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Doctoral Dissertation

Soile Nymark

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Dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the Faculty of Information and Natural Sciences for public examination and debate in Auditorium D at Helsinki University of Technology (Espoo, Finland) on the 6th of June, 2009, at 12 noon.

Helsinki University of Technology
Faculty of Information and Natural Sciences
Department of Biomedical Engineering and Computational Science

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Soile Nymark

This thesis for the degree of Doctor of Science in Technology has been carried out at two departments:

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Department of Biomedical Engineering and Computational Science

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Opponent(s): Professor Carter Cornwall
Supervisor: Professor Ari Koskelainen
Instructor: Professor Ari Koskelainen and Professor Kristian Donner

Abstract

The photoreceptor cells of the vertebrate retina share a common morphological design and molecular scheme for phototransduction. Within this framework, there are great functional differences with respect to response amplification, kinetics, and adaptability to different mean light levels, first, between the two main classes of photoreceptors, rods and cones, and second, between different taxonomic groups. The present thesis analyses functional differences and similarities between i) mammalian and amphibian photoreceptors, and ii) rods and cones by studying effects of temperature on electrophysiological response properties.

The research is based on characterisation of sensitivity and photoresponse kinetics in rods and cones of two mammals (rat and mouse), and two amphibians (frog and toad). Photoresponses to light pulses of incremental strength were recorded by the electroretinogram (ERG) technique across isolated aspartate-treated retinas at different temperatures in the range 2 - 37 ^{o}C, and at different levels of mean illumination.

One objective was to investigate how the major functional differences between different vertebrate photoreceptors can be explained without assuming large differences in the properties of the phototransduction molecules. A general conclusion is that at the same temperature photoreceptors of mammals and amphibians exhibit similar functional properties. In rods, the remaining differences in the electrophysiological properties can largely be explained by differences in outer-segment size and morphology. In cones the picture is more complex due to the highly folded structure of the outer segment as well as the presence of thermal isomerizations of visual pigment, which may occur at a rate possibly high enough to 'light-adapt' cones in darkness.

Another objective was to relate the capacity for temporal integration of dark-adapted rod photoreceptors to the integration time of vision and absolute visual sensitivity. A strong correlation was found between temporal integration in rods and in a visually guided behaviour of toads at different temperatures. The results allow the conclusion that temporal integration is mainly set by the rods and explains a considerable part of differences in absolute visual sensitivity between amphibians and mammals.

The thesis also includes a project in which the aim was to develop a method where the vertebrate retina could be used as a biosensor for monitoring controlled drug release from temperature-sensitive polymeric carriers. The developed method enabled accurate concentration determinations of the model drug 3-isobutyl-1-methylxanthine (IBMX) based on the square root dependence of photoresponse kinetics on [IBMX] discovered in the work. Moreover, the biocompatibility of drug carriers can be assessed by the degree to which rods retain stable function in the presence of the carrier molecule, or its monomers.

Keywords photoreceptor, retina, temporal integration, vision, controlled drug release, biocompatibility

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Työn ohjaaja	Professori Ari Koskelainen ja Professori Kristian Donner

Tiivistelmä

Verkkokalvon näköaistinsolut ovat morfologian ja fototransduktion (valon muuttaminen sähköiseksi signaaliksi) puolesta pääpiirteissään hyvin samankaltaisia kaikilla selkärankaisten ryhmään kuuluvilla eläimillä. Toiminnallisista eroista merkittävimmät liittyvät valovasteen vahvistukseen ja kinetiikkaan sekä näköaistinsolujen sopeutumiseen eri valaistusolosuhteisiin. Tässä väitöstyössä selvitettiin toiminnallisia eroja i) tasalämpöisten nisäkkäiden ja vaihtolämpöisten sammakkoeläimien näköaistinsolujen välillä sekä ii) yleisesti sauvasolujen ja tappisolujen välillä tutkimalla lämpötilan vaikutusta näköaistinsolujen valovasteisiin.

Tutkimus pohjautuu näköaistinsolujen herkkyyden ja vastekinetiikan karakterisoimiseen kahdella nisäkäslajilla (rotalla ja hiirellä) sekä kahdella sammakkoeläinlajilla (sammakolla ja rupikonnalla). Näköaistinsolujen sähköisiä vasteita intensiteetiltään kasvaviin valopulsseihin rekisteröitiin eristetyn verkkokalvon elektroretinogrammitekniikalla (ERG). Mittaukset suoritettiin laajalla lämpötila-alueella (2 - 37 ^{o}C) ja useilla taustavalointensiteeteillä.

Yksi väitöstyön päätavoitteista oli selvittää, onko välttämätöntä olettaa merkittäviä eroja fototransduktion molekyylitason mekanismeissa selittämään näköaistinsolujen toiminnallisia eroavaisuuksia. Yleisenä johtopäätöksenä todettiin, että samassa lämpötilassa nisäkkäiden ja sammakkoeläinten näköaistinsolut toimivat hämmästyttävän samankaltaisesti. Havaitut eroavaisuudet selittyvät sauvoissa suurelta osin solujen kokoeroilla. Tapeissa asia on huomattavasti monimutkaisempi mm. tappien ulkojäsenen rakenteen takia. Lisäksi tapeissa näköpigmentin termisten aktivaatioiden taajuus on niin suuri, että se voi toimia sisäisenä taustavalona.

Toisena päätavoitteista oli verrata pimeäadaptoituneessa tilassa temporaalista integraatiota näköaistinsolujen ja käyttäytymistason näön välillä. Tulosten pohjalta voitiin päätellä, että temporaalinen integraatio hyvin vähäisessä valossa määräytyy pääasiassa sauvojen integraatiosta ja se selittää suurelta osin sen, miksi sammakkoeläinten näöaisti viileänä kesäyönä on n. 10 kertaa nisäkkäiden näköaistia herkempi.

Työn oheisprojektina kehitettiin menetelmä, jossa verkkokalvoa käytettiin biosensorina kontrolloidussa lääkeaine-vapautuksessa lämpötilaherkistä polymeereistä. Menetelmä perustuu työssä havaittuun neliöjuuririippuvuuteen valovastekinetiikan ja mallilääkeaineen 3-isobutyyli-1-metyyliksantiinin (IBMX) pitoisuuden välillä ja se mahdollisti hyvin tarkat IBMX:n pitoisuusmääritykset. Lisäksi havaittiin, että lääkeaineen kuljettajamolekyylien mahdollinen toksisuus näkyy sauvasolujen toiminnan epästabiiliutena, mitä voidaan käyttää yhtenä indikaattorina bioyhteensopivuustestauksessa.

Asiasanat Näköaistinsolu, verkkokalvo, temporaalinen integraatio, näköaisti, kontrolloitu lääkeainevapautus, bioyhteensopivuus

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To Mom

Preface

This thesis is based on research work carried out at the Department of Biomedical Engineering and Computational Science at the Helsinki University of Technology and at the Department of Biological and Environmental Sciences at the University of Helsinki. The financial support was granted by the Helsinki University of Technology, the Finnish Cultural Foundation, the International Graduate School in Biomedical Engineering and Medical Physics, the Academy of Finland, the Finnish Foundation for Technology Promotion and the Emil Aaltonen Foundation.

The above institutions are gratefully acknowledged for the financial support. In addition I am very thankful to number of individuals that have contributed to this thesis project. First and foremost I wish to express my gratitude to both of my supervisors Professor Ari Koskelainen and Professor Kristian Donner. I want to thank Professor Koskelainen for initiating my interest in vision research as well as for providing excellent research facilities. I am also thankful to Professor Koskelainen for his remarkably skilful guidance in the experimental work. My deepest gratitude to Professor Donner is for his help and encouragement during the writing of this thesis as well as all the scientific publications included in it. I appreciate the number of discussions with Professor Donner in which he has shared his wide knowledge in the field of vision research. Moreover, I want to thank both of my supervisors for many good laughs and for providing an exceptionally friendly atmosphere in our research group.

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Special thanks are addressed to co-authors of the publications enclosed in this thesis: Ms. Hanna Heikkinen, Ms. Charlotte Haldin, Dr. Ann-Christine Aho and Professor Heikki Tenhu. Ms. Heikkinen has been a wonderful colleague through all these years and it is fair to say that without her being there to help and discuss about all sorts of issues I would not have this thesis. I am also very thankful to past and present members of our research group for the great time together. I especially want to thank Dr. Petri Ala-Laurila, Mr. Frans Vinberg and Mr. Rauli Albert for their help and friendship as well as Dr. Mikhail Firsov, Ms. Hanna Mäki, Ms. Elina Sahala, Mr. Janne Räsänen, Ms. Anna Moroz, Ms. Jenny Vesterlund and Ms. Tiina Saloniemi. Very warm thanks are extended to Dr. Johan Pahlberg and Dr. Maureen Estevez for all the support and friendship, and many thanks also to Ms. Pia Saarinen, Ms. Mirka Jokela-Määttä and Dr. Nanna Fyhrquist.

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To my dear husband Lasse I express my deepest gratitude. Without his love, wonderful sense of humor, practicality and endless patience I could not have gotten to this place. I am also exceedingly thankful to have three lovely children Niklas, Lilian and Bettina delighting my life.

Finally, I dedicate this thesis to my late mother Ritva who lost her vision already early in life.

Pirkkala, May 2009

Soile Nymark

Contents

List of publications

The present thesis is based on the following five 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 Haldin, C.*, Nymark, S.*,Aho, A.-C., Koskelainen, A., and Donner, K. (2009). Rod phototransduction determines the trade-off of temporal integration and speed of vision in dark-adapted toads. *The Journal of Neuroscience* **29**, 5716-5725.
* Equal contributions

III 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*, (in press).

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

V Nymark, S., Haldin, C., Tenhu, H., and Koskelainen, A. (2006). A new method for measuring free drug concentration: Retinal tissue as a biosensor. *Investigative Ophthalmology and Visual Science* **47**, 2583-2588.

Author’s contribution

The author has had a significant role in all the research reported in papers I-V. She has participated in the planning and execution of the electrophysiological experiments as well as the analysis and interpretation of the data of both electrophysiological and behavioural experiments. The author has also constructed a method to measure axial absorbance of rat rods presented in paper I and she has developed the method of paper V in which retina was used as a biosensor. In papers I, II and V she was the corresponding author. The author also participated actively in the writing of papers III and IV.

List of abbreviations and symbols

A	amplification constant of phototransduction
a	specific absorbance
A1	visual pigments containing 11-*cis*-retinal as chromophore
A2	visual pigments containing 11-*cis*-3,4-dehydroretinal as chromophore
α	weighting coefficient
cGMP, cG	guanosine 3',5'-cyclic monophosphate
ERG	electroretinogram
GC	guanylate cyclase
GCAP1&2	guanylate cyclase activating proteins
GTP	guoanosine triphosphate
HFBMA	hexafluorobutylmethylacrylate
I	light intensity
I_B	background light intensity
I_0	background light intensity required to reduce fractional sensitivity by half
$I_{½}$	flash intensity required to elicit a half-maximal response
I_{abs}	intensity of light absorbed in the absorbing media
IBMX	3-isobutyl-1-methylxanthine
I_R	circulating current in rods
I_C	circulating current in cones
L	long-wavelength-sensitive
l	length of the outer segment
LCST	lower critical solution temperature
MSP	microspectrophotometry
n	number of stages
NIPAAm	N-isopropylacrylamide
PDE	phosphodiesterase
R	response amplitude
R_{max}	amplitude of the saturated response
Rh*	photoisomerisations per cell
S	fractional sensitivity
S_{Dark}	fractional sensitivity in darkness
S_f	flash sensitivity
t_i	integration time
t_p	time-to-peak of the response
τ	general time constant
V	volume
VCa	vinylcaprolactame
λ	wavelength
Φ	photoisomerisations per cell due to short light pulse

1 Introduction

Vision is arguably the most important sense for humans and for other quickly moving animals, including other vertebrates as well as arthropods and molluscs. The importance of vision to human behaviour is seen in the structure of the brain, where more than half of the cortical area is dedicated to processing visual information captured by our eyes. The coordinated operation of eyes and brain together allows us to see amazingly varying objects in very different ambient conditions: we can discern objects that are still or fast moving, objects that contain different colours or small, detailed characters, and identify them in greatly varying illumination.

In vision, as for all our senses, the system must trade-off between sensitivity and resolution (see Fig. 1). Furthermore, this trade-off must consider different types of resolution, which in human vision include spatial, temporal, and chromatic resolution. This "optimisation space" is necessarily constrained by the properties of the input neurons, the photoreceptors. The anatomical array of photoreceptors sets one inexorable limit to the spatial resolving power (visual acuity) (for a review, see e.g. Williams, 1986): with increased cell diameter and center-to-center distance the spatial resolution is degraded (Miller & Bernard, 1983; Snyder & Miller, 1977), although simultaneously the quantum catch of a single photoreceptor is increased. Similarly, the response kinetics of photoreceptors sets limitations to temporal resolution. Finally, the numbers and distribution of different types of cones set limits to chromatic resolution.

An additional feature of vision in a large spectrum of vertebrates, including humans, is that it operates over at least a billion-fold range of light intensities, ranging from a moonless starry night to bright midday sunshine. This sets great demands on the photoreceptors, which should encode the information into neural signals despite their relatively narrow dynamic range, which may span ~ 3 orders of magnitude at best.

The optimisation problem is somewhat facilitated by the use of two classes of photoreceptors in parallel: very sensitive, slow rods that are used in dim light and somewhat less sensitive, faster cones of several spectral types that underlie vision in bright light conditions. Rods are of a single kind (sacrificing chromatic resolution) and give large and slow responses to photons in dim light. They do have a limited capacity for light-adaptation, i.e., for shifting their operating range to higher mean light intensities by desensitization coupled to acceleration of responses. Cones, on the other hand, have a striking ability to light-adapt and to function in very bright light as they do not electrically saturate at any steady light intensity (Barlow, 1972).

The present thesis focuses on the role of photoreceptors in constraining the optimization space of vision, particularly on the sensitivity versus temporal resolution axis. The original papers are concerned with the physiological and molecular mechanisms that set the mode of operation of the photoreceptor cells, as well as the functional importance of temporal integration in rods in determining the absolute sensitivity of vision. Temperature is used as an experimental tool to manipulate the time scale of photoresponses and the intrinsic thermal activity of the photoreceptors, which is

presumed to contribute to setting dark-adapted sensitivity as well as response kinetics. The experimental work is mainly based on photoreceptor electrophysiology (ERG across the isolated retina), but also includes a study of toad behaviour. Mammalian (rat and mouse) and amphibian (toad and frog) rods and cones were selected for study with a view to provide comparisons between photoreceptors that differ with respect to the temperature and light level at which they normally operate, as well as the size and morphology of the outer segments.

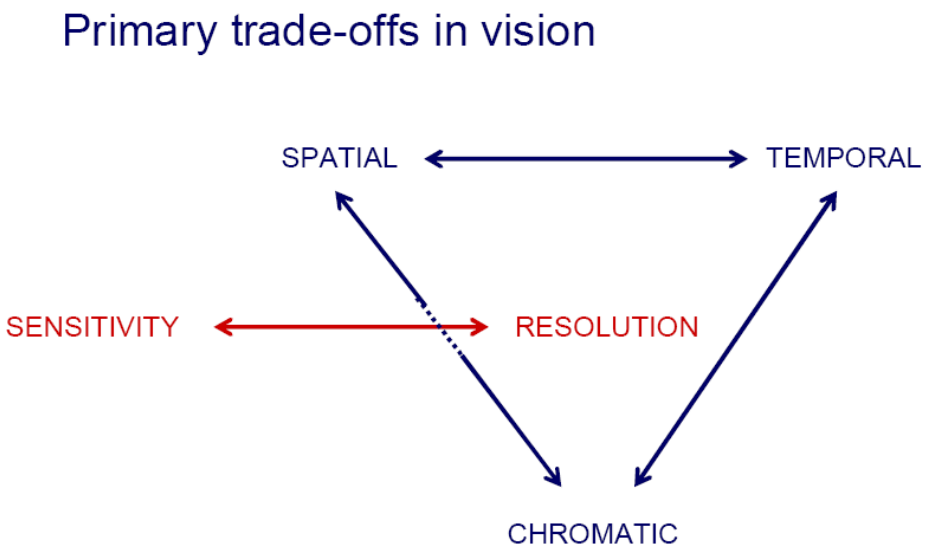

Figure 1. *The "optimisation space" of vision. Compromises have to be made between sensitivity and resolution and between different kinds of resolution (spatial, temporal and chromatic) in order to optimise the use of the information carried by light at different ambient illuminations.*

2 Review of the literature

2.1 Vertebrate photoreceptors and their function

Vision is a highly complex process that requires the coordinated activity of numerous components in the eye and brain. The initial steps are carried out in the retina, which is part of the brain, forming a layered sheet of neurons residing in the back of the eye. The retina has two main functions: (i) to act as a transducer that converts photon absorptions into neural signals and (ii) to perform the first steps of neural analysis and encode the end result into spike trains that are sent to the brain. The first task is accomplished by two types of photoreceptors, rods and cones, of which rods operate in dim light and cones in brighter light.

Almost all vertebrates have at least two cone types but only a single rod type. The different cone types are mainly distinguished by the part of the light spectrum to which each is most sensitive. The spectral sensitivity of a photoreceptor is determined by the light-absorbing properties of a so-called visual pigment (sometimes modified by absorbing structures in front of the pigment). The absorbance spectrum of a pigment describes the probability of a photon to be absorbed as function of its energy, inversely proportional to its wavelength. Once absorbed, the effect of each photon on the photoreceptor is independent of the wavelength (Rushton, 1972). Thus wavelength discrimination always requires comparison of signals from different cone types, providing the basis for colour vision. With three different cone types in the retina, humans have trichromatic vision in bright light but no colour vision in dim light, as there is only one rod type in the retina.

2.1.1 Morphology

Rods and cones of most vertebrates can be morphologically distinguished from each other. The first observations of these cells were likely made as early as 1722 by Anthonie van Leeuwenhœk who studied human retina by microscopy (Østerberg, 1935). The proper discovery of “light sensitive photoreceptors” and understanding of their functional meaning, however, had to wait until the studies of many different animals by Gottfried Treviranus in 1834 (Finger, 2001).

The cellular organization of rods and cones is broadly similar. Both consist of an outer segment where vision is initiated and which contains the visual pigment and other transduction molecules, an inner segment where metabolic processes take place, and a synaptic terminal transmitting the visual signals to second-order neurons. The morphological features making rods and cones of many vertebrates appear different is the generally ‘rodlike’ shape of rod outer segments versus the slightly tapered shape of cone outer segments (Fig. 2). In rods, the shape is related to maximizing quantum catch by packing a high density of visual pigment in a large number of separate membranous discs resembling a stack of coins. This differs from cones, where the membrane that holds the visual pigment typically forms a smaller number of infoldings contiguous with the external cell membrane. (Rodieck, 1998).

In rod and cone outer segments the visual pigment molecules constitute about 95 % of all the membrane proteins (Nathans, 1987). The different outer segment morphology of rods and cones might affect the level of their intrinsic noise. It has been suggested that the isolation of the outer segment discs from the extracellular medium in rods may be one factor allowing the great stability of the rod visual pigment (Burns & Lamb, 2003).

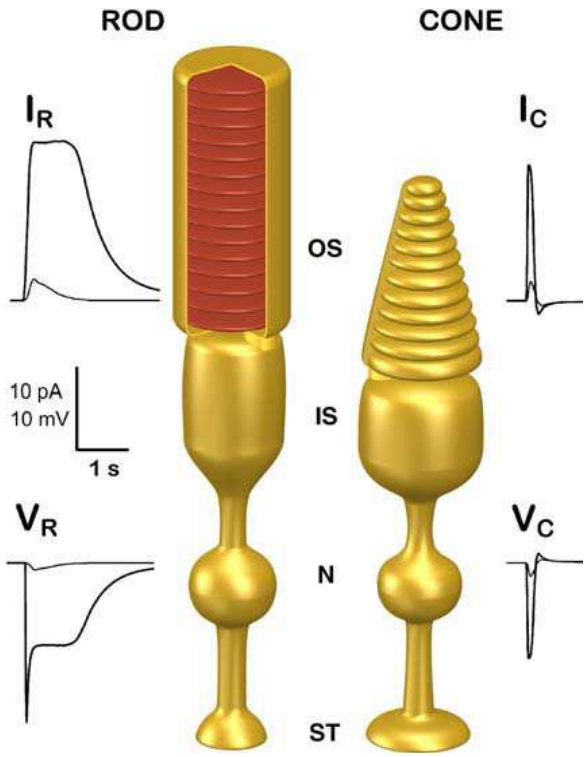

Figure 2. *A schematic illustration of rod and cone photoreceptors. In rods the 'rodlike' outer segments (OS) have separate membranous discs holding visual pigments and other phototransduction proteins whereas in cones the slightly tapering outer segments contain these proteins in infoldings of the cell membrane. In both photoreceptors there is an inward current into the outer segment and outward current out of the inner segment (IS) forming a current loop in darkness. Light induces a change in this circulating current (I) which in rods lasts considerably longer than in cones (see traces I_R for rods and I_C for cones). The resulting membrane hyperpolarization (V_R for rods and V_C for cones, modified by voltage-sensitive conductances in the inner segment compared with the outer-segment current response) modulates transmitter release from the synaptic terminals (ST). The figure is reprinted with minor modifications from Burns & Arshavsky, 2005, Copyright (2005) with permission from Elsevier.*

Rod and cone morphology differs also in the inner segment, which is thicker in cones to enable funnelling of light into the outer segment. In both cell types the contents of the inner segment appears to be quite similar, including the nucleus and other usual

organelles e.g. for protein synthesis and processing and cell metabolism (Rodieck, 1998). Synthesised proteins needed in the outer segment are moved from the inner segment through a narrow connecting cilium (Wolfrum & Schmitt, 2000). This structure, with microtubules and molecular motors, is the only intracellular link between the morphologically and functionally distinct compartments of the inner and the outer segments and is, therefore, crucial for photoreceptor functioning (Schmitt & Wolfrum, 2001).

The ending of the inner segment, the synaptic terminal, is known as the pedicle in cones and spherule in rods. The names refer to the synapse morphology: cone pedicles are large and conical with flat end-feet whereas rod spherules are small and round. Both types of endings are filled with synaptic vesicles containing the neurotransmitter glutamate (Rodieck, 1998).

2.1.2 Phototransduction and photoresponses

Vision begins with the absorption of light by a molecule of visual pigment and its transduction into an electrical signal, a photoresponse, via a G-protein cascade in the photoreceptor outer segment. This process, called phototransduction, is similar in all vertebrate rods and cones (Pugh & Lamb, 2000). Of all G-protein mediated signalling cascades, phototransduction is arguably the one that is known in greatest detail.

Visual pigments have been under intense investigation since the discovery by Franz Boll about 130 years ago, that light acts directly on a chemical substance in the retina, “Sehpurpur” or “rhodopsin”, which is “bleached” when illuminated (Boll, 1877; for a review see Luo *et al.*, 2008). Knowledge about rhodopsin and its decomposition by light increased rapidly by the findings of Wilhelm Kühne in the late 1870s as well as of George Wald, Ruth Hubbard and others in the 1930s and 1940s (Kühne, 1878; Wald, 1968). The absorption spectrum of rhodopsin was determined as early as 1894 by A. Koenig (Koenig, 1894), and was correlated with dim light vision in 1922, when Selig Hecht and R. Williams determined the relation between the frequency of light and the intensity needed to produce a colourless sensation in the human eye (Hecht & Williams, 1922). Ragnar Granit and Carl M. Wrede (1937) importantly correlated the psychophysical data with retinal neurophysiology by electroretinogram (ERG) recordings of the spectral sensitivity of rods. Wald (1950, 1951) showed that the initial event in visual excitation was the isomerisation of the vitamin A aldehyde prosthetic group (the “chromophore”) of the visual pigment molecule from the 11-*cis* to the all-*trans* form.

After Granit’s ERG experiments it was not until the mid-1960s before the picture of how the absorption of a photon by a visual pigment triggers an electrical signal in the photoreceptor cell started to become clear. By 1970 it was understood that in darkness there is an inward movement of positive charge into the outer segment and an outward movement of positive charge from the inner segment creating a current loop known as the “dark current” or the “circulating current”, which is suppressed by light (Hagins *et*

al., 1970). Soon after these findings it became evident that light-induced changes in the current were mediated by an intracellular second messenger. Whether this messenger was Ca^{2+} or cyclic guanosine monophosphate (cGMP) was the subject of a long and intense debate. In the mid-80s the question was definitely resolved by the patch-clamp experiments of Fesenko *et al.* (1985), showing that the light-sensitive channel is directly gated by cGMP (without phosphorylation, the first such channel characterized) (cf. also Yau & Nakatani, 1985). Thus Ca^{2+} is not an intracellular second messenger mediating the light-induced change in circulating current but, instead, it constitutes *ca*. 15 % of the primarily Na^{+}-carried ion current flowing into the outer segment. This current is maintained by Na^{+}/K^{+} ATPases located in the inner segment cell membrane and its suppression by light leads to a decrease in intracellular Ca^{2+} concentration due to continuous extrusion from the outer segment via Na^{+}/Ca^{2+} - K^{+} exchangers.

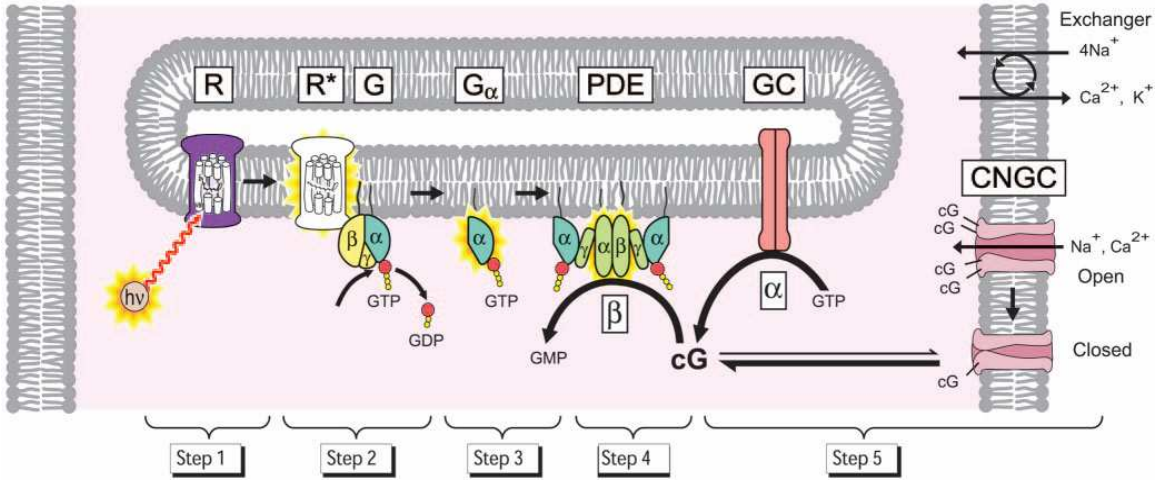

Figure 3. *The phototransduction cascade. The first step in the cascade is the absorption of a photon by a visual pigment molecule (here rhodopsin, R). The activated rhodopsin R* contacts G-proteins (G) and catalyses the reaction in which active form of G-protein, G_{α}, is produced (Steps 2 and 3). G_{α} can bind to the inhibitory γ subunit of the phosphodiesterase (PDE) and binding of G_{α} to both of the γ subunits of PDE activates its α and β subunits which are then able to catalyze the hydrolysis of cyclic guanosine monophosphate cG (cGMP in the main text) (Step 4). The decrease in cG concentration leads to closure of the cyclic nucleotide gated channels (CNGC) of the outer segment and decrease in the circulating ion current (Step 5). The figure is reprinted from Lamb & Pugh, 2006, Copyright (2006) with permission from The Association for Research in Vision and Ophthalmology.*

Activation

The very first step of phototransduction is the activation of visual pigment molecules acting as light detectors in photoreceptor cells (see Fig. 3). Visual pigments consist of a light absorbing "chromophore", which is a form of vitamin A aldehyde (retinal),

covalently bound to a 7-transmembrane G-protein coupled receptor protein called opsin. Absorption of light isomerizes the chromophore from the 11-*cis* configuration to the all-*trans* configuration on an extremely short (*ca*. 200 fs (Schoelein *et al.*, 1991, Wang *et al.*, 1994)) time scale leading to conformation changes in the protein part of the molecule (Wald, 1968). As a result of these changes, the protein transforms to its activated (catalytic) form, which initiates the phototransduction cascade. The activated form of visual pigment binds and activates the G-protein transducin, which in turn activates phosphodiesterase (PDE). Activated PDE hydrolyses cyclic guanosine monophosphate (cGMP, cG in Fig. 3), which acts as a cooperative channel gatekeeper in the outer segment of photoreceptors so that there is a cubic dependence of membrane conductance on the concentration of cGMP (Fesenko *et al.*, 1985). The concentration of free cGMP in darkness is high holding cGMP-gated channels open. Light-induced PDE activity leads to a decrease in cGMP concentration, which reduces the probability of cGMP binding to the cation channels, causing channels to close.

As the cation channels in the outer segment close, there is a reduction in the dark current which leads to hyperpolarization of the cell and a decrease in neurotransmitter (glutamate) release from the synaptic terminal. The electrical signal generated in this manner is the result of a massive molecular amplification: in rods a single photoisomerization may cause hydrolysis of 10^4 molecules of cGMP and interrupts the flow of at least 10^5 cations into the cell (Burns & Baylor, 2001). The amplification, which is qualitatively similar although lower in cones, arises mainly from the following three processes. First, a photoisomerized rhodopsin activates many transducin molecules. Second, each PDE molecule activated by transducin hydrolyses a large number of cGMP molecules. And third, multiple channels, each allowing the influx of cations at a high rate, close in response to the drop in cGMP concentration. This cascade creates considerable amplification: in a human retinal rod a single photoisomerisation reduces the circulating current by approximately 2 % at peak (Rodieck, 1998). In cones, however, the molecular gain of phototransduction is much lower than in rods (Kefalov *et al.*, 2003; Kawamura&Tachibanaki, 2008). The lower gain and faster response recovery in cones results in lower cone light sensitivity: a single photoisomerisation reduces the circulating current by 0.1 % or less at peak in primate cones (Schnapf *et al.*, 1990).

Deactivation

The visual system signals continuously about changes in the light environment, and for this to be possible, termination of the response to light in a timely manner is as important as the onset of the response. Thus, all the activation steps induced by light have to be terminated soon after their onset to enable photoreceptors to respond to subsequent light stimulation. This deactivation of the response is an active process and it requires shutdown of the visual pigment, transducin and PDE, as well as restoration of the cGMP level by activation of guanylyl cyclase. On a longer time scale, visual pigment has to be regenerated, as the deactivated pigment cannot be activated by light. The first step in termination of the light response is the shutdown of the visual pigment. It is initiated by phosphorylation of the active pigment at several sites by rhodopsin

kinase (Kühn & Wilden, 1987; Newton, 1997). These phosphorylations reduce the catalytic activity of the active visual pigment in stepwise manner (Wilden *et al.*, 1986) but for complete deactivation, the binding of arrestin is also needed. This process eventually shuts off the visual pigment activity, preventing further binding and activation of transducin molecules.

Deactivation of the visual pigment alone is not sufficient for rapid termination of the light response. Transducin and PDE activity need also to be shut off. In its active form transducin has guanosine triphosphate (GTP) bound to one of its subunits. The transducin-PDE –complex is inactivated when GTP is hydrolysed in a process requiring an accelerator protein RGS9-1 and a protein Gβ5L (He *et al.*, 1998; Makino *et al.*, 1999).

Ca^{2+} feedback is important for several steps of the deactivation processes. Upon closure of the outer-segment cation channels by light, the extrusion of Ca^{2+} ions by Na^{+}/Ca^{2+} - K^{+} exchangers leads to a decrease in intracellular Ca^{2+} concentration. One important Ca^{2+}-dependent deactivation mechanism is the restoration of the concentration of cGMP to the dark level. This is achieved by synthesis of cGMP by guanylate cyclase (GC), whose activity is regulated by the calcium dependent guanylate cyclase activating proteins GCAP1 and GCAP2 (Pugh *et al.*, 1997). In addition to cGMP synthesis, the regulatory negative feedback mediated by Ca^{2+} influences rhodopsin kinase activity and reduces the affinity of the light-sensitive cation channels for cGMP through calmodulin. In all these processes, the drop of Ca^{2+} concentration acts to restore the receptor dark current.

For photoreceptors to restore their full dark-adapted sensitivity, the visual pigment must be regenerated. The all-*trans* retinal is detached from the opsin and transported to the retinal pigment epithelium (Saari *et al.*, 1982; Bunt-Milam & Saari, 1983; Jones *et al.*, 1989), or to Müller cells for cones (Mata *et al*., 2002), where it is reisomerized to the 11-*cis* form and delivered back to the photoreceptors, where it binds to opsin and reconstitutes the visual pigment (for a review, see Lamb & Pugh, 2004)

Modelling photoresponses

Responses of photoreceptors to light are produced by the transient decrease in cGMP concentration in the outer segment described above. The resulting change in the circulating current (see I_R for rod and I_C for cone in Fig.2) depends on the light intensity such that at low intensities the amplitude of the photoresponse increases linearly with the stimulus intensity, retaining the response waveform. At high intensities, when the number of open channels and thus the circulating current approaches zero, photoreceptors saturate, indicating that the response amplitude can no longer increase with increases in light intensity, although the time spent in saturation does. Temporal parameters of responses in all these light regimes can be used to constrain molecular models of phototransduction.

The current knowledge about the molecular scheme of activation is sufficiently detailed that the time course of the onset of photoresponses can be predicted accurately by physiologically-realistic mathematical models. One widely used model of this kind is the 'activation model' developed by Lamb and Pugh (1992). The model does not include inactivation reactions and is, therefore, valid only at relatively short times after the stimulus. A model covering all aspects of photoresponses does not yet exist (see e.g. Hamer, 2000). However, there are models describing the full waveform of rod photoresponses in restricted conditions (such as Nikonov *et al.*, 1998) obtained by including certain simplifications of the phototransduction cascade.

2.1.3 Light adaptation

The vertebrate visual system operates over a very wide range of light levels: from a dark night to bright daylight the intensity increases even by a factor of 10^8. On the other hand, the working range of neurons is much narrower being about 2 log units on average. The problem arising from this difference is solved by adaptation acting to adjust the operating range of visual system to be all the time within a physiologically useful region of light intensities, i.e. being able to respond in graded manner to the most commonly occurring contrasts. Primarily, this adaptation has been observed to be of retinal origin and a property of photoreceptors. However, a big part of the extension of the dynamic range of rod vision is a result of neural convergence occurring in the rod pathway.

As described in the previous chapter, the phototransduction cascade allows dark-adapted rod photoreceptors to respond reliably to a single photon. Yet, photoreceptors need also to retain function in conditions where the mean light level is increased by several orders of magnitude. By way of light adaptation, the sensitivity of the phototransduction cascade is rapidly decreased so that saturation does not occur and the receptor can respond to light stimuli in the presence of the background illumination (see Fig. 4). Adaptation that occurs in the presence of steady illumination of relatively low or moderate intensity is termed light adaptation or background adaptation. In electrophysiological recordings, light adaptation is characterized by (i) a reduction in cell's sensitivity (measured as a decrease in the amplitude of the single-photon response) and (ii) acceleration of response recovery.

The molecular mechanisms of light adaptation are not yet fully understood but their ultimate effect is to keep a fraction of the cGMP-gated channels open in varying background illuminations to retain responsiveness. The findings of Nikonov *et al.* (2000) showed that the gain of the activation phase of phototransduction is unaltered under light adaptation. This means that molecular mechanisms of light adaptation do not operate via the gain of the activation steps but rather via the lifetimes of active substances. The cellular events known to mediate light adaptation are (1) calcium-dependent mechanisms, (2) increased steady-state PDE activity, (3) protein translocation and (4) pigment bleaching. Response compression (decrease in the number of open channels under steady background light) is also often characterized as an

adaptation phenomenon although it is more a consequence of working against a limit where all the channels are closed. (Burns & Lamb, 2003)

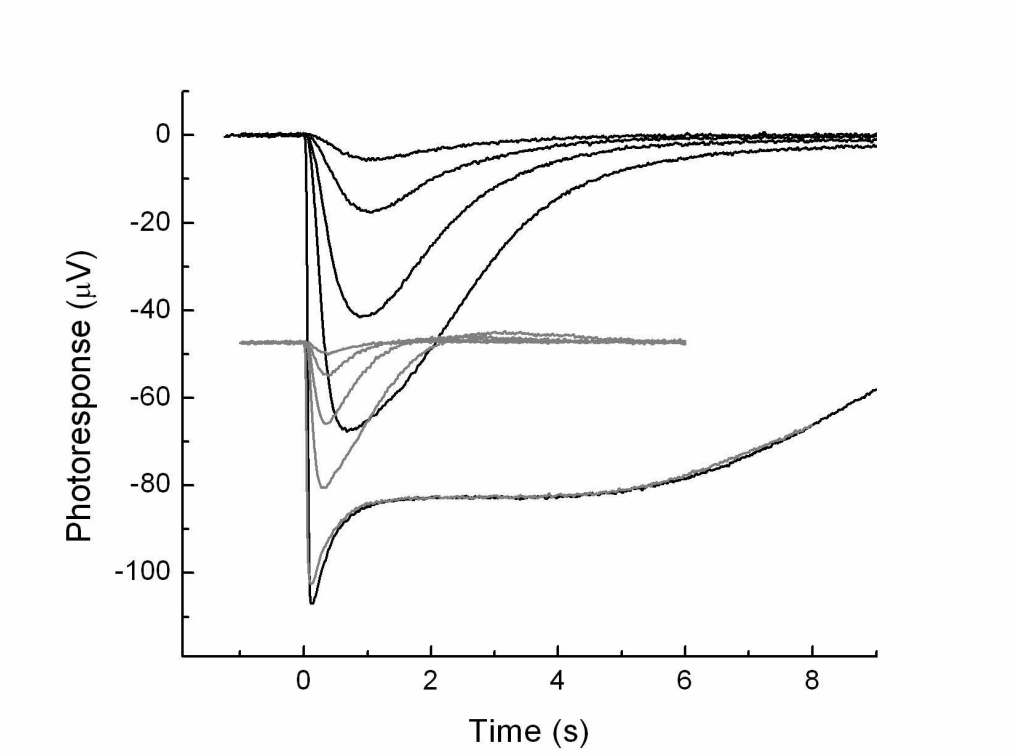

Figure 4. *Responses to brief (20 ms) flashes of light covering the working range of rat rods in darkness (black traces) and at an illumination activating ca. 22 rhodopsin molecules per rod per second (Rh*/s) (gray traces). Due to light adaptation the intensity of the stimulus light needed to produce a response of the same fractional size was 5-fold higher at the background illumination than in darkness. In darkness the stimulus intensities were 1.7, 5.4, 17, 54 and 5400 Rh* and under the background illumination 8.5, 27, 85, 270 and 27 000 Rh*.*

Calcium and light adaptation

In darkness the inward movement of positive charge through the cGMP-gated cation channels is mainly carried by Na^+, but also Ca^{2+} ions, so that there is a continuous influx of Ca^{2+} ions to the photoreceptor outer segment. Ca^{2+} is extruded from the cell via Na^+/Ca^{2+} - K^+ exchangers (Hodgkin *et al.*, 1987; Nakatani & Yau, 1988). Light closes the channels and decreases the influx of Ca^{2+} ions. The extrusion of Ca^{2+}, however, continues and this leads to a decline in intracellular Ca^{2+} concentration.

The light induced decrease in Ca^{2+} concentration affects at least three inhibitory processes in rod outer segments. The mechanisms may be similar also in cones although

much less is known about them. Most important of these negative feedback mechanisms is the Ca^{2+}-dependent regulation of GC activity (Koch & Styrer, 1988). It is mediated by GCAPs, which stimulate rapidly cGMP synthesis by GC in response to the fall in intracellular Ca^{2+} (Palczewski *et al.*, 1994; Palczewski *et al.*, 2004). Here it is noteworthy that in addition to affecting the recovery of the flash response, Ca^{2+}-dependent regulation of GC activity also controls the steady state level of cGMP under continuous illumination.

The second negative feedback mechanism is the regulation of rhodopsin activity by the Ca^{2+}-binding protein recoverin. Ca^{2+}-bound recoverin inhibits the activity of rhodopsin kinase and thus prevents it from phosphorylating rhodopsin (Kawamura, 1993; Gorodovikova *et al.*, 1994). The decrease in intracellular Ca^{2+} in light relieves this inhibition and leads first to a faster rhodopsin inactivation and consequently to a lower level of active PDE molecules (Gray-Keller *et al.*, 1993; Koutalos *et al.*, 1995; Matthews, 1997).

The third Ca^{2+}-dependent feedback mechanism is the calcium regulation of the cGMP channels by calmodulin or calmodulin-like proteins (Hsu & Molday, 1993; Nakatani *et al.*, 1995; Bauer, 1996). The reduced Ca^{2+} concentration in the presence of light is followed by calmodulin dissociating from the channel which increases its affinity to cGMP (Gordon *et al.*, 1995). This feedback mechanism may be minor in rods but, instead, it is quite probably significant in cones (Rebrik *et al*., 2000).

Contribution of elevated steady-state PDE activity to light-adaptation

The steady-state PDE activity is increased in constant illumination. Although this is a direct consequence of phototransduction it provides a major contribution to photoreceptor light-adaptation behavior (Nikonov *et al.*, 2000). In darkness, PDE activity is low and the absorption of even a single photon in a rod produces a large relative change in cGMP concentration giving rise to a sizable electrical response. In the presence of steady illumination, the PDE activity is higher and, accordingly, the activation of the same amount of PDE by one photon produces a smaller relative increase in PDE activity. This translates to a smaller relative decrease in cGMP concentration and also a smaller electrical response amplitude (Hodgkin & Nunn, 1988). In addition to smaller response amplitude the higher PDE activity in light also makes the response recovery faster (Nikonov *et al.*, 2000; Govardovskii *et al.*, 2000). The contribution of elevated steady-state PDE activity to light-adaptation is remarkable in rods but less important in cones due to ~10-fold higher dark PDE activity (Holcman & Korenbrot, 2005).

Contribution of pigment bleaching to light-adaptation

At higher background light intensities, bleaching of the photopigment starts to contribute to light-adaptation (Rushton & Henry, 1968). This adaptation mechanism is based on the loss of visual pigment, which decreases photoreceptor sensitivity by reducing the probability of successful photon absorption. In rods, pigment bleaching has

a negligible role in light-adaptation whereas in cones, which are capable of functioning even when a substantial fraction of their pigment is bleached (Burkhardt, 1994), this mechanism is particularly important. In cones, pigment bleaching, together with the fast regeneration of the bleached pigment provides means to avoid saturation at any illumination (Barlow, 1972; Shichida *et al.*, 1994). Furthermore one must consider in both rods and cones that the bleached visual pigment, i.e. chromophore-free opsin, is known to activate transducin at a low level thus desensitising the cells (Cornwall *et al.* 1994&1995).

Light-adaptation and protein translocation

Light-adaptation is characterised by the fact that up to intensities eliciting even 70 % suppression of the dark current in rods the amplification of the activation phase of phototransduction remains invariant (Nikonov *et al.*, 2000; Sokolov *et al.*, 2002). At higher intensities, however, reduction in the initial amplification has been observed (Lagnado & Baylor, 1994; Kennedy *et al.*, 2001; Sokolov *et al.*, 2002). This reduction is correlated with the massive translocation of transducin from rod outer segment to the inner segment but it is not yet clear whether this mechanism mainly serves to rescue rods from saturation in bright light or to act as a means to minimize metabolism by preventing excess energy consumption (Sokolov *et al.*, 2002; Burns & Lamb, 2003).

Recoverin and arrestin also translocate in rods upon illumination: recoverin moves from the outer segment to the inner segment and arrestin moves in the opposite direction (Brokhyuse *et al.*, 1985; Brann & Cohen, 1987; Philp *et al.*, 1987; Whelan & McGinnis, 1988; Strissel *et al.*, 2005). The functional consequences of this translocation are still under investigation but they probably include accelerated rhodopsin inactivation and thus faster response recovery and lower light sensitivity (Burns & Arshavsky, 2005). It has also been suggested that one effect of moving recoverin to the inner segment is to speed up synaptic transmission (Sampath *et al*., 2005).

2.2 Impact of physical variables other than light

In all vertebrates studied the photoreceptor cells function in the same general way (see Pugh & Lamb, 2000). Yet the electrical responses differ in kinetics, amplitude, and sensitivity. The differences are likely to arise in part from the properties of the different isoforms of the phototransduction molecules between animal species and between rods and cones. However, there are two important physical factors that have to be considered when comparing photoreceptors from different species: temperature and photoreceptor morphology. In the present work, rods and cones of both amphibians and rodents were studied. These photoreceptors differ significantly with respect to normal working temperature and/or morphology. Temperature is also a useful experimental variable.

2.2.1 Temperature

On an early autumn day, the outside temperature can easily change by 10 or even 15 ºC between night and daytime. The body temperature of amphibians changes with the ambient temperature, whereas in mammals body temperature remains constantly near 37 ºC.

In amphibians, temperature has many effects that are discernible both at the behavioural cellular level. The active behaviour of frogs and toads, for example, depends on the ambient temperature so that movements get slower at lower temperatures (Putnam & Bennet, 1981). These animals also stop feeding at temperatures below 7-8 ºC (Larsen, 1992; Aho, 1997).

Changes at the cellular level reflect the general effects of temperature on molecular motion and reaction times. Baylor *et al.* (1983), Lamb (1984) and Donner *et al.* (1988) studied the effects of temperature on photoreceptor function by recording rod photoresponses in toad and frog retina at different temperatures. They found that kinetics accelerated and the maximal response amplitude increased monotonically with warming over the whole range studied from 5 to 30 ^{o}C. Fractional sensitivity stayed essentially constant up to *ca*. 10-11 ^{o}C in frog and *ca*. 13 ^{o}C in toad and then decreased with increasing temperature. They concluded that the increase in amplitude is a result of an increased dark conductance and the change in the response kinetics a result of the change in diffusion of molecules within the disk and plasma membrane (Lamb, 1984).

The effects of temperature on phototransduction

The phototransduction reactions occur both in the membranes and in the cytoplasm and are, thus, dependent on the rates of diffusion of the phototransduction molecules in the lipid and water phases. As temperature is a measure of the kinetic energy of a system, higher temperatures imply higher average kinetic energy of molecules and more collisions per unit time. Therefore, it is evident that all the reactions involved in phototransduction are accelerated when temperature is increased.

Furthermore, at higher temperatures spontaneous (thermal) activation of any component of the phototransduction reaction chain will become more frequent. Especially important will be the spontaneous activation of visual pigment molecules, because the signal triggered by such an event will be amplified through the transduction cascade to produce a quantal electrical response indistinguishable from the response to a single photon. The idea of activation of visual pigment by thermal energy was proposed by Autrum (1943) but the first direct experimental evidence came from suction pipette measurements of dark current fluctuations in single toad rods by Baylor *et al.* (1980). They showed that discrete decreases of the light sensitive current (dark events) that are indistinguishable from photon-triggered events occur in absolute darkness. It is now commonly accepted that these events are initiated by thermal activation of visual pigment molecules. In all vertebrate photoreceptors studied so far their frequency of

occurrence is observed to be temperature dependent with a *ca.* 3-4 fold rise per 10 ^{o}C (Baylor *et al.* 1980; Matthews, 1984; Sampath & Baylor, 2002).

In addition to visual pigment, other proteins of the phototransduction cascade (G protein, PDE) can also be activated by thermal energy. Their activation produces so-called "continuous" noise, the relative importance of which varies among photoreceptors. It now seems that the dominant intrinsic noise in many cone types and even in some rod types may originate downstream from the visual pigment (Luo *et al.*, 2008; Ala-Laurila *et al.*, 2007). A basic difference, however, is that only pigment-originated noise can be truly "photon-like", since the amplification of any events initiated downstream in the transduction cascade must, in principle, be smaller.

2.2.2 Geometric factors

Vertebrates form a highly diverse group of animals. Correspondingly, within the framework of a common structural scheme, photoreceptor cells show enormous variation in detailed morphology (see e.g. Walls, 1942), and the rod/cone dichotomy was established in early histological studies (Schultze, 1866). It is obvious that such differences generally have a functional "meaning". Any attempt at understanding function and modelling photoresponses must take into account simple size parameters like outer segment diameter, length, surface and volume, and especially the surface-to-volume ratio.

Rods and cones differ widely in size among species. In mammals and birds the outer segments are generally slender compared to photoreceptors of poikilothermic vertebrates: mammalian rod outer segments are 25-30-fold smaller than those of amphibians (Lamb & Pugh, 2006). Furthermore, the size difference between rods and cones in amphibians is often quite remarkable: the volume of the outer segment of cone photoreceptors may be 30-70 times smaller than that of the rod outer segment (Donner *et al.*, 1998; Kawamura & Tachinabaki, 2008). In mammals, by contrast, rod and cone outer segments are more similar in size.

The functional consequences of the size differences are not yet fully understood. Advantages of having thin outer segments are the short diffusion distances from disks to the plasma membrane, and the smaller volumes where decrese in cGMP concentration in response to light have to be achieved. In phototransduction this means that the same rate of PDE activation will produce a faster change in cGMP concentration in thinner outer segments. In addition, the transduction signal spreads longitudinally further in thinner photoreceptors compared to thicker ones (Hemilä & Reuter, 1981; Holcman & Korenbrot, 2004). On the other hand, as the concentration of the visual pigment is quite constant in all photoreceptors, long outer segments allow very efficient photon catch, but a large outer segment volume also implies a high content of potentially noise-producing pigment molecules.

Another important photoreceptor parameter is the surface-to-volume ratio. Thicker cells provide lower surface-to-volume ratios and are thus energetically more economical. Expressed somewhat differently, the metabolic cost per retinal area of maintaining the light-sensitive current is smaller for few thick outer segments than for many thin outer segments. The difference in surface-to-volume ratio appears especially pronounced when comparing cones with rods. Due to the folded outer segment membrane cones have a greatly increased surface-to-volume ratio which is important in speeding up the rate of change of intracellular ion concentrations, especially calcium (Burns & Lamb, 2003). Furthermore, the higher surface-to-volume ratio provides faster recovery of the cell after a bleaching exposure (Ala-Laurila *et al.*2006).

2.3 Rods and the absolute sensitivity of vision

Any visual performance is ultimately constrained by the information provided by the retina. A dynamic electrical image of the outside world is created in the retina based on the signals of photoreceptors. In dim light, where the light sensitivity of cones is too low for these cells to signal the arrival of single photons, vision relies on the rod system. On the other hand, bright light saturates rods and in such illumination the electrical image of the outside world is based entirely on cone photoreceptors. At low light levels where the small number of photons and their stochastic nature limit the amount of information gathered by rod photoreceptors, retinal convergence is essential: rod signals are pooled in a series of convergent connections to ganglion cells sending signals from thousands of rods to higher visual centres (see e.g. Aho *et al.*, 1993), providing a way to obtain visual sensitivity much higher than the sensitivity of individual rod cells. In mammals this pooling occurs in a specialized circuitry referred to as the rod bipolar pathway and it transmits light-evoked rod signals to the retinal ganglion cells via rod bipolar cells, AII amacrine cells and cone bipolar cells (Dacheux & Raviola, 1986; Smith et al., 1986). Another important factor facilitating high absolute visual sensitivity is the slowness of electrical responses of rods, which implies that the neural trace left by each photon in each individual rod persists and is summed with the traces of photons arriving earlier/later over long times. This temporal summation, together with spatial summation, is how the rod system maximizes photon capture and thus sensitivity, although spatial and temporal resolution is simultaneously degraded (Barlow, 1982).

As described above, at low light levels, vision depends on rods being able to signal single photoisomerizations reliably. This feature of the rod system, however, implies that ‘false’ photon-like events due to thermal activations of visual pigment are also processed as visual information. The idea that vision at very low light levels may be limited by a “dark noise” due to spontaneous activations of visual pigments is from the middle of the twentieth century (Autrum, 1943; Barlow, 1956). It was shown by Aho *et al.* (1988, 1993) that absolute dark-adapted sensitivity in toads is indeed close to that allowed by the rates of thermal activations measured in single rods. Thus thermally-generated photon-like events produce “dark light” or “inner background light” that light signal must exceed to become detected. Viewed from this perspective, visual detection can be seen as a statistical problem: the photon flux when a light stimulus is present

must differ significantly from the range of photon fluxes plus dark light when the stimulus is absent (Barlow, 1956; Barlow, 1957).

In individual rods, thermal activations do not represent a notable source of noise due to the extremely stable nature of the rod visual pigment: on average, each molecule of e.g. toad rhodopsin is thermally activated once in *ca.* 3000 years at room temperature (Baylor *et al.*, 1980). By contrast, in cones the rate of spontaneous activations of visual pigments may be 3-4 log units higher than in rods (Barlow, 1958; Baylor *et al.*, 1984; Rieke & Baylor, 2000; Sampath & Baylor, 2002). Therefore, in L-cones of at least some species thermal activations of visual pigment may form the dominant source of noise, especially at higher temperatures (Rieke & Baylor, 2000). In primate L-cones, however, only a minor fraction of the very considerable dark noise appears to be due to thermal activation of visual pigment (Schnapf *et al.*, 1990; Fu *et al.*, 2008).

One component of photon-like noise that also needs to be considered is the "continuous" dark noise (Baylor *et al.*, 1980) due to spontaneous activation of transducin (Lamb, 1987) and PDE (Rieke & Baylor, 1996) molecules. In mammalian rods, unlike to amphibian rods, this form of noise has been observed to constitute the main component of the dark noise (Baylor *et al.*, 1984). However, in the mammalian scotopic system the continuous component is suppressed by a non-linear signal transfer from rod photoreceptors to rod bipolar cells (Field & Rieke, 2002; Sampath & Rieke, 2004; Field *et al.*, 2005). This threshold-like nonlinearity greatly improves the absolute sensitivity of the rod signals in the rod bipolar pathway. In amphibian rod vision it is unlikely for this kind of thresholding nonlinearity to occur due to extensive electrical coupling (Copenhagen *et al.*, 1990) and high signal-to-noise ratio (see Okawa & Sampath, 2007). Thus, in toads the retinal ganglion cells underlying the behavioral response are likely to have access to a linear sum of the full number of single-photon responses estimated from absorptive quantum efficiency.

3 Aims of the study

Basic parameters (sensitivity and kinetics) of photoresponses in the dark-adapted state and under adapting light were studied in rods and cones of two rodents (rat and mouse) and two anuran amphibians (toad and frog). The first objective was to investigate factors that determine the major functional differences between different species of vertebrate photoreceptors, given the fundamental similarity of the phototransduction cascade in all (rods and cones). The second objective was to relate the capacity for temporal integration in mammals and amphibians to rod photoreceptor kinetics and to the differences in visual sensitivity. In a separate project the goal was to develop a method using retina as a biosensor in experiments of controlled drug release.

In **paper I**, the purpose was to investigate how far temperature (and outer-segment size) can explain differences in rod sensitivity, response kinetics and light adaptation between mammals and amphibians, assuming phototransduction molecules in all vertebrate rods to be functionally similar. For this objective the effects of temperature and steady background light on rat rod photoresponses were studied by electroretinogram (ERG) recordings. For amphibian rods, the information from the literature was supplemented by ERG recordings from toad retina at different temperatures.

In **paper II**, the main objectives were (i) to clarify to what extent differences in the absolute visual sensitivity of amphibians and mammals (including humans) depend on differences in the capacity for temporal integration and (ii) to study whether temporal integration at the behavioural level can be explained by the response kinetics of the rod photoreceptors. Sensitivity, integration time (t_i), and temporal accuracy of vision were measured psychophysically in toads by recording snapping at worm dummies moving at different velocities at two temperatures (15 and 25 ^{o}C). To compare behavioral data to information at the visual input the integration time (t_i) of rods was determined by electroretinogram (ERG) recordings across the isolated toad retina at the same two temperatures (15 and 25 ^{o}C).

In **paper III**, the main purpose was to estimate the importance of thermal activations of visual pigment as a source of ‘dark light’ (activity of the phototransduction machinery in darkness) in frog long-wavelength-sensitive (L) cones. For this purpose both L-cone and rod photoresponses were studied by electroretinogram (ERG) recordings across frog retina at different intensities of steady background light, using temperature changes as an experimental tool.

In **paper IV**, the purpose was to characterise physiological properties of mouse cones intact in the isolated retina and thus make a bridge between electroretinogram studies of living animals and single cell suction pipette recordings of mouse cones. For this purpose mouse cone photoresponses were recorded by electroretinogram technique from isolated retinas and the basic parameters of cone photoresponses were determined from the recordings.

Paper V presents a practical application of the preparation, and the electroretinogram (ERG) recording technique combined with current knowledge of phototransduction. The idea was to use the (rat) retina as a biosensor for evaluating controlled drug release and the biocompatibility of polymers and polymeric nanostructures used as drug carriers. For this purpose ERG recordings across the isolated rat retina were performed at different concentrations of the model drug 3-isobutyl-1-methylxanthine (IBMX) and in the presence of polymers or monomers alone.

4 Materials and methods

The main experimental technique used in this thesis was electroretinogram (ERG) recording across the isolated aspartate-treated retina (I-V). This is a useful technique, especially in experiments requiring long and reliable recordings with treatments that are stressful for the cells, such as repeated temperature changes and use of a wide range of background light intensities. Such experiments would be very challenging – or even impossible – to perform with e.g. single-cell recording techniques.

In the ERG recordings, the flat-mounted retina was perpendicularly illuminated from the receptor side by brief light pulses or adapting background lights. The objectives of the studies required light intensities to be accurately converted into photoisomerisations per photoreceptor per second, and the absorbance properties of the rat retina were not well known. For this purpose, the axial absorbance of rat rods was measured by a method set up by the author (I). For calibration in amphibian retinas and mouse cones, for methods in the behavioural experiments (II), and for modelling of dim-flash rod responses (I), the reader is referred to the Methods sections of the original papers.

4.1 Animals, preparations and chemicals

Table 1. List of animal species and experimental temperatures used in studies I-V.

Species	**Photoreceptor type**	**Temperature (°C)**	**Reference**
Rattus norvegicus	rod	5, 12, 20-40	I, V
Bufo bufo	rod	15, 25	II
Rana temporaria	L-cone & rod	7, 11, 15, 20, 25	III
Mus musculus	M-cone & rod	37	IV

Animals and preparations

The animal species used in the research of this thesis are listed in Table 1. The rodents were lab-raised Wistar rats (*Rattus norvegicus*) and mice (*Mus musculus*, C57Bl/6). The amphibians were caught in the wild in the autumn, either in Leningrad Region, NW Russia (common frogs, *Rana temporaria*) or in SW Finland (common toads, *Bufo bufo*).

Before the experiment, the animals were dark-adapted usually for at least 12 h but for shorter durations in some rat and mouse experiments (at least for 3 h). The rodents were killed by CO_2 inhalation and decapitation. After decapitation (and double-pithing for the amphibians) the eyes were enucleated and bisected along the equator. The retinas were isolated from the open eyecups and the pigment epithelium removed in cooled Ringer solution (for Ringer compositions, see Methods in paper I) under dim red light.

Chemicals and polymeric materials used in the application of paper V

This thesis includes also an application of the ERG technique used in the other works, using the retina as a biosensor to study controlled drug release from polymeric structures. The model drug in the release experiments was 3-isobutyl-1-methylxanthine (IBMX), purchased from Sigma. The polymeric materials used as model drug carriers in the application were synthesised from monomers N-isopropylacrylamide (NIPAAm) and vinylcaprolactame (VCa) in Professor Heikki Tenhu's research group at the University of Helsinki. In addition, hexafluorobutylmethylacrylate (HFBMA) was used for copolymerisation with NIPAAm to modify the lower critical solution temperature (LCST) and to increase the hydrophobicity of the polymers (Lowe, Virtanen & Tenhu, 1999a, 1999b). All these monomers were purchased from Polysciences Inc. (Warrington, Pennsylvania, USA). The thermoresponsive polymers PNIPAAm and PVCa were synthesised from the monomers and the latex particles prepared as described earlier (Lowe, Benhaddou & Tenhu, 1998).

4.2 Electroretinogram (ERG) recordings (I-V)

The basic principle in ERG recordings was to stimulate the isolated retina by light and measure the changes in transretinal potential by two Ag/AgCl electrodes placed at the distal and proximal sides of the retina. The retina was placed in the specimen holder (Fig. 5) so that the illumination came from the photoreceptor side, which was continuously perfused by Ringer. The recorded DC signal was amplified 10 000×, digitized at 200 Hz (I, II, III and V) or 1000 Hz (IV) and stored on a computer hard disk for further analysis. During the experiment, the retinal temperature was controlled by a heat exchanger placed below the specimen holder and monitored continuously with a thermistor close to the retina.

Stimulus system

The retina was stimulated by a dual-beam optical system (Donner *et al.*, 1988). The homogeneous illumination was provided by a 50 W tungsten lamp with either 519 and 503 nm interference filters (papers I and V) or 552, 642 and 621 nm interference filters (paper III) (interference filters Melles Griot, half-transmission bandwidth *ca*. 10 nm). In part of the experiments (papers II and IV and toad experiments of paper I) the tungsten lamp with interference filters was replaced by 543.5 nm He-Ne laser (Melles Griot 05 LGR 173, 0.8mW). In both cases, light pulses were provided by a computer-controlled Compur shutter allowing a minimum pulse length of 16 ms. To get shorter stimuli (*ca.* 1.4 ms) for mouse cones, light pulses were generated with a computer-controlled xenon flash gun (Minolta 360PX), spectrally limited by a long-pass edge filter (GG495 by Schott, $\lambda > 495$ nm). The intensity of the light of each of these sources was controlled separately with calibrated neutral density filters and wedges.

The intensity of the light covering homogenously the whole measurement area was measured regularly by a calibrated photodiode (EG & G HUV-1000B; calibration by the

National Standards Laboratory of Finland). The homogeneity of the light spot was verified by measuring the intensity of light passing through a small aperture positioned at different points of the field.

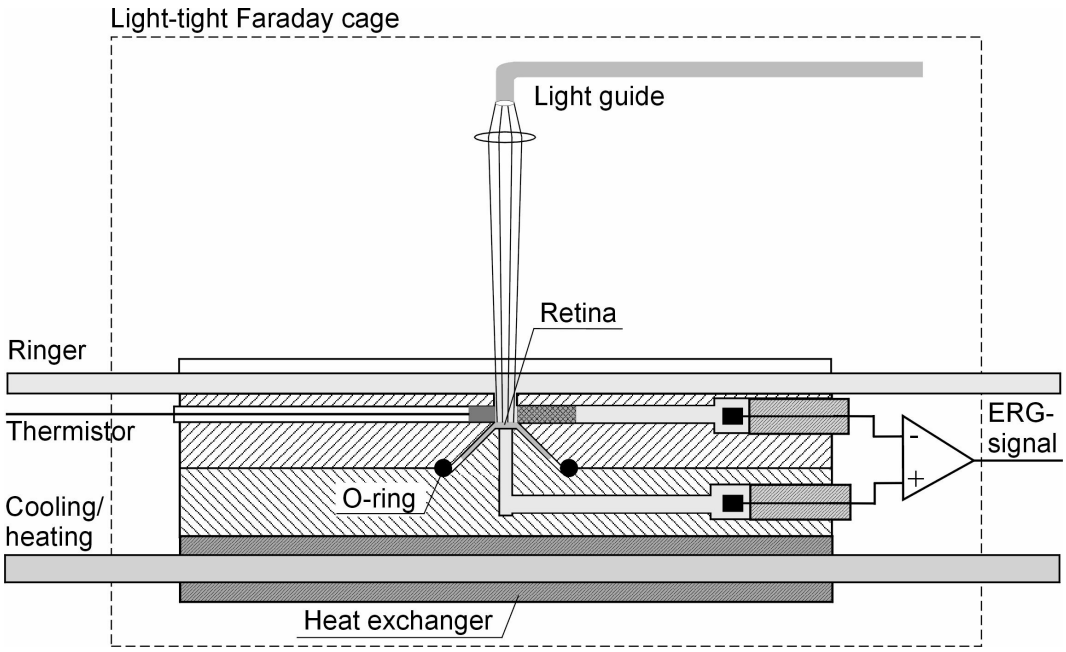

Figure 5. *A schematic representation of the setup used to record transretinal ERG.*

Isolation of the appropriate response component

The ERG voltage signal reflects changes in all radial currents that occur due to light in the resistive extracellular space of the retina. For this reason the measured signal may contain components not only from different types of photoreceptors but also from other cells, notably bipolar cells and glial cells. In the studies of this thesis, it was essential to isolate as reliably as possible the signal form a single photoreceptor type. Post-receptoral signal components were eliminated by using different chemicals. To isolate the signal of the photoreceptor type under study, different experimental protocols were used (see section iii) below).

i) Removal of response components from second- and third-order neurons

The information from photoreceptors to second-order neurons (bipolar and horizontal cells) is conveyed by chemical synapses through modulation of glutamate release. If this synaptic transmission is blocked, the recorded ERG-signal is practically free from postsynaptic components although glial responses to changes in extracellular ionic concentrations remain (see below). In most of the experiments, adding 2 mM sodium-L-aspartate (an agonist for glutamate) to the perfusing Ringer solution was enough to block glutamatergic transmission. In some experiments – especially when retinal

metabolism was high due to high experimental temperature – this concentration had to be doubled to remove the response of bipolar cells.

ii) Removal of glial components

Glial (Müller) cells extend radially across most of the thickness of the retina They serve as a retinal potassium buffer, releasing K^+ ions near the photoreceptor inner segments as the local K^+ concentration is decreased by illumination. This extrusion of K^+ is balanced by K^+ influx near the endfeet of the Müller cells in the inner retina to form a current loop. The ERG component due to this current is big and slow, but it can be eliminated by blocking potassium channels located mainly at the endfeet of Müller cells. (see e.g. Newman, 1993). In the experiments of this thesis, blocking was done by adding a high concentration of $BaCl_2$ in the lower electrode space of the specimen holder, from where it could slowly diffuse, mainly to the proximal side of the retina (Bolnick *et al.*, 1979; Donner & Hemilä, 1985; Newman, 1989), with minimal effects of Ba^{2+} on the photoreceptors themselves. In rodent experiments, the concentration was 10 mM, in anuran experiments a concentration of 3 mM was adequate.

iii) Isolation of the photoresponse of rods or L-cones

Mammals have only one type of rod photoreceptors in the retina. Frogs and toads have, in addition, blue-sensitive so-called "green" rods which are really morphologically modified cones. The proportion of the latter is small, however, and as their peak sensitivity lies at ca. 431 nm, their contribution to the ERG signal when stimulated with lights > 500 nm is negligible. This made the isolation of the rod component from the ERG-signal easy in all the animals used here: it could be done solely based on the slower kinetics and higher sensitivity of rods compared with cones.

The anuran retina may contain three cone types: short-, middle- and long-wavelength sensitive S, M and L cones (Liebman & Entine, 1968; Harosi, 1975; Koskelainen *et al.*, 1994). Of these, M cones have never been reliably identified in *Rana temporaria* and *Bufo bufo*, and the response contribution of S cones is negligible at the stimulus wavelengths used. To isolate the L-cone response from the rod response two different experimental procedures were used. One method giving confidently dark-adapted cone responses was a subtraction method (paper III) based on the spectral sensitivity difference between rods and cones and described in detail by Donner *et al*. (1998). In this method, the "pure" rod response to 552 nm light (rod favouring stimulus) is subtracted from the response to 642 nm light with an equivalent rod component but a much larger L-cone component (for illustration and more information see Fig 1. in paper III). The small cone component in the 552 nm response used for subtraction is then restored by adding the response to a cone-equivalent 642 nm flash to obtain a 'pure' L-cone response. The other method (paper IV) allowing distinguishing of cone responses from responses of spectrally similar rods is a double-flash technique (Koskelainen *et al*., 1994) where a rod-saturating flash is followed by a cone stimulating flash. The time between the flashes is defined so that cones have recovered from the first flash while rods remain saturated.

General experimental protocol

The goal of the experiments in this thesis was to characterise certain functional properties of rods and/or cones by recording response families to flashes of light with increasing intensity covering the dynamic range of the photoreceptor type studied. Responses were recorded (i) in the dark-adapted state at different temperatures (I-III) or at fixed temperature (IV), (ii) under a series of steady adapting lights of increasing intensities at different fixed temperatures (I, III), or (iii) in the dark-adapted state at different temperatures and at several concentrations of IBMX as well as in the presence of polymeric structures (V). Furthermore, in paper II responses to dim light pulses of successively increasing duration (from 20 ms up to 25 000 ms) keeping intensities in the linear response range were recorded to determine the integration time analogously to the "critical duration" in psychophysics.

4.3 Analysis

The recorded photoresponse families were used for further analysis to obtain parameters describing the functional properties of photoreceptors. Dim flash responses as well as intensity *versus* peak response amplitude (*I–R*) data extracted from response families were fitted with appropriate model functions. In addition, intensities of criterion responses of paper II were plotted against pulse duration on log-log axes and the integration time (t_i) was determined by accurately mapping the *end* of the time range where the stimulus intensity needed for a criterion response changed inversely to stimulus duration and the *beginning* of the time range where it was duration-independent (see paper II for more details).

Fractional sensitivity S, i.e. the fraction of the light-sensitive current turned off per photoisomerization was obtained by fitting *I-R* data with a weighted sum of an exponentially saturating function (Lamb *et al*., 1981)

$$\frac{R}{R_{\max}} = 1 - e^{-SI} \qquad (1)$$

and a Michaelis function

$$\frac{R}{R_{\max}} = \frac{I}{I + I_{½}} . \qquad (2)$$

In (1) and (2) $R_{\max}$ is the amplitude of the saturated response and $I_{½}$ is the flash intensity required to elicit a half-maximal response. The parameters S and $I_{½}$ are related by $I_{½} = 1/S$. If the weighting coefficient is α ($0 \leq \alpha \leq 1$) the weighted sum of (1) and (2) can be written as

$$\frac{R}{R_{\max}} = \alpha\left(1 - e^{-SI}\right) + (1-\alpha)\frac{I}{I + S^{-1}}. \qquad (3)$$

It is noteworthy that for amphibian photoreceptors and mammalian cones, α is typically $\alpha = 0$ but for mammalian rods (paper I) a smooth transition from Michaelis ($\alpha = 0$) to exponential saturation behaviour ($\alpha = 1$) was observed when changing the temperature from 12 to 37 °C. Thus to get a good fit of Eq. (3) to *I–R* data at all temperatures in rodent rods, the weighting coefficient was allowed to change with temperature.

Time-to-peak of the flash response (t_p), the parameter describing the time scale of the photoresponse if the waveform stays constant, can be directly measured from responses to dim, brief flashes if the signal-to-noise ratio is good enough. If this is not the case, a better estimate for t_p is obtained by fitting responses to brief ($\leq$ 20 ms) flashes with the phenomenological multi-stage filter models that describe the time course of linear-range responses of both rods and cones. The version mainly used in the present thesis is the 'independent activation' model of Baylor *et al.* (1974, 1979)

$$R(t) = IS_f \frac{n^n}{(n-1)^{n-1}} n^{-t/\tau}(1 - n^{-t/\tau})^{n-1}, \qquad (4)$$

where $R(t)$ is the response, S_f is flash sensitivity (in μV / (photons / μm^2) if I is in photons / μm^2), n is the number of stages (here $n = 4$), and τ is the general time constant

$$\tau = \frac{t_p}{\ln(n)}. \qquad (5)$$

The integration time t_i, the parameter describing the capacity of photoreceptors to temporally integrate photon signals, is by definition (Baylor and Hodgkin, 1973):

$$t_i = \int_0^\infty \frac{R(t)}{R} dt, \qquad (6)$$

where R is again the peak amplitude of the response $R(t)$. The integration time for responses that can be described by the independent activation model is obtained by substituting $R(t)$ from equation (4) into equation (6) and integrating:

$$t_i = \tau\left(\frac{n}{n-1}\right)^{n-1}. \qquad (7)$$

Substituting τ from equation (7) into equation (5) allows the integration time to be calculated directly from the time-to-peak t_p.

The amplification constant of activation A, a parameter describing the onset phase of the photoresponse, was obtained by fitting fractional response families (r(t) = $R(t)/R_{max}$) with a delayed Gaussian function (Lamb & Pugh, 1992)

$$\frac{R(t)}{R_{\max}} = 1 - \exp\left[-\frac{1}{2}\Phi A(t - t_d)^2\right], \tag{8}$$

where Φ is the flash intensity (number of photoisomerisations per photoreceptor) and t_d is the effective delay time putting together several short delays in the activation phase of transduction. In Eq. (8) all inactivation reactions are not considered. Therefore, fitting is restricted to relatively early times before shut-off reactions come into play. Defined in this way, *A* describes the effect of activation of a single visual pigment molecule after passing through the overall amplification of the phototransduction cascade. Taking the inverse square root of *A* provides us with a parameter corresponding to the time it would take for a flash response to reach a criterion amplitude in the absence of deactivation processes. This parameter ($A^{-½}$) depends only on activation, whereas t_p depends on deactivation processes as well.

For the analysis of *background light adaptation* in photoreceptors, the reader is referred to the description in paper III. The traditional fitting of the Weber function

$$S = S_{Dark}\frac{I_0}{I_B + I_0}, \tag{9}$$

to the fractional sensitivity *S* versus background light intensity I_B data gives the background intensity that depresses sensitivity to half its dark-adapted value (I_0). In estimating the intrinsic light-like activity in photoreceptors ("dark light") the Weber function has limited usefulness, since the dependence is generally shallower and, especially in L-cones, much more complex than envisaged by simple functions like eqn. (9) (Donner *et al.*, 1998).

4.4 Absorbance measurements

The intensity of the light incident on the retina was measured (usually in each experiment) with a calibrated photodiode (EG & G HUV-1000B; calibration by the National Standards Laboratory of Finland) in units of photons / mm^2 / s. The conversion of this value into photoisomerisations per photoreceptor per second (Rh* / s) requires knowledge of photoreceptor dimensions as well as their optical density, reflecting the specific absorbance of the visual pigment. For the anuran species used here, these values were readily available (Aho *et al.*, 1993; Donner *et al.*, 1995 & 1998). For rodent

rods, however, the absorption coefficient values used in earlier studies were found to be unrealistically low (0.01 μm^{-1}, Penn & Hagins (1972); 0.0085 μm^{-1}, Robinson *et al.* (1993)) compared with values for both rods and cones of many other vertebrate species (e.g. *Bufo marinus* 0.0161 μm^{-1} (Hárosi, 1975)). Therfore, the author set up a method for careful measurement of axial absorbance in rat rods in the intact isolated retina.

After trying to measure pigment density in isolated rat rod outer segments by microspectrophotometry (MSP) and finding that it was difficult or impossible to measure reliable absolute values in such thin cells as rat rods (outer segment diameter in our strain about 1.7 μm), the device illustrated in Fig. 6 was set up. The goal was to measure light transmission transversely through the isolated intact retina before and after bleaching the visual pigment.

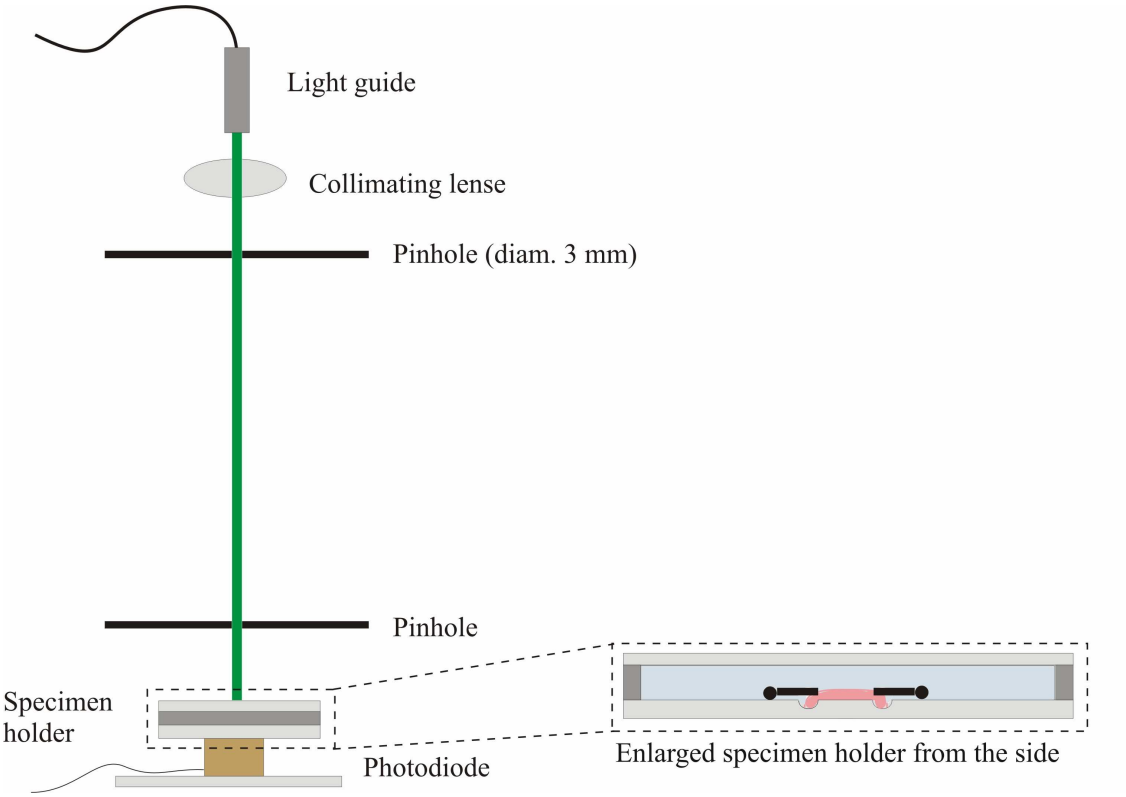

Figure 6. *Experimental arrangement for measuring axial absorbance of rat rods in the isolated retina.*

The dark-adapted retina was flat-mounted photoreceptors side up, on a transparent circular area (diam. 3 mm) at the bottom of a horizontal glass chamber (76x26x5 mm) (Fig. 6). The retina was held in place by a black metal ring (inner diameter 3 mm), which also served as a mask against light scattered from the 1 mm deep and 2 mm wide groove surrounding the measurement area. The chamber was filled with Ringer's solution containing 50 mM hydroxylamine to prevent pigment regeneration. The test light was a 501 nm unpolarized full-field exposure perpendicular to the retinal plane and its intensity was measured by the calibrated photodiode placed below the glass chamber.

The absorbance was determined by comparing the mean intensity of 3-4 dim 2-s test light pulses transmitted through the retina before and after a “total bleach”. The bleaching exposure was estimated to bleach more than 99.7 % of the pigment, whereas the 2-s test light pulse was estimated to bleach less than 0.01 % of the visual pigment.

An estimate for the absorption coefficient of rhodopsin in rat rods was calculated from the percentage of light absorbed in the retina and from the equation

$$I_{abs} = I \cdot 10^{-al}, \qquad (10)$$

where I is the intensity of light without the absorbing medium, a is the absorption coeffiecient and l the length of the rod outer segment. It was assumed that rods are packed hexagonally and that a fraction of light proportional to the retinal area uncovered by the rods will pass unattenuated through the retina. The very sparse population of cones in the rat retina was neglected.

4.5 An application for drug delivery tests (V)

The electroretinogram provides a useful tool to study the properties of the photoreceptor light response. The phototransduction cascade is intricate, and susceptible to pharmacological manipulation, thus we developed the retina as a model system for targeted drug delivery. In targeted (or controlled) drug delivery, a drug is released from carrier structures rapidly and locally by external stimuli (here by warming). When assessing the suitability of candidate carriers, the amount of drug released in living tissue needs to be carefully measured and the biocompatibility of the carrier material tested. The retina has properties that make it a promising biosensor for evaluating carriers. First, the photoreceptor cells are sensitive to any general cytotoxic effects and will reveal acute toxicity by changes in response sensitivity or kinetics, or even the disappearance of photoresponses. Second, drug release from the carrier can be tested with a model drug selected to target a specific step in phototransduction, whereby changes in the parameters of photoresponses could provide a sensitive and quantitative measure of the concentration of drug released in the living tissue. Here, the membrane-permeable PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) was used as model drug and effects on rod photoresponses were measured by ERG in rat retina

4.5.1 Testing the biocompatibility of molecules

A biocompatibility test was performed as follows. Response families of rat rods to 20 ms pulses of light with intensities covering the dynamic range of rods were repeatedly recorded in standard Ringer perfusion until a stable state had been reached (i.e., responses did not change between families). The polymer to be tested was then added to the Ringer and the retina was perfused with this solution for as long as needed to achieve a new steady state (unless the effects of the polymer were too pronounced). This was followed by washout with standard Ringer solution (see Fig. 4 in paper V).

4.5.2 Measuring the concentration of free IBMX

The method for determining the concentration of released model drug (not tied to carrier molecules, i.e., 'free') in the retina was based on its effects on phototransduction. The model drug IBMX is a PDE inhibitor that increases the time it takes for activated G-protein to find a PDE molecule that can be activated. This leads to slowing of rod responses in a dose-dependent manner, so that responses decelerate monotonically with increasing IBMX concentration (Capovilla *et al.*, 1982). The effects are fully reversible.

First, a calibration curve was constructed, plotting the time-to-peak (t_p) of dim-flash responses against the concentration of free IBMX in the perfusate. This curve had to be determined for each retina and temperature separately. Response families were recorded at different IBMX concentrations (ranging from 3 to 100 or 300 μM), then again in standard Ringer solution, and finally in Ringer solution containing the carrier polymer loaded with IBMX. With every solution it was essential to record rod responses until a new physiological steady state was achieved and responses remained constant with respect both to amplitude and kinetics.

The IBMX calibration data were fitted by a second-order polynomial

$$[IBMX] = b_1 + b_2 t_p + b_3 t_p^2 \qquad (11)$$

with temperature-dependent factors b_1, b_2 and b_3. Thus, the concentration of IBMX released from the polymeric drug carriers could be determined by measuring the time to peak t_p of linear-range flash responses of rods (see Figs. 5 and 6 in paper V).

5 Results

5.1 Physiological properties of photoreceptors affected by temperature

5.1.1 Photoresponse kinetics (I-IV)

The effect of temperature on photoresponse kinetics was qualitatively similar in all photoreceptors studied. The time to peak of dim-flash responses (t_p), which could be used as an index of response kinetics as the waveform stayed essentially constant, increased with decreasing temperature. The change in t_p was steepest in rat rods at lower temperatures being 5.4-fold over the lowest 10 ºC interval (6-16 ºC). This change became shallower towards higher temperatures so that over the highest 10 ºC (26-36 ºC) the change in t_p was comparable to that of anuran photoreceptors at all experimental temperatures (2-25 ºC).

While the overall shape of the dim-flash response remained approximately constant under temperature changes, in the sense that responses could be made to coincide by appropriate scaling of the time and amplitude axes, the speed of activation was less steeply dependent on temperature than the deactivation phase. In frog L-cones this phenomenon was quite remarkable: the parameter describing 'pure' activation $A^{-½}$ was observed to change 1.5-fold per 10 ºC whereas the change in t_p, which reflects both activation and inactivation, was ca. 2.5-fold per 10 ^{o}C. In rods, in comparison, the retardation of the deactivation phase compared to activation phase was only slightly stronger.

5.1.2 Rod response kinetics and visual time scale (II)

The kinetics of rod photoresponses analyzed at the cellular level in paper (I) was shown in paper (II) to be of immediate functional importance for vision. The integration time of rod vision, determined by the snapping behaviour of dark-adapted toads (details in paper II) decreased with increasing temperature in a manner well-correlated with the acceleration of the kinetics of small-stimulus rod photoreceptors determined by ERG recordings from the isolated toad retina. The values for behavioural integration time at the experimental temperatures 15 and 25 ºC were 4.3 s and 0.9 s, respectively. Rod t_i estimated from ERG recordings was 4.2-4.3 s at 15 ^{o}C and 1.0-1.3 s at 25 ^{o}C. The difference in integration time at these two temperatures correlated with ca. 5-fold higher sensitivity for seeing persistent targets at 15 ^{o}C compared with 25 ^{o}C. It is particularly noteworthy that "warm" toads were unable to integrate light over longer times than their rods, although the sensitivity advantage from doing so would have been at least as great as for the "cool" toads. The constraint on visual time scale set by the rod photoreceptors will affect all temporal properties of scotopic vision. This was demonstrated in paper (II) as a deterioration of the spatio-temporal precision of snapping with decreasing temperature.

5.1.3 Fractional sensitivity (I, III)

The fractional sensitivity (*S*) of rat and toad rods, and of frog rods and L-cones showed a monotonic decrease when temperature was increased from 10 to 25 ºC in the amphibians and from 12 to 36 ºC in rats. Below these temperatures *S* remained roughly constant. The decrease in fractional sensitivity was quite similar in all rod photoreceptors studied, the change being ca. 2-fold per 10 ºC. In frog L-cones the temperature dependence was comparable up to about 15 ºC but got steeper at higher temperatures (15 – 25 ^{o}C), where *S* decreased by a factor of 4 per 10 ºC. Thus, there was extra desensitization in frog L-cones versus rods at temperatures higher than 15 ºC. In the waveform of flash responses, the extra desensitization correlated with an extra acceleration of the deactivation phase in L-cones compared with rods upon warming from 15 to 25 ^{o}C. This phenomenon resembles light adaptation (see below).

Interestingly, fractional sensitivities in rodents at 36 ºC and in anurans at 15 ºC were very similar (see Table 2) although values in t_p differ by up to one log unit. At the same temperatures, on the other hand, t_p values were quite similar, while *S* at any given experimental temperature was about 0.7 log-units higher in rat rods than in the anuran rods.

Table 2. Some dark resting electrophysiological properties and outer segment sizes of rods and cones studied in this thesis at mammalian body temperature (36-37 ºC) and a typical temperature of frogs and toads on a summer night in Finland (15 ºC).

	Rod			**Cone**		
	t_p (s)	S (Rh*$^{-1}$)	V (μm^3)	t_p (s)	S (Rh*$^{-1}$)	V (μm^3)
Rat (at 36 ºC)	0,15	0,017	50			
Frog (at 15 ºC)	1,4	0,015	1400	0,26	0,0033	20
Toad (at 15 ºC)	2,4	0,013	1900			
Mouse (at 37 ºC)	0,117	0,013	37	0,051	0,0023	15

5.1.4 Light adaptation (I, III)

Exposure to steady adapting lights had qualitatively similar effects at any fixed temperature on the dim-flash responses of all photoreceptors studied. Increasing the intensity of the adapting light above a certain level caused photoreceptor desensitization coupled to response acceleration. Furthermore, as might be expected from the temperature dependence of fractional sensitivity in darkness, the desensitizing effect of the adapting light also set in at lower intensities the lower the temperature. Thus, in all photoreceptors studied, the intensity I_0 where background light started to affect sensitivity increased strongly with temperature. The I_0 values of frog L-cones were 1.5-2.5 log-units higher overall than those of rods (in frog as well as rat).

Comparing changes in I_0 over the temperature range studied reveals that the change in background light intensity required to reduce fractional sensitivity by half is steeper in

frog L-cones than in rods of frogs and rats. This phenomenon is most pronounced at temperatures higher than 15 ºC, i.e. over the temperature interval where the extra desensitization in dark-adapted cones versus rods was observed. Furthermore, if the change in integration time (proportional to t_p) with increasing adapting light intensity is factored out (see paper III for details) the increase in I_0 with warming disappears in rods but not in cones.

5.2 Retina in testing candidate materials for controlled drug release

The method using retina for testing candidate carrier materials with respect to biocompatibility and drug release properties comprises two main aspects. First, rod photoresponses are likely to reveal sensitively any acute cytotoxic effects of a general nature that the carrier structure or its constituents may have. The results show how toxicity can, in principle, be estimated from desensitization, deceleration, (and disappearance) of rod photoresponses, validating rat retina as potentially useful in a battery of fast biocompatibility screening tests.

Second, when a molecule with a precise action on the phototransduction machinery (here IBMX) is used as the drug to be released, it is possible to use changes in rod photoresponse parameters as quantitative measures of the fraction of drug released by the carrier, available in the tissue. Thus the release properties of the candidate carrier and its responsiveness to the control signal (e.g. temperature) may be easily studied. The accurate quadratic relation between [IBMX] and the time to peak of rod dim-flash responses (equation 11) is a central result of paper V. It suggests that IBMX is a useful model drug for testing any carrier that is broadly neutral with respect to the chemical nature of the drug to be released. In case there are specific interactions between the carrier and the drug molecule, however, IBMX cannot of course be used as a “general model”, and any quantitative testing of carriers using the retina as a biosensor is limited to release of molecules that have a very specific action on phototransduction.

6 Discussion

6.1 Phototransduction in mammals vs. amphibians (I-IV)

Our present understanding of vertebrate photoreceptors is largely based on experimental studies in two groups of animals, amphibians and mammals. The data derived from amphibian photoreceptors shows that their electrical responses are much slower than recorded from mammals (Baylor *et al.*, 1974, 1979, 1984; Lamb, 1984; Matthews, 1991; Miller & Korenbrot, 1994; Kraft *et al.*, 1988, 1993; Friedburg *et al.*, 2001&2004; Nikonov *et al.*, 2000&2006). In addition, amphibian rods are reported to exhibit extensive light adaptation starting at low light intensities (Fain, 1976; Hemilä, 1977; Baylor *et al.*, 1980; Donner *et al.*, 1990a), whereas rods of mammals have been shown to adapt only in a narrow range of high light intensities (Tamura *et al.*, 1989; Matthews, 1991; Hood & Birch, 1993; Kraft *et al.*, 1993; Silva *et al.*, 2001; Friedburg *et al.*, 2001). Thus, the data in literature suggests important molecular differences in phototransduction and light adaptation between "cold-blooded" vertebrates and mammals, including humans. This thesis shows that significant generic differences in the functional properties of the transduction proteins between these groups are not necessary to explain their functional differences: at similar temperatures photoreceptors of mammals and amphibians work in a remarkably similar way, when differences in outer-segment size and morphology are also taken into account.

Kinetics and fractional sensitivity

As described earlier (see chapter 2.2.1) higher retinal temperature brings along faster reaction kinetics and increased PDE activity. In photoreceptors, this is seen as a speeding of response kinetics and decrease in fractional sensitivity. Viewed from this perspective, it is not surprising that photoresponses of mammalian and amphibian rods show strikingly similar values of t_p when referred to the same temperature. The plot of t_p vs. temperature (Fig. 7), as well as our preliminary data on mouse cones at 25 ^oC, suggests that it is approximately true of cones as well. Changes in t_p of rat and toad rods and frog rods and cones can be described by similar Q_{10} values (2-3) in agreement with earlier reports of amphibians (*Bufo marinus*: Baylor *et al.* 1983; Lamb, 1984; *Pseudemys scripta elegans*: Baylor *et al.* 1974). This requires, however, that the profoundly steeper change in t_p of rat rods at temperatures lower than *ca.* 15 ^oC is not given full weight. The observed excessive retardation of the recovery phase compared with the activation phase of the photoresponse at very low temperatures is consistent with the observations of Robinson *et al.* 1993 and may be a phenomenon of warm-blooded animals only, reflecting for instance a phase transition in the cell membrane leading to significantly slower diffusion of molecules. This discrepancy between mammals and amphibians may be quite natural as these are temperatures far below those that the visual cells of a living rat or other mammal ever experience. However, from a molecular point of view, the implication of the observed excessive retardation of the recovery phase is that different mechanisms for response shut-off become rate limiting at the lowest temperatures.

Activation and recovery phases of responses were affected differently by temperature also in frog cones, but at higher temperatures (above *ca*. 15 °C). In rat rods, the strong retardation of the recovery phase upon cooling to very low temperatures was accompanied by only a shallow change in fractional sensitivity. In frog cones, on the contrary, warming induced excessive acceleration of the recovery phase compared with the activation phase, resulting in a steeper change in fractional sensitivity than evident at lower temperatures. Rieke and Baylor (2000) concluded that in salamander L-cones (using a mixture of 11-*cis*-retinal (A1) and 11-*cis*-3,4-dehydroretinal (A2) chromophores) thermal activations of visual pigment effectively light-adapt the cells. Our main hypothesis in paper III was that this may also be the case in frog L-cones, although A1 chromophore they use is likely to produce a much less stable pigment than the A2 chromophore (Donner *et al.*, 1990b, Ala-Laurila *et al.*, 2007) The excessive acceleration in response shut off coupled to desensitization observed in frog L-cones at temperatures higher than *ca*. 15 °C is probably at least partly due to spontaneous thermal isomerisations of visual pigment light-adapting the cones.

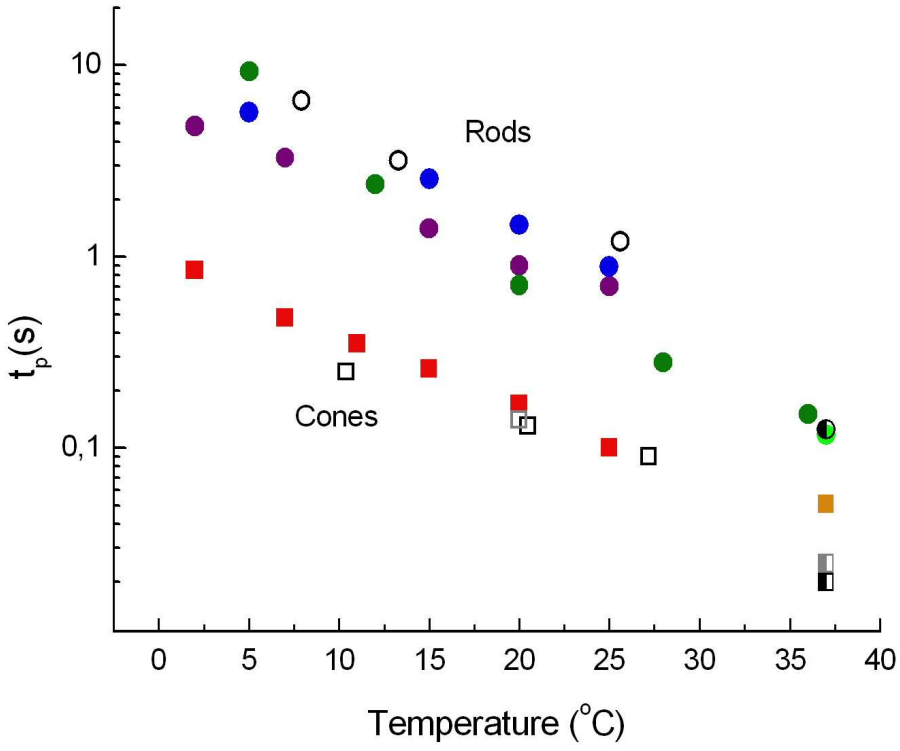

Figure 7. *Acceleration of dim-flash responses with increasing temperature in rods (circles) of frog (purple solid), toad (blue solid), rat (green solid), mouse (light green solid), cane toad (black open, Lamb, 1984) and human (black half open, Friedburg et al., 2001) as well as cones (squares) of frog (red solid), mouse (orange solid), turtle (black open, Baylor et al. 1974), salamander (gray open, Perry & McNaughton, 1991), ground squirrel (gray half open, Kraft et al., 1988) and human (black half open, Friedburg et al., 2004). All t_p values of our recordings are means of at least three experiments*

In all photoreceptors studied fractional sensitivity changed in a qualitatively similar way: it increased monotonically with cooling down to *ca*. 10-12 ^{o}C and remained rather constant at even lower temperatures. Our values of *S* in rodents at body temperature and anurans over the temperature range 10-25 ^{o}C were consistent with values reported in the literature (see e.g. Baylor *et al.*, 1984; Kraft *et al.*, 1993; Baylor *et al.*, 1979; Donner *et al.*, 1995) although the fractional sensitivity of anuran rods in our work was somewhat lower than reported earlier, probably reflecting our choice of using solely HEPES as a buffer instead of a mixture of HEPES and bicarbonate (see Donner *et al.*, 1990c). It is noteworthy that a close agreement was found in the fractional sensitivity of rodent photoreceptors (rods and cones) at body temperature and the corresponding type of anuran photoreceptors at the temperature (around 15 ^{o}C) where frogs and toads in the northern hemisphere most naturally are active. Moreover, our results suggest that the rate of thermal isomerisations of visual pigment molecules is not high enough to light-adapt frog L-cones at temperatures below ca. 15 ^{o}C, and the situation appears to be similar in human cones at body temperature: Fu *et al.* (2008) recently concluded that thermal isomerisations of human L-cone pigments at body temperature occur at such a low rate (ca. 10 Rh*/s) that it is unlikely to light-adapt the cones.

Light adaptation

Light adaptation in rat and frog rods agreed well at similar temperatures. This means that rat rods adjusted their fractional sensitivity as efficiently as rods of frogs and other amphibians in general (see papers I&III as well as Hemilä, 1977; Donner *et al.*, 1995). Still, at 36 ºC rat rods showed light adaptation behaviour very typical to mammalian rods, including humans (Tamura *et al.*, 1989; Nakatani *et al.*, 1991a&b; Kraft *et al.*, 1993). The somewhat higher fractional sensitivity of rat rods compared with frog rods at similar temperatures was not evident as a higher susceptibility to densensitization by backgrounds. This is consistent with our suggestion (paper III) that, in rods, differences in the weakest background light that begins to affect sensitivity simply reflects differences in temporal integration of the photoactivations from the background light. Temporal integration in frog and rat rods, as judged by the time to peak of shape-invariant dim-flash responses (cf. Fig. 7), was comparably similar at any given temperature.

As for rods, steady background light in frog cones also had qualitatively similar effects on the linear-range responses at any experimental temperature. The adaptation behaviour of cones at all temperatures thus agreed with earlier reports (Hood & Hock, 1975; Donner *et al.*, 1998). The “dark light” of frog cones as determined by background adaptation was found to increase more steeply with temperature above 15 ºC than below that temperature, supporting the notion that L-cone dark sensitivity may truly be limited by an intrinsic thermal “dark light” at higher temperatures. One probable source of this intrinsic thermal “dark light” is thermal activity of visual pigment. In addition, spontaneous activations of phosphodiesterase molecules may provide another source, although this effect seems not to be strongly temperature-dependent (Baylor et al., 1983). Also, one possible source comparable in its effect to the effect of thermal activity of visual pigment, is the chromophore-free opsin shown to activate transduction at a low

level (Cornwall et al., 1995; Kefalov et al., 2005), but neither the absolute level nor the temperature-dependence of this activity in dark-adapted cones *intact* in the retina is known.

The size of outer segments

The data on mammalian and amphibian photoreceptors presented here indicates that it is not necessary to assume remarkable differences in phototransduction molecules between mammals and amphibians. In paper I, it was shown that the observed difference in fractional sensitivity between rat and toad rods can be fully explained by the 39 times smaller outer segment volume of rat rods compared with toad rods. This conclusion reflects the fact that the same rate of PDE activation will produce a faster change in cGMP concentration in the smaller volume of a rat rod compared with a toad rod, and, since it is a change in concentration that modulates the channels, the response per R* will be larger in rat than in toad.

In mammalian rods, it is possible that the very thin outer segments are a result of adaptation to high and stable body temperatures, allowing the generation of large single-photon responses within the compressed time scale afforded by the high temperature. Furthermore, keeping outer segments long (although narrow) retains high quantum catch (although it is far from that of most amphibians). Thus, the long and thin outer segments of mammalian rods enable simultaneous achievement of high dark-adapted sensitivity and fast response kinetics

The effect of outer segment size in cones is more complex due to the folded structure of the outer segment affecting greatly e.g. the surface-to-volume ratio of these cells. Qualitatively, both small size and large surface will support efficient recovery of processes dependent on calcium feedback.

6.2 The impact of photoreceptor properties on visual performance

6.2.1. High visual sensitivity is a result of long integration times and low intrinsic noise

Lowering body temperature in amphibians increases the absolute sensitivity of vision as well as fractional sensitivity of photoreceptors – the same phenomenon being apparent also in mammalian photoreceptors (Papers I-III). Is the reason for this the decrease in intrinsic noise or the increase in temporal integration following slowing down of responses of neurons to light?

Thermal activations of photopigment produce intrinsic thermal noise indistinguishable from “background” flux of photons. The role of this noise in limiting the absolute sensitivity of vision is shown to be particularly important (Baylor *et al.*, 1980; Aho *et al.* 1988, 1993; Fyhrquist *et al.*, 1998; Firsov *et al.*, 2002). In the present thesis (paper

II), the contribution of temporal integration to the absolute sensitivity of vision was assessed directly. It was found that in a dark-adapted toad visual sensitivity grows proportionally to exposure time up to a limit, the integration time. The integration time measured from behavioral studies was found to be five-fold higher at 15^{o}C than at 25^{o}C and correlated closely with the integration time measured in rod responses

6.2.2. Setting the trade-off of temporal integration vs. temporal resolution

Increasing visual sensitivity by means of temporal integration leads to losses in temporal resolution. Is there then any benefit in getting higher sensitivity if vision simultaneously becomes very slow? The answer depends on the targets of interest to the animal. For toads hunting at night under very dim illumination the increase in sensitivity is truly valuable. At low temperatures the worms or woodlice they feed on are quite slow (see paper II) so that toad vision remains in the biologically relevant temporal range (see Warrant, 1999). On the other hand, slow vision at the low temperature was associated with deterioration in the spatio-temporal accuracy of visually guided snapping (II).

In mammalian rod bipolar pathway high-pass filtering occurring during signal transfer from rods to second-order cells sharpens the temporal resolution of the light response (Field & Rieke, 2002). This filtering accelerates rod single photon response downstream the retina (Field & Rieke, 2002) and, in addition to mammals, it has also been observed in turtle (Schnapf & Copenhagen, 1982) and salamander (Capovilla *et al*., 1987; Bialek & Owen, 1990; Armstrong-Gold & Rieke, 2003) but *not* in toad (Belgum & Copenhagen, 1988).

7 Conclusions

1. Temperature and the structural features of photoreceptors can explain much of the difference in their functional properties across mammals and amphibians.

2. In rods, the differences in electrophysiological properties between mammals and amphibians disappear if temperature and outer segment size are taken into account. In cones other factors must also be present.

3. Lowering temperature increases the absolute sensitivity of vision in amphibians as well as fractional sensitivity of photoreceptors (also in mammals). Measurements suggest this increase is due to the increase in temporal integration following slowing down of photoresponses.

4. The method developed for controlled drug delivery enables accurate concentration determinations of the model drug IBMX.

5. Biocompatibility of the drug carrier molecules can be rapidly tested by following the degree to which rods retain stable function in the presence of the carrier molecules.

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