for their skillful contribution to the various experiments, and Jenny Vesterlund for her endeavors on the preliminary experiments on mouse cone light adaptation.

Collaboration, meetings and conferences have brought plenty of good memories and introduced me to a large number of great people in the vision research community. I want to thank Dr Simo Hemilä and emeritus Professor Tom Reuter for their inspirational expertise and ongoing enthusiasm on visual science, and many fruitful discussions and critical comments on some of the present work. Thanks to Pia Saarinen, Dr. Mirka Jokela-Määttä

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I am grateful to the pre-examiners Dr Sergei Nikonov and Professor Juha Voipio for taking the time to help me improve this manuscript. I greatly appreciate your expert comments on it. I also wish to thank Professor Voipio for providing me with fun and challenging teaching opportunities (and allowing some of that teaching to bounce back in a very practical sense).

Throughout these years, friends have been there, ready to listen and support through success and challenges. I wish to thank you all full-heartedly. Specifically, Dr Camilla Lindholm has been an exceptional support to me through her friendship and our shared enthusiasm for both science and fast little sighthounds.

I have been blessed to have a family that has always supported my endeavors and aspirations. My parents, Martti Suominen and Marja Kuosmanen, and my siblings, Pasi and Sanni have never expressed any doubts over my capabilities to achieve whatever I wish to nor the good sense of going for it. Throughout the years, Reino Kuosmanen and Eila Vainio have added to the parental troops ready and willing to listen and discuss my joys and troubles, always encouraging and supporting. My parents-in-law, Irma and Raimo Heikkinen, are people made of gold. Thank you for being there, for your support and help! During the progress of this work, also something much more important came along into my life. Joona and Aino, you make my day and to speak the truth of it, you made finishing this thesis possible. And throughout it all, good days and bad, my husband Tommi has been there. I thank you for your love and support, and foremost for your friendship.

Espoo, May 19th, 2010

Hanna Heikkinen

List of publications

The present thesis is based on the following four publications referred to by their Roman numerals in the text:

I Nymark, S., Heikkinen, H., Haldin, C., Donner, K., and Koskelainen, A. (2005): Light responses and light adaptation in rat retinal rods at different temperatures, *Journal of Physiology,* 567:923-38.

II Heikkinen, H., Nymark, S., Donner, K., and Koskelainen, A. (2009): Temperature dependence of dark-adapted sensitivity and light-adaptation in photoreceptors with A1 visual pigments: a comparison of frog L-cones and rods, *Vision Research,* 49:1717-1728.

III Heikkinen, H., Nymark, S., and Koskelainen, A. (2008): Mouse cone photoresponses obtained with electroretinogram from the isolated retina. *Vision Research,* 48:264-272.

IV Heikkinen, H., Vinberg, F., Nymark, S., and Koskelainen, A. (2010): Rods suppress cone flash responses of mouse electroretinogram in darkness through a mechanism that is removed by mesopic light or by blocking gap junctions. Department of Biomedical Engineering and Computational Science Publications, Report A17, 18 pages.

Author's contribution

The author has had a significant role in all stages of the research reported in these papers, as described in detail below. She is the first as well as the corresponding author of papers II-IV.

Paper I is an experimental comparative study on phototransduction and adaptation in mammalian and amphibian rods. The author has contributed to planning and executing the experiments, analysis of the data and writing the manuscript. She did the feasibility analysis on phototransduction models, and implemented the semi-physiological model used to analyze photoresponse data. She analyzed the model results and wrote the corresponding sections of the manuscript concerning the methodology, implementation and analysis of the model results.

Paper II presents an analysis of experimental data on frog and cone light-adaptation in the framework of the underlying sensitivity setting factors. The author developed and calibrated the automated dual-beam stimulus system (used also in Papers I), planned and performed the experiments and analyzed the data. She further developed the analysis methods for probing the activation state of photoreceptors with background light, as well as the theoretical analysis of thermal activation in frog cones. She wrote the first draft of the manuscript and edited the final version together with the co-authors.

Paper III is a basic characterization of mouse cone flash responses. The author adjusted and calibrated the stimulus system to suit the experimental program, performed the experiments, analyzed the results and wrote the manuscript.

Paper IV is a study of rod-cone interactions shaping the cone electroretinogram. The author made the original observation of cone signal suppression in darkness and planned the experiments, performed major part of the experiments and data analysis, interpreted the results and wrote the manuscript.

List of abbreviations and symbols

[X]	Free concentration of substance X
A1	11-*cis* retinal
A2	11-*cis* dehydroretinal
A	Activation coefficient
a_c	*C*ollection area
$[Ca^{2+}]_I$	Intracellular calcium concentration
CB	Cone bipolar cell
cGMP	cyclic GMP, the internal messenger of phototransduction
CNG channels	cGMP-controlled (cyclic nucleotide –gated) channels
D-*AP5*	D-amino-5-phosphonovalerate
DL-*AP4*	DL-2-amino-4-phosphonobutyric acid
ERG	Electroretinogram
E_i	Nernst potential of ion i
GCAP	Guanylate cyclace activating protein
GC	Guanylate cyclace
GPCR	G-protein-coupled receptor
I_F	Flash intensity
$I_{F,½}$	Half-saturating flash intensity ($1/S_f$ in Michaelis relation)
I_0	"Dark light", as determined from SVI or t_pVI data
I_{Bg}	Background light intensity
I_{th}	"Dark light" from thermal visual pigment activation
IS	Inner segment
L-	Long wavelength sensitive
MFA	Meclofenamic acid
M-	Middle wavelength sensitive
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
OS	Outer segment
*P**	Active (cone) visual pigment
PDE	Phosphodiesterase
*Rh**	Active (rod) visual pigment
RB	Rod bipolar cell
S-	Short wavelength sensitive
S	Flash sensitivity
S_f	(Fractional) flash sensitivity
SEM	Standard error of mean
SNR	Signal-to-noise ratio
SPR	Single photon response
SPR_f	Fractional single photon response
SVI	(Fractional) sensitivity vs. background intensity
t_i	Integration time
t_p	Time to peak of the linear response
t_p*VI*	Time to peak vs. background intensity
V_m	Membrane potential

1. Introduction

The information we humans receive about the outside world is largely based on our eyes. Vision's special importance among our senses is reflected in countless metaphors of every day language. "I see", conveys the message that one understands, while "shades of gray", or "black and white" may well refer to a person's viewpoints towards life instead of the prevalent contrasts and luminance levels in the visual scene. Colors are not just informative features of the surrounding landscape; they also bear strong emotional and communicational significance to most people.

Consequently, vision-related diseases have enormous significance in health related research. Retinitis pigmentosa, one of the leading causes of visual handicap and blindness, affects a million people yearly (Hartong *et al.,* 2006). Endeavors to cure or delay retinal degeneration span multiple approaches, from artificial retinal transplants to conventional medication and genetic manipulations. Thorough knowledge of retinal function in its healthy state is essential for any of these treatments. Also tools are needed for detailed monitoring of retinal function at varying stages of disease and treatment.

This dissertation concentrates on the very first stage of vision: how the photoreceptor cells transform information about light entering the eye into electrical signals, and how this transformation is regulated. All vertebrate photoreceptors share qualitatively common steps in this process, phototransduction. Yet they differ greatly in appearance and performance. Much of the information available on photoreceptor function and retinal processing has been acquired from "cold-blooded" vertebrates such as fish, frogs and salamanders. Yet it is not completely understood how this information relates to humans and other mammals. Functionally, mammalian photoreceptors differ from their amphibian counterparts at least in their sensitivity, kinetics and adaptational properties. It is possible that these differences stem from modified protein function or expression levels. Some adaptation mechanisms may exist only for mammals (*e.g.* Krispel *et al.,* 2003). However, there are also simple physical factors, such as size and morphology, that may account for much of the observed differences between photoreceptors. Another major factor is temperature: the mammalian body temperature implies biochemical reaction rates that are drastically different from those coped with by the amphibian visual system. The role of these physical factors can be estimated through a functional comparison of selected photoreceptors from cold-blooded and mammalian animals, representative of differences in both morphology and function. This reveals the invariant features of vertebrate phototransduction, and may point out some true differences in the underlying molecular machinery.

The amount of light reaching the eye varies over 10 log-units from starlit night to sunny winter day, an enormous functional range for any receptor device. Vertebrate vision has divided the task of light-detection on two broad classes of photoreceptors: the rods and cones. Rod-based vision is highly sensitive: rods can transform information about individual photons into neuronal signal, mediating vision at very dim light levels. Increase in the overall light level causes a gradual switch to cone-based vision, which can adapt effectively and swiftly to wide range of light levels. The cone system provides also preconditions for color

vision for most vertebrates, when multiple cone types with different wavelength sensitivities are present. The two classes of photoreceptors, rods and cones, use qualitatively similar machinery for their phototransduction process despite their highly different functional properties. Deciphering the differences of rod and cone phototransduction on the molecular level has been one of the longstanding questions in photoreceptor research.

Like all sensory receptor systems, the photoreceptors deal with optimizing their signal to noise ratio. Under very dim illumination, the randomness in the flow of photons onto the retina creates an unavoidable variance in the visual signal. In addition, thermal energy of the molecules initiates spontaneous activity at various stages of the biochemical cascade responsible for phototransduction (Barlow, 1956; Baylor *et al.,* 1980). The need to limit thermal noise may have been a significant factor in the photoreceptor adaptation to mammalian body temperature. Specifically, this applies to a fundamental limiting factor in vision: the stability of the visual pigment. Instead of activation by a photon, a visual pigment molecule can sometimes also be activated by thermal energy. As the resulting cellular response can not be distinguished from the response initiated by a photon, the intrinsic noise from these spontaneous activation events may limit visual performance either at the photoreceptor (Rieke and Baylor 2000) or neural network level (Copenhagen *et al.,* 1987).

This thesis concentrates on the role of physical factors behind functional differences among vertebrate photoreceptors. Photoresponse data from representative mammalian and amphibian rods and cones was obtained under varying adapting conditions and analyzed for common and distinct determinants of sensitivity and kinetics. Specifically, the dark- and light-adapted cone function of the mouse retina is investigated, as it has remained elusive to thorough characterization despite its importance as a model for mammalian visual function. Finally, the interaction between mammalian rod- and cone photoreceptor signaling is addressed and a novel adaptational mechanism within this interaction is suggested.

2. Vertebrate photoreceptors

The vertebrate retina, located at the back of the eye, is organized into distinct neuronal layers. The photoreceptors' light-detecting structures, outer segments, form a dense mosaic in the outermost layer, nourished by processes from the pigment epithelium (Steinberg, 1985). After light has entered the eye and traversed most of the retina, much of it gets absorbed by visual pigment molecules in this layer. About 2/3 of the photon absorptions trigger a biochemical amplification cascade that subsequently leads to hyperpolarization of the photoreceptor (Rodieck, 1988). The process of translating information about an incoming photon into an electrical neural signal is called phototransduction. To an extent, the photoreceptors can be viewed as a mosaic of detectors with different sensitivity properties. However, the picture transferred to the brain is far from a set of pixels: the neural network of the retina combines information from multiple photoreceptors at a time to enhance temporal and spatial information on luminance and chromatic contrast borders. The photoreceptors, rods and cones, provide the basic information for this task, and thus many fundamental limits for visual performance originate in these cells.

A dark-adapted rod photoreceptor transforms information about individual absorbed photons into a discrete electrical signal with quite an impressive precision (Baylor *et al.*, 1979b; Field and Rieke, 2002a). Functioning under brighter conditions sets different requirements on phototransduction: without adaptive mechanisms the high amplification necessary for single photon detection would lead to receptor saturation already at dim twilight conditions. This dilemma is avoided partly by the existence of two complementary receptor systems: the rods are optimized for dim-light vision while cones mainly function under daylight. In addition, both receptor types adjust their molecular amplification and response kinetics according to the mean illumination (Ch. 3.3). This process - light-adaptation - enables rod-based (scotopic) vision to span several decades of intensity above the dark-adapted state, at which it is mediated by responses of individual rods to single photons. As the rod photoresponses are progressively suppressed and finally saturated with increasing mean light intensities, the visual system is gradually transferred into the cone-based (photopic) state.

Besides its importance in vision research, the biochemical amplification cascade of the rod light response is a well characterized model for cellular signaling more generally: the visual pigment represents the large and important superfamily A of G-protein coupled receptor molecules (GPCRs, Hargrave and McDowell, 1992). The GPCRs are encountered in a wide variety of signaling pathways in eukaryotic cells, such as other sensory systems or regulation of behavior, mood and the immune system. They are also the molecular target of *ca*. one third of modern medicinal drugs (Filmore, 2004). The photoreceptors provide a conveniently approachable preparation in which the signaling of this particular class of GPCRs can be studied using its natural stimulus, light, which can be administered with exceptional precision. The entire biochemical sequence of events leading from activation of a single molecule to closure of thousands of ligand gated ion channels in the plasma membrane has been modeled to a degree of quantitative detail (Lamb and Pugh, 1992; Hamer *et al.*, 2005) not attained in any corresponding system.

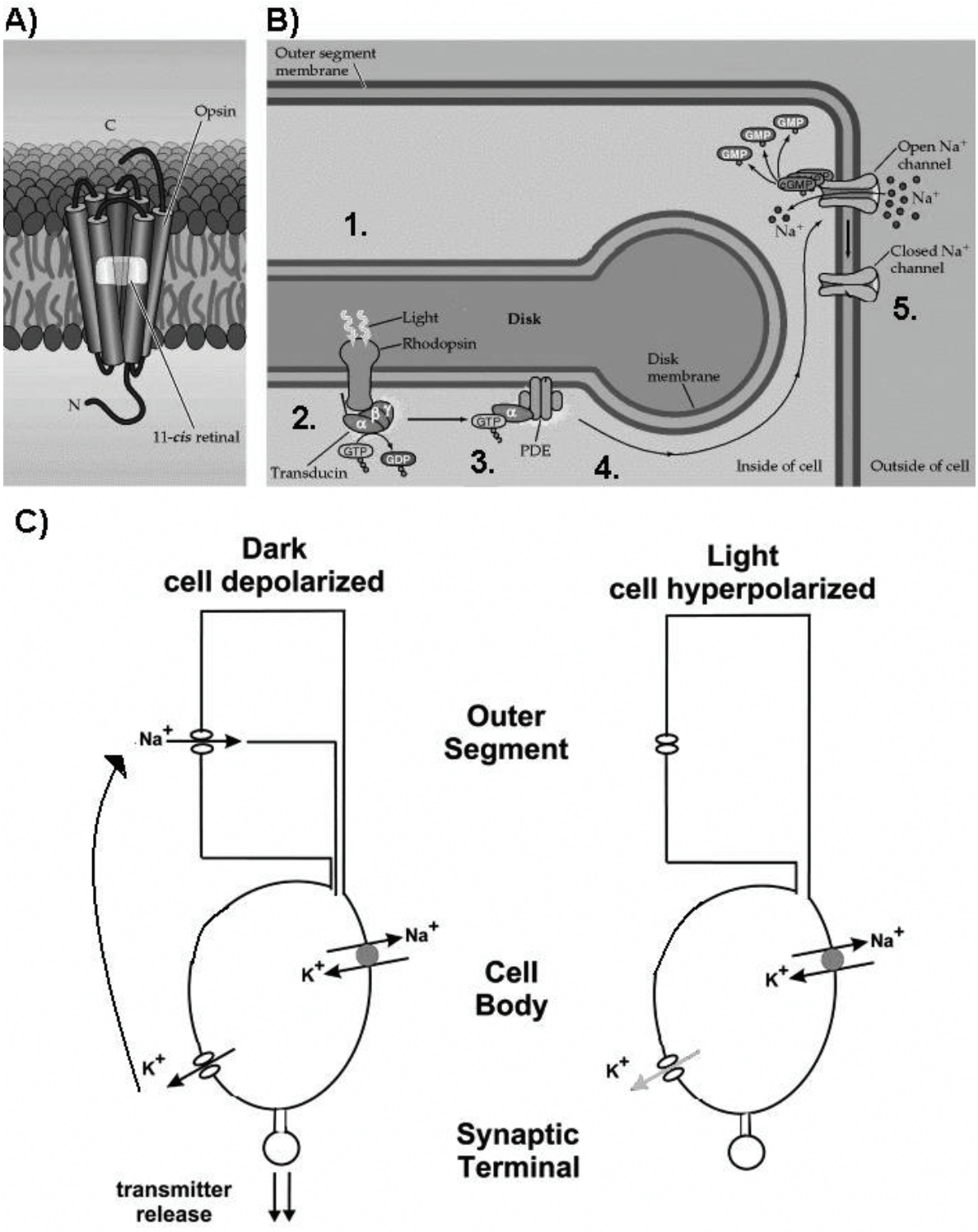

Figure 1. A) The membranous discs within the rod outer segment are packed with visual pigment molecules, rhodopsins. B) Absorption of a photon by a visual pigment molecule (1.) leads to activation of dozens of transducin (2.), binding to phosphodiesterase (3.), leading to hydrolysis of internal messenger cGMP (4.) and closure of cation channels in the outer segment (5.). C) In darkness, the flow of Na^+ into the outer segment maintains the cell in a depolarized state. Closing of the cation channels in the outer segment leads to hyperpolarization and subsequent decrease in the transmitter release. A) modified from Purves et al. (2008), with permission.

2.1. Photoreceptor function

Photoreceptors from different subclasses or animal species vary in size, morphology and function. The quantitative details of their biochemical function vary accordingly. Nevertheless, the overall process can be described in a rather prototypical manner (Pugh and Lamb, 2000). The most detailed information about vertebrate phototransduction has been collected from the rods of the larval tiger salamander, *Ambystoma tigrinum*, and existing photoresponse models are based largely on this species although they are supplemented by experiments on other preparations. With the rise of genetic modification as a research tool, the mouse has emerged as the main mammalian model for phototransduction studies.

2.1.1. Light absorption in the photoreceptor outer segment

Efficient capture of incoming photons is a prerequisite for high visual sensitivity. It occurs in the photoreceptor outer segments (OS). These elongated structures are packed as a tight mosaic to form a light-absorbing layer in the distal part of the retina. In some animals this layer is almost isomorphic, but usually several photoreceptor types with different morphologies and visual pigments vary spatially on the retina. The spatial variation of photoreceptor types and densities is especially pronounced in and around the fovea of the primate retina, a center of high visual acuity achieved by a locally increased density of cones.

Visual pigments (rhodopsins, *Fig.* 1A), like all G-protein coupled receptor molecules are membrane bound, ligand-gated proteins with 7 α-helices traversing the membrane (therefore also known as 7-TM receptors). Visual pigments differ from the bulk of GPCRs by the nature of their ligand. Instead of activation by ligand binding, the apoprotein (opsin) binds a cofactor, chromophore, already in its inactive state. The chromophore regulates the protein's catalytic activity, rendering it inactive while in 11-*cis* –form. Upon absorbing a photon the chromophore is isomerized to the all-*trans* form, which activates the rhodopsin. The chromophore, which in vertebrate rhodopsins is either an 11-*cis* retinal (A1, always in mammals) or an 11-*cis* dehydroretinal (A2) also partly determines the spectral absorption properties of the protein (see Ch. 3.4.2).

Located on folded membrane structures in the outer segment, visual pigment molecules remain free to diffuse laterally in the disk membrane. In most photoreceptors light traverses the outer segment axially; the transmembrane helices of the opsins lock the chromophore in the plane of the membrane, which is favorable for absorption of axially incident light regardless of polarization.

The tight packing of photoreceptors and pigment molecules allows incoming light to be absorbed efficiently. The 20-40 μm thick outer segment layer of the retina absorbs more than 50% of axially incoming photons (*e.g.* frog: Gyllenberg *et al.,* 1974; rat: paper I) at wavelengths near the absorbance maximum of the rod visual pigment. The visual pigment density in the disc membranes is so high that crowding of molecules actually limits the maximal activation speed of phototransduction (Calvert *et al.*, 2001). The funneling properties of some photoreceptors' inner segments, as well as the reflective tapetum behind the retina of many nocturnal animals increase the photon catch even further.

2.1.2. Phototransduction

The information about an absorbed photon is transformed into an electrical photoresponse through several successive stages of molecular amplification (*Fig. 1B,* reviewed e.g. by Pugh and Lamb, 2000). An active rhodopsin (Rh*) activates repeatedly G-proteins before being quenched by multiple phosphorylations and subsequent arrestin binding (Kuhn, 1984; Wilden *et al.* 1986). Each active transducin in turn may bind and activate one subunit of a phosphodiesterase molecule (PDE), leading to enhanced hydrolysis of the internal messenger cGMP in the cytoplasm. This constitutes a second stage of molecular amplification. In darkness, a steady mainly Na^+-mediated current flows into the outer segment via cGMP-gated cation (CNG-) channels in the outer segment plasma membrane. As the third stage of amplification, the fall in the cGMP concentration closes some of the CNG channels, which diminishes the sodium current and allows the cell to hyperpolarize towards E_K (Fesenko *et al.*, 1985).

Shutdown of the light response requires deactivation and restoration of each component of the activation chain. In addition to deactivation of Rh*, this includes break-down of the transducin-PDE –complex and restoration of the cGMP level by synthesis. Several calcium-coupled feedback mechanisms modulate the efficiency of these processes and are reviewed along with the light adaptation in Ch. 3.3.

2.1.3. Electrical photoresponses

Vertebrate photoreceptors differ from most neurons by hyperpolarizing when activated (see *e.g.* Pugh and Lamb, 2000 for a review). The resting potential of a prototypical neuron is mostly defined by the Nernst potential for K^+, and activation involves an increase in Na^+ conductance and thus depolarization towards the sodium equilibrium. In contrast, a dark-adapted rod or cone maintains a constant cation current carried by Na^+ and to a lesser extent by Ca^{2+} flowing into the outer segment through the CNG channels. Together with the (mainly) K^+ -driven outward currents of the inner segment, the inward cation current of the outer segment maintains the cell in a relatively depolarized state with membrane potential of *ca*. -35 mV. When CNG-channels close in response to light, the cell is hyperpolarized towards potentials more typical of other neurons. The hyperpolarization reduces release of the excitatory neurotransmitter glutamate in the synaptic cleft. Thus in a sense, light acts as an inhibitory stimulus to the photoreceptors.

The photoreceptor's membrane voltage is thus controlled by two sets of conductive mechanisms: the light-sensitive CNG-channels of the outer segment and numerous voltage sensitive conductances in the inner segment (*e.g.* Bader *et al.*, 1982; Cia *et al*., 2005). Due to the laminar organization of the retinal tissue, the gradient of the photoreceptor's membrane currents in darkness effectively produces a current sink in the outer segment layer and a source close to the inner segments (Penn and Hagins, 1969). The changes in these sinks and sources will change the radial extracellular currents in the retina and thus form the basis of the photoreceptor signals recorded with the electroretinogram technique (see Ch. 2.2.2).

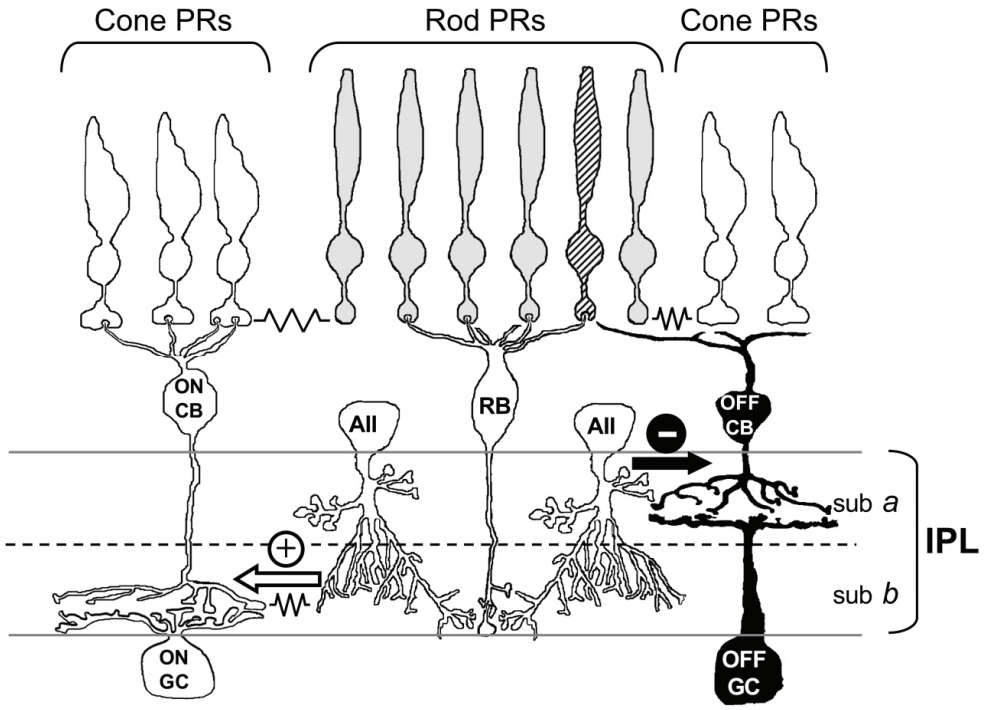

*Figure 2. Forward connections from the photoreceptors (PR) in the mammalian retina. Cones contact depolarizing (ON CB) and hyperpolarizing (OFF CB) bipolar cells via glutamatergic synapses. The rods contact depolarizing rod bipolar cells (RB), which feed into the cone pathways through A*II*-amacrine cells forming sign-conserving (electrical) synapses on cone ON-bipolars and sign-reversing (glycinergic) synapses on cone OFF-bipolars. Connection to the cone pathway is also mediated by gap junctional coupling to the cone pedicles. The horizontal cells are not depicted in the figure. Reprinted from Protti et al (2005), with permission.*

2.1.4. Transmission of the photoreceptor signal to other neurons

In darkness, the photoreceptors release continuously glutamate vesicles from their synaptic terminals. Hyperpolarization reduces the release. The bipolar cells, which transmit the signals to the proximal retina, may respond in two opposite ways to the light-induced decrease in the release of excitatory transmitter. One class of bipolar cells responds by hyperpolarization. These bipolars originate the OFF system, which signals dimming of the light by an excitatory response. The other main class of bipolar cells, ON bipolars, responds to the light onset by depolarization through a transduction cascade initiated by activation of their metabotropic glutamate receptors. This segregation of bipolar cells into two classes basically enables the various combinations of ON and OFF-responses in the ganglion cell receptive fields and subsequent visual processing.

In cold-blooded vertebrates, rods and cones generally connect to the same ON and OFF bipolar cells, although with varying efficiency (Werblin and Dowling, 1969; Lasansky, 1973). In mammals (*Fig. 2*, reviewed *e.g*. by Bloomfield and Dacheux, 2001), there is only one rod bipolar cell type (an ON bipolar) but many cone-specific types of bipolar cells (Kolb and Famigilietti, 1974). The rods connect to the cone pathways both via chemical synapses and gap junctions at various stages of the pathway, as shown in *Fig. 2* (Nelson, 1977; deVries and Baylor, 1995; Schneeweis and Schnapf, 1999; Tsukamoto *et al.*, 2001).

In addition to the forward connections to the bipolar cells, both cones and rods synapse on horizontal cells spanning laterally on the retina. These mediate both feedforward and feedback lateral inhibitory connections (Baylor *et al.*, 1971; recently reviewed by Fahrenfort *et al.*, 2009).

2.2. Electrophysiological methods for studying phototransduction

Electrophysiological studies oriented towards *visual function* focus on the photoreceptors' role in transmitting information about incoming light to higher-order neurons. This comprises the route from photoisomerization via changes in light-sensitive current to the hyperpolarization of the photoreceptor cell and subsequent modulation of transmitter release. In this context the photoreceptor's membrane voltage response is often the relevant measure of photoreceptor function. It is shaped by both the sensitivity and kinetics of the phototransduction cascade and the voltage-dependent processes of the inner segment.

Thus, when electrophysiology is used to elucidate *phototransduction* as an intracellular signaling process, the most relevant signal is the light-sensitive current of the outer segment. When combined with information obtained by biochemical methods, current recordings can provide a quantitative description of events at the molecular level in the phototransduction cascade (Lamb and Pugh, 1992; Nikonov *et al*., 2000; Hamer *et al*., 2005, see also Ch.4).

Specific research methods and recording techniques yield information on different aspects of photoreceptor function, each with their own advantages and drawbacks. In brief, the electrophysiological methods can be divided into: 1. direct measurements of membrane potential or currents crossing the cell membrane (intracellular recordings; patch clamp), 2. indirect measurement of the light-sensitive outer-segment conductance by recording the extracellular current between inner and outer segments (suction pipette), or 3. electroretinography, which records changes in the field potential arising as the ohmic voltage associated with radial currents between sources and sinks in the retina. Electroretinogram (ERG) recorded across the isolated, intact retina is the main method used in this thesis. Its advantages compared with more invasive techniques are listed in Table 1. They are particularly valuable for studies of adaptation and response cut-off kinetics, properties that are easily compromised under "unphysiological" conditions.

2.2.1. Recording from single cells

Intracellular recording

The early electrophysiological data from photoreceptors and retinal neurons was mainly obtained by intracellular recording, *i.e.* with a very fine tipped glass micropipette driven into the inner segment of the cell (*Fig*. 3.1.). This method was used to obtain the first recordings of photoreceptor membrane potential by Bortoff (1964). Bortoff and Norton (1965) combined intracellular recordings with electroretinogram (see Ch. 2.2.2.), obtaining direct evidence of the relation of the fast PIII component of the ERG and photoreceptor light

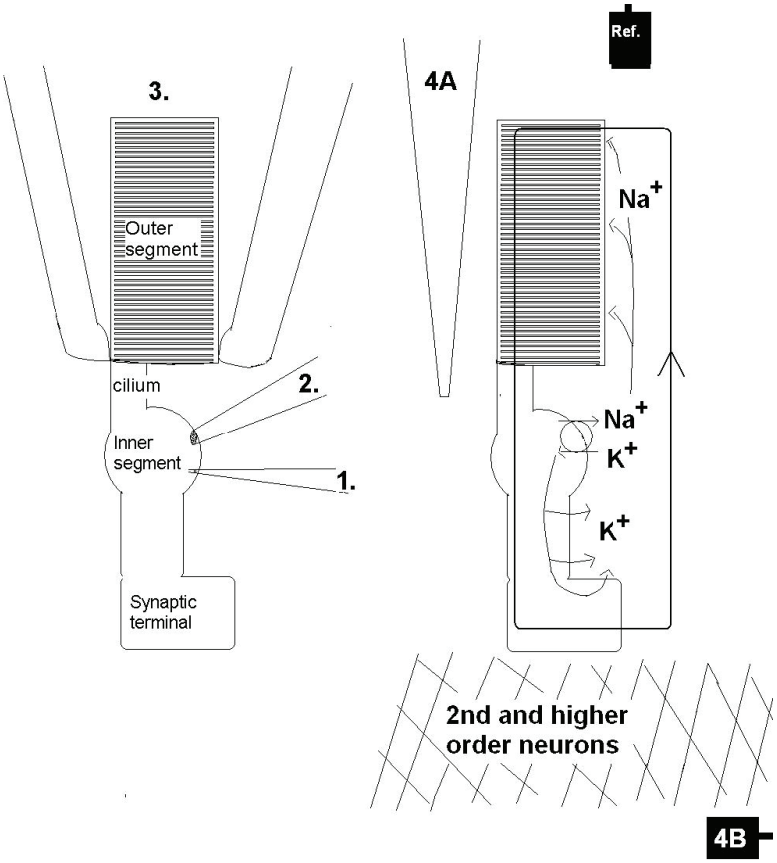

Figure 3. Four electro-physiological methods for studying photo-transduction: 1. Intra-cellular recording (membrane current or voltage), 2. Patch clamp (membrane current or voltage; patch or whole cell geometry), 3. Suction pipette, 4. Electroretino-gram, recorded either with a microelectrode inserted to a certain depth in the retina (4A) or trans-retinally (4B). The right-hand panel shows the main current sinks and sources of the rod, relevant to the suction pipette as well as the ERG recordings.

response. The use of intracellular recording is limited by the time an individual cell can be held, as well as the small size of many cells. Thus only selected cell types can be recorded from intracellularly.

Patch clamp

In patch clamp methods (*Fig*. 3.2.) a microelectrode with *ca*. 1 μm open tip diameter is brought in contact with the cell membrane until a high resistance seal is formed around a small patch of membrane. Current or voltage can then be recorded in several configurations either through intact membrane (current only) or after rupturing it. The main variations of patch clamp used for photoreceptor research are the whole cell configuration and perforated patch. In the whole cell mode the membrane inside the pipette opening is mechanically ruptured and recording is made over the rest of the membrane. This geometry also allows control of the composition of the cytoplasm. In the perforated patch technique, the membrane is chemically perforated with pore forming antibiotics, providing electrical access to the intracellular medium without allowing large molecules to leak out of the cell. Patch clamp recording across isolated patches of the rod outer segment membrane led to the discovery of the cGMP-dependent modulation of the photoreceptor CNG-channels (Fesenko *et al.*, 1985).

Suction pipette

Suction pipette recording (*Fig* 3.3.; Baylor *et al*., 1979a) has been the method of choice for electrophysiological studies of phototransduction for about three decades. A glass pipette

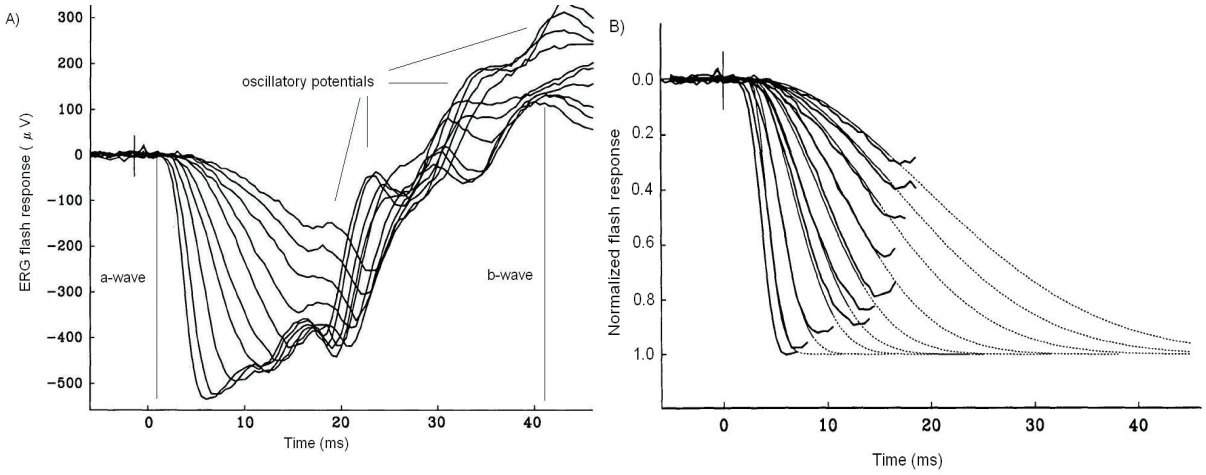

Figure 4. A) The ERG flash responses of dark-adapted human eye to flashes of incremental intensity. The amplitudes of the cornea-negative a-wave and the positive b-wave are indicated. The rising phase of the b-wave is partly masked by the superimposed oscillatory potentials. B) The normalized a-waves of the responses in A) fitted with the molecular photoresponse activation model by Lamb and Pugh (1992). Adapted from Breton et al. *(1994), with permission.*

with a rounded tip is shaped to match the dimensions of a photoreceptor outer or inner segment. The segment is drawn into the pipette by gentle suction, so that the current flowing extracellularly between the outer and inner segments is recorded. This current represents largely the light-sensitive current through the CNG channels in the outer segment, and can be related to the conductance changes produced by the biochemical phototransduction cascade (see Ch.4, about modeling photoresponses)

The suction pipette method can be combined e.g. with patch clamp or intracellular recordings to obtain photocurrent responses under voltage clamp, or to manipulate the chemical composition of the cytoplasm. While the suction pipette method may seem to be the ideal choice for studying phototransduction, it is in some regards compromised by disruption of the cells' connections to the retinal network, as well as affecting their adaptational capabilities (*e.g.* Donner *et al.*, 1990a). On the other hand, it can provide a "pure" model of the phototransduction events in the outer segment. The suction pipette method can be applied either on isolated photoreceptors or on cells attached to the retinal tissue.

2.2.2. Field potential photoresponses (ERG)

The retina is a highly layered tissue with alternating nuclear and synaptic ("plexiform") layers. The straight vertical pathway to the output neurons, ganglion cells, comprises two cell levels with strict radial orientation: the photoreceptors and the bipolars, interspersed with likewise radially oriented Müller glial cells. The signal flow is basically unidirectional at these levels, so that light induces largely synchronized activity proceeding successively towards the inner retina. This activity is accompanied by extracellular radial currents. Thus at some depths in the retina an event creates a current sink while at others there are current sources, each with characteristic temporal behavior. The radial currents flowing from sources to sinks within the extracellular space are associated with radial potential

gradients in the retina, which add up into the signal known as the electroretinogram (ERG). The electroretinogram can be recorded from the intact eye *in vivo*, transretinally from the isolated retina, or intraretinally with microelectrodes (see also *Table 1*).

Electroretinogram is the first bioelectrical signal recorded from human subjects (see Granit, 1933). Today it is commonly used as a clinical diagnostic tool of retinal function, as well as in the research of retinal physiology. However, the complexity of the ERG signal makes its interpretation a challenge: it rises as a combination of superimposed components due to the electrical activity of individual cell types. *Fig. 4 A*) shows a typical ERG response to a brief full-field flash of light recorded from the dark-adapted human eye. It is commonly accepted (see *e.g.* Pugh *et al.,* 1998) that the leading edge of the initial cornea-negative phase of the ERG flash response, a-wave, reflects the photosensitive currents of the rod and/or cone outer segments rather accurately.

Fig. 4B) shows the a-waves of the records in *Fig. 4A*) fitted with a model of phototransduction activation (Lamb and Pugh 1992, see Ch. 4). The subsequent phases of the photoreceptor response are masked by waveforms due to the activity of other retinal cells. The most prominent of these subsequent signals is the cornea-positive b-wave originating from the activity of ON-bipolar cells (Stockton and Slaughter, 1989). At least in mammalian photopic ERG, inner retinal function also adds to the later stages of the a-wave (primate: Bush and Sieving, 1994; mouse: Shirato *et al.*, 2008). Additional waveforms rise from the function of OFF-pathway (*e.g.* d-wave, Xu and Karwoski, 1995), ganglion and amacrine cells (*e.g.* the oscillatory potentials, Wachtmeister and Dowling, 1978), pigment epithelium (slow, cornea positive c-wave, Oakley and Green, 1976) and glial cells of the retina (a slow negative component, slow PIII, Bolnick *et al.*, 1979).

There are three principal ways to isolate the photoreceptor component from ERG flash response:

1. *Pharmacological isolation* (see Ch. 7.2.2 in Materials and Methods). Blocking the glutamatergic synaptic transmission from the photoreceptors to higher order neurons and removing the component due to the retinal glial cells yields the ERG component generated by the photoreceptor cells. This method can be used freely on isolated retinas and to some extent on experimentation on laboratory animals.

2. *Intraretinal recording* (Tomita, 1952, Penn and Hagins, 1969, 1972). In isolated retina, potentials generated exclusively at the OS layer can be recorded with extracellular microelectrodes.

3. *Paired-flash –method* (Pepperberg *et al*., 1997). The light-sensitive OS current *not* extinguished by a test stimulus is probed with another flash intense enough to drive the photoreceptors rapidly into saturation. Subtracting the response amplitude R_{probe} from the amplitude of the response to probe flash delivered alone yields an estimate for R_{test} (t_k), response to the test stimulus at t_k, time of delivery of the probe flash. A pointwise presentation of the photoreceptor response to the test flash can be obtained by varying the inter-stimulus intervals. The paired-flash method is a useful tool, but time-consuming. It is also not very precise due to the limited temporal resolution, as well as lack of certainty on

the true level of steady state photosensitive current (which is partly masked by the intervening postreceptoral components).

Additionally, genetic modification provides new powerful animal models that facilitate photoreceptor research especially in mouse. For example the nyx^{nob}-mouse ("no b-wave") lacks the metabotropic (ON-pathway), while its photoreceptor function appears to be normal (Pardue *et al.*, 1998).

2.2.3. Evaluation of the electrophysiological methods

The first question to pose when choosing an experimental method is: can it provide an answer to the problem at hand? A method may be inapplicable to a given preparation, or the parameters determined with a method may be only indirectly coupled to the process under study. For example, the membrane voltage response affects synaptic transmission directly, while its connection to the phototransduction cascade is less direct due to voltage sensitive conductances in the inner segment.

The experiment itself may also unduly affect the observed phenomena. With the exception of the electroretinogram from a live subject without any pharmacology, every method described above is more or less invasive to the cells under study. Disrupting the photoreceptor membrane, subjecting the cells to varying pharmacological manipulations, or mechanically detaching cells from the tissue are bound to challenge natural photoreceptor function. Reduction of the system may be useful, but it may also compromise the validity of the obtained results.

The suction pipette method records the current through the CNG channels directly (albeit supplemented by the lesser $Na^+/Ca^{2+},K^+$ –exchange current), but subjects the inner and outer segments to unnatural and often different chemical environments. It may also disrupt the photoreceptor's connections to neighboring cells and the retinal network. These manipulations may maim especially the adaptational processes of the photoreceptor cell itself (Donner *et al*., 1990a), and disregard many processes relevant to photoreceptor physiology *in vivo*, such as cone pigment regeneration (see Ch. 3.4). Electroretinogram recorded from a live subject or an isolated retina is complicated by signals from other cells, as well as the complexities of measuring the photosensitive currents only indirectly through extracellular voltage. Genetic manipulations aimed to separate a given pathway or cell type in retinal signaling (e.g. mice with dysfunctional rods) may seem like a "clean cut", dissecting certain functions from the system, but may lead to unexpected changes in retinal wiring and function. Given the drawbacks and advantages of different methods, which are summarized in *Table 1*, full understanding of photoreceptor function can be reached only if experimental results from different approaches are compared and combined into a coherent description, together with data from biochemical studies. Then each method presents a different level of magnification or a different viewpoint towards understanding the system under study.

Table 1. Electrophysiological methods for studying photoreceptor function

Method	***Description***	***Advantages***	***Drawbacks***
Single cell methods		**- direct recording of photoreceptor function** **- allows significant reduction of the system**	**- always invasive to the cells, only in vitro use**
patch clamp, whole cell, perforated patch, Intracellular	Microelectrode attached to the photoreceptor membrane. Records membrane voltage or current.	- recording either currents from a membrane patch, whole cell currents or the membrane voltage - enables manipulation of the cytoplasmic composition - enables voltage and current clamp	- very invasive - not readily applicable to all photoreceptors
Suction pipette	The inner or outer segment is drawn into a pipette. The current flowing into/out of the pipette is measured.	- robust and direct recording of the photosensitive current - the photoreceptor can be separated from the other retinal cells - can be combined with voltage clamp by another electrode	- invasive - the photoreceptor's connection to the retinal network is often disrupted and the external milieu is unnatural - the pigment regeneration and adaptational properties may be compromised
Field potential recordings (ERG)		**- photoreceptors not detached from the retinal network** **- the signal is averaged over a mass of photoreceptors** **- in vivo and transretinal ERG are very robust and stable methods allowing long recording sessions**	**- interference of signals from other retinal cells** **- the photoreceptor signal may reflect both directly light sensitive *and* voltage dependent currents** **- signals of various photoreceptor types must be isolated from each other** **- depends on the extracellular resistance traversed by radial currents**
In vivo	Potential changes between corneal electrode and a reference point are recorded on a living subject.	- practically non-invasive - can be used clinically - gives measures of retinal function as whole (as well as that of the pigment epithelium) in completely natural condition - applicable to freely moving subjects - the photoreceptor flash response can be determined using the pair flash –method	- the signal is very complex: non-photoreceptor components intervene at an early stage - the paired flash –method is tedious and lacks resolution - calibration of stimulus intensities in terms of photoisomerizations is demanding - difficult to sort out the relative contributions of different cones at different eccentricities
Transretinal	Potential changes recorded across an isolated retina.	- very stable recordings, easily accessible to pharmacological manipulations - the photoreceptor cells remain embedded in a reasonably natural environment - excellent signal-to-noise ratio	- possible contamination by residual components when isolating the photoreceptor light response - the relation between the ohmic voltage generated by extracellular currents and the current through the CNG channels is not straightforward
Local with microelectrodes	Photoresponses recorded locally with microelectrodes placed at selected layers in the retinas	- records exclusively from the desired layer - quite direct ly reflects the outer segment photosensitive current, without disrupting the photoreceptor's connections to other retinal cells	- slightly invasive: may mechanically damage outer segments - changes in photon catch due to positional changes of the cells in the recording area may alter the observed photoreceptor sensitivity - stable recording is difficult to achieve

3. Regulation of photoreceptor sensitivity and kinetics

Rod-mediated vision emphasizes high sensitivity, while the less sensitive cone vision is coupled with better temporal resolution. These differences stem largely from the properties of the respective photoreceptor cells. In photoreceptor light-adaptation, sensitivity and temporal resolution are tightly coupled over a certain range of adaptation levels, as first demonstrated in *Limulus* lateral eye by Fuortes and Hodgkin (1964) and in vertebrate cones by Baylor and Hodgkin (1974). This implies a tradeoff in which one of the two must be favored on the expense of the other. The exact difference between rod and cone function and composition is still subject to intense research.

This chapter reviews the factors determining the amplification and kinetics of phototransduction, with an emphasis on differences between rods and cones. Particular attention is paid to the role of temperature, both in regulating enzymatic reaction rates and as a modulator of noise in photoreceptor signaling. First, some of the physical determinants of dark-adapted sensitivity are reviewed (Ch.3.1), followed by the differences between rods and cones (Ch.3.2.). Ch.3.3. addresses light-adaptation and Ch.3.4. considers the hypothesis that the low sensitivity of cones originates partly from desensitization due to thermal activation of the cone visual pigment. Finally, intrinsic activity of the phototransduction proteins downstream from the visual pigment is briefly addressed in Ch.3.5.

3.1. Physical factors

The major physical factors that affect phototransduction, besides the availability of light, are temperature, cell size and morphology. Temperature affects every chemical reaction. Cell size affects diffusion distances and the relative effect of a single molecule's catalytic activity on the cellular level. Morphology affects the metabolic budget and the efficiency of molecule transport both within the cell and across the plasma membrane.

3.1.1. Temperature

Temperature is a major physical factor that necessarily affects any cellular signaling. The mammalian and avian visual systems have adapted to a constant, physiologically rather high temperature, while poikilothermic ("cold-blooded animals") must deal with variation over wide temperature ranges: for example, a common frog (*Rana temporaria*) may be active at temperatures ranging from 5 to 20 ^{o}C (278-293 K) during a single day. Temperature-dependence of features in a process such as phototransduction, based on a complex biochemical reaction chain, is not straightforward to predict, as both forward and reverse rates of individual catalytic reactions as well as activation and inactivation steps are affected. Yet typical biochemical reaction rates are accelerated by 2 to 4-fold with warming of 10 degrees, so transfer from 5 to 37 ^{o}C may accelerate a reaction by more than 60-fold! Thus temporary changes in the temperature encountered by an individual poikilotherm, or a population-wide adaptation to an altogether different temperature regimen, must involve considerable changes in both sensitivity and kinetics of phototransduction as well as molecular adaptations (Baylor *et al.*, 1983; Lamb 1984; Donner *et al.*, *1988*). On an evolutionary time scale, this may drive adjustments in cell size and morphology coupled

with the biochemical changes. The temperature-dependent features of visual performance sometimes lead into optimizing some important feature at the expense of others: the fast-hunting swordfish has a specific heating organ behind its eye to achieve greater speed of vision (Fritsches *et al.,* 2005), while the common toad (*Bufo bufo*) takes advantage of its low hunting temperatures to achieve high visual sensitivity due to low thermal noise and long integration time (Aho *et al.,* 1988; Haldin *et al.,* 2009).

3.1.2. Cell size and morphology

Rod sensitivity and function

If two outer segments differ only in size, *i.e.* their molecular densities are equal, a photoisomerization must have a larger fractional effect on the light-sensitive current of the smaller one: with equal catalytic activity, an active PDE subunit (and thus each Rh*) removes a larger fraction of the smaller amount of cGMP in the OS (Lamb and Pugh, 1992). This tends to cause larger quantal responses, *i.e.* increase fractional sensitivity in small outer segments. If the visual pigment molecules are prone to "false alarms" due to spontaneous activation (see Ch.3.5), their incidence is also lower in the smaller photoreceptor (the density of visual pigment molecules appears to be roughly constant in all vertebrate photoreceptors).

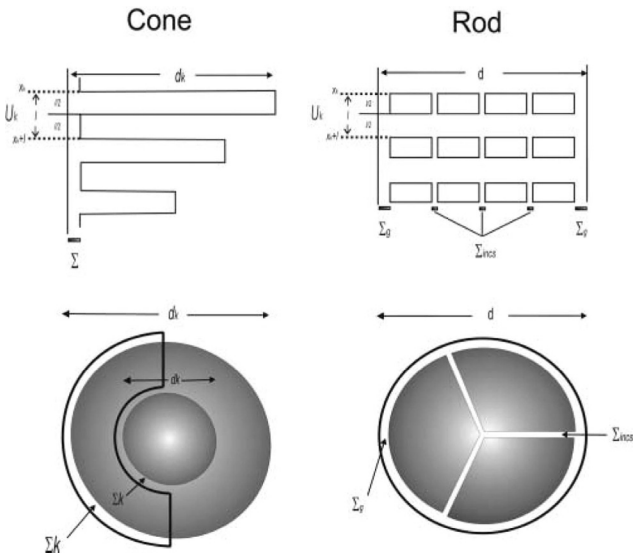

Figure 5. Schematic presentation of rod and cone outer segment (OS) geometry: longitudinal (top) and transverse (bottom) cross-sections. The membrane foldings of the cone OS (left) of most species are contiguous with the plasma membrane. In contrast, the disks within rod OS are separate from the plasma membrane with the exception of the most proximal part of the OS in which the disks are generated from folding plasma membrane. Discs are disrupted with incisures. From Holcman and Korenbrot (2004), with permission.

Structural differences between rods and cones

Pigment-containing lipid bilayer membrane disks fill the rod OS as a stack of coins. At the base of the inner segment, they arise as folds of the plasma membrane, from which they are subsequently completely disconnected (*Fig. 5.*). Oriented incisures in the stacks (Papermaster *et al.*, 1978) facilitate longitudinal diffusion of molecules in the cytoplasm of the tightly packed outer segment. In cones, the membrane folds remain contiguous with the plasma membrane, although to a varying degree in different species (Cohen, 1968). Thus rod phototransduction begins in the disk membranes, surrounded by cytoplasm and requiring transmission of the signal through the water phase to the channels embedded in the plasma membrane. In the cones, all the membrane-bound enzymatic reactions happen in the same membrane that also contains the CNG-channels. This shortens diffusion

distances and at least in principle allows direct interaction between the participating proteins. Also the chemical environment of the proteins may differ in the two cell types: whilst the membrane-spanning proteins in cones are partly exposed to the extracellular medium, the composition of the environment within the rod disks, although not fully known, is regulated and different from the extracellular medium. The folded structure of the cone plasma membrane also provides a higher surface-to-volume ratio than in rod outer segments. Thus a given current density leads to a faster change in the cytoplasmic concentration of the current carrier, such as calcium. As a downside effect, the large surface area also results in larger membrane capacitance, increasing the time constant of the membrane and effectively slowing down voltage photoresponses (*e.g.* Perry and McNaughton, 1991; see also papers II and III).

The precise significance of the different outer segment structure in rods and cones is not fully understood. However data and calculations from Holcman and Korenbrot (2004) suggest that longitudinal diffusional spread of small aqueous molecules is about 4 times faster in rods than in a cone OS composed of fully contiguous plasma membrane foldings, indicating that the prototypical cone OS structure hinders diffusion. In rods, the electrical response to a single photon spreads 3-4 μm along the outer segment, far beyond the initial location of absorbance (Lamb *et al*., 1981; Gray-Keller *et al.*, 1999). The rod outer segment structure may thus have developed partly to facilitate large single photon responses.

3.2. Phototransduction in rods and cones

The main bulk of information on phototransduction reaction kinetics originates from rods, as material can be obtained in large amounts for biochemical assays (see *e.g.* Tachibanaki *et al.*, 2007). To date, cones have been more elusive to such characterization, mainly due to their much lower share of total outer-segment volume in most retinas. Molecular cloning of phototransduction proteins, as well as immunolabelling specific to known phototransduction participants in both photoreceptor types have shown that specific isoforms of the same protein families exist in rods and cones (*e.g.* PDE: Gillespie and Beavo 1988, Hamilton and Hurley 1990; G-protein: Lerea *et al.,* 1986, Ryan *et al*., 2000; CNG-channels: Paillart *et al.*, 2006; $Na^+/Ca^{2+},K^+$ exchanger: Sheng *et al.,* 2000). As the electrophysiological data also suggest similar photoresponse activation in rods and cones, it has been concluded that the main features of the phototransduction process are qualitatively similar in the two cell types. Furthermore, cone opsins expressed in a rod photoreceptor (even in different species) can couple to the phototransduction cascade of the host cells and vice versa (Opsins: Shaaban, 1998; Kefalov *et al.,* 2003; Fu *et al.,* 2008). Likewise, the rod α-transducin subunit is fully functional in a cone transduction environment (Deng *et al*., 2009).

Why then are the cones so much faster and less sensitive? One qualitative difference appears to concern pigment regeneration and stability. Beyond these pigment properties, addressed in Ch.3.4., a number of quantitative differences underlie the fast and small photoresponses in cones.

Molecular amplification

Different concentrations and catalytic efficiencies of enzymes may lead into differences in molecular amplification rates in rods and cones (cf. Pugh and Lamb, 1993, Tachibanaki *et al.,* 2001). However, it is still debated whether the activation reactions of the phototransduction cascade are less amplified in cones. Some studies state that the catalytic efficiency of the reactions leading to cGMP hydrolysis is of the same order in cones and rods (activation of transducin by the visual pigment Vissers *et al.,* 1998; PDE activity and concentration Gillespie and Beavo, 1988). However, Tachibanaki *et al.* (2001, 2007) deemed activation less amplified in cones. Whether the discrepancies are due to limits in experimental techniques or actually reflect variation of functional properties in different cones is so far unknown. Nevertheless, there seems to be no major generic difference between initial molecular amplification in rod and cone.

Calcium homeostasis and feedback

The light sensitive current of the OS contains a fraction of Ca^{2+} (Ohyama *et al*., 2000). Closure of CNG-channels decreases the influx of Ca^{2+} to the OS, while extrusion of Ca^{2+} by the $Na^{+}/Ca^{2+},K^{+}$ exchanger continues (Schnetkamp, 1995). The decrease in intracellular Ca^{2+} serves as a feedback signal on many calcium-dependent regulating proteins, accelerating the photoresponse deactivation. In cones, the greater fraction of light-sensitive current carried by Ca^{2+}, along with the larger surface-to-volume ratio leads to relatively larger and faster changes in $[Ca^{2+}]_i$ enabling effective feedback mechanisms (Picones and Korenbrot, 1995; Korenbrot, 1995; Ohyama et al., 2000). This hastens the recovery of the cone light response, evident as lower peak amplitude and shorter time-to-peak of flash responses.

Voltage-dependence of the light-sensitive current

The current flowing through the CNG-channels in the rod outer segment is thought to be quite independent of voltage in the physiological range (Baylor and Nunn, 1986). This is not so in the cones, in which the light-sensitive current increases with hyperpolarization (Attwell *et al.,* 1982*)*, although the physiological consequences of this are not clear.

One cone is not like another

The cones as a general class are more heterogeneous than rods. It is common, that there are multiple classes of cones on the retina, distinguishable either by their visual pigments, by morphological and functional differences or a combination of these. Cones can exist either as single cones, or as double cones in which two outer segments either with same or different pigment share a common inner segment, or at least connected inner segments. In some species, there are two types of double cones with one having much faster kinetics and lower sensitivity than the other despite sharing a common pigment. A representative example of variation in cone types is striped bass, for which the functional parameters of flash responses from all photoreceptors have been extensively characterized by Miller and Korenbrot (1993).

3.3. Light-adaptation

Under prolonged light exposure, when a photoreceptor's quantal responses overlap in time, maintained closure of CNG-channels tends to reduce the light-sensitive current. This reduction decreases the cell's dynamic range, *i.e.* compresses its responses to luminance changes. Both rods and cones have mechanisms for preventing compression under maintained stimulation, either by opposing directly the effects of accelerated cGMP-hydrolysis on the light-sensitive current or indirectly through desensitization. Rods have limited capacity for light-adaptation, and their light-sensitive current is suppressed under bright light. This may be advantageous for a duplex retina, as it reduces energy consumption under photopic conditions (*see* Okawa *et al.,* 2008). Instead, powerful compression-opposing mechanisms maintain cones functional at virtually all light levels (Matthews, *et al*., 1990a; Burkhardt, 1994).

Two photons absorbed by a rod may contain relevant information about a visual object in darkness. Even in a dimly lit environment the same excitation would merely present noise in the average rain of photons onto the retina. Then detecting the salient temporal and spatial contrasts requires graded response to much larger amounts of photons, sufficient to saturate a dark-adapted cell. Controlled desensitization with increased mean luminance shifts the dynamic range of the photoreceptors with respect to relevant stimuli in each situation. Specifically, the contrasts of reflecting objects remain constant irrespective of illumination, and at higher light levels the functional goal of vision might be to maintain constant contrast sensitivity (Weber adaptation).

Readjusting to dark-adapted state after strong light exposures is referred to as dark-adaptation. Intuitively it may seem simply as reversal of adaptation to increased light levels. However, it is a different process, based on partly separate mechanisms mostly requiring much longer time scales than light-adaptation. Dark-adaptation is not dealt further in the present work (see *e.g*. Leibrock *et al.*, 1998 and Lamb and Pugh, 2004).

3.3.1. Phenomenology: Light-adaptation in electrical photoresponses

Light-adaptated flash photoresponses are both smaller and faster than dark-adapted responses to the same stimuli (*e.g.* Baylor and Hodgkin, 1974; Fain, 1976; Nakatani *et al.,* 1991*;* Matthews *et al.*, 1990b, 1991). Yet despite desensitization, the activation phase (molecular amplification) of fractional photoresponses remains constant through a wide range of intensities both in rods (Nikonov *et al.*, 2000) and cones (Soo *et al.,* 2008, *cf.* also papers I and II), implying the smaller amplitude is achieved by accelerated deactivation. This enables countering compression and maintaining responsiveness to relevant stimuli without compromising the speed of activation (and thus visual reaction times).

Fig. 6 illustrates the role of controlled desensitization in light-adaptation. As explained in Ch. 3.3.2., photoreceptor light- adaptation depends largely on changes in OS calcium level. With $[Ca^{2+}]_i$ clamped to its dark-adapted level, response compression drives salamander rods into saturation at 1000-fold lower intensities than under more physiological conditions (Fig. 6A; Matthews *et al.*, 1990a,b). The same applies to flash sensitivity (*Fig. 6B*): the controlled desensitization that depends on changes in $[Ca^{2+}]_i$ actually *rescues* the differential sensitivity of the photoreceptor.

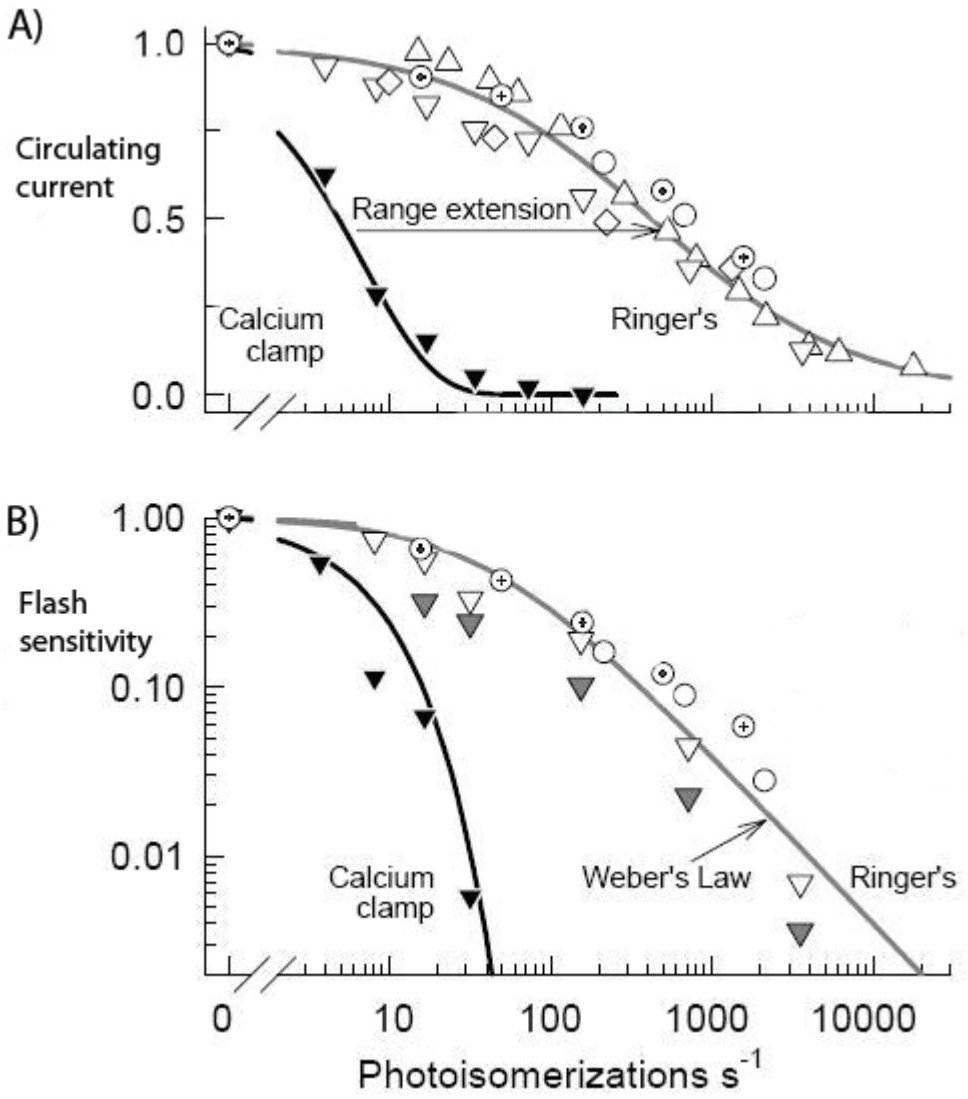

Figure 6. Changes in cytoplasmic calcium extend the operating range of photoreceptors. A) Normalized circulating (light-sensitive) current of salamander rods as a function of steady background intensity, expressed in terms of isomerized pigments per rod. $[Ca^{2+}]_i$ *is either clamped to its level in darkness (closed black symbols) or free to change (open grey symbols). B) Flash sensitivity as a function of background intensity with* $[Ca^{2+}]_i$ *clamped to its dark-adapted level (black triangles) or free to change (open symbols). Values normalized to sensitivity in darkness. Adapted from Pugh and Lamb (2000), with permission.*

Fig. 7A illustrates the characteristic changes in salamander rod flash responses under light-adaptation. The fractional responses to a fixed stimulus get smaller with increasing backgrounds, but strictly via accelerated shutdown of the response: the rising edges follow initially a common course from which the more light-adapted responses start "peeling off" earlier. *Fig*. 7B summarizes how the time-to peak and sensitivity of frog cones change under varying backgrounds in a similar experiment. It is common to describe background-induced desensitization of photoreceptor flash responses with the Weber relation $S = S_{dark} (I_0 / (I_{Bg} + I_0))$, as used in *Figs.* 6B and 7B. According to this relation, background intensity I_{Bg} begins to

have an effect on the photoreceptor sensitivity S only when it is sufficiently strong relative to a hypothetical dark light, I_0, describing the intrinsic adaptational state of the cells. At sufficiently strong backgrounds, the sensitivity depends linearly on the background intensity, implying preserved contrast sensitivity. However, it is clear from *Fig*. 7B that the Weber relation provides only a rough approximation to actual data (Donner *et al.*, 1998). Given the amount and complexity of known mechanisms underlying neural light-adaptation in photoreceptors, it is hardly surprising that the background induced desensitization appears to involve multiple regions with different intensity dependencies.

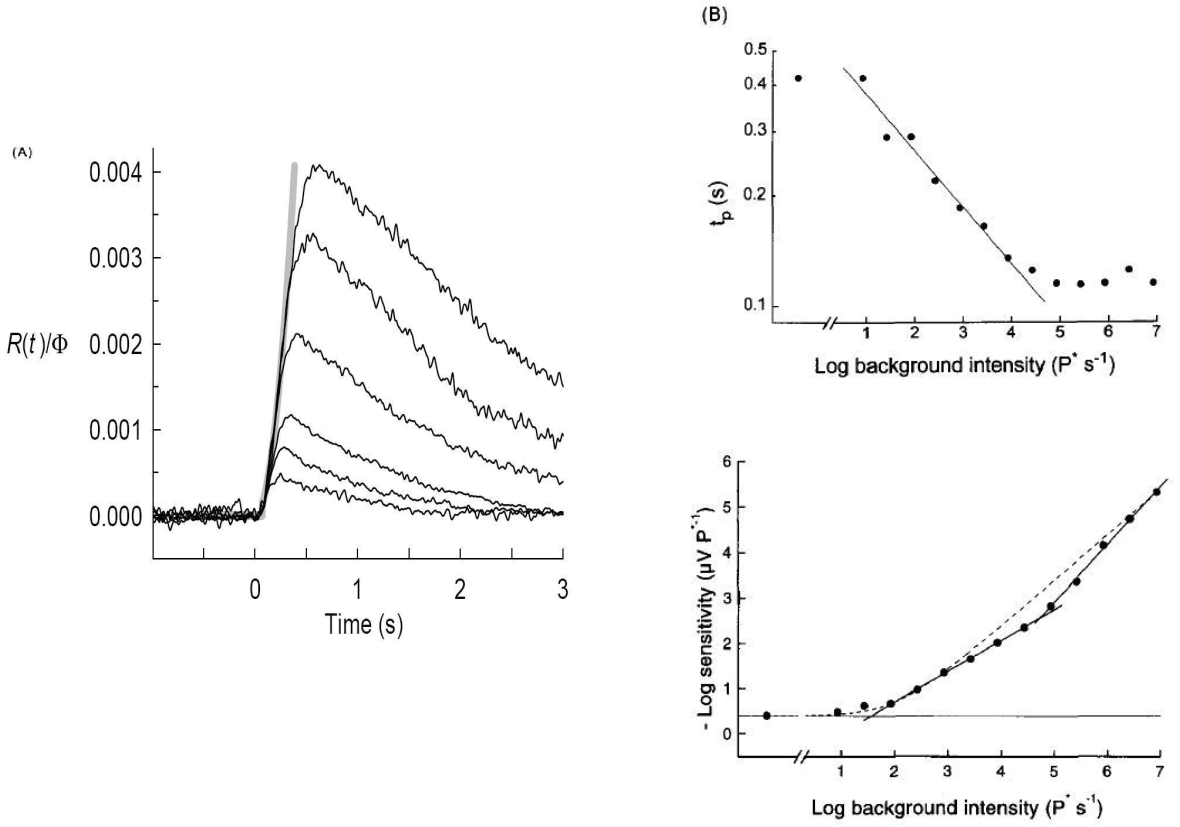

Figure 7. A) Responses to a brief flash of constant intensity (in the linear response range) recorded from a salamander rod under steady adapting backgrounds of different intensities. The largest response has been recorded in darkness and the smaller ones under progressively increasing backgrounds. The responses have been normalized to the saturated amplitude and the number of isomerized pigments by the stimulus (ϕ) to reveal the fraction of the circulating current shut off by a single photopigment isomerization. (Pugh and Lamb, 2000, reprinted with permission) B) Changes in the time-to-peak and sensitivity of frog cones as function of the background intensity (isomerized photopigments, P^*s^{-1}). The dashed curve fitted to the sensitivity data represents Weber adaptation, while the continuous lines on the log-log scale are linear fits to subsets of the data, emphasizing the different adaptational regimes with varying intensity dependencies (Donner et al., 1998, reprinted with permission).

3.3.2. Molecular mechanisms of light-adaptation

Much of photoreceptor light-adaptation is mediated by changes in the outer segment calcium level $[Ca^{2+}]_i$, (Nakatani and Yau, 1988; Fain *et al*., 1989). The relative effect of individual feedback mechanisms depends on the cell type and luminance range (Koutalos and Yau, 1996), although the exact contribution of each has not been fully characterized. The individual processes complete with varying time scales, ranging from seconds to a few minutes (Calvert *et al.*, 2002; Sokolov *et al.*, 2002).

A reduction of the light-sensitive current decreases $[Ca^{2+}]_i$ (Yau and Nakatani, 1985) by reducing influx of Ca^{2+} ions while the outflux via the Na^+/Ca^{2+}, K^+ exchanger (NCKX) is maintained. The decrease in $[Ca^{2+}]_i$: 1. enhances cGMP-synthesis via GCAP-proteins facilitating the function of guanylate cyclase (Haeseleer *et al*., 1999; Mendez *et al.*, 2001), 2. reduces the lifetime of active rhodopsin (Kawamura and Murakami, 1991) and 3. increases the affinity of the CNG channel to cGMP (through calmodulin in rods: Hsu and Molday, 1993; and an analogous CNG-modulin in cones: Rebrik and Korenbrot, 1998, Rebrik 2010). All three mechanisms serve to counter compression and maintain the functional range of phototransduction.

In cones, a larger fraction of the light-sensitive current is carried by calcium than in rods, and the turnover of $[Ca^{2+}]_i$ is faster. Therefore, the calcium-dependent feedback on phototransduction tends to have more significance in cones than in rods. cGMP synthesis acceleration is the main physiologically relevant Ca-dependent adaptation mechanism in rods (Koch and Stryer, 1988; Koutalos *et al*., 1995). It also happens following small, transient stimuli, hastening the flash response deactivation in dark-adapted rods (Burns *et al*., 2002). On the other hand, tuning the affinity of the CNG channels for cGMP has physiological significance only in maintaining the functional range of cones. (Rebrik and Korenbrot, 1998, 2003).

In salamander rods, the dominant desensitizing mechanism during exposure to weak and moderate light is the maintained catalytic activity of phosphodiesterase (Nikonov *et al*., 2000). Increasing the steady-state turnover of cGMP will lead to faster quenching of a photoresponse through faster restoration after any displacement of [cGMP]. In cones with a higher overall PDE-activity level even in the dark-adapted state (Holcman and Korenbrot, 2005), the Ca^{2+}-related feedback mechanisms are dominant.

Pigment bleaching

When the photon absorption rate exceeds the visual pigment regeneration capacity, the reduced total absorption efficiency of the remaining pool of visual pigments becomes a desensitizing factor. Pigment bleaching is considered the dominant reason for cone desensitization at high illumination levels, where the remaining pigment content and thus the absorption by the cone outer segments finally becomes inversely proportional to the intensity of mean illumination (Rushton and Henry, 1968).

Protein translocation*.*

Phototransduction proteins are translocated between the inner and outer segments both following a diurnal rhythm and following light-adaptation. Under bright adapting backgrounds, up to 90% of transducin initially found in rod outer segments is transported

into the inner segment to reduce metabolic stress and possibly also lower the molecular gain (Philp *et al.*, 1987; Whelan and McGinnis, 1987; quantified by Sokolov *et al*., 2002) along with recoverin (Strissel *et al.*, 2005). Most of the arrestin is relocated into the outer segment (Broekhuyse *et al.*, 1985), also in cones (Zhu *et al*., 2002). Protein translocation happens in rods on a time scale of minutes (Elias *et al.*, 2004) and is initiated only at near-saturating background intensities.

Electrophysiological studies have also suggested additional mechanisms of photoreceptor sensitivity regulation that are yet hypothetical or possibly entirely unknown. In some photoreceptors an initial *rise* of sensitivity can be seen at low background intensities (Monkey cones: Dunn *et al*., 2007, see also Ch. 8.3 in results), the mechanism for which is not understood. As a somewhat better characterized phenomenon, background-induced regulation of transducin turnoff (Krispel *et al*., 2003; Woodruff *et al*., 2008) has been suggested to occur in mouse rods.

3.3.3. Light-adaptation in mammalian photoreceptors

In contrast to amphibian rods, which light-adapt over significant ranges of background intensities, adaptation in mammalian rods has generally been considered to be quite limited, if occurring at all (Penn and Hagins, 1972; Baylor et al., 1984a). More recent studies have shown that adaptation does occur, but at relatively high light intensities compared to most cold-blooded species (30-40 Rh* s^{-1} per cell; Tamura et al., 1989; Nakatani et al., 1991). The limited capability for light-adaptation has long been accepted as a fundamental property of the biochemical machinery in mammalian rods, although it is noted that the different kinetics and operating temperature may underlie much of the difference. The present study compares rat and toad rod function at different temperatures to further clarify the role of OS size and operating temperature behind the differences between the rod function in mammals and “lower” vertebrates.

3.4. Pigment regeneration and stability

The main functional properties of a visual pigment are its 1) spectral absorbance, which determines the photoreceptor’s quantum catch in a given light environment, 2) thermal stability, a major determinant of the “dark light” of the photoreceptor, and 3) regeneration, which affects the light-dark adaptation of the photoreceptor. These properties are partly interdependent (*Fig.* 8), so optimizing performance in one respect often affects the others. For example, rods achieve a highly stable visual pigment (*i.e.* few false transduction events) at the cost of relatively slow regeneration after activation. Also tuning a pigment’s spectral absorbance to maximize quantum catch in certain light environment is generally coupled to changes in thermal stability, *i.e.* shifting a pigment’s absorbance to longer wavelengths may lead to an increase in the spontaneous activation rate. Compared with rod pigments, cone pigments on the whole seem to opt for fast and efficient regeneration at the expense of stability. Paper II investigates the idea originally proposed by Barlow (1958) and supported by later electrophysiogical studies (Rieke and Baylor, 2000) that dark-adapted L-cones are desensitized by an intrinsic “dark light” due to spontaneous activity of their visual pigments.

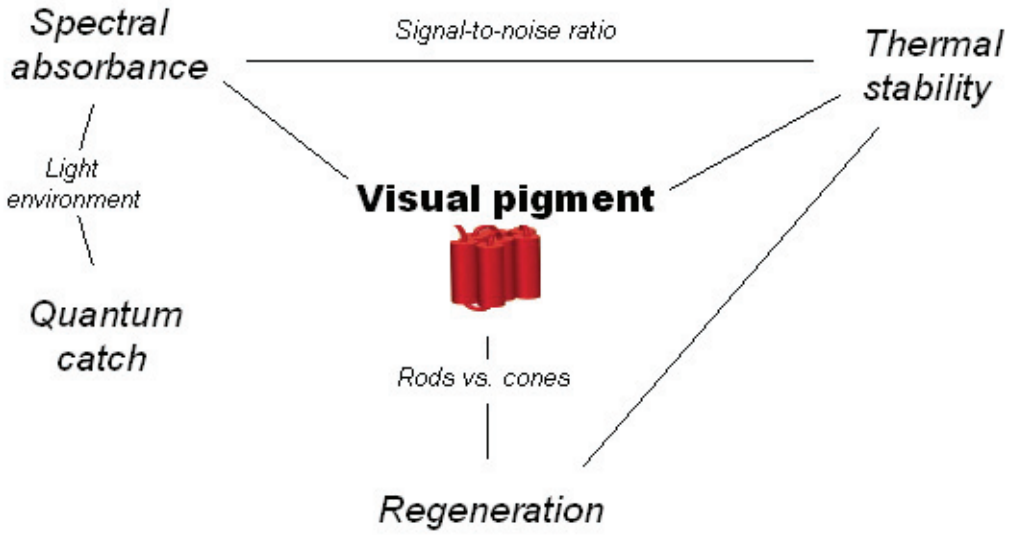

Figure 8. Visual pigment function can be optimized regarding several partly interdependent properties (see text for details).

3.4.1. Pigment regeneration

Much of a photoreceptor's performance after light-evoked activity depends on how the chromophore-opsin complex is broken down and recycled after deactivation of visual pigment, to be available again for photon absorption and activation. The complex dissociates much faster in cone pigments, where the covalent bond is easily broken and the chromophore pocket much more "open" (see Sampath and Baylor, 2002). This is likely to have consequences for thermal stability of the pigment (ch 3.4.3.). Both rods and cones need other cells to complete this so-called "visual cycle" – dissociation and regeneration of activated pigments - but cones have access to a faster and more efficient route for recycling the chromophore (Mata *et al.*, 2002; Wang *et al.*, 2008). Rods cannot regenerate their visual pigment in the isolated retina beyond using their limited chromophore reserve, but are supplied with fresh *11-cis*-retinal by the pigment epithelium. The cones, however, are supplied with 11-*cis*-retinol by the Müller glial cells of the retina. Unlike rods, they are able to uptake and oxidize it into retinal, even in the isolated retina.

3.4.2. Optimizing the absorbance spectrum: quantum catch and pigment stability

Thermal activation of the visual pigment

Two out of three photons absorbed by a visual pigment initiate a visual signal (Dartnall, 1968). Generally, this requires that the incident photon has sufficient energy to isomerize the chromophore, but part of the needed energy may also originate from the molecule's different vibrational modes (deVries, 1948; Koskelainen *et al.*, 2000). Occasionally, thermal energy can initiate a spontaneous isomerization of the chromophore even when no photon has been absorbed (Yau *et al.,* 1979; Ala-Laurila *et al.,* 2004). A thermal isomerization will trigger a "photoresponse" indistinguishable from a real single photon response (Yau *et al.,* 1979, Baylor *et al.,* 1980). As the rods and rod pathways are regularly used for vision at very low light levels, where single photons count, there is clear evolutionary pressure towards minimizing "false alarms" by developing highly stable pigments. On the whole, spontaneous pigment activations are extremely rare especially in rods with A1 chromophore: the

expected lifetime of a (A1) rhodopsin molecule of a toad is about 3000 years (Baylor *et al.*, 1980). However, as a rod outer segment contains *ca*. 10^9 pigment molecules, thermal pigment activations will still occur in each rod about once per minute on average. These events can be observed as discrete events mimicking the true single photon response in the circulating current of isolated dark-adapted rods.

Thermal pigment isomerizations are so infrequent in rods that they can not "light-adapt" these cells. However, they do provide a random source of photon-like noise to the retinal circuitry. Any real light must be detected against this intrinsic background activity. The notion that sensitivity of rod vision is limited by visual pigment noise was suggested by Autrum (1943) and Barlow (1956) and has gained support from recordings at ganglion cell level (Copenhagen *et al.* 1987) and behavioral experiments (Aho *et al*., 1988).

Tuning spectral absorbance

The spectral absorbance of a visual pigment is defined by its opsin sequence and modified by the choice of chromophore. The apoprotein can utilize either A1 or A2 retinal, the extra double bond in the latter generally shifting the absorption spectrum of the resulting complex into longer wavelengths (smaller energies). This helps to maximize photon catch in certain light-environments but comes with a trade off of decreasing the thermal stability of the pigment (Donner *et al.*, 1990b*;* Ala-Laurila *et al.*, 2004). Thus mammals use exclusively the A1-chromophore, A2-chromophore is commonly used by many "cold-blooded" animals. Several amphibians and fishes adjust the A2/A1 ratio in their photoreceptors during their lifetime to better match the spectrum of light available in their habitats: for example larval salamanders (*A. tigrinum*) as well as common frogs (*R. temporaria*) use the red-shifted A2-pigment in their aquatic habitats and shift into a A1-based pigments during maturation, adapting into their land-based lifestyle as adults (Crescitelli, 1959).

3.4.3. Spontaneous photopigment activity in cones

Due to their role in daylight vision cone pigments have not, presumably, been subject to similar evolutionary pressure towards high stability as rod pigments. On the other hand, the efficient regeneration of bleached cone pigment may require a looser binding of chromophore by the opsin, rendering cone pigments generically less stable than rod pigments. Additionally, the correlation between the wavelength of maximal absorption and the rate of spontaneous pigment activation in rods suggests that thermal pigment activity may be especially pronounced in the long-wavelength sensitive cones (L-cones). Indeed, it has been a long-standing hypothesis (Barlow, 1957, 1958) that L-cones may be effectively "light-adapted" by intrinsic light-like activity even in darkness.

Electrophysiological studies of spontaneous visual pigment activation

The large quantal response of dark-adapted rods allows direct studies of thermal rhodopsin activation from the fluctuations in the OS current of dark-adapted rods: They can be analyzed through the asymmetry of the current histogram, isolation of the low-frequency, "light-like" component of the noise power spectrum, or in some case with simple counting of the "photon-like" events exceeding criterion amplitude, possibly after matched filtering based on the observed waveform of the single photon response. The various approaches can be used jointly, to lessen the uncertainty in identification of pigment-evoked events against the overall noise in the circulating current. Additionally, pharmacological methods

such as buffering intracellular calcium changes can be used to improve their discrimination by increasing the quantal response.

In cones, the single photon response is too small to distinguish individual, presumably temporally overlapping events in the light-sensitive current. The remaining method with wild type cones is to study the frequency composition of the current fluctuations, matching the lower end of the spectrum to that calculated from the real small-stimulus photoresponse. Scaling of the power spectra yields an estimate for the spontaneous pigment activation rate that would generate similar noise to that observed in the relevant frequency band. This method requires that the spectral composition of "photon-like" and "continuous" noise is sufficiently distinct, as in the cones of the larval tiger salamander (*Ambystoma tigrinum,* studied e.g. by Rieke and Baylor, 2000; Sampath and Baylor, 2002). It has also been applied to other species, although the estimates have often been uncertain due to the mismatch in the power spectrum between the noise recordings and the light responses (turtle by Lamb and Simon, 1977; macaque by Schneeweis and Schnapf, 1999). In such cases, the obtained dark event rate must be regarded as an upper limit.

Lately, another approach has emerged: expressing cone opsins in rods of transgenic animals of the same or even different species (Kefalov *et al.,* 2003; Sakurai *et al.,* 2004; Fu *et al.,* 2008). Cone pigment expressed in a rod can apparently initiate phototransduction cascade similarly to the native pigment. Thus the high gain of rod phototransduction can be utilized to make activation of individual cone pigment molecules directly detectable by electrophysiology. As the cone opsins are also expressed at relatively low levels in these transgenic systems (thus producing a lower rate of thermal isomerizations than if they were expressed at similar levels as in the wild type cells), the straightforward analysis methods generally used in rods can be applied. These works have yielded substantially lower estimates for the spontaneous pigment event rates than analysis of the noise power spectra (see below).

Do thermal isomerizations shape the cone photoresponse?

Rieke and Baylor (2000) showed that nominally dark-adapted salamander L-cones are likely to be desensitized by an intrinsic "dark light", as suggested decades before. They showed that the power spectrum of the OS current noise recorded from these cells had a low-frequency component highly resembling the frequency composition of the single photon response. This suggested that the noise in this frequency band originated mainly in spontaneous isomerizations of the visual pigment, occurring at a rate of *ca.* 600-700 events in a cone per second. This agreed well with the amount of real background light required to desensitize these cones by half from their dark-adapted sensitivity. The authors did not propose that desensitization due to thermal "dark light" would account for the different sensitivity and kinetics of rods and cones, but their comparative experiments on the short-wavelength sensitive S-cones did suggest that L-cones differ from S-cones only by the degree of "light-adaptation" due to their (thermal) intrinsic dark light.

However, expressing the salamander L-cone pigment in a rod (Kefalov *et al.*, 2003) yielded over 3-fold lower estimate for its thermal activation rate. Moreover, the estimates for thermal pigment activation rate obtained with dark noise recordings of monkey cones and through expression of human cone pigments in mouse rods differ more than 100-fold (Fu *et*

al., 2008). The obtained thermal isomerization rate for salamander L-pigment is still sufficiently high to desensitize the L-cones to some extent in this species. However, if the rate of dark events in ectopically expressed human L-cone pigment represents the pigment's properties in its natural surroundings, it must be concluded that thermal visual pigment isomerizations do not limit the sensitivity of human cones.

The L-cones of larval salamander use the A2 chromophore to enable absorption at relatively long wavelengths. In rods, the A2-based pigments are known to be more instable than those with A1 (Donner *et al.*, 1990b; Ala-Laurila *et al.*, 2007). There is likely to have been evolutionary pressure towards highly stable visual pigments in mammalian L-cones, partly due to the higher body temperature. Given a 4.5-fold increase in thermal isomerization rate per 10 ^{o}C (Sampath and Baylor, 2002), a pigment as instable as the salamander pigment would yield several thousands of P* s^{-1} in a primate cone. This would be incompatible with the sensitivity and reliability of dark-adapted human cone vision (Donner *et al.*, 1992).

The A2-based visual pigments of aquatic animals and the stable (A1-based) mammalian pigments may represent two extremes of L-cone pigment stability. However, very little is known about the wide variety of L-cones of other terrestrial animals, with pigments spectrally similar to those of mammals. There are currently no direct recordings available of photocurrent noise from amphibian L-cones with A1 chromophore, nor estimates of its effect to the photoreceptor function. This sets the scene for the indirect physiological approach to the problem taken in paper II.

Free opsin

The chromophore is covalently bound to the opsin via a protonated Schiff base. In cones this binding is reversible and there is continuous traffic of chromophore in and out of the chromophore pocket. Thus there is a dynamic equilibrium leaving a certain proportion of the opsin in the outer segment chromophore-free. In isolated salamander cones, the fraction of free apoprotein can be as much as 10% of the total opsin content (Kefalov et al., 2005). Free opsin is known to activate phototransduction, although with only 1/10 000 of the efficiency of the light-activated visual pigment (Cornwall *et al*, 1995). Kefalov *et al.* (2005) deduced that the excitation by free opsin will cause light-adaptation of similar magnitude as previously proposed by Rieke and Baylor (2000) for the thermal pigment isomerizations. The noise effect of large numbers of very small activations, on the other hand, would be negligible. The proportion and significance of free opsin in the intact retina is unknown, however. In the perfused, isolated cones the free *11-cis*-retinal may well have been continuously washed out, and this may not reflect the natural situation of these cells which are normally embedded in tissue and supplied with chromophore by the Müller cells.

3.5. Activity of phototransduction molecules downstream from the visual pigment

The enzymes participating phototransduction downstream from the visual pigment may also exhibit spontaneous activity (Baylor *et al.*, 1980). Missing at least the first stage of amplification, this activity produces electrical fluctuations different from fully amplified

photoresponses. However, it can regulate photoreceptor sensitivity in a manner analogous to light-adaptation if the level of activation is sufficiently high (Nikonov *et al.*, 2000). It may also hinder postsynaptic detection of small photoresponses if its variance is large compared to the quantal response. Rieke and Baylor (1996) found spontaneous phosphodiesterase activity to be the dominant source of the non-discrete component of noise in the light-sensitive current of dark-adapted toad rods. They concluded that a PDE-molecule activates independently of transducin on average once per hour, resulting in thousands of active PDE subunits at any given time in the amphibian rod OS. They also stated that a spontaneously activated and light-activated PDE have similar catalytic activity levels, although the molecular activation mechanism is different.

In rods, the spontaneous PDE activity sets the sensitivity of phototransduction (Baylor *et al.*, 1986) and is the dominating source of noise, other than photon-like events (Baylor *et al.*, 1984a). In mouse rods the variance of continuous noise is so high that the rod synapse has a threshold for passing signals that is estimated to reject not only continuous noise but also a substantial fraction of photon responses from the lower end of their amplitude distribution (Field and Rieke, 2002b).

Spontaneous PDE-activity is likely to play a role also in setting the sensitivity of many cone types. The different shapes of the frequency spectra of noise and single-photon response in macaque (Schneeweis and Schnapf, 1999) and turtle cones (Lamb and Simon, 1977) imply a significant role of continuous noise in these cells. Holcman and Korenbrot (2005) concluded that PDE-activity is the dominant source of the continuous noise also in bass cones, suggesting similar mechanisms underlying noise in rods and cones, although with varying proportions depending on the properties of the visual pigment.

4. Modeling phototransduction

Quantitative models of the photoreceptor light response provide a bridge between electrophysiological and biochemical data, at best connecting electrical photoresponse parameters to the underlying events at the molecular level. A complete model of a photoreceptor would span its function from the absorption of a photon to changes in the quantal release of the synaptic transmitter. Although most subprocesses in rod photoreceptor signaling have been modeled at least to some extent, there is yet no comprehensive "model photoreceptor" combining all processes into one functional entity. Still, existing models of the individual processes are informational for interpreting the electrophysiological data and suggesting new hypotheses and experiments.

The early photoresponse models were based on a top-down approach similar to the highly successful Hodgkin and Huxley (1952) model on the origins of the action potential in the squid giant axon. In these phenomenological models the flash responses were described with a series of linear filters with parameters suitable for producing the observed response, without specific knowledge on the underlying biochemical events. This kind of modeling has provided predictions for subsequent research, but is no longer informative for comparing phototransduction of different cell types on the molecular level. On the other hand, the "Poisson" and "independent activation" models of Baylor *et al.* (1974) are still useful as general descriptors of the flash photoresponse shape and the latter was used here to probe the general quality of the mammalian ERG data (paper I).

Photoresponse models with different levels of detail and coverage were needed in this thesis. Balancing between minimizing the level of detail and maximizing the explanatory power was necessary, as the experimental conditions involved were intrinsically complex and involved multiple unknown variables. The model selections included phenomenological descriptions of response-intensity functions (all papers), an exact quantitative model for the response activation phase (papers II and III), a single parameter model for the response recovery (paper III), and a simplified flash response model adapted to linear range photoresponses (paper I).

4.1 Flash response amplitude: stimulus-response functions

A photoreceptor's flash responses to sufficiently weak stimuli scale linearly with flash strength, suggesting no interference between individual quantal responses within the outer segment. With stronger stimuli, the intensity-response relation gradually saturates, due to local saturation of the phototransduction mechanisms, and finally full closure of all CNG channels. The relation between the fractional photoresponse amplitude R/R_{sat} and stimulus intensity is often described with the Michaelis relation originally applied to visual cells by Naka and Rushton (1966) and to vertebrate photoreceptors by Baylor and Fuortes (1970):

$$\frac{R}{R_{sat}} = \frac{I}{I + I_{½}}, \qquad (4.1.)$$

in which $I_{½}$ depicts the half-saturating flash intensity, and is inversely proportional to the sensitivity parameter S_f (see below). The Michaelis-relation is an empirical description, but if interpreted mechanistically, it is consistent with the assumption of unrestricted spatial spread of the internal transmitter of phototransduction. It has been successfully applied to rods and cones of "cold-blooded" species (*e.g.* turtle cones: Baylor *et al.* 1974; toad rods: Baylor *et al.* 1979a).

The mechanistic implication of the Michaelis relation is not really valid in any photoreceptors, as the spread of the photoresponses has been shown to be restricted to a small fraction of the outer segment (Lamb *et al.*, 1981). In such a case, when the local excitation is near saturation, the response-intensity relation is better described by the exponential relation

$$\frac{R}{R_{sat}} = 1 - e^{-S_f I}, \qquad (4.2.)$$

which has been found to apply well to dark-adapted mammalian rods (monkey rods: Baylor et al. 1984a; guinea pig: Matthews, 1991; rat: Robinson et al. 1993; human, Kraft et al. 1993). Forti *et al*. (1989) point out that the assumption of locally restricted responses is expected to break down in light-adapted rods, in which the responses to photons absorbed at distinct sites in the OS continuously overlap spatially.

For small stimuli and sufficiently large $I_{½}$, both response-intensity relations (4.1.) and (4.2) are linear. They yield comparable sensitivity parameters $I_{½}^{-1}$ and S_f for the fraction of light-sensitive current extinguished by a single absorbed photon ("derived single photon response").

4.2. Models of phototransduction

4.2.1. Activation only -model

The activation model of Lamb and Pugh (1992) is the most widely applied photoresponse model. It has found routine use in photoreceptor research, ranging from single cell current recordings to mass potential (ERG) recordings in the clinical settings. The model is based on the known diffusion rate of activated visual pigment in the OS disk membrane, followed by the series of enzymatic activation reactions leading to closure of the cGMP-gated channel in the OS membrane. The deactivation reactions, as well as the feedback mechanisms involved in regulating the photoresponse are ignored, with the consequence that the model is applicable only at the initial stages of the real light response. The model assumes that ϕ visual pigment molecules are fully activated with a small delay t_d after a brief flash administered at time t=0 (also subsequent reaction steps with very short time constants are all considered as simple delays included in t_d). The following enzymatic reactions are then quantified, resulting into an analytical expression for the rising phase of the photoresponse. The response form, a delayed Gaussian, accounts for the initial phases of the fractional flash responses R_f throughout a photoreceptor's functional range with only one parameter:

$$R_f = e^{-\frac{1}{2}A\phi(t-t_d)^2} . \quad (4.3.)$$

This "amplification factor", A, can be directly connected to the underlying molecular reactions:

$$A = \nu_{RP}\beta_{sub}n, . \quad (4.4.)$$

ν_{RP} is the rate of activation of PDE subunits by a single R*, β_{sub} the catalytic efficiency of a single activated PDE subunit and n is the co-operativity index of the cGMP-gated channel opening. Specifically, other parameters remaining equal, β_{sub} scales with the inverse of the outer segment volume $\beta_{sub} \sim 1 / V_{cyto}$.

The amplification factor was initially presented by Lamb and Pugh in its inverse square form, $A^{-½}$, as the characteristic time constant of the photoresponse activation. This is a useful notation: the time required for a photoresponse to reach its peak reflects the balance between activation and inactivation reactions, while the time for the initial activation phase of the response to reach a criterion level reflects purely the activation kinetics.

The amplification factor can be combined with other parameters of the photoresponse including time-to-peak, sensitivity and dominant time constant of recovery (Pepperberg *et al*., 1992) for a comprehensive description of photoreceptor function in a given state. It can also be used to quantify photoreceptor function in ERG studies from live subjects, when only the initial portion of the photoreceptor light response is visible in the signal due to interference from the rest of the retinal network (*Fig. 4 B)*.

4.2.2. Models covering the whole flash response

The details of phototransduction are understood at a highly quantitative level, and several models covering the full photoresponse have been based on this knowledge (*e.g.* Forti *et al.* 1989; Nikonov *et al.*, 1998, 2000; Hamer *et al*., 2005; van Hateren, 2005). While the activation phase of the photoresponse can be described with a limited set of parameters (*Eq. 4.3.*), the number of factors underlying the full photoreceptor light response and its adaptation make unified model of phototransduction difficult to achieve. Nikonov *et al.* (1998) presented a model for dark-adapted salamander rod photoresponses, and extended it to cover much of the known features of light-adaptation in (2000). The most comprehensive model of rod phototransduction has been presented by Hamer *et al*., (2005). This model combines stochastic simulation of the initial activation and deactivation reactions involving the pigment molecules into deterministic equations describing the subsequent stages of phototransduction. The model captures many key features of phototransduction in both wild-type and specific knockout mouse rod models, and where it fails, it points to some remaining questions in the field of phototransduction. The model contains 46 parameters, with data obtained from varying species and methods. This kind of detailed modeling is quite informative for mapping the extent of existing knowledge on photoreceptor function, as well as testing hypothetical mechanisms. In practice, the key features of the existing models need to be selected and the level of abstraction adjusted to fit a specific experimental question at hand, such as in paper I (Ch. 7.4.2).

5. Rod-cone interactions and the mammalian electroretinogram

Previous chapters have discussed photoreceptors as independent units, suggesting their outputs represent "pixels" in a matrix analyzed further by the retinal network. However, in the intact retina each photoreceptor takes part in a network of lateral connections that affect its synaptic output. The two known electrical inputs to photoreceptors are the interphotoreceptor gap junctions through which voltage signals spread from one photoreceptor to another (Raviola and Gilula, 1973) and the inhibitory feedback from horizontal cells (Baylor and Fuortes 1971). In addition, photoreceptor function is regulated by neuromodulatory substances released in the inner retina, such as dopamine and melatonin (reviewed by Witkovsky, 2004 and Wiechmann and Summer, 2007, respectively). These substances tune the retinal circuitry and lateral connectivity both through a circadian clock and in response to changes in actual light levels.

Rods and rod pathways are generally said to take care of vision in dim lights, cone pathways taking over when the rods saturate under bright lights (in most vertebrates including humans, this is accompanied by a shift in the overall spectral sensitivity of vision, the Purkinje shift, following the change of dominant photoreceptor type). In reality, there is a more than 1.5 log-unit range of illumination levels where both rods and cones are responsive (Yang and Wu, 1989), the so-called mesopic range. A complex set of adjustments in the transmission pathways, reviewed by Krizaj (2000), serves to sharpen the transition between rod- and cone-dominated state. Transmission of cone signals is suppressed in the dark-adapted retina and released from suppression upon sufficient light-adaptation. This is apparent also at the behavioral level. For example threshold for human cone-mediated flicker detection is lowered by light-induced suppression of rods (Goldberg *et al.*, 1983; Frumkes *et al.*, 1983). Rod-originated signals, on the other hand, are suppressed even at light levels where the rod photoreceptors are not yet saturated.

5.1. Mammalian cone-mediated electroretinogram under background illumination

In several mammalian species, both a- and b-waves of the cone-driven ERG grow significantly during first minutes after exposure to a background light after a dark-adapted state (Burian 1954; Armington and Biersdorf 1958; Gouras and McKay, 1989; Peachey *et al.*, 1993). The growth is still seen in the photoreceptor responses when the glutamate-mediated transmission to second and higher-order retinal neurons is blocked, and has thus been thought to originate in the cones themselves (Bui and Fortune, 2006).

However, some evidence suggests rods are involved in the cone signal enhancement. First, the phenomenon is absent or altered in rodless mouse models (Tanikawa *et al.,* 2004, Cameron and Lucas, 2009). Second, rods are known to tonically inhibit cone-mediated signaling in the dark-adapted retina (*e.g.* Goldberg *et al.*, 1983; Arden and Frumkes, 1986; Peachey *et al.*, 1991), although this may be entirely associated with postreceptoral mechanisms and not related to photoreceptor function as such. Thirdly, in human multifocal ERG, where only a subregion of the retina is stimulated at the time, the growth in both a-

and b-waves is maximal in the rod-dominated peripheral retina (Kondo *et al.,* 1999).

The precise origin of the light-induced enhancement in the cone-driven ERG is not known, and its relation to the light adaptation of the cone phototransduction or to other intrinsic cone function is not clear. In this thesis, modulation of direct electrical coupling to rods is studied as a possible alternative explanation. The present work (Paper IV) investigates the role of an external regulatory input in the light-induced enhancement of cone light responses. A hypothetical relation to rod function is investigated by blocking gap junctions between the photoreceptors, in addition to the transmission over the glutamatergic synapses. Other regulatory inputs to the cones require functional synaptic transfer from photoreceptors to bipolar and horizontal cells and are not discussed further.

5.2. Electrical coupling between rods and cones

In most vertebrate species, both rods (Schwartz, 1975a) and cones (Baylor, 1971) generate photoresponses to stimuli spanning larger retinal areas than their own collection area. This is because they are electrically coupled to neighboring photoreceptors via gap junctions (Raviola and Gilula, 1973), small pores composed of proteins of the connexin family.

Direct electrical connections exist also between neighboring rods and cones. Rod-cone coupling is relatively weak in amphibian and reptilian retinas, in which cone signals are mediated through a few "gatepost" rods to the rod pathway (toad: Attwell *et al.*, 1984; clawed frog: Krizaj *et al.*, 1998; turtle: Schwartz, 1975b, Firsov and Green, 1998). In mammals the rod-cone coupling is thought to be extensive, presumably allowing the rods to connect directly into the ON and OFF cone bipolar cells independently of the primary rod bipolar - AII amacrine cell pathway (macaque and rabbit: Raviola and Gilula, 1973; cat: Nelson, 1977; macaque: Schneeweis and Schnapf, 1999; mouse: Tsukamoto *et al.,* 2001). This relatively insensitive but fast alternate pathway would transmit rod responses to stimuli intense enough to saturate the highly sensitive rod bipolar cells, yet not intense enough to produce very large photoresponses in cones (Schneeweis and Schnapf, 1999). In salamander and clawed frog, the rod-cone coupling has been reported to increase under weak background light as well as with addition of dopamine, a transmitter released by the neurons in the inner retina to mediate overall retinal light-adaptation (Yang and Wu, 1989; Krizaj *et al.*, 1998). Dopamine is also an agent that mediates the circadian rhythm, allowing rods access to cone pathways at night and closing it during the day (Wang and Mangel, 1996; Ribelayga *et al.*, 2008). While it is not presently known whether there are differences between species in this regard, there are examples of both coupling and uncoupling of retinal gap-junctions with light (reviewed by Bloomfield and Völgyi, 2009). Also the significance of gap junctions in exchanging messenger compounds between rods and cones is unclear. Thus both electrical and chemical coupling via gap junctions form a hypothetical route for rod-mediated depression of cone photoresponses in darkness.

6. Aims of the study

Pharmacologically isolated photoreceptor flash responses were recorded by transretinal electroretinogram (ERG) from rods and cones of model mammal and amphibian species. They were studied in different states of light-dark adaptation and at different temperatures to address the following questions:

1. Is rod phototransduction in mammals intrinsically different from that of poikilotherms? (papers I, III, and IV**)**
Much of what is known about phototransduction mechanisms originates from studies of poikilothermic animals. The different sensitivity, kinetics and apparently limited capability of mammalian rods to light-adapt suggest that mammalian rods may be intrinsically different from rods of "lower" vertebrates. Toad, frog and rat rod photoresponses (paper I; paper II) were studied at different temperatures to analyze the effect of temperature differences on phototransduction in these cells. A simplified physiological model was applied to parametrize the linear-range flash responses and to resolve the effects in terms of initial molecular amplification and deactivation and of intrinsic PDE-activity. The goal of the work was to determine to what extent differences in the flash responses and adaptation properties of mammalian and amphibian rods could be explained on the basis of temperature and outer segment dimensions.

2. What is the role of molecular amplification in the sensitivity differences of rods and cones? (paper II and III**)**
Current data about the molecular amplification during the pure activation phase of rod and cone photoresponses is ambiguous, and the contribution of this factor to rod-cone differences is not clear. Rod and cone responses of rodent (mouse, rat) and amphibian (toad, frog) models were studied under the same experimental protocols and recording geometries to address this question. The purpose was to find how much differences in the initial molecular amplification rate, as determined by the model of Lamb and Pugh (1992), contribute to the sensitivity differences of rods and cones within a species.

3. Do thermal isomerizations set the "dark" state of adaptation and sensitivity of L-cones using the A1 chromophore? (paper II**)**
The long-standing hypothesis that long-wavelength sensitive cones may be in a "light-adapted" state even in darkness due to a "dark light" from thermal activation of their visual pigments has been challenged by recent evidence indicating that some L-cone pigments are much more stable than was previously thought. The direct evidence of significant adaptation due to thermal isomerizations comes from salamander cones with a large proportion of A2 chromophore. In this study the threshold for desensitizing effects of real light (hypothetically titrating an intrinsic "dark light") was probed at different temperatures to obtain upper-bound estimates for the rate of thermal pigment activations in A1 L-cones of frog. The purpose was to find out the role of thermal activation of A1 L-cone pigment in setting the sensitivity of the cells.

4. What is the mechanism underlying the light-induced growth in the a-wave of the mammalian cone electroretinogram? (paper IV**)**

The cone-driven flash responses of human and murine ERG initially grow under weak background illumination. It is not known whether this growth is intrinsic to cones and related to their phototransduction process. Cone flash responses were studied in the dark-adapted state, followed by weak to moderate background lights or rod-saturating flash trains to find how this enhancement is related to rod saturation and cone desensitization. The purpose was to clarify the role of rod-cone coupling in the cone response enhancement, and to find indications about the mechanism behind the cone enhancement. This analysis also served aim (5).

5. Is the transretinal electroretinogram useful as a tool for studies of light-adaptation in wild-type mouse cones? (papers III and IV)

The mouse is a central experimental model also in vision research, but the function of mouse cones is difficult to study. Cones represent only a small fraction (3%) of photoreceptors in the wild-type mouse retina and are not very resilient to single-cell recordings. The cones of transgenic "rodless" mice models, on the other hand, may present distorted physiological properties. The goal in this study was to quantify background-induced changes in sensitivity and kinetics of mouse cones and compare them to existing data from transgenic mouse models and other mammals.

7. Materials and methods

The experimental methods are described in detail in the original papers. This chapter concentrates on clarifying selected central points in the methodology with emphasis on the author's own contribution.

In brief, the main body of data was obtained with transretinal ERG (Ch. 7.2.1.-2.) from isolated retinas of selected amphibian and mammalian species (Ch. 7.1.). The photoreceptor component of the retinal response to brief flashes of light was isolated by pharmacological means. The flash responses of selected photoreceptor types were distinguished from the combined photoreceptor response based on their known spectral and physiological properties (Ch. 7.2.3.). Photoresponses were studied at several temperatures in the dark-adapted state and under steady adapting background lights. Special attention was paid to calibrating the stimulus intensities both in terms of photon flux incident on the retina and photoisomerization rates in individual photoreceptor types (Ch. 7.3.). Standard and novel parameters were extracted from the dark- and light-adapted photoresponse data (Ch. 7.4.) to quantify the sensitivity, molecular amplification and intrinsic activity levels of the photoreceptors, as well as the deactivation kinetics of the light responses.

7.1. The model photoreceptors

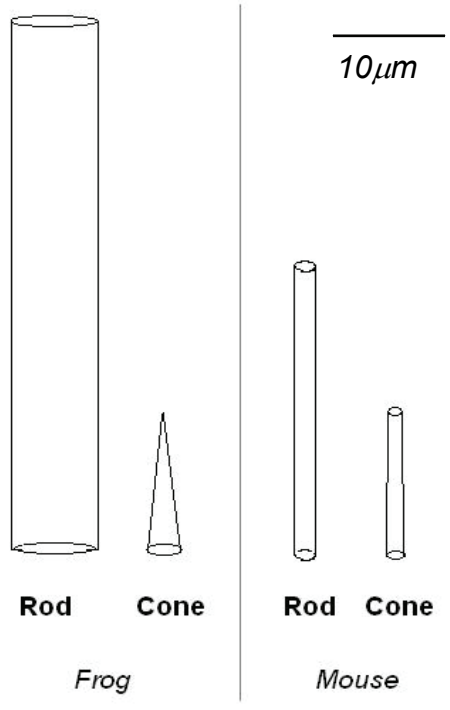

Figure 9. The relative sizes of the rod and cone outer segments of the common frog and the mouse.

Two amphibian and two rodent species were used in the study. Experiments on adaptation at different temperatures were performed with rat, toad and frog rods (papers I and II) and long wavelength sensitive (L-)cones of the frog (paper II). Rod and cone flash responses were also characterized in rods and middle-wavelength sensitive (M-) cones of mouse (paper III). Mammalian ERG in the mesopic region was studied in the mouse retina (paper IV).

Amphibian photoreceptors

The amphibian (poikilotherm) data was collected from frog (*Rana temporaria*) and toad (*Bufo bufo*). The (red) rods and (red-sensitive) cones of these species are prototypical "textbook examples" morphologically and size-wise in these species and thus supply a good "baseline model" for the rod and cone function (*Fig.* 9). The rods have long (>40μm) and sturdy (diameter 6-7μm), cylindrical outer segments. In contrast, the cone outer segments are conical and small: their volume is only 1/70 of the rod OS and membrane folds open to the extracellular environment along

the whole length. Thus any difference stemming from differences in morphology and size between rods and cones should be evident in these species. Both species are also naturally active at the large range of temperatures (3 - 25 ^{O}C) studied, and are thus ideally suited for probing photoreceptor function with temperature.

The common frog has two types of cones (S- and L-) and two types of rods ("green" and "red", the latter resembling more the mammalian rods). The signals generated by the "red" rods (λ_{max} = 502 nm) and L-cones (λ_{max} = 562 nm) can be readily distinguished in the electroretinogram (Donner *et al.,* 1998). Their prevalence in the retina highly exceeds that of the short wavelength sensitive cells (S-cones and green rods), favoring their contribution to the ERG. Also due to the different absorption spectra of short- and long wavelength sensitive pigments, S-cones are stimulated negligibly by the wavelengths used in the present work (643 nm for cones, 519 nm for rods).

Mammalian photoreceptors
Rat and mouse retinas are both commonly used as models in vision research. These nocturnal animals have rod-dominated retinas: cones carrying a mixture of short- and long wavelength sensitive visual pigments (Szel and Rochlich, 1992, Applebury et al., 2000) comprise only 3% of the photoreceptors (Carter-Dawson and LaVail, 1979). Like most mammalian cones, murine cones do not bear the traditional hallmarks of cone-like appearance but their outer segments are instead only slightly conically tapered, and volume ¼ of the rods' (*Fig.* 9; Carter-Dawson and LaVail, 1979). The selection of mouse as a model for mammalian cone function, despite significant differences between the nocturnal and diurnal retinas of mouse and human, was motivated by the emergence of mouse as the most important animal model for transgenic research on physiology and disease.

7.2. Obtaining photoreceptor flash responses

The electroretinogram signal is a response generated by the extracellular radial current components across the retina, weighed by the local resistance profile. Any given component is an average of signals generated by a large number of cells, which reduces variability and the need for averaging over trials. The function of multiple cells can be monitored simultaneously, as far as the components of the recorded signal can be traced to individual sinks and sources and their relation to specific cell types.

7.2.1. Transretinal electroretinogram (ERG)

The electroretinogram signal was recorded with Ag/AgCl pellet electrodes on each side of an isolated retina. After detaching the retina from the eyecup, it was placed distal (photoreceptor) side up in a closed recording chamber on a dome covered with porous filter paper. Throughout the experiment, the retina was superfused on the photoreceptor side with physiological saline solution containing salts and nutrients essential for cell function. Homogenous full-field light stimuli with controlled intensity, wavelength and duration were brought to the retina with a light cable from separate light sources (tungsten lamp in (papers I and II), flash gun (paper III) or laser (papers III and IV). Two separately controlled stimulus channels enabled paired-flash stimulation with lights of different spectral

compositions, as well as independent control of flash stimuli and steady adapting background lights.

7.2.2. Pharmacological isolation of photoreceptor flash responses

Chemical synapses

The photoreceptor flash responses were isolated by blocking the glutamate-mediated synaptic transmission from photoreceptors to bipolar and horizontal cells.

In the amphibian retinas, as well as most of the work on mammalian retinas, it was sufficient to use the glutamate analog aspartate to non-selectively block all glutamatergic transmission. In the light-adaptation study on mouse cones (paper IV), more specific glutamate receptor blockers were used:

- DL-AP4 (DL-2-amino-4-phosphonobutyric acid, Slaughter and Miller, 1981) at 20-80 μM to block the sign-reversing synapses from photoreceptor to ON-bipolar cells (based on metabotropic glutamate receptors). This removed the b-wave from the ERG flash response, but left the initial negative phase (a-wave) intact (*Fig.* 10 A).

- NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione) was used at 10 - 40μM to block AMPA and kainate ionotropic glutamate receptors (Yu and Miller, 1995), which should remove contributions from horizontal and OFF-bipolar cells. This led to a loss of amplitude compared to the DL-AP4 -isolated responses and removed much of the positive overshoot at the end of the responses (Fig 10 B).

- Subsequent addition of a blocker of NMDA receptors, the third major class of ionotropic glutamate receptors, (10 μM D-AP5, D-amino-5-phosphonovalerate, Coleman and Miller, 1988) did not further affect the obtained cone flash response. This was to be expected, as the NMDA receptors reside on ganglion and amacrine cells (Yang, 2004), i.e. at more proximal synapses and thus should not affect flash responses after blockade of the photoreceptor synapses.

Glial contribution to the electroretinogram

Blocking synaptic transmission to higher-order retinal neurons reveals a negative extension of the a-wave, named PIII by Granit (1933). It consists of two main components, one of which is the photoreceptor response. The rest is of non-neural origin, generated by the Müller glial cells (Bolnick *et al.*, 1979). The glial component, "slow PIII", has the same polarity as the photoreceptor light response and results from the Müller cells buffering light-induced changes of $[K^+]$ in the extracellular space. Ba^{2+} inhibits the slow PIII by blocking influx of K^+ to the Müller cells (*Fig.* 11). However, adding BaCl to the solution on the photoreceptor side of the retina will also block K^+ currents in the photoreceptor inner segments, thus affecting photoresponses. In the present work, BaCl (10 mM) was therefore added on the proximal side of the retina, and the efficient perfusion on the photoreceptor side served as a sink for barium, minimizing the effects on photoreceptors.

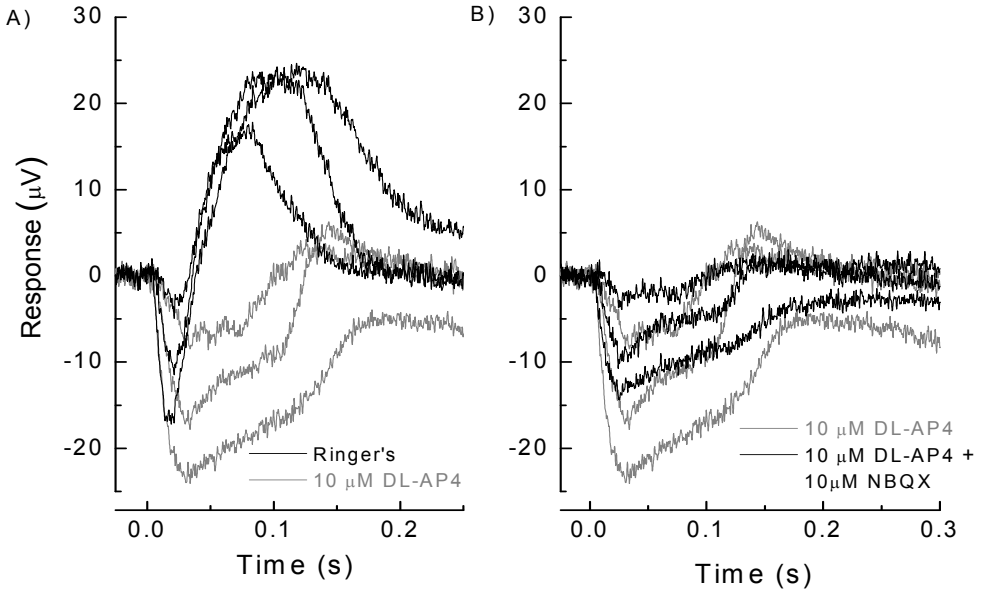

Figure 10. Pharmacological isolation of mouse cone flash responses. A) Transretinal ERG responses of dark-adapted mouse cones to brief flashes of light presented at three intensities before (black) and after (grey) blocking transmission from cones to ON-bipolars with DL-AP4). B) The dark-adapted cone responses from the same retina to the same stimuli before (gray, same traces as in A) and after (black) subsequent addition of NBQX to block AMPA/kainate receptors (glutamatergic transmission from cones to OFF-bipolars and horizontal cells).

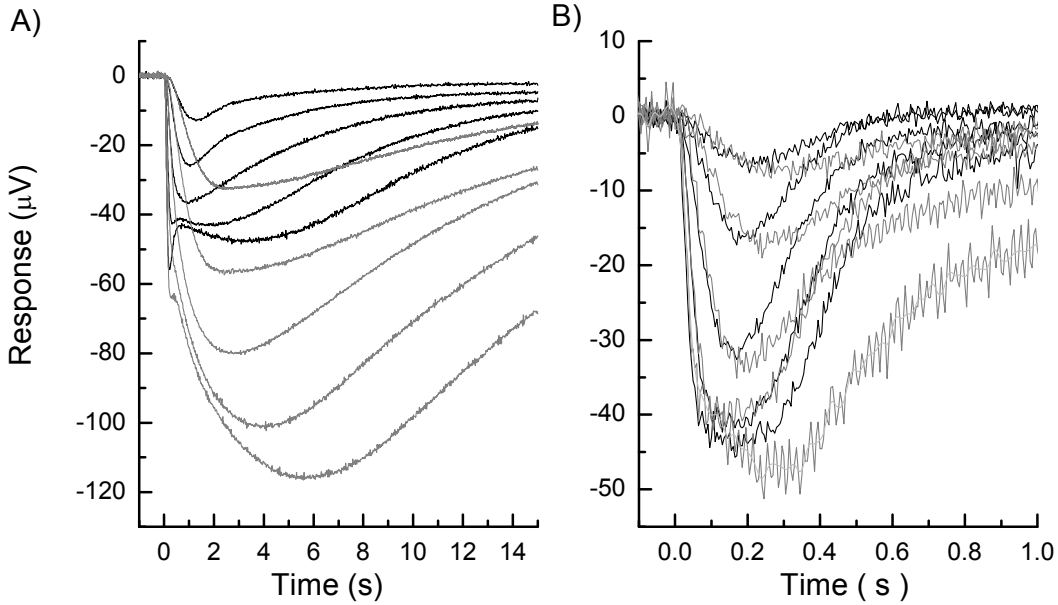

Figure 11 Removal of glial contributions (slow PIII) to the transretinal ERG flash responses of A) rat rods and B) frog L-cones by addition of 10mM BaCl in the subretinal space of the specimen holder. Grey traces represent responses in the absence of BaCl, black traces with BaCl added. All response families were recorded in the dark-adapted state.

Electrical synapses
Three structurally unrelated substances were used to block gap junctions in paper III: Carbenoxolone (100 μM), Meclofenamic acid (MFA, 50μM) and Octanol (1 mM). Repeating the experiments with different compounds was chosen to avoid uncertainties due to the generally non-specific nature of gap junction blockers (Rozental *et al.,* 2001).

7.2.3. Isolation of the cone and rod flash responses

The cone component can be separated from the mixed photoreceptor light response with three methods, based on the different spectral absorption, sensitivity and kinetics of individual photoreceptor types. The resulting cone component may still be a mixture of responses from several cone types, depending on the retina and stimulus. In the present work, the desired cone types (L-cones in the frog and M-cones in mouse) were stimulated selectively with red and green flashes that excited S-cone pigments (blue sensitive in frog and UV-pigment in mouse) negligibly.

1) *Spectral subtraction* (*Fig*. 12 A., Donner *et al*., 1998; paper II). This method is based on (and requires) different absorption spectra of rods and cones, univariance of the photoresponse regarding the wavelength of absorbed photons, and linear summation of different components. When applicable, it is the best approach for obtaining cone photoresponses without significantly affecting their state of adaptation. However, it is time-consuming and sensitive to exact matching of rod- and cone-equivalent stimuli at two different wavelengths. Further, it cannot be applied to rods and cones with very similar absorption spectra, as in mouse.

2) *2-flash technique* (*Fig.* 12 B), *e.g.* Koskelainen et al. (1994), papers III and IV). When the absorption spectra of rods and cones are similar, as in rodents, cone responses can be isolated based on the different sensitivities and response kinetics of rods and cones. After a strong flash, rods remain saturated much longer than cones. Due to their lower sensitivity and faster deactivation, cone flash responses recover fully while the rods are still saturated. Thus a second “test” flash shortly after the rod-saturating pre-flash generates electrical photoresponse only in cones. This method is sensitive to the flash interval: too short intervals produce partly light-adapted cone responses, while too long intervals produce responses contaminated by partially recovered rod responses.

3) *Rod-saturating steady adapting background light.* This method is not explicitly used in the present work as it does not allow the study of truly dark-adapted cones.

Verdon *et al.* (2003) have compared the three cone-isolation techniques in the human ERG. At low stimulus energies, they find the subtraction method and the paired-flash –method to converge on dark-adapted cone responses. At high stimulus energies they note the failure of a simple subtraction method due to the excitation of cones also by the photopically less effective stimulus. This has been accounted for in the present work (paper II). They also note slight differences in the postreceptoral contributions to the ERG, which are of little importance in the present work. Rod-saturating background is found the most problematic method for approximating the dark-adapted cone-driven flash responses, as the responses are changed both by enhancement of the a- and b-waves and by desensitization due to the background.

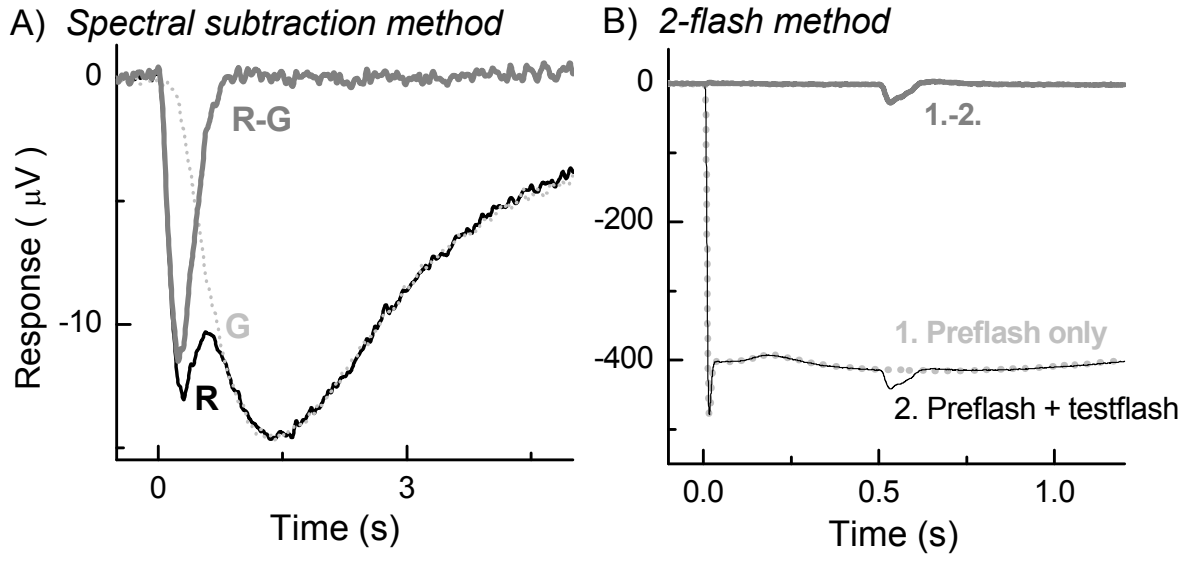

Figure 12. The methods 1) and 2) for isolating the cone flash response in transretinal ERG. A) Recordings from a dark-adapted frog retina. A response to a red flash (R) stimulating both rods and red-sensitive cones (black trace) and a response to a green flash (G) chosen to stimulate rods equally (dotted trace). The cone flash response is obtained by subtraction R-G. With higher stimulus intensities, G also elicits a small cone response component that is lost in the subtraction. This is compensated by addition of an isolated cone photoresponse to a dim red flash chosen to match G in cone stimulating efficiency. B) Recordings from a dark-adapted mouse retina. The response to the test flash (at 2) is superimposed on the response to a rod-saturating preflash (shown without test flash by trace 1). The inter-flash interval is sufficient for allowing the cones to recover from the preflash, thus the cone flash response is obtained by subtracting the response to preflash only from the response to the flash pair (1.-2.).

7.3. From incident photons to evoked photoisomerizations

To compare phototransduction in different photoreceptors, differences in their photon catch have to be factored out, i.e. one must estimate the stimulus intensities in terms of activated pigments molecules. This enables estimation of the unit response and the amplification of the phototransduction process.

7.3.1. Rods

Collection area

In rods, the conversion is straightforward. When light arrives from the distal side of the retina and travels axially through the cylindrical outer segments, the number of pigments (Rh*) activated by a stimulus (photons/μm^2) is given by multiplying it with the effective collection area of the cell (Baylor *et al.*, 1979b)

$$a_c(\lambda) = \frac{\pi d^2}{4}\left[1 - 10^{-\Delta D(\lambda)\cdot l}\right]\gamma, \qquad (7.1.)$$

in which d and l are the diameter and length of the outer segments and γ is the quantum efficiency of photoisomerization, i.e. the probability that an isomerization activates the pigment molecule. $\Delta D(\lambda)$ is the specific optical density of the outer segment at wavelength λ. For ease of comparison, the collection areas are usually expressed for λ_{max}, the wavelength of maximal absorbance, and converted to the wavelength used for stimulus as needed with empirically determined templates (*e.g.* Govardovskii *et al.,* 2000).

Specific absorbance

The estimates found in the literature for specific optical density of rat rods appeared unrealistically low (*e.g.* 0.01 μm^{-1} by Penn and Hagins, 1972 and 0.0085 μm^{-1} by Robinson *et al*., 1993), when compared with typical values reported for photoreceptors of other species, such as 0.016 μm^{-1} for the toad Bufo Marinus (Hárosi, 1975). This may be due to experimental limitations in measuring absorbance in the small and slender mammalian rod outer segments. After unsuccessfully trying microspectrophotometric (MSP) measurements from individual outer segments, direct measurements of absorption by rods in the intact rat retina were devised to resolve the issue.

The absorbance measurement is described in detail by Nymark (2009). In brief, transmission of dim 501 nm light pulses through the central region of the flat mounted rat retina was compared before and after bleaching about 99.7 % of the visual pigment. Regeneration of the visual pigments was prevented by addition of hydrozylamine to the physiological saline. Specific absorbance within the rod outer segments was then calculated from the measured difference in optical density between dark-adapted and bleached states, assuming tight hexagonal packing of the cylindrical outer segments in the retina and neglecting the small fraction of cones. The value obtained, 0.0159 μm^{-1} agreed well with the specific absorbance in amphibians (see above), and was used for calculation of photoisomerizations in the mammalian photoreceptors in this study.

7.3.2. Cones

In the intact eye, light approaches the photoreceptors from the proximal side, through the inner segments. They collect axially incoming light from an area larger than the outer segment. Uncertainties due to light-collecting properties were avoided in the current recording geometry, where light enters from the distal side. Yet the conversion of intensities to isomerised visual pigments (P*) in cones is somewhat more complicated in cones, for which the detailed conversion is presented below.

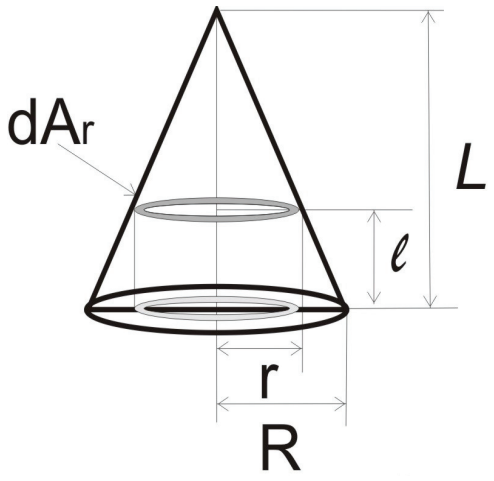

Figure 13. The collection area of frog cones was estimated by integrating the absorption into concentric differential elements.

Frog

L-cones of the frog *Rana temporaria* are almost perfectly cone-shaped (Donner *et al.,* 1998). Their collection area was calculated by

integrating absorption into concentric differential elements with area dA_r and height $l(r)$ (see Fig. 13):

$$a_c(\lambda) = \int_{A_r(0)}^{A_r(R)} \left[1 - 10^{-\Delta D(\lambda)\cdot l(r)}\right] \gamma \, dA_r \qquad (7.2.)$$

Mouse
Unlike frog cones, murine cones taper only slightly into a conical shape and can be approximated as cylinders with equal volume. On the other hand, conversion from photons to photoisomerizations is complicated by the coexpression of two visual pigments in the mouse cones (Applebury *et al.,* 2000).

Mouse cones contain a mixture of UV-sensitive ("S-") and green sensitive ("M-") visual pigments (Applebury *et al.*, 2000), which both contribute to their photoresponses (Nikonov *et al*., 2006). The present study concerns only photoresponses driven by the M-pigment, and the UV-pigment absorbs the stimulating green light only negligibly. Thus an estimate for a_c based on an average fraction of 35 % M-pigment in cones in the central retina (the active area under recording) was made based on the work of Applebury *et al.* (2000).

Additionally, the small and scarce cones are screened by twice as long surrounding rod outer segments (Carter-Dawson and LaVail, 1979). In our recording geometry, unlike the situation in the intact eye, there is considerable attenuation of incident light due to absorption in rods before the light reaches the cones. It was estimated that the incident light first has to pass one half of the absorbing layer of rod outer segments. This attenuation factor, 0.64 at λ_{max} of the rods, was included in calculations of the effective collection area for a single cone.

7.4. The flash response data

The photoreceptors were stimulated with brief (2 or 20 ms) full-field flashes, either in the dark-adapted state or superimposed on a steady background. In papers III and IV, the cone-stimulating flash was preceded by a rod-saturating pre-flash. The inter-stimulus intervals between flashes or flash pairs were chosen so that both rods and cones could fully recover from the flash-evoked activity before subsequent stimulation.

Photoreceptor function in any given state was characterized by recording flash responses to a number of stimulus intensities covering the dynamic range of the photoreceptor (*Fig.* 13). The response amplitudes of thus obtained flash response families were fitted with a linear combination of Michaelis-Menten and exponential relations (*Eqs. 4.1* and *4.2)*, weighed to give the best fit to the data regardless of the preparation, state of adaptation and temperature.

7.4.1. Flash response parameters

Several common parameters quantifying phototransduction were deduced from the flash responses (*Table* 2). The analysis concentrates on the amplitude vs. intensity data (*Fig.* 14)

and the kinetics of flash responses at the lowest 10-20% of the dynamic range. Photoresponses in this range scale linearly with stimulus strength, with constant kinetics. Linearity breaks down with stronger stimuli. Response kinetics becomes intensity-dependent due to increased Ca^{2+} feedback and amplitude compression. Finally, with stimuli intense enough to temporarily close all the CNG-channels, the response amplitude saturates. Then increasing the stimulus intensity further only prolongs saturation.

Table 2. The parameters used to characterize flash photoresponses.

Parameter	***Significance***	***Method of determination***
Saturated amplitude, U_{sat}	*Reflects the light-sensitive current at steady state and thus the dynamic range of the photoresponses.*	*The amplitude of the saturated flash response.*
Flash sensitivity, S	*Amplitude of the single photon response. The term is also used more generally for the amplitude of photoreceptor responses to brief linear-range flashes, divided by flash intensity.*	*In the present work, by interpolation from the response vs. intensity data, based on the linearity of the small stimulus responses (eqs. 4.1. and 4.2.).*
Fractional sensitivity, S_f	*The fraction of the light-sensitive current closed by one photo-isomerization, i.e. the fractional single photon response. Factors out the effect of compression and relates directly to the properties of the photo-transduction process in the outer segment.*	$S_f = S/U_{sat}$
Time to peak, t_p	*The time a photoresponse in the linear range takes to reach its maximum amplitude. A standard parameter describing the time scale of photoresponses.*	*From the linear range photoresponses.*
Integration time, t_i	*The time window during which the responses to two subsequent photoisomerizations add to each other. Separated by a longer interval, two absorbed photons will produce independent photoresponses.*	1. *From the integral of the single photon response, scales with time-to-peak when the response waveform remains unchanged (Baylor* et al.*, 1979a)* 2. *The time interval, during which an extension of stimulus duration increases the amplitude of the photoresponse in the linear range.*
Amplification factor, A	*A parameter measuring the molecular activation of phototransduction.*	*By fitting the model of photoreceptor activation (Eq. 3., Lamb and Pugh, 1992) to the flash response data.*
Dominant time constant of recovery, τ_{rec}	*The time constant of a postulated rate-limiting step of rod photoresponse recovery at saturating stimulus intensities.*	*By analyzing the delay in the photoresponse recovery induced by increasing the stimulus intensity at saturating stimulus levels.*

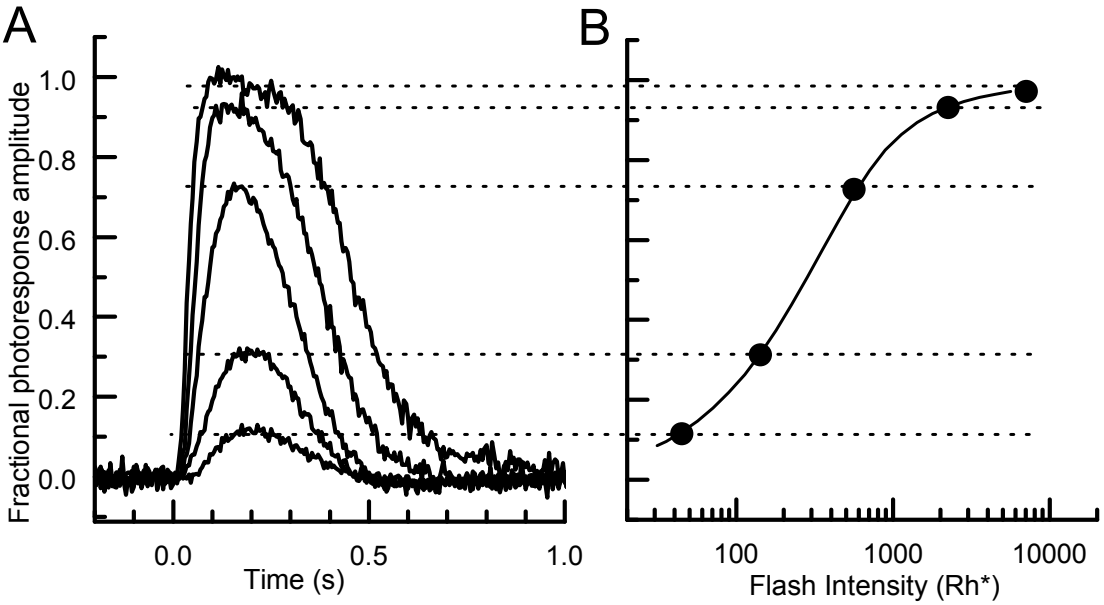

Figure 14. A) A family of dark-adapted, aspartate-isolated L-cone flash responses from an isolated frog retina. B) The corresponding stimulus-amplitude data (dots), fitted with the Michaelis-Menten relation (continuous line, Eq. 4.1).

7.4.2. Modeling the linear rod flash responses

Probing differences between mammalian and amphibian phototransduction with temperature (paper I) posed challenges for the analysis. A photoresponse model was needed to break down changes in the response sensitivity and kinetics into parameters that could be interpreted in relation to the underlying molecular machinery. This involved optimizing the level of complexity: enough detail had to be obtained with a minimum of adjustable parameters. A formulation first used by Fain *et al.* (2001) to demonstrate the qualitative features of phototransduction was chosen for the task. The model was based on the following assumptions and simplifications, through which the system of differential equations was reduced into one differential equation with a handful of parameters.

- Only the dark-adapted linear-range photoresponses were modeled to exclude most of the Ca^{2+}-dependent reactions associated with background adaptation and responses to intense flashes. The only Ca^{2+}-coupled feedback included was the accelerated cGMP synthesis during the light response.
- As the perturbations in the phototransduction machinery in response to the dim flashes in darkness could be assumed to be small
    - the reaction equations were linearized around the dark-adapted state, and

    - the catalytic efficiencies of the reaction participants were calculated based on the steady state concentrations of the substrates
- Calcium kinetics was assumed to be fast enough for $[Ca^{2+}]_i$ to follow the light sensitive current with a negligible delay.
- Calcium buffering by the outer segment was ignored.
- Deactivation reactions were modeled with exponential time course, without an initial delay. The stochastic nature of inactivating Rh* is thus neglected, which is reasonable when analyzing the average response of hundreds or thousands of rods as provided by the ERG signal.

The three last simplifications were highly useful for addressing the problem at hand, but also compromised the physiological validity of the model. The model was easily fitted to the dark-adapted linear-range photoresponses. However, a qualitative disagreement was met regarding the light-adapted flash responses, at least partly due to the assumption of immediate Ca^{2+} equilibrium. On the other hand, the more complete photoresponse model of Hamer *et al.* (2005) also fails to capture the qualitative features of light-adaptation in the linear range responses.

Changes in $[cGMP]_i$

The fractional current response r(t) results from the light-induced perturbation g(t) in the cGMP concentration of the outer segment, G(t), which is continuously hydrolyzed and synthesized:

$$\frac{dG(t)}{dt} = \alpha_0 + \alpha(t) - (\beta_0 + \beta(t)) \cdot G(t) \quad , \qquad (7.3)$$

Here α_0 and $\alpha(t)$ denote the basal rate and light-induced perturbation in cGMP synthesis and β_0 is the basal cGMP hydrolysis rate, supplemented by a transient light-induced PDE activity $\beta(t)$. cGMP synthesis is temporarily accelerated during the light-response due to the changes in $[Ca^{2+}]_i$. Linearized around the operating point:

$$\alpha(t) = a \cdot (\left[Ca^{2+}\right]_{i,0} - \left[Ca^{2+}\right]_i(t)) = a \cdot c(t), \qquad (7.4.)$$

where

$$a = \left. \frac{\partial \alpha(t)}{\partial \left[Ca^{2+}\right]_i(t)} \right|_{\left[Ca^{2+}\right]_{i,0}} \qquad (7.5.)$$

and c(t) is the change in $[Ca^{2+}]_i$ due to change in the light-sensitive current. As the basal activity in cGMP synthesis and hydrolysis in the steady state must be related by $\alpha_0 = G_0 \cdot \beta_0$, (7.3.) becomes

$$\frac{dG}{dt} = \beta_0 (G_0 - G(t)) + a \cdot c(t) - \beta(t) \cdot G(t) \qquad (7.3')$$

and the perturbation g(t) = G_0 – G(t) is

$$\frac{dg}{dt} + a \cdot c(t) + \beta_0 g(t) = \beta(t) \cdot G_0$$

, (7.3'')

Given that g(t) is sufficiently small for β(t) to operate on the initial concentration G_0.

Changes in $[Ca^{2+}]_i$

Ca^{2+} is carried into the OS through the CNG-channels, with Ca^{2+} -ions constituting fraction F_{Ca} of the light sensitive current flux J(t). It is extruded by the NCKX –exchangers with rate $K_{ex} \cdot [Ca^{2+}]_i$, so the total change of $[Ca^{2+}]_i$ is governed by:

$$\frac{d\left[Ca^{2+}\right]_i}{dt} = F_{Ca} \cdot J(t) - K_{ex} \cdot \left[Ca^{2+}\right]_i \quad (7.6.)$$

Before the flash-evoked activity, $F_{Ca} \cdot J_0 = K_{ex} \cdot [Ca^{2+}]_{i,0}$. Thus the flash-evoked perturbation in $[Ca^{2+}]_i$, c(t) = $[Ca^{2+}]_{i,0}$ - $[Ca^{2+}]_i$

$$\frac{dc(t)}{dt} + K_{ex} c(t) = F_{Ca} J_0 - F_{Ca} J(t) = F_{Ca} j(t) \quad (7.6'),$$

In terms of the current response, j(t) = J_0 – J(t).

The current response

The probability of the open state of the light-sensitive channels is co-operative with Hill constant n_{Hill}, and the current J(t) scales approximately with $G(t)^{n_{Hill}}$, given that G(t) is much smaller than K_D of the Hill equation. So that $J(t)/J_0 = G(t)^{n_{Hill}} / G_0^{n_{Hill}}$. The first two terms of Taylor's expansion for the light-evoked response j(t) = J_0-J(t) yield:

$$j(t) \approx \frac{n_{Hill} \cdot J_0}{G_0} g(t), \quad (7.7.)$$

which combined with (7.3'') becomes

$$\frac{dj}{dt} + ac(t) + \beta_0 j(t) = n_{Hill} \cdot J_0 \beta(t) \quad (7.8)$$

With the assumption of fast equilibrium of Ca^{2+} in the OS, the light-evoked deviation c(t) in $[Ca]_{in}$ (7.6' and 7.7) is

$$c(t) \approx \frac{n_{Hill} F_{Ca} J_0}{K_{ex} G_0} g(t). \quad (7.9.)$$

And (*Eq. 7.8*) becomes

$$\frac{dj}{dt} + a\,\frac{n_{Hill}\,F_{Ca}\,J_0}{K_{ex}\,G_0}\;j(t) + \beta_0\,j(t) = n_{Hill}\cdot J_0\beta(t)$$

. (7.8')

cGMP synthesis is related to $[Ca^{2+}]_i$ in a co-operative manner so that $\alpha = \alpha_0[Ca^{2+}]_i^{\,m_{Ca}} / [Ca^{2+}]_{i,0}^{\,m_{Ca}}$, so definition (7.5) becomes

$$a = \frac{m_{Ca}\alpha_0}{\left[Ca^{2+}\right]_{i,0}} = \frac{m_{Ca}\alpha_0 K_{ex}}{J_0 F_{Ca}} = \frac{m_{Ca}\beta_0 G_0 K_{ex}}{J_0 F_{Ca}}$$

, (7.11.)

and

$$\frac{dj}{dt} + m_{Ca}n_{Hill}\;\beta_0\,j(t) + \beta_0\,j(t) = n_{Hill}\cdot J_0\beta(t)$$

(7.8'')

The fractional flash response r(t) = j(t) / $J_{0,}$ *i.e.*

$$\frac{dr(t)}{dt} + \left(m_{Ca}n_{Hill} + 1\right)\beta_0 \cdot r(t) = n_{Hill}\beta(t)$$

(7.12.)

An analytical expression for the light-evoked phosphodiesterase activity β(t) derived by Lyubarsky et al. (1996) was applied to (7.12.) to obtain the time derivative for the small-stimulus flash response

$$\frac{dr(t)}{dt} = \left(n_{Hill}\cdot\nu_{RE}\cdot\beta_{sub}\cdot\frac{\tau_{R^*}\tau_{PDE^*}}{\tau_{R^*}-\tau_{PDE^*}}\cdot\max\left[0,\left(e^{-(t-t_d)/\tau_{R^*}} - e^{-(t-t_d)/\tau_{PDE^*}}\right)\right]\right)$$
$$-\left(m_{Ca}\cdot n_{Hill}+1\right)\beta_0\cdot r(t)$$

(7.13.)

Eq.(7.13.) was solved numerically and fitted to the estimated single photon responses, obtained from linear-range flash responses by dividing by the stimulus intensity (in Rh*) and the saturated amplitude.

The model contained altogether 7 parameters, four of which were allowed to vary freely while the rest were fixed with representative values obtained from literature. The model parameters are described in *Table 3*.

Table 3. The parameters used in the model for linear range flash responses of rods

Parameter	Unit	Allowed to vary? (value)	Physiological counterpart
$\nu_{RE}\beta_{sub}$	$1 / s^2$	Yes	Molecular amplification of the photoresponse at the disc membrane
β_{dark}	$1 / s$	Yes	The intrinsic PDE-activity
τ_1	s	Yes	The shorter of the apparent deactivation time constants for R* and PDE*
τ_2	s	No ($\tau_2 = 3.75\ \tau_1$)	The longer of the apparent deactivation time constants for R* and PDE*
n_{Hill}	-	No (2.5)	Hill's coefficient of cGMP binding to CNG-channels
m_{Ca}	-	No (2)	Hill's coefficient for the calcium dependence of cGMP synthesis
t_d	s	Yes	A delay lumping together the effect of several processes with short time constants in the activation of the phototransduction cascade

7.5. The experimental conditions

The isolated retina is a very stable preparation for electrophysiological recordings, as the photoreceptor responses do not show deterioration even during 10-12h recordings provided the experimental conditions are suitable. Modified Ringer's solutions tailored for amphibian or mammalian photoreceptors sustained stable functioning of most photoreceptors throughout the lengthy experiments on light-adaptation and temperature changes (papers I and II). Previous experimentation (Lamb, 1984; Donner *et al.*, 1990c) indicates that the lack of bicarbonate leads to slightly decreased sensitivity and slower response kinetics, but with sufficient general buffering capacity, the effect is comparatively modest. On the other hand, mammalian cone function turned out difficult to maintain stable in long adaptation experiments without CO_2(5%)/O_2 –bubbled bicarbonate-based solution. Additionally, a relatively high perfusion flow rate was found necessary to support stable cone function in these experiments. The change in the experimental conditions led to a significant acceleration of cone flash responses with *ca*. 25% decrease in the time-to-peak, but did not much affect cone sensitivity.

8. Results

8.1. Consequences of homeothermy

Dark- and light-adapted flash responses of rat, toad and frog rods were studied at temperature ranges 7-25 ^{o}C (toad and frog) and 5-36 ^{o}C (rat). The lower susceptibility of rat rods to light adaptation and their faster response kinetics were accounted for by their smaller OS size and higher physiological operating temperature. No outstanding differences in the functioning of the transduction molecules had to be assumed to explain the data.

8.1.1. Sensitivity and kinetics

The fractional sensitivity of all model rods was quite similar near their respective physiological operating temperatures, their derived fractional single-photon responses (SPR_f) comprising *ca*. 1-2 % of the saturated amplitude. However, in all three species, as well as in mouse rods (data not shown) the estimated SPR_f decreased monotonically with warming with Q_{10} of 3-4. Thus when recorded at the same temperature, the rodent rods were more sensitive than their amphibian counterparts.

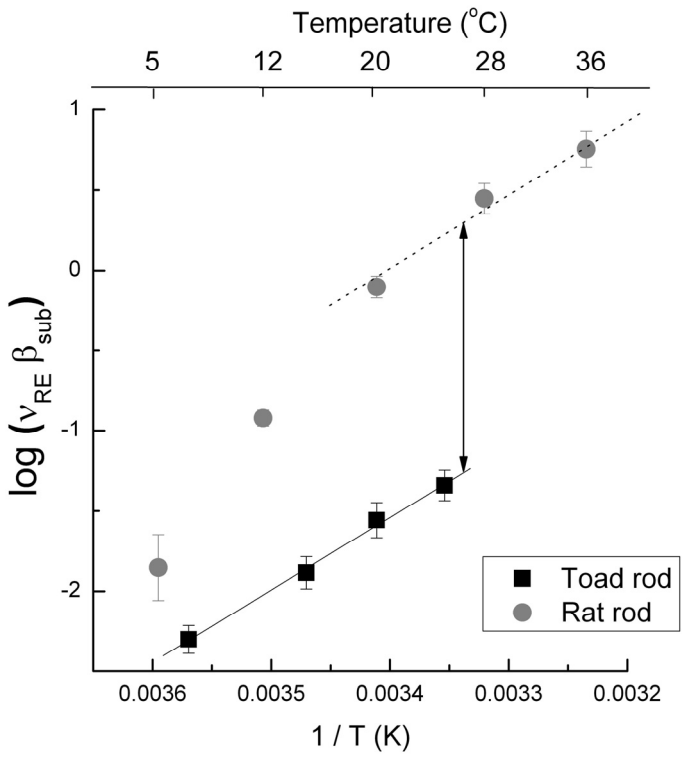

Figure 15. The activation parameter of toad (squares) and rat (circles) rod SPR_Fs on Arrhenius coordinates. A straight line fitted to the toad rod data also fits the upper range of the corresponding rat rod data when shifted 1.5 log-units vertically (values multiplied by 32-fold).

Response kinetics accelerated with warming, in accordance with Baylor *et al.* (1983), Lamb (1984) and Donner *et al.* (1988). Both activation and deactivation were accelerated approximately equally in the rod flash responses, and the time-to-peak in all species decreased with Q_{10} of *ca*. 2. The flash responses of rat and toad rods had broadly similar t_p when recorded at the same temperature, allowing rodent vision at mammalian body temperatures the benefit of significantly faster photo-responses: t_p in rodent rods was 100-150 ms near mammalian body temperature, while the flash responses of toad and frog rods peaked at *ca*. 1 s near 15^{O}C, a typical foraging temperature for these animals.

8.1.2. Small outer segment size provides high amplification in rat rods

By fitting the adopted phototransduction model (Ch. 7.4.2.) to the dark-adapted SPR_f data from rat and toad rods, respectively, the observed temperature dependence was resolved into hypothetical contributions of 3 principal parameters: molecular amplification, basal PDE-activity and deactivation time constant.

Despite the great simplifications of the phototransduction model, the obtained results were in reasonable agreement with comparable parameters obtained by other, more direct means (see paper I for detailed discussion). The largest deviation from the existing literature values was observed in the deactivation time constant, which is not surprising, given that the simplifying assumptions regarding pigment deactivation and calcium dynamics in the OS mainly affect late stages of the flash response.

All parameters exhibited clear monotonic trends with temperature: activation-related rate parameters increased with warming, while delays and time constants decreased (*Fig. 7* in paper I). All parameters behaved broadly similarly in rat and toad rods, except for the activation parameter $\nu_{RE}\beta_{sub}$. Log($\nu_{RE}\beta_{sub}$) is redrawn in *Fig. 15* as function of 1 / T(K), and the toad data has been fitted with a straight line. When transferred vertically by 1.5 log-units, the same relation describes well the three points at the warm end of the rat rod data. The two points below 12 ^{o}C fall below the trend, consistent with clear changes in the shape of the flash response observed in this region (*Fig 5* in paper I). Closer to the physiological temperature the flash responses are simply scaled in amplitude and time axis when warmed or cooled. The simplest interpretation for the 1.5 log-unit vertical shift on logarithmic coordinates, corresponding to a 32-fold difference in the parameter, presumably reflecting molecular amplification in rat and toad rods, would be that it comes from the 40-fold difference in their OS volumes. This factor alone would also suffice to explain the 5-6 fold sensitivity difference between the model cells, except for the lower end of the temperature range studied, where it must be coupled with retarded deactivation.

8.1.3. Light adaptation

The relatively modest capacity for light-adaptation previously found in mammalian rods (*ch 3.3.3.)* was reinvestigated under the general hypothesis that the differing light-adaptation properties between rat and toad rods – similarly to sensitivity and kinetics – might be largely explained by strictly physical variables: temperature and dimensions. The efficacy of background light in inducing changes in phototransduction may depend on three factors: the amplitude of the single-photon response (sensitivity), the time scale of the response (integration time), and the potency of the system to adjust its gain in response to ongoing stimulation. The first two are directly affected by warming and cooling, as the integration time is proportional to t_p as long as the response form does not change much (Baylor *et al.*, 1979a). The third, the capability of the phototransduction machinery to regulate its sensitivity, might underlie any additional difference between mammalian and amphibian light-adaptation. The 5-6 -fold sensitivity difference and opposite but approximately equal difference in t_p between the model photoreceptors suggest that light-adaptation might be broadly similar in rat and toad rods when brought to similar temperature. New recordings from toad rods were here carried out only in the dark-adapted state, as there is extensive literature data on light-adaptation in frog rods (Hemilä *et al.*, 1977; Donner *et al.*, 1995; paper II) suitable for comparison with rat rods. *Fig. 16* shows the SVI-data from two rat

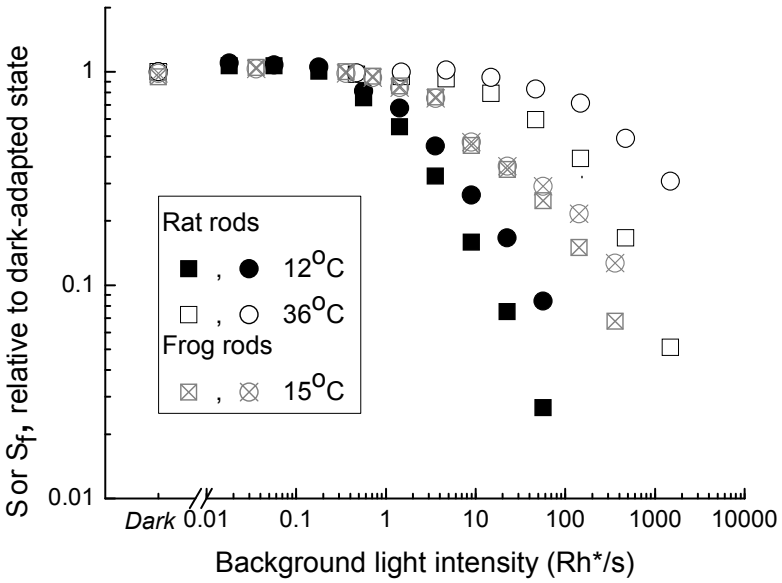

Figure 16. Light-adaptation of rat rods at two tempera-tures (filled symbols: 12 °C and open symbols: 36 °C) and frog rods at 15 °C (grey cross-filled symbols). Circles depict fractional sensitivity S_f and squares flash sensitivity S. Each dataset (S and S_f) is from a single representative retina included in the analysis of papers I and II.

retinas (one at 12°C and one at 36°C) and one frog retina (at 15°C), each representative of the data and parameters presented in papers I and II. The open symbols depict fractional sensitivity as function of steady background intensity. Indeed, the rat rods desensitize quite similarly to frog rods with background light when brought to similar temperature. Thus their apparent low capability to adjust sensitivity in response to background light at physiological temperature is likely to reflect mainly their shorter integration time at body temperature. Instead there seems to be a slight difference in the absolute flash sensitivity (closed symbols), which begins to deviate from the fractional sensitivity at a relatively lower background in rat. In frog rods, the two sensitivity parameters overlap until sensitivity has fallen to *ca*. 1/3 relative to the dark-adapted value, after which response compression begins to separate them. The flash sensitivity of rat rods begins to slightly deviate from the fractional sensitivity already at the lowest effective backgrounds, indicating response compression is not fully prevented even at these low stages of adaptation. This lesser capability to prevent compression is most evident near physiological temperature, possibly contributing to the former interpretation of mammalian light-adaptation data as indicative of poor rod adaptation.

8.2. Amplification factor in rods and cones

To evaluate activation efficiency as a source of the lower sensitivity of cones, the amplification factors (ch 4.2.1.) were determined for rods and cones of mouse and frog (papers II and III).

In frog, cones had a higher amplification factor than rods throughout the temperature range investigated (filled symbols in *Fig 17*). This complies with the view that the smaller fractional sensitivity in these cells depends on faster deactivation kinetics alone. In contrast, the amplification factor in mouse cones (crossed symbols in *Fig* 17) was slightly smaller than in

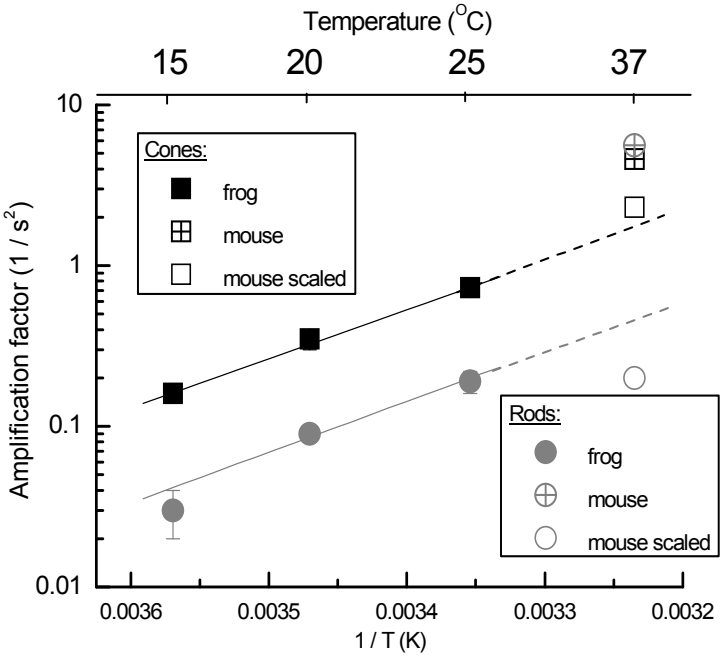

Figure 17. The amplification factors determined for dark-adapted rods and cones of frog (filled symbols) and mouse (crossed symbols). The murine values can be approximately predicted from the temperature-dependence of the respective frog values if the differences in OS volume between species are factored out (open symbols, see text for details).

rods of the same species, in line with the analysis of human ERG by Smith and Lamb (1997), and mouse photoreceptors Nikonov *et al.*, (2006). Also in this case the amplification factor was too high to explain the much larger sensitivity difference between rods and cones.

The apparent difference between mouse and frog with respect to the ratio of rod/cone amplification factors is again explicable simply with the OS size. If all other factors are assumed to be equal, $A_{murine,\ scaled} = A_{murine} \cdot V_{OS,\ murine} / V_{OS,\ frog}$ (open symbols in *Fig* 17) will represent the hypothetical amplification factor of a murine rod or cone scaled to the OS volume of the corresponding frog photoreceptor. The predicted relation is in reasonably good agreement with the data, indicating that molecular activation in rods and cones is specific to the photoreceptor type rather than to the species.

It should be noted, however, that outer segment size can*not* account for the differences in the amplification factor between rods and cones. Although the amplification is higher in frog cones than in frog rods at the *cellular* level, the 70-fold larger volume of rods implies that the *molecular* amplification is higher in rods.

8.3. Sensitivity regulation in amphibian cones

The objective in paper II was to modulate the thermal pigment isomerization rate I_{th} in frog L-cones by varying temperature. If I_{th} is sufficiently high to set the sensitivity of these cells, raising temperature should desensitize the cones even more, and also increase the intensity of background light needed for further desensitization. The rods of the same species were used as a "baseline" for the study, as the infrequent thermal pigment activations in rods are too rare to modulate sensitivity or light adaptation (Baylor et al., 1980; Donner *et al.*, 1990b). Thus any temperature-dependent changes in flash responses that were *similar* in cones and rods could be attributed to other mechanisms than visual-pigment stability. The study produced extensive general data about sensitivity regulation in frog L-cones, and

suggested thermal pigment isomerizations have at most a minor role in setting the dark-adapted sensitivity of these cells.

8.3.1. Warming "light-adapts" frog L-cones

The initial hypothesis for paper II was that adding thermal energy to the system would desensitize L-cones by increasing I_{th}, the rate of thermal pigment isomerizations. As a decrease of S_f with warming turned out to be a general trend in both rods and cones (papers I and II), this feature as such proved non-informative regarding the question of pigment stability.

The idea of a qualitatively different mechanism regulating rod and cone sensitivity was thus further evaluated with detailed comparison of the temperature dependence of S_f and the kinetics of flash responses. Although temperature affected S_f similarly in rods and cones below *ca*. 15 ^{o}C, cones desensitized more with warming than rods at 15-25 ^{O}C. The stronger desensitization was coupled with faster deactivation (see *Fig* 5 of paper II). This suggested that warming had an additional effect on cones that resembled light-adaptation. In this range, cone desensitization might reflect an "adapting" mechanism activating phototransduction with relatively strong temperature dependence, such as thermal pigment isomerizations for which Q_{10} = 4-5. In the lower temperature range other mechanisms, presumably common to cones and rods, apparently determine dark-adapted sensitivity. To investigate this hypothesis, the "adaptational state" of dark-adapted cones was probed with real background light. This allowed us to evaluate "dark light", the overall level of intrinsic phototransduction activity in frog rods and cones.

8.3.2. Probing dark light with background illumination

Background light can have an effect on cone phototransduction only it is approximately as strong as or stronger than the dark light within the cells. Thus a reliable measure for the dimmest background light sufficient to modulate phototransduction was needed to provide an upper limit for I_{th} in the cones.

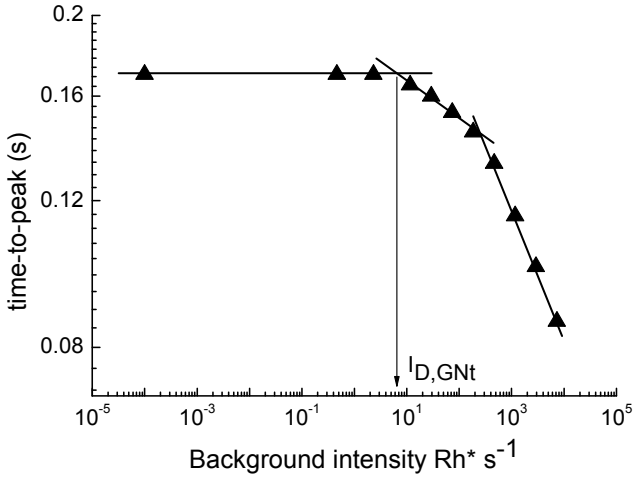

Figure 18. Method for estimating the "Greatest Non-adapting Background" from t_p of the linear range flash responses, see text for details.

Light adaptation is often described with the Weber-relation, $S / S_{Dark} = I_0 / (I_{Bg} + I_0)$, where I_0, represents dark light. Donner *et al.* (1998) stated that light-adaptation in frog cones is more complex, and the fractional sensitivity vs. background intensity (SVI) function contains multiple regions. These regions are best described with different functions, likely to reflect different dominating mechanisms of adaptation.

Paper II confirmed the complexity of light-adaptation in frog L-

cones, and observed that in addition, the shape of the SVI function changes with temperature. Thus a single forced fit of the Weber function to the SVI data at different tempera-tures did not yield a reliable estimate on the temperature-dependence of light-adaptation. Also in many retinas, an *increase* in sensitivity was observed at backgrounds too low to desensitize the retina. Similar phenomenon has also been observed in the photocurrent of macaque cones by Dunn *et al.* (2007), and cannot be accounted for by existing models of light-adaptation.

Due to the complexity of the SVI-data, a simpler and more sensitive measure of dark light was adopted: the "Greatest Non-adapting Background" ($I_{0,\,GN}$) , which could be determined from the effects of background light both on response kinetics (t_pVI data) and sensitivity (SVI data) (*Fig.* 18). It was defined as the intersection between a horizontal asymptote describing the dark-adapted data and a least-square fit to the points falling between the horizontal asymptote and a second asymptote, fitted to the data at the highest background intensities at each temperature. The time-to-peak of linear-range responses began to shorten even at lower background intensities than those that began to decrease sensitivity; in the initial stage shortening of t_p could often be associated with a slight increase in sensitivity (see above). Thus the dark light values determined from t_pVI-data were smaller than those determined from SVI data. However, the temperature-dependence of both parameters was surprisingly similar, and provided a key to the underlying factors.

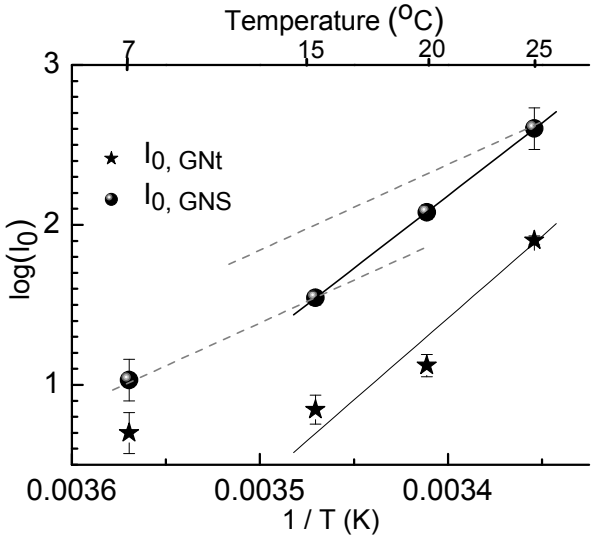

Figure 19. The greatest non-adapting background light in frog L-cones determined from sensitivity (circles) and t_p (stars). Continuous lines represent linear fits to the points at 15-25°C. Dashed lines represent the temperature-dependence of thermal isomerizations in salamander L-cones (Sampath and Baylor, 2002). Data from Fig. 8 of paper II.

The greatest non-adapting background can be expected to increase rather trivially with warming, as S_f and t_p (and thus the integration time) decrease. Indeed, both measures for $I_{0,\,GN}$ were found to increase >10–fold with warming from 15 to 25 °C in L-cones and *ca*. 5-fold in rods. In rods, the increase was mainly accounted for by the decrease in integration time, (see *Fig.* 9 in paper II) emphasizing the requirement of multiple single-photon events to coincide within one integration time. In cones, there was a component not explicable by the change in integration time and thus dependent on sensitivity. However, the idea of thermal isomerizations as the chief determinant of I_o in this region had to be rejected based on the temperature-dependence of I_{th}, for which reported Q_{10} lie within range 3.8-4.7 (Baylor *et al*., 1980; Sampath and Baylor, 2002). *Fig.* 19 presents the two $I_{0,\,GN}$ -measures of dark light in

frog L-cones at temperatures 7-25 ^{o}C: determined either from sensitivity (circles) or t_p (stars). The slope of the two dashed lines represents the highest measured temperature-dependence of thermal isomerizations in salamander L-cones, positioned, respectively, to coincide with the lowest and highest point of $I_{0,\ GNS}$. It appears that I_0 above 15 ^{O}C are not likely to be determined by thermal activations of visual pigment, as I_{th} with the cooler region would then exceed the I_0 determined from the effect of real light. On the other hand, if I_0 below 15 ^{o}C is based on thermal activations of visual pigment, then at >15 ^{o}C it must be determined mainly by other factors. It thus appears that thermal activations of the visual pigment play at most a minor role in regulating the sensitivity of frog L-cones. The I_0 determined by probing with real light (Table 1 in paper II) must be regarded as upper limits for I_{th} in these cells.

8.4. Dark- and light-adapted mouse cone electroretinogram

Flash responses of mouse cones were studied in the dark-adapted state and under weak and moderate backgrounds. The growth of the cone signal previously shown to occur in rod-saturating conditions was found to be initiated already at low background intensities, expected to light-adapt only rods. Removing electric coupling between dark-adapted rods and cones by pharmacologically blocking gap junctions induced enhancement similar to that induced by background light, and no further growth of the cone flash responses was then observed with light-adaptation.

8.4.1. ERG flash responses from wild type mouse cones

Flash responses of dark-adapted wild type mouse cones recorded with transretinal ERG in HEPES-buffered perfusion (paper III) resembled those recorded with suction pipette (Nikonov *et al.*, 2006). They were monophasic, peaked at *ca*. 50 ms, and had an amplification factor similar to rods (see *Ch. 8.2*). Under rapid perfusion with HCO_3^--buffered medium (paper IV) the flash responses were faster, peaking at *ca*. 40 ms. They also occasionally exhibited a biphasic waveform, yet the fractional sensitivity was similar to the HEPES-buffered case.

Under background light, the cone flash responses changed: while the initial 5-10 ms of the response remained unaltered, the rest of the response grew to a degree dependent on the intensity of the background light. The maximal enhancement, on average 60%, was obtained with background sufficient to practically saturate rods. These backgrounds typically caused little or no desensitization in cones. Increasing the background intensity further led to characteristic desensitization of the cone photoreceptors.

8.4.2. Rod-cone coupling and cone light-adaptation

The apparent dependence of cone enhancement on the rod saturation level suggested that gap-junctional coupling between rod and cone synaptic regions (see Ch. 5 and discussion) plays a role in this phenomenon. Three potent gap junction blockers were used to test this theory. Carbenoxolone (CBX, 100μM), a commonly used antagonist of gap junctional coupling, prevented light-induced enhancement. However, CBX can have unspecific toxic effects on neuronal function (Pan *et al.*, 2007; Tovar *et al.*, 2009), and it desensitized cones

and diminished their photoresponses. Thus two other structurally unrelated substances were tested: meclofenamic acid (MFA, 50-100μM; Veruki and Hartveit, 2009) and octanol (1mM; Rozental *et al*., 2001). They boosted dark-adapted flash responses, and the changes in response waveform were qualitatively similar to those induced by background light. Subsequent exposure to background light had no further enhancing effect. When applied in light-adapted retinas, MFA was found to have no significant effect on the already enhanced cone responses.

8.4.3. Light-adaptation in wild type mouse cones

Studies of light-adaptation in wild type mouse cones have turned out to be challenging. In the ERG, the substantial enhancement of cone responses under background light masks adaptation at the phototransduction level. The enhancement is absent in rodless mouse models, *e.g*., α-transducin knockouts, where cone function can be readily studied (*e.g.* Wang and Kefalov, 2009), but it remains unclear to what extent their cone function is representative of wild-type cones. Thus cone flash responses isolated pharmacologically from the transretinal ERG turned out a useful model for studying light-adaptation in wild-type cones. After subjecting the dark-adapted retina to MFA or octanol, the cones were found to desensitize with background light much like other mammalian cones. The sensitivity-halving background intensity was *ca.* 5000 P^*s^{-1}, similar to that measured for macaque cones (Schneeweis and Schnapf, 1999).

9. Discussion

9.1. Methodological considerations

Transretinal ERG as a method enables complex and long experiments on photoreceptor adaptation. The number of necessary repetitions of a given experimental sequence is generally small, as the ERG signal in itself averages light responses from thousands of cells and has excellent stability and signal-to-noise ratio. On the other hand, the ERG flash response recorded across an intact retina is a complex signal not bearing a straightforward one-to-one relation to any single function within or following phototransduction. However, with some reservations (*e.g.* Donner et al., 1992), the early rise of the a-wave can be rather directly related to the activation phase of phototransduction in the rod and cone photoreceptors (see below). Pharmacological block of glutamatergic signaling to higher-order neurons, together with removal of the glial slow PIII, reveals the light response originating in photoreceptors (fast PIII) as an extension of the a-wave.

The rod and cone contributions to the fast PIII can often be separated (see Ch. 7.2.3). Assuming that a pure flash response originating in a single photoreceptor type can be obtained by ERG, the question remains: how is it related to phototransduction and the subsequent changes in the light sensitive current of the outer segment?

9.1.1. Photocurrent, photovoltage and the electroretinogram

The fast PIII originates mainly in changes of the (predominantly) Na^+ current sink in the outer segment and the K^+ current source in the inner segment region (*Fig 1.*). The relative contributions of the inner and outer segment (IS and OS) currents to the transretinal voltage response depend on the geometry of the photoreceptors and the resistance profile of the extracellular space (Hagins *et al.,* 1970; Arden 1976). In the mammalian retina, with ISs and cell bodies spanning a great fraction of the photoreceptor length, the component generated in the IS represents a larger fraction of the transretinally recorded fast PIII (Arden, 1976), which is thus shaped by the voltage-dependence of the IS currents. In amphibian photoreceptors with relatively shorter ISs, the contribution of the corresponding currents is likely to be smaller.

The contribution of the driving force and conductance changes of the IS currents to the fast PIII may in part explain differences in photoresponse parameters obtained with ERG and suction pipette (paper III), although much of the variation may be explicable by different experimental conditions (*cf.* Doan *et al.*, 2009). The membrane voltage response initially follows changes in the outer segment current, but is at later stages modified by the numerous voltage-dependent currents of the IS (Fain *et al.*, 1978, Werblin, 1979, Baylor *et al*., 1984b). The high-pass filtering properties of the inner segment may hasten the recovery of small-stimulus ERG responses compared to the light-sensitive current kinetics.

The relative contributions of OS and IS currents to the fast PIII in the different preparations used in this thesis have not been determined. However, the differences in sensitivity and kinetics as measured from membrane voltage vs. photocurrent lie in the range 10-50 % (Baylor *et al*., 1984b, Schneeweis and Schnapf, 1999), which will usually have little effect on parameters determined on logarithmic scales. Also several features of the data are invariant

against the relative OS/IS contribution to the light response, specifically two important measures used in this thesis: 1. The initial activation phase of the photoresponses follows the changes in light-sensitive current. 2. The determination of the "dark light" from the lowest intensity of adapting background light that starts to affect responses will reflect only changes in the photosensitive current as long as the membrane potential is not affected by neural feedback or inter-photoreceptor coupling.

9.1.2. The pharmacological approach

Pharmacological approaches in neurophysiological studies have been progressively supplemented and partly replaced by the use of transgenic animal models. Photoreceptor function and retinal signaling pathways are commonly investigated with animals with reduced retinal function, such as "rodless" and "coneless" mice (Calvert *et al*., 2000; Chang *et al.*, 2006). Phototransduction is studied by manipulating the expression levels of individual participating proteins (examples reviewed in the context of the photoresponse model by Hamer *et al.*, 2005).

Is the pharmacological approach then outdated? Rather, it is a necessary complement to the genetic manipulations, especially the knockout models. Pharmacological treatments may have unspecific actions, such as the direct inhibitory action of the gap junction blocker carbenoxolone on neuronal signaling (see Ch. 8.4.2). They may also be less than fully reversible. On the other hand, a knockout model is always irreversible, the control being another individual (commonly, a wild type sibling). Also, as the development of the nervous system is generally known to be highly plastic, the question arises: how well does a neural network with a specific part knocked out represent its wild-type counterpart? Just like in the case of choosing experimental methods to solve a problem (ch 2.2), a combined approach is called for, with pharmacological and genetic methods complementing each other.

9.2. Phototransduction in rods and cones

A major objective of the present work was to clarify how far differences in photoreceptor function across subtypes and species could be accounted for by two simple physical factors, temperature and cell morphology. Several studies have previously addressed temperature effects on phototransduction (amphibian rods: Baylor *et al*., 1983; Lamb, 1984; Donner *et al.*, 1988; turtle cones: Baylor *et al*. 1974*;* guinea-pig rods: Demontis *et al*., 1997). Based on these existing studies it appears that acceleration of photoresponses with warming is common to all photoreceptors, although the magnitude of the effect varies somewhat. Sensitivity is somewhat more difficult to analyze from many of the earlier studies, which concentrate on absolute rather than fractional sensitivity. Nevertheless, more or less monotonic decreasing of S_f with warming seems to be a general trend among vertebrate photoreceptors. Demontis *et al.* (1997) reported a quite similar decrease of S_f in guinea-pig rods upon warming as shown for rat rods in paper I.

The current study used temperature changes as an experimental tool for a specific purpose: to probe common and distinct features of phototransduction between photoreceptors types. The results (re-)emphasize the importance of temperature as a variable that affects

practically all photoresponse parameters. They shed light on sensitivity regulation in rods and cones as well as on differences in mammalian and amphibian phototransduction.

9.2.1. Rod and cone sensitivity

Despite the current controversy in the literature about differences in molecular amplification between rods and cones, it seems that to achieve "textbook" rods and cones, it is not *necessary* to assume significantly lower amplification in the activation phase of cone photoresponses (Ch. 8.2).

The fractional sensitivity of the rods in the current study was only 5-10 –fold higher than that of the cones of the same species. This reflects an overall similarity of function, despite the faster response deactivation and highly efficient light-adaptation of cones. The much greater sensitivity difference between rod and cone *vision* in the investigated species arises partly from the differences in the collection areas of rods and cones: The ratio $a_{c,\,rod}$ / $a_{c,\,cone}$ is 6-7 in mouse and 26 in frog in the current recording configuration (referred to the λ_{max} of each cell type). Naturally, it is still possible that a significant difference in molecular amplification underlies the even larger rod/cone sensitivity difference found in many species. Whether the relatively lower amplification in the fish cones reported by Tachibanaki *et al*. (2001) reflects true differences between cones of various species or methodological differences remains to be seen.

9.2.2. Temperature, outer segment size and sensitivity

The difference between mammalian and amphibian rod OS size is striking. The high area/volume –ratio of the slender mammalian OSs subjects them to high metabolic stress due to the need to maintain high steady-state ion fluxes in darkness. Paper I suggests that the small OS size in mammals enables generation of relatively large single photon responses within the accelerated time scale associated with mammalian body temperature.

The small OS size in mammals may also be viewed as a means of limiting noise from thermal activation of transduction molecules at the high body temperature. The level of continuous noise is somewhat higher in mouse rods than in toad rods (Field and Rieke, 2002) and the difference would be even greater if the outer segments were similar in size. Additionally, assuming that Q_{10} for thermal pigment isomerizations is *ca*. 4 (Baylor *et al*., 1980), there would be a > 10-fold difference in the molecular rate constant of rhodopsin thermal activation between room temperature and mammalian body temperature. On the other hand, however, the molecular rate constant for thermal pigment activation of mouse rhodopsin appears (Burns *et al.*, 2002) to be only twice that of toad's (Baylor *et al*., 1980). This suggests that mammalian rhodopsins may have developed exceptional stability as an adaptation to the high temperature. Similarly, rhodopsins of animals using both A1 and the noisier A2 chromophore during their life cycle are, in their A1 form, more stable than toad rhodopsin by at least one order of magnitude (bullfrog: Donner *et al.*, 1990b; salamander: Ala-Laurila *et al*., 2007).

A third factor that favors slender rods with small collecting areas in mammals is the narrow operating range of the rod-rod bipolar cell synapse, which saturates even for responses to just a few photons (*e.g.* Field and Rieke, 2002).
Decreasing the cross-sectional area of the rods allows the benefits of larger SPR_f, faster

exchange of cytoplasmic compounds, and low noise. Functional analysis of OS *length* must additionally take into account that the OS layer has to be sufficiently thick to ensure reasonable quantum catch. However, the absorption probability for axially incident light increases as a decelerating function of OS length (*Eq.* 7.1.), while the noise from thermal activation of transduction molecules depends linearly on it. Thus there is theoretically an optimal OS length, depending on the level of spontaneous phototransduction activity. Leibovic and Moreno-Diaz (1991) analyzed the OS length of rods in several species from the viewpoint of the information content of a single-photon response (SPR), and found that the true OS lengths in toads and model mammals were indeed rather "optimal" observing the experimentally determined SPR amplitude and noise levels. However, this kind of optimization analysis contains much uncertainty in the relevant parameters, and the optimization criteria may also depend on the light environment of the species. Moreover, the relatively short mammalian rod outer segments may simply result from metabolic and structural constraints associated with the decrease in thickness.

9.2.3. Visual pigment stability in (A1) L-cones

Measuring dark lights of frog L-cones with ERG (paper II) is a very indirect approach to studying the stability of their visual pigment. However, measurements of noise in A1-based amphibian L-cone pigments were not (and are still not) obtainable. Concurrently with preparation of paper II, results on human L-cone pigment ectopically expressed in mouse rods were published, indicating that pigment stability is unlikely to be a significant determinant of human L-cone sensitivity (Fu *et al.*, 2008). The authors suggested that one reason for the great stability of the human L-cone pigment is the A1 chromophore: at least in frog and salamander rods the A1 based pigments are 10-40 times more stable than the same pigments with A2. It is also possible that human (or mammalian in general) cone pigments have evolved particularly high stability to compensate for their high operating temperature. The results of the current study support the idea that only L-cones using A2-based pigments are intrinsically desensitized by pigment-generated "dark light".

Theoretical estimates of spontaneous pigment isomerization rates in frog L-cones (paper II) comply with the conclusions from the physiological data. The expected I_{th} are too low to constitute a factor that limits the desensitizing effect of real background light. Yet it is worth noting that the first subtle changes in response kinetics at low background intensities coincide with the estimated I_{th}, at background lights 100-fold lower than those given by the Weber relation.

Two questions immediately arise from the results of paper II and other recent literature, awaiting future research: 1. Would the temperature dependence of dark light in salamander (A2) L-cones, physiologically determined as in paper II, comply with that of I_{th}, as determined from dark noise analysis by Sampath and Baylor (2002)? 2. If isolated salamander (A2) L-cones were successfully bleached and regenerated with A1-chromophore, would they become "frog-like", *i.e.*, is the higher stability of A1 pigments previously demonstrated in rods also a general property of cone pigments? A third question concerns the changes in cone sensitivity and kinetics at low mesopic background lights, and will be addressed in Ch.*9.3*.

9.2.4. Light-adaptation in mouse cones and other photoreceptors

Light-adaptation of phototransduction in mouse cones was partially masked by the prominent growth of the flash responses observed under dim background lights (paper IV). However, an estimate thought to reflect mainly "pure" light-adaptation of phototransduction was obtained after subjecting the retinas to gap junction blockers MFA or Octanol. The background light then needed to depress cone sensitivity by half was 5000 ± 1000 P^*s^{-1} (N=4).

The sensitivity-halving background intensity, $I_{0,\ Weber}$, is a robust measure much used in the literature and can be used for comparison of the susceptibility of different photoreceptors to background desensitization (*Fig.* 20). Despite the range of species and experimental techniques involved, $I_{0,Weber}$ is well predicted by the time integral of dim flash responses

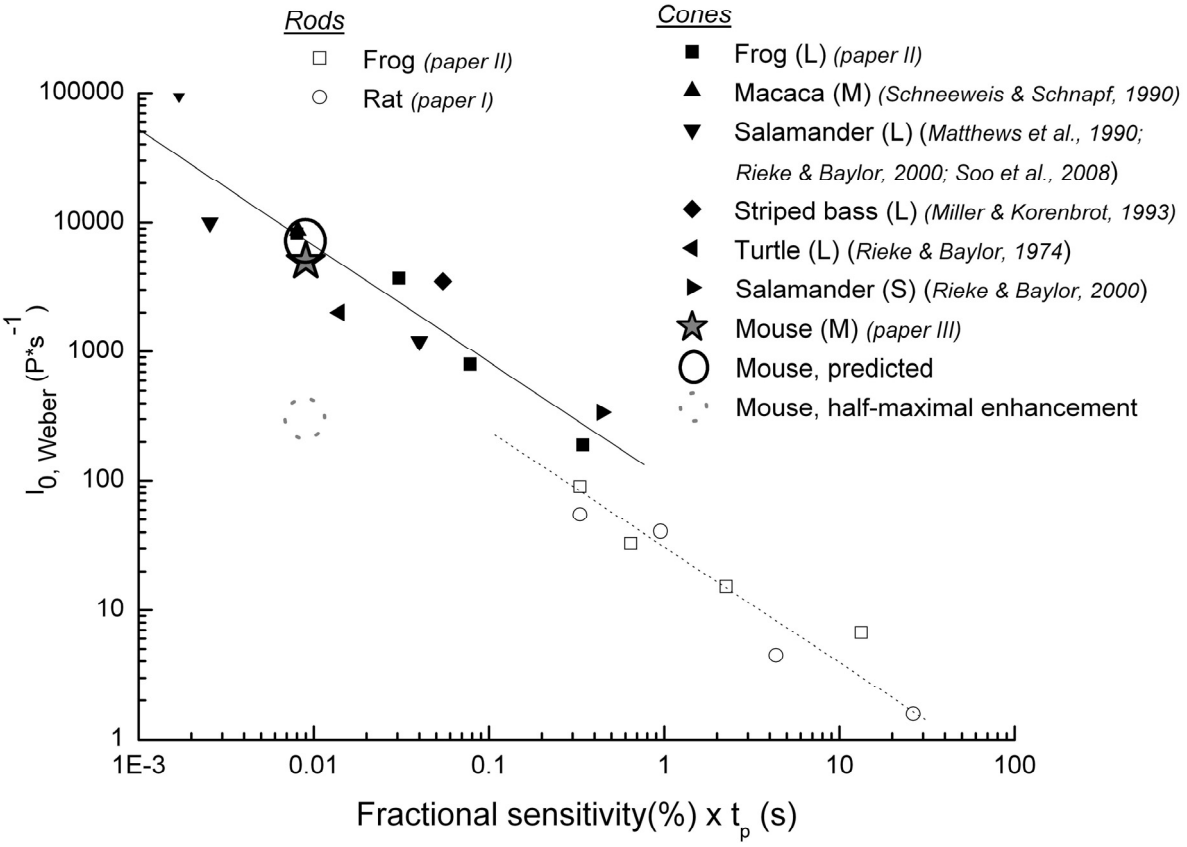

Figure 20. The sensitivity-halving background light (in terms of isomerized pigments per cell per second) for rods and cones of several species shows a strong correlation with the integrated fractional dim-flash response. The time integral of the response is here approximated with the product of fractional sensitivity and time-to-peak (see Text). Cones: closed symbols, rods: open symbols. The solid line is a least-square linear fit to the logarithmic cone data (slope 0.9); the dotted line is the same slope displaced vertically ca. 0.3 log-units to fit the rod data. Large open circle: $I_{0,\ Weber}$ predicted from the average sensitivity and t_p of dark-adapted mouse cones, large dashed circle: background intensity producing half-maximal enhancement of mouse cone responses (paper IV).

(here approximated by the product of fractional sensitivity and the time to peak t_p, which is proportional to integration time if the response waveform is constant). The sensitivity-halving background of mouse cones (star) agrees well with the prediction from a linear fit to the logarithmic data with slope 0.9 +/- 0.1. The relation of *Fig*. 20 is hardly surprising as such, since the time integral of the (fractional) linear-range response is likely to reflect the overall molecular activation of the phototransduction machinery per photoisomerization. It suggests that the adapting signal at low light levels is proportional to this activation, but of course says nothing about the mechanisms underlying sensitivity and time scale in each case. However, deviations from the relation can be considered as indicative of "novel" mechanisms not observed in traditional descriptions of light-adaptation.

9.3. The mesopic range

Light-adaptation, described with sensitivity vs. intensity (SVI) curves, is far more complex than predicted by the Weber relation. Especially in cones, the SVI shows several regions evidently reflecting the dominance of different mechanisms (Donner *et al*., 1998). Papers II and IV investigated changes in cone responses in the mesopic range, at light levels too low to desensitize the cones, yet sufficient to markedly alter their light responses. The results were indicative of i) a background-induced interplay of sensitizing and desensitizing mechanisms in cone adaptation at significantly lower intensities than has been generally thought and ii) an involvement of rod-mediated suppression in the cone responses of the mouse retina in darkness.

9.3.1. Increase of frog L-cone sensitivity at low backgrounds

A sensitivity increase coupled to response acceleration was observed in frog L-cones (paper II) at background intensities insufficient to depress sensitivity. This was often coupled to a slight general increase in cone response amplitudes, as judged from the saturated responses. Is this truly a property of phototransduction, then, or is it related to changes in voltage-dependent currents in the IS similar to those invoked to explain sensitization in the mouse retina? There are some indications that the initial fastening and sensitization of the photoresponses may reflect real behavior of the cone phototransduction machinery. 1. The analogous data by Dunn *et al.* (2007) represents direct recordings of the photocurrent in monkey cones. 2. Amphibian rod-cone electrical coupling may be differently modulated from its mammalian counterpart, as at least in salamander it seems to be enhanced rather than depressed under backgrounds (Yang and Wu, 1989). It is also generally weaker in amphibians (see *Ch. 5.2*). Thus the mechanisms to which we ascribe cone enhancement in mammalian ERG (paper IV) may not be present in the amphibian retina. 3. Features reminiscent of the light-induced sensitization can be observed in some of our rod recordings, too, suggesting an interplay between sensitizing and desensitizing factors also within these cells. 4. The change in frog cone ERG responses appears different from that in mouse cone ERG, as frog cones show a monotonic decrease in t_p accompanied by a slight decrease in the saturated response, whereas light-enhanced dim-flash ERG responses of mouse cones commonly peak later than in the dark-adapted state and the saturated response amplitude grows significantly.

Yet adaptation of frog L-cones in the mesopic range deserves further attention in the light of the findings in paper IV. The experiments were performed in the steady state, >10 minutes

after turning on the background light, so the time course of sensitization vs. desensitization is not presently known. Pharmacological blocking of gap junctions might shed light on the origin of sensitization at low backgrounds. Nevertheless, it seems plausible that the photoreceptor sensitization at low backgrounds reflects true events in phototransduction. The background intensities needed to desensitize frog L-cones by half are so high (*Fig. 20*) that even much weaker backgrounds are estimated to sustain a significant continuous phototransduction activity.

9.3.2. Rod-cone coupling and the mammalian ERG

Paper IV shows that ERG responses of mouse cones recorded soon after a rod-saturating pre-flash from a dark-adapted retina are suppressed relative to responses recorded by a similar protocol under dim to moderate adapting background light. A similar release of suppression could be induced by superfusing the retina in darkness with pharmacological agents known to block gap junctions. Taken together, these results suggest that electrical coupling to rods is involved in the cone suppression.

In mouse, the pedicle of each cone is electrically connected to *ca*. 30 rod spherules, while each rod contacts on average only one cone (Tsukamoto *et al*., 2001; *cf.* Smith *et al*., 1986 for cat retina). It thus seems plausible that rods "piggy-backing" on the cone pathway (Ch. 2.1.4, *Fig. 2*) substantially affect the membrane potential in the cone synaptic region. The method of separating cone signals in papers III and IV always involves hyperpolarizing the rods before obtaining the cone response, regardless of the state of "steady" light-adaptation. Then, if the electric coupling between rods and cones is assumed to weaken in a graded manner with light-adaptation, the rod hyperpolarization will spread less efficiently to the cone pedicles the stronger the steady background light. This would leave the cones in a relatively depolarized state at the moment the cone test flash is delivered, and thus allow a larger functional response range for the cone signals in the synaptic region.

Paper IV supports this interpretation, although the exact mechanism of enhancement in the ERG signal remains unknown. Simply doubling the circulating current seems unlikely. The changes in response kinetics suggest mechanisms distinct from the primary response in the CNG-channel current. A possible hypothesis would be a contribution of calcium-controlled K^+-currents around the proximal parts of the cones to the ERG flash responses. A moderate depolarization would be expected to change these currents significantly, both in terms of their driving force and level of activation by changes in $[Ca^+]_{in}$.

Rod-cone coupling is also known to be regulated diurnally, so that rod signals should reach the cone pathway more efficiently at night (Ribelayga *et al*., 2008). The experiments in paper IV were performed mostly before the subjective dawn, and it would be interesting to see if the cone suppression/enhancement is affected by the circadian clock.

10. Conclusions

The following conclusions are grouped according to the questions formulated in Chapter 6 (Aims of the study):

1. Is rod phototransduction in mammals intrinsically different from that of poikilotherms? (papers I and II**)**
Comparison of toad (*Bufo bufo*), frog (*Rana temporaria*), rat (*Rattus norvegicus*) and mouse (*Mus musculus*) rod photoresponses over wide ranges of temperatures and states of light adaptation indicated no need to assume qualitative generic differences of the phototransduction machinery, including light-adaptation, between mammalian and amphibian rods. The observed functional differences were accounted for by temperature and cell dimensions.

2. What is the role of molecular amplification in the sensitivity differences of rods and cones? (papers II and III**)**
Activation coefficients of rods and cones were determined from experiments on mouse and frog retinas. The photoresponse amplification was found to be of the same order in mouse rods and cones, while initial amplification in frog cone responses was higher than in rod responses. Thus the lower sensitivity of cones does not require lower activation efficiency. However, when activation is corrected for the small size of the cone outer segments in frog, it appears that the molecular amplification in cones is indeed lower than in rods.

3. Do thermal isomerizations set the "dark" state of adaptation and sensitivity of L-cones that use the A1 chromophore? (paper II**)**
Temperature changes were used to vary the rate of thermal activation of visual pigment as well as other transduction molecules in frog L-cones. Effects on flash sensitivity were measured, and the state of adaptation was probed with real background lights. Based on i) the overall similarity of sensitivity changes in rods and cones, ii) the discrepancy between the observed temperature-dependence of intrinsic activation probed with background light and that reported in studies where rates of thermal isomerization-like events have been estimated from the dark noise of single cells and iii) theoretical estimates of the thermal isomerization rate in frog L-cones, it was concluded that thermal isomerizations play at most a minor role in setting the dark-adapted sensitivity of these (A1) L-cones.

4. What is the mechanism underlying the light-induced growth in the mammalian cone electroretinogram? (paper IV**)**
Growth of the photoreceptor component in mouse ERG was found to be elicited even by very weak backgrounds. The magnitude of the effect was dependent on the background intensity, saturating at intensities sufficient to saturate rods. Cone response growth could also be induced by selective stimulation of rods with flash trains. Further, a qualitatively similar effect could be elicited in darkness with three different gap junction blockers, and then no further growth under background light was observed. The results i) provide physiological evidence for existence of rod-cone electrical coupling in mouse and ii) suggest that the light-induced enhancement of the cone component in mouse ERG is related to gradual closure of gap junctional coupling between rods and cones with increasing

intensities of steady adapting backgrounds. The latter would be a novel adaptational mechanism not previously described in the mammalian retina.

5. Is the transretinal electroretinogram useful as a tool for studies of light-adaptation in wild-type mouse cones? (papers III and IV)
When the background-induced growth in the cone flash responses (see above) was blocked, cone mass responses recorded across the isolated, aspartate-treated mouse retina were found to light-adapt like most cones with sensitivity-halving background intensity typical to mammalian cones. It was found that the technique can be used for studying light-adaptation in wild type mouse cones, although its interpretation is complicated due to the effect described above.

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