The Photopigment Content of the Teleost Pineal Organ

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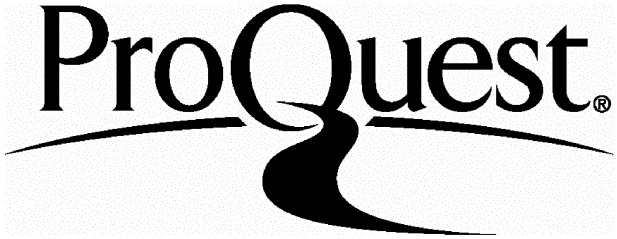
A thesis submitted to the University of London for the degree of
Doctor of Philosophy (PhD)
2000



ABSTRACT

Numerous studies have addressed the issue of the photopigment complement of the retinae of different vertebrate species. However, in most non-mammalian vertebrates, extraretinal photoreceptive sites also exist, of which the pineal complex is the most evident. The pineal organ of lower vertebrates contains photoreceptive cells, similar to the cones of the retina, and is thought to play an important role in the temporal organization of the animal, through both a neural output to the brain and the synthesis of the photoperiod-mediating hormone melatonin.

The visual system of teleost fishes has been studied extensively, particularly in regard to its adaptations to different photic environments. Although several studies have addressed extraretinal photoreception in teleosts, little is known as to the exact nature of the photopigments found in the teleost pineal organ, in terms of the number of photopigments present, their spectral sensitivity and their relationship to the visual pigments of the retina.

Microspectrophotometry (MSP) was used to determine the absorbance characteristics of the pineal photoreceptors of the goldfish (*Carassius auratus*), revealing a single photoreceptor population with a λ_{max} of 511 nm. Because of the variable A_1/A_2 chromophore content in these photoreceptors, the pineal photopigment was also bleached and regenerated with a single artificial chromophore, 9-*cis* retinal. Comparison with 9-*cis* data from goldfish retina suggested the opsin present in the pineal was most similar to the retinal rod opsin, although slightly blue-shifted. MSP was also conducted on the native pineal photoreceptors of another cyprinid, the golden orfe (*Leuciscus idus*) and a characid, the Mexican tetra (*Astyanax fasciatis*) in which similar pineal–specific photopigments were identified.

The goldfish pineal was screened for retinal opsins, and during this process a novel rod-like opsin, exo-rod opsin, was identified, which has since been demonstrated to occur uniquely within the teleost pineal organ. The full coding sequence of the goldfish exo-rod opsin was obtained, including gene structure.

DECLARATION

I declare that this thesis submitted for the degree of Doctor of Philosophy is my own composition and that the data presented herein is my own original work, unless otherwise stated.

Stuart Neil Peirson, B.Sc.

This work is dedicated in loving memory of my grandparents, who provided continual encouragement and support throughout my studies

ACKNOWLEDGEMENTS

I would like to thank a great number of people for their assistance and support, both scientific and moral, throughout the course of this work.

Firstly, a huge thanks to my principal supervisor Jim Bowmaker, for his continual support and advice, and most of all for the time he has spent neglecting his own work to sit and discuss my ideas, not to mention suffer my complaints and laments. Thanks are also especially due to my second supervisor, David Hunt, for his expertise and patience.

A great number of people at the Institute have made my time there a pleasure, especially the fellow students with whom I have had the luck to share an office. So a big thank-you to Matt, Emma and Jen for making day-to-day life at the Institute both entertaining and informative. Thanks also to Tony, Roger, Aachal and Luke for their general sociability and good humour (and apologies to Fred for distracting them from their work so often). Many thanks are also due to Juliet for her unwavering interest and the numerous discussions and advice relating to this project, and to Glen for all his assistance with the histological aspects of this work (and the taunting this entailed). I also owe a debt of gratitude to Jay for all his assistance, especially during writing up.

Thanks to all the people in Molecular Genetics who have made an alien visual scientist welcome, and especially to Phillippa for her excellent tutelage and unflagging patience, to Suba for the assistance and constant mockery she has provided, and to Suzanne, Inez, Naheed, Jill and everyone else in the group for making the lab a friendly and pleasant place to work, even, god forbid, at weekends.

A big thanks also to all the sociable folks who have participated in the many forays to the safety of the pub – Glen, Tony, Luke, Roger and Jay (again), Juan, Phillipe, Damian, Helen and Nacho, to name but a few. Thanks are also due to all my friends, especially to Hannah, Andrea, Jim and Steve, for taking my mind off work and reminding me that there is a world outside of science.

I would especially like to take this opportunity to thank to all my family for their ever-present support throughout my time at university, and for their interest and patience in the face of my scientific ramblings. So to Mum, Ron, Dad, Joan, Andy and Em, cheers!

CONTENTS

Results and Discussion

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A_1	11-*cis* retinal
A_2	11-*cis* 3-dehydroretinal
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dNTPs	Deeoxynucleotidetriphosphates
EPG	Electropinealogram
ERP	Electroretinogram
gDNA	Genomic DNA
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HIOMT	Hydroxyindole-O-methyl transferase
IPTG	Isopryl-B-D-thiogalactoside
Kb	Kilobase
λ_{max}	Wavelength of maximum absorbance
LWS	Long-wave sensitive
M	Molar
µg	Microgram
µl	Microlitre
ml	Millilitre
mRNA	Messenger RNA
MSP	Microspectrophotometry
MWS	Middle-wave sensitive
NAT	N-acetyl transferase
nm	Nanometer
PCR	Polymerase chain reaction

PDE	Phosphodiesterase
RACE	Rapid amplification of cDNA ends
RPE	Retinal pigment epithelium
rpm	Revolutions per minute
SWS	Short-wave sensitive
Taq	*Thermus aquaticus*
TM	Transmembrane
UV	Ultraviolet
UVS	Ultraviolet-sensitive
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

INTRODUCTION

CHAPTER 1

BACKGROUND

1.1. BIOLOGICAL CLOCKS

Organisms ranging from unicellulars to vertebrates exhibit daily fluctuations in biochemical, physiological and behavioural parameters. In fact, such temporal rhythms are so prevalent that for many years they remained unstudied, and were assumed to simply reflect a response to the changing conditions of the environment. However, when faced with constant conditions these rhythms persist, demonstrating the presence of an endogenous biological clock, usually referred to as an endogenous oscillator. These oscillators are entrained by environmental stimuli (*zeitgebers* or 'time-givers'), such as light and temperature, allowing the organism to synchronise its body clock with periodic environmental events, typically the day/night cycle. Biological rhythms that persist with an endogenous period (τ) of approximately 24 hours are usually referred to as circadian (from the Latin *circa* 'about' and *dies* 'day').

The primary zeitgeber for most organisms is light, which acts to synchronise the oscillator to the precise light cycle of the environment (photoperiod), although thermal cues may also influence the oscillator. Other important biological rhythms include *ultradian* rhythms (shorter than 1 day) and *circannual* rhythms (yearly cycles such as seasonal behaviours).

Why do virtually all organisms possess biological clocks? The obvious benefit a circadian oscillator confers to an organism is the ability to anticipate periodic environmental events, and consequently to allow a realignment of physiology to exploit the changing environment for the maximum benefits. An activity rhythm arising from a purely driven response to environmental stimuli results in a time delay between stimulus and response, and particularly with complex behavioural adaptations these delays may be quite significant. Perhaps it is a reflection of the importance of anticipation or 'being prepared' that biological clocks are found in such a wide variety of organisms (Takahashi and Zatz, 1982, Pittendrigh, 1993 and Foster and Provencio, 1999).

A) CIRCADIAN ORGANIZATION

An efficient circadian time-keeping system requires three components:

1) Photoreceptor cells to measure the light environment
2) An endogenous oscillator to keep time
3) Neuroendocrine or neuronal outputs to modulate temporal physiology

In most vertebrates the core of the circadian system consists of three elements: the retina, the pineal complex and the suprachiasmatic nuclei of the hypothalamus. Together, these structures make up a vertebrate "circadian axis" (Menaker *et al.*, 1997). The circadian system has often been represented as a simple pathway connecting photoreceptor-to-oscillator-to-output, particularly due to studies concentrating on the mammalian system, where the individual components appear to be dispersed. In mammals the photoreceptor input lies within the eye, a central oscillator resides within the suprachiasmatic nuclei, and a neuroendocrine melatonin signal is elaborated by the pineal organ (Takahashi and Zatz, 1982 and Reuss, 1996). However, such a model is an obvious oversimplification, and in most vertebrates each structure may independently contain photoreceptors, oscillators and output mechanisms, and in many cases a single structure may contain all three (see Korf *et al.*, 1998 and Falcon, 1999).

The organisation of the circadian axis appears to vary considerably between vertebrate classes. The presence of photoreceptors, oscillators and output mechanisms within each structure, the relative importance of each structure within the circadian hierarchy, the precise functions mediated (be they local or systemic), and the interconnections between the elements of the circadian axis may all vary considerably, even within each vertebrate class (Underwood and Groos, 1982 and Underwood, 1990).

B) CLOCK GENES

The last few years have seen an explosion in the field of circadian biology, primed by the isolation of numerous so-called 'clock' genes (Reppert, 1998). At the simplest level these clock genes are transcribed to produce rhythmic levels of clock gene mRNA, which in turn produce rhythmic levels of clock protein. These proteins are themselves elements that act to regulate their own transcription, typically resulting in a negative

feedback loop. It is this form of transcriptional feedback loop that is thought to form the basis of the circadian oscillator at the cellular level (Takahashi, 1991).

However, it has become evident that numerous complex interactions of various different genes and their products also occur, fine-tuning and carefully regulating this rhythmic transcription process. The pace and depth of this expanding field are beyond the scope of this study, but a basic awareness of the transcriptional nature of circadian oscillators is essential when considering the role of photopigments in the regulation of circadian systems, and specifically how these pigments may interact with the oscillator to bring about photoentrainment. Several papers are available reviewing the recent advances in the clock gene field, to which the reader is referred, including (Foulkes *et al.*, 1997, Sassone-Corsi, 1998, Dunlap, 1999 and Lakin-Thomas, 2000).

1.2. EXTRA-RETINAL PHOTORECEPTION

As well as the lateral eyes most vertebrates possess additional extra-retinal photosensory organs, including photoreceptors in the pineal complex, deep brain, skin, cornea and iris (for review see Shand and Foster, 1999). These extra-retinal photoreceptive sites mediate a variety of physiological functions, as discussed below. The pineal complex, as the basis of this study, is covered separately in the following section.

A) EXTRA-RETINAL PHOTORECEPTOR SITES

Dermal photoreceptors (melanophores, chromatophores and iridocytes) mediate direct responses to light such as colour changes in chromatophores or iridocytes, and pigment aggregation in melanophores. In addition, dermal photoreceptors may mediate behavioural responses such as triggering locomotor activity (Wolken and Mogus, 1979 and Shand and Foster, 1999).

Non-retinal photoreceptors in the eye may mediate iris constriction and corneal coloration, the latter acting as a filter to reduce chromatic aberration or to enhance contrast perception (Shand and Foster, 1999).

Deep-brain photoreceptors occur in the hypothalamus or periventricular organs of most non-mammalian vertebrates. These photoreceptors appear to be important for the

regulation of seasonal reproduction and circadian physiology, as well as non-rhythmic responses to light (see Underwood and Groos, 1982, Foster *et al.*, 1994, Yoshikawa and Oishi, 1998, Foster and Soni, 1998 and Shand and Foster, 1999).

B) CIRCADIAN PHOTORECEPTION

One of the immediate questions posed by the presence of extra-retinal photoreceptive sites is what advantage do these multiple non-visual photoreceptors provide? The photoreceptors of the deep-brain and pineal appear to play an important role in circadian photoreception, but why can circadian responses not be mediated by ocular photoreceptors as they appear to be in mammals? (see Foster, 1998 for evidence). This may be explained by the differences in the spatial and temporal aspects of visual and non-visual photoreception. Vision requires rapid responses and precise analysis of stimuli from across a highly organized photoreceptive surface to provide a spatial representation of the outside world. However, such a system provides a measure of brightness from a particular direction (radiance) rather than from the whole environment (irradiance). For circadian photoreception irradiance provides a far better means of monitoring the level of overall environmental light. As such most vertebrates appear to have evolved extra-retinal photoreceptive sites, which lacking the focusing mechanisms and precise photoreceptor arrays of the retina, are perfectly adapted for irradiance detection (see Foster *et al.*, 1994 and Foster and Provencio, 1999).

The presence of extra-retinal photoreceptors may therefore reflect adaptations for irradiance detection, as well as providing advantages by tuning to respond to different aspects of the light environment, or by providing specific photic information to the individual tissues in which they reside (Menaker *et al.*, 1997).

1.3. THE PINEAL ORGAN

The pineal organ is a small midline structure found in the brain of most vertebrates. Until about a century ago very little experimental evidence existed as to the function of the pineal, although its actual existence has been documented for over 2000 years. The history of this small organ has in many ways reflected the leading medical and philosophical ideas of the day, receiving particular acclaim due to its central role in

Descartes dualistic approach to the mind-body problem. Reviews tracing the fascinating history of the pineal are provided by (Wurtman and Axelrod, 1965, Kappers, 1971, Kappers, 1979 and Zrenner, 1985).

Although both Studnicka and Von Frisch inferred that the pineal organ in lower vertebrates may be photosensitive almost a century ago, the first direct evidence of pineal photosensitivity came from the work of Dodt and Heerd, who demonstrated that the pineal organ of frogs and trout reacted to illumination with a recordable electrical response (Dodt and Heerd, 1962 and Dodt, 1963). Subsequently ultrastructural studies demonstrated that the major cellular component of the pineal of most non-mammalian vertebrates shared many features with the photoreceptors of the retina (Oksche, 1971, Collin, 1971 and Flight, 1979).

A) Comparative Physiology of the Pineal

A comparative approach to pineal research has highlighted the remarkable difference in pineal organs between species. In the progression from lower to higher vertebrates the pineal undergoes a remarkable change from a photosensory organ to an endocrine gland, as shown in Figure 1.1 (Oksche, 1965, Hamasaki and Eder, 1977, Vollrath, 1979 and Korf, 1994).

The pineal complex may consist of two components, variously referred to as a) the frontal organ, parietal eye or parapineal organ, and b) the more caudal epiphysis cerebri or pineal organ proper. These multiple terminologies stem from the different species in which they were identified, and this distinction is mentioned here to differentiate between studies concerning these two anatomically separate, though related tissues.

The major cellular constituents of the pineal, the pinealocyte, displays striking variations among the different vertebrate classes, and have been divided into three main categories: 1) true pineal photoreceptors, 2) modified pineal photoreceptors and 3) pinealocytes *sensu stricto*. All represent cells of the receptor line, and can be readily differentiated from the supportive cells and neurons of the pineal parenchyma (Collin, 1971 and Korf, 1994).

B) ROLE OF THE VERTEBRATE PINEAL

The pineal has been implicated in a broad range of functions, mostly revolving around the regulation of circadian and seasonal physiology. These functions include regulation of locomotor activity, pigmentation, thermoregulation, metabolism, seasonal breeding and reproductive maturity (see Wurtman and Axelrod, 1965, Hastings *et al.*, 1989, Underwood, 1990, Cassone, 1990, Reppert and Weaver, 1995 and Arendt, 1995). The role of the pineal in these processes appears to be primarily mediated through the synthesis of melatonin, which acts as a neurohormonal indicator of darkness (See section 1.4). However, in addition to the melatonin output of the pineal a neural projection to the brain also occurs in many poikilotherms. This neural projection is poorly understood but may convey precise photoperiodic signals regarding the amount and composition of environmental light, as well as being involved in behavioural responses such as thermoregulation and phototaxis (Collin *et al.*, 1989, Oksche, 1984 and Korf, 1994).

In mammals it has been demonstrated that artificial administration of melatonin mimics the effect of short days on the reproductive system, suggesting that melatonin secretion acts as a mediator of the photoperiodic control of annual reproductive cycles. As well as reproduction, melatonin has been implicated in other seasonal behaviours such as fasting, thermoregulation and hibernation (Reiter, 1973 and Arendt, 1995). In non-mammalian species, the role of the pineal is less well understood, though the effect of melatonin on reproductive parameters in these species is unsupported (Mayer *et al.*, 1997).

Melatonin has been demonstrated to be involved in the entrainment of circadian oscillators to the prevailing environmental conditions (Reppert *et al.*, 1994). An important role of melatonin in many vertebrates may therefore be the synchronisation of the multiple oscillators of the circadian axis (Underwood, 1990).

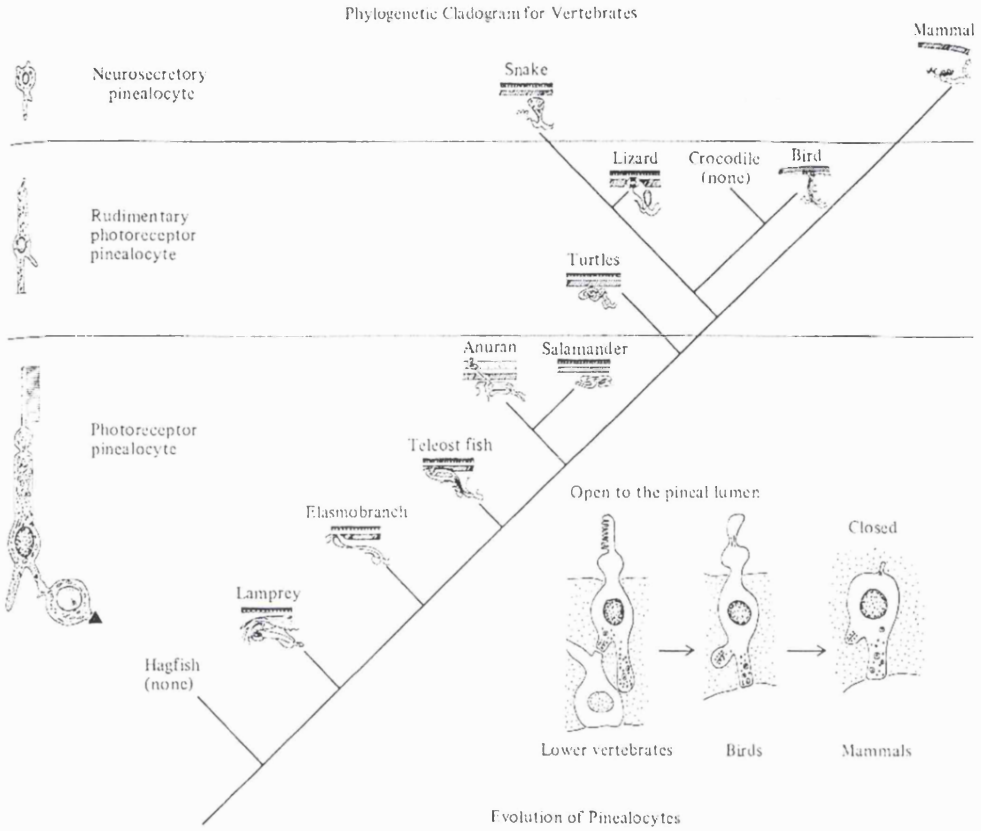


FIGURE 1.1. Schematic diagram illustrating the evolution of the vertebrate pineal complex. In lower vertebrates the pineal is photoreceptive, but undergoes a regression of photosensory characteristics in the progression to the endocrine pineal found in mammals. From (Withers, 1992).

1.4. MELATONIN

One factor common to the pineal organ of all vertebrate species so far studied is the synthesis and secretion of the indoleamine hormone melatonin (Korf *et al.*, 1998). Melatonin was first isolated from the bovine pineal by Aaron Lerner and co-workers in 1958. Extracts from pineal glands were noted to produce a lightening of melanocytes in frog skin, the primary chromatic effect, and it was therefore suggested that the pineal compound responsible be named melatonin. The structure of melatonin and its neurohormonal nature was elucidated shortly thereafter (Lerner *et al.*, 1958 and Lerner, 1959).

Cells of the vertebrate pineal are the major site of melatonin synthesis. The production and release of melatonin demonstrates a remarkable daily rhythm, with high levels of production during the dark phase (*scotophase*) and suppressed production during the light phase (*photophase*). As such, melatonin is thought to act as a neurohormonal signal for the ambient photoperiod (see Axelrod, 1974 and Korf *et al.*, 1998).

A) MELATONIN SYNTHESIS

Melatonin (N-acetyl, 5-methoxytryptamine) is synthesized in the pineal from serotonin (5-hydroxytryptamine, or 5-HT). Serotonin is converted to N-acetylserotonin by the enzyme N-acetyltransferase (NAT), which is in turn converted to melatonin (N-acetyl, 5-methoxytryptamine) by the action of hydroxyindole-O-methyltransferase (HIOMT). The rate-limiting step in this synthesis is the N-acetylation of serotonin by NAT. This biosynthetic pathway is summarized in figure 1.1 (for further details see Wurtman and Axelrod, 1965, Axelrod, 1974, Sugden, 1989 and Borjigin *et al.*, 1999).

Melatonin is not stored, and once synthesized it is secreted into the bloodstream. As such, the synthesis and secretion of melatonin are dependent upon the level of the rate-limiting enzyme NAT within the pineal (Korf *et al.*, 1998). By regulating the level of NAT expression melatonin synthesis may be modulated, although recent evidence suggests that post-translational regulation also appears to be important (Gastel *et al.*, 1998).

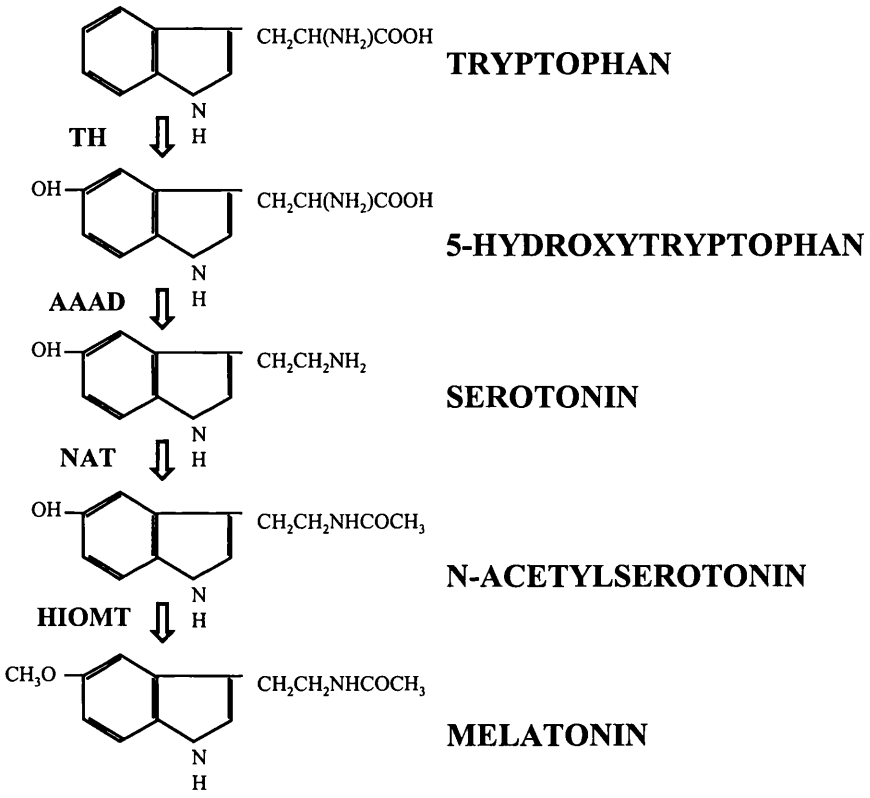


FIGURE 1.2. The melatonin biosynthetic pathway. See text for details. Abbreviations: TH (Tryptophan hydroxylase), AAAD (Aromatic amino acid decarboxylase), NAT (N-acetyltransferase) and HIOMT (Hydroxyindole-O-methyltransferase). Based on Wiechmann, 1986 and Sugden, 1989).

B) EXTRA-PINEAL MELATONIN

Melatonin is not produced exclusively by the pineal, and in many vertebrates is also synthesized by the retina (Gern and Ralph, 1979, Wiechmann, 1986 and Krause and Dubocovich, 1990), as well as the parapineal organ and Harderian glands where present (Underwood, 1990).

Retinal photoreceptors have been shown to possess a circadian oscillator regulating melatonin synthesis in a variety of vertebrate species (Cahill and Besharse, 1995, Cahill, 1996 and Niki *et al.*, 1998). Retinal melatonin has been implicated in the modulation of many retinal rhythms, including dopamine release, effects on horizontal cell sensitivity, retinomotor movements, and regulation of photoreceptor disc-shedding and phagocytosis (Dubocovich, 1983, Wiechmann, 1986 and Cahill and Besharse, 1995).

The extent to which extra-pineal melatonin sources contribute to circulating melatonin levels is not entirely clear. In mammals and birds, the retina may contribute towards plasma melatonin levels, especially following pinealectomy where the retina may compensate for the lack of pineal melatonin. In lower vertebrates the pineal and plasma melatonin profiles are in phase, whereas the retinal melatonin synthesis profile is not, suggesting that the pineal is the major source of circulating melatonin. In addition, cultured teleost retinas do not release melatonin into the culture medium, and pinealectomy has been shown to suppress plasma melatonin levels. As such it appears that, certainly in lower vertebrates, retinal melatonin does not significantly contribute to plasma melatonin levels in the normal physiological state, instead exerting a local autocrine or paracrine effect before being metabolised *in situ* (for review and references see Falcon, 1999).

CHAPTER 2

2. THE TELEOST VISUAL SYSTEM

2.1. TELEOST FISHES

The teleosts are one of three groups of Actinopterigii, or ray-finned fishes. These three groups are the Chondrostei, Holostei and Teleostei, meaning primitive, intermediate and advanced forms respectively. Teleosts represent the most advanced level of ray-finned fishes, and are the dominant fishes in the world today. Teleosts are found in virtually every possible marine habitat, from the strand line to the abyssal depths, as well as throughout most freshwater habitats from fast flowing rivers and streams to lakes and underground caves.

The dominance of the teleost fishes is apparent in the fact that they are the most abundant vertebrates on the planet, with an estimated 20, 000 different species. The success of teleosts can be attributed to their efficient body organization and their remarkable fecundity. Another factor contributing to the success of the teleosts is their remarkable versatility, displaying a vast diversity in size, shape, behaviour and adaptation to their ecological niche (Romer and Parsons, 1986 and Nelson, 1994).

A number of reasons exist for the use of the fish visual system as a model. Firstly, the similarity between the eyes of mammals and fishes allows cross-species comparisons to be made, and as such invasive techniques for studying the visual system can be routinely conducted. A second reason, central to the studies conducted on the visual pigments of fishes lies in the variety of habitats in which fish are found, and the consequent diversity the visual system of fishes demonstrates in adapting to these habitats. The aquatic environment provides "a unique ready-made natural laboratory for vision research" (Douglas and Djamgoz, 1990).

When considering the photoreceptive function of the teleost pineal, analogies between retinal and pineal structure and function are inevitable, so the following aims to provide a primer on the teleost visual system. For further coverage of this subject the reader is referred to Douglas and Djamgoz, 1990 and Nichol, 1989.

2.2. THE UNDERWATER PHOTIC ENVIRONMENT

The aquatic environment provides a great diversity of light conditions, differing in aspects such as the brightness, scattering and spectral composition of the available light. At the waters surface the majority of radiation from the sun (about 80%) is restricted to a range between 300 nm in the ultraviolet, up to 1100 nm in the infrared. Wavelengths below 300 nm, and above 1100 nm are absorbed or scattered by the atmosphere, primarily by water vapour and the ozone layer. The exact spectral bandwidth at the water's surface will also depend upon other factors such as altitude, cloud cover and solar elevation (Lythgoe, 1979 and Douglas and Djamgoz, 1990).

The major difference in the spectral range of available light in aquatic environments is produced by water, which acts as a monochromator, absorbing both long- and short-wavelength light. The maximum transmission of pure water is located in the blue region of the spectrum around 460 nm. However, natural aquatic environments very rarely consist of pure water, and usually contain impurities influencing the spectral characteristics. For example, suspended particles will further scatter short wavelengths, and dissolved particles may colour the water. Chlorophyll containing phytoplankton can have an important influence on nutrient-rich water, especially in the summer months. The yellow products of vegetable decay (referred to as *gelbstoffe*) when combined with the absorbance characteristics of pure water produce a green coloration, and in highly stained waters the transmission maximum is forced towards the infrared. Depth has another important effect on the transmission of water, with the spectral bandwidth of available light become more and more restricted with increasing depth (Lythgoe, 1984).

Due to the variety of underwater habitats in which teleosts are found, it is perhaps not surprising that their visual systems correspondingly vary to reflect this diversity of light environments. Numerous reviews of visual ecology are available of which good accounts are provided by Lythgoe, 1972, Munz and McFarland, 1977, Lythgoe, 1979, Knowles and Dartnall, 1977 and Lythgoe and Partridge, 1989.

A) TWILIGHT

Changes in the amount and spectral composition of environmental light occur throughout the diurnal cycle. As well as the obvious changes occurring in irradiance at twilight (approximately 6 log units), changes in the spectral composition also occur, known as the Chappius effect (Lythgoe, 1979). As the sun's rays must pass through a thicker layer of the atmosphere, and the absorption by ozone in the yellow-orange region of the spectrum (500-650 nm) becomes significant. The result of this absorption is in effect a relative enrichment of shorter-wavelength light (<500 nm) around this time. Furthermore, due to the attenuation of longer wavelengths by water, this effect is accentuated in the underwater photic environment, even more so with increasing depth. This twilight blue-shift is not as pronounced in freshwater environments as in clear, tropical seas, and increasing depth may actually produce a long-wave shift (Munz and McFarland, 1977).

The changes occurring in the spectral composition of light at dawn and dusk may serve as a reliable marker for the phase of twilight. Animals possessing two spectrally distinct photoreceptors may be capable of discerning these changes, which could provide vital information for the entrainment of the circadian axis (Roenneberg and Foster, 1997).

2.3. THE TELEOST EYE

In general form the teleost eye is similar to a mammalian eye, with the necessary modifications for underwater vision (see figure 2.1, overleaf). Whereas the lens of land vertebrates is somewhat flattened and convex on both sides, the teleost lens is spherical, this extreme convexity being necessary due to the similarity in density between the lens tissue and the water in which the fish lives. The cornea in teleosts is flat, and the iris (which may be brightly coloured) has little power of contraction compared with the land vertebrates, in which it regulates the amount of light entering the eye.

In terrestrial vertebrates accommodation is usually accomplished by altering the convexity of the lens through the action of the ciliary muscles. In fishes accommodation is instead accomplished by changing the position of the lens in relation to the retina (Norman and Greenwood, 1963).

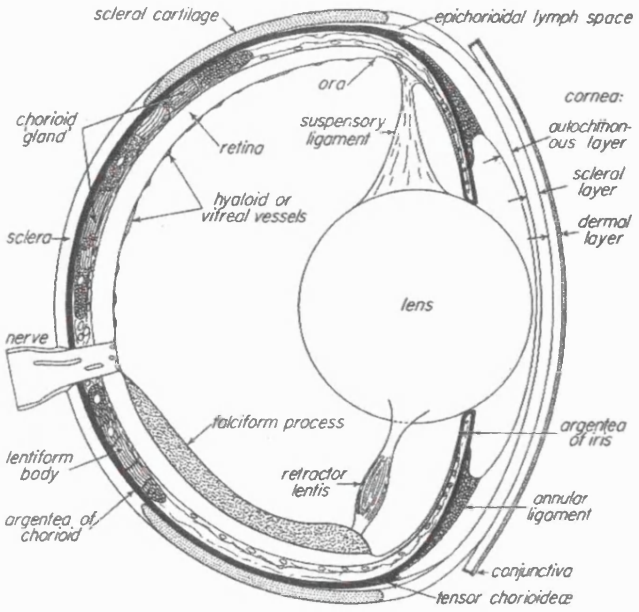


FIGURE 2.1. Vertical section through the eye of a teleost fish. From Walls, 1942.

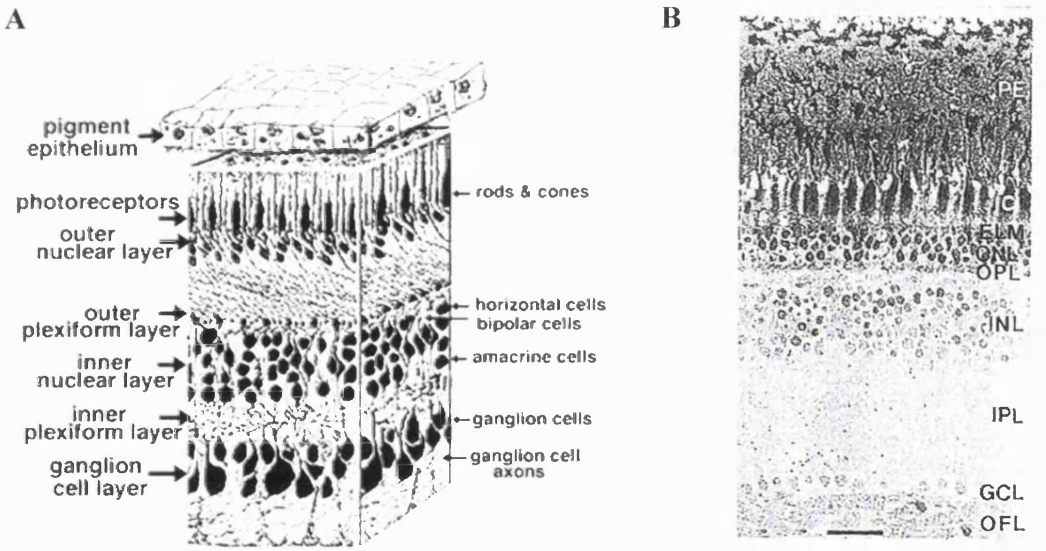


FIGURE 2.2. Structure of the retina. **A**) Schematic diagram of a typical vertebrate retina. **B**) Radial section through the retina of the cichlid, *Aequidens pulcher*, demonstrating the characteristics of the typical teleost retina. Scale bar is 1μm. Abbreviations - PE: pigment epithelium, C: photoreceptor layer, ELM: external limiting membrane, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer, OFL Optic fiber layer (Douglas and Djamgoz, 1990).

2.4. THE TELEOST RETINA

The structure of the teleost retina is much like that of other vertebrates, with a layered structure first identified by Ramon y Cajal (1892 – see Rodieck, 1973). The retina can be subdivided into eight "layers" and two "membranes", as shown in figure 2.2. These divisions are, from sclera to vitreous:

1. Epithelial layer
2. Visual cell layer (rods and cones)
3. External limiting membrane
4. Outer nuclear layer
5. Outer plexiform layer
6. Inner nuclear layer
7. Inner plexiform layer
8. Ganglion cell layer
9. Nerve fibre layer
10. Internal limiting membrane

Of particular interest to the initial stages of vision is the visual cell layer, which contains the photoreceptor cell outer segments (rods and cones – see section 2.5).

The external limiting membrane is often the dividing line between the myoids and nuclei of the photoreceptor cells, and is made up of the Mullerian cell branches. The external nuclear layer consists of the cell bodies of the photoreceptor cells, which project axons to the inner nuclear layer. These projections, along with the dendrites of the horizontal and bipolar cells form the external plexiform layer. The external nuclear layer consists of the cell bodies of the horizontal, bipolar and amacrine cells. Also part of this layer are the Mullerian cells, which project cell branches to form the external limiting membrane. These cells are the main supporting cells of the retina. The internal plexiform layer is composed of the axons of the bipolar and amacrine cells, as well as ganglion cell dendrites. The ganglion cell axons form the nerve fibre layer, and these fibres converge from across the retina to at the region of the optic disc to form the optic nerve which projects to the visual centres of the brain. Finally, the internal limiting

membrane, like the external limiting membrane, is a product of the expansion of Mullerian cell fibres. For further information regarding the teleost retina see (Ali and Anctil, 1976, Rodieck, 1973 and Nichol, 1989).

2.5. TELEOST PHOTORECEPTORS

Teleosts, like the majority of vertebrates, possess a duplex retina consisting of two distinct photoreceptor classes, rods and cones. The photoreceptor cells consist of an outer segment with numerous membrane lamellae, attached to the inner segment by a connecting cilium. The inner segment consists of an ellipsoid containing mostly mitochondria, and a myoid. The myoid is thought to be involved in the movements of the photoreceptor outer segments with respect to the pigment epithelium - a process known as retinomotor movement. The nucleus and perikaryon lie beyond the myoid, and the photoreceptor cell terminates in an axon, the terminal pedicle of which contains synaptic vesicles and ribbons.

Rod photoreceptors typically possess long and slender outer segments, cylindrical in form, whereas the cones possess shorter tapering outer segments (see figure 2.3). Cones may be morphologically of two varieties, single and double. Double cones may possess similar outer segments, or unequally sized outer segments, in which case the larger is termed principle and the smaller termed accessory.

Scotopic vision is mediated through the use of the more sensitive rod photoreceptors, whereas photopic vision is mediated primarily through the cones. Colour vision, the ability to discriminate between wavelengths of light, requires at least two classes of spectrally distinct photoreceptor. Although wavelength discrimination is possible with just two photoreceptor classes with broadly overlapping spectral sensitivities, many species make use of the whole spectral range of their photic environment and utilize three or even four colour channels (termed trichromacy and tetrachromacy, respectively).

Due to their varied habitats the photoreceptor complement of teleosts varies considerably. Many teleost species living at depth (marine or freshwater) possess pure rod retinae. In contrast, shallow-living species occupying a spectrally broad light environment may be trichromatic or tetrachromatic. In the middle ground are those

species occupying spectrally limited light environments, such as coastal waters, which are usually only dichromatic. Obviously these examples are gross generalisations, and the photoreceptor complement may vary between species occupying the same photic environment. For a more detailed summary of the photoreceptor complement of fishes, the reader is referred to (Bowmaker, 1995 and Muntz, 1990).

A) The Receptor Membrane

The outer segments of rod and cone photoreceptors differ at the membrane level as well as morphologically. The lamellae of cone photoreceptors are continuous with the plasma membrane - formed by invaginations of the cell membrane. As such their internal space is filled with extracellular fluid. Rods, however, have outer segments consisting of stacks of internalised disks, pinched off from the plasma membrane with each disk forming a closed intracellular vesicle (Fein and Szuts, 1982).

The photopigment molecules lie within the membrane of the lamellar outer segments of the photoreceptor cells, arranged so that the long axis of the chromophore is parallel to the lamellar membrane surface. The absorbance of light by the photopigment (more specifically, the chromophore) is the primary event in vision (see section 3), and this absorbance requires an electronic transition to occur. For efficient absorbance of light to occur the light must approach the photopigment molecule in the correct direction, coupling the wave motion of the light with the electronic transition. If the outer segment is viewed transversely in polarized light, the absorbance will be seen to differ when the plane of polarization is rotated, that is, the photoreceptor exhibits dichroism. Measurement techniques such as microspectrophotometry make use of this effect, using light of E-vector perpendicular to the to the long axis of the outer segment to produce a maximal absorbance measurement (Knowles and Dartnall, 1977).

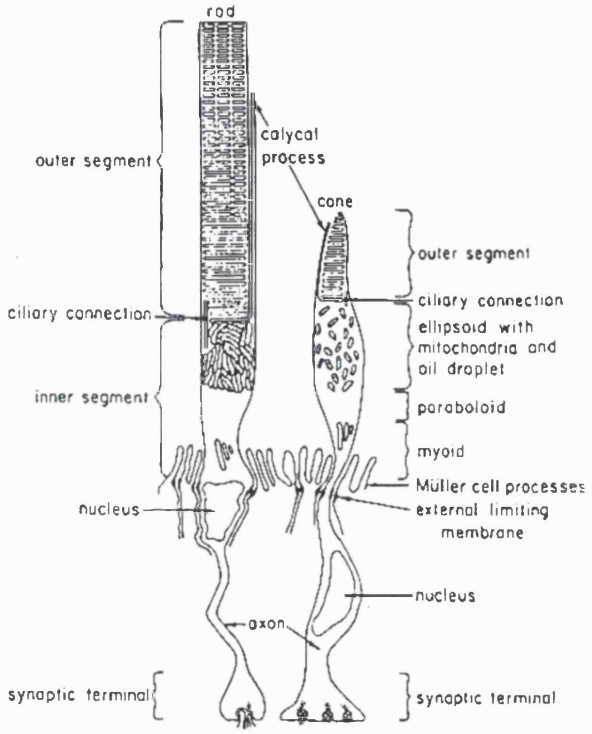


FIGURE 2.3. Schematic diagram of 'typical' vertebrate rod and cone photoreceptors, representative of teleost retinal photoreceptors. The teleost retina may also contain double cones (Fein and Szuts, 1982).

CHAPTER 3

VERTEBRATE PHOTOPIGMENTS

3.1. THE PHOTOPIGMENT MOLECULE

All vertebrate visual pigments are based on a similar plan with a membrane-embedded protein, opsin, within which is covalently bound a light sensitive chromophore (Bownds, 1967). In the majority of vertebrates this chromophore is the aldehyde of Vitamin A, retinal. However, Vitamin A naturally occurs in two forms, A_1, resulting in retinaldehyde (retinal), and A_2, producing 3-dehydroretinaldehyde (3-dehydroretinal). This gives rise to two families of vertebrate visual pigments, those utilizing retinal (rhodopsins) and those containing 3-dehydroretinal (porphyropsins). Any opsin may combine with either form of retinal, and as such there can be a pigment pair for each opsin protein (Wald, 1968 and Knowles and Dartnall, 1977).

Invertebrate photopigments have also been shown to possess pigments based on retinal, and in some species 3-dehydroretinal. However, in addition to these chromophores, insects have been shown to possess pigments based on 3-hydroxyretinal, and a fourth vitamin A derivative, 4-hydroxyretinal, has been demonstrated in the firefly squid (see Kito *et al.*, 1992 and Kirschfeld and Vogt, 1986).

In recent years it has become apparent that visual pigments may mediate not only vision, but also other non-visual functions such as neurohormone synthesis and the entrainment of circadian oscillators. As such the term photopigment is perhaps better applied to describe these molecules.

3.2. RETINAL

Retinal is the chromophore of the majority of vertebrate photopigments, comprising a terminal β–ionone ring and a polyene chain terminating in an aldehyde group (see figure 3.1). The photoisomerisation of 11-*cis* retinal to its all-*trans* isomer (shown in figure 3.1) is the primary event in vision. In the *cis-trans* isomerisation of retinal no formal chemical bonds are broken, - the isomerisation energy is required to overcome the energy barrier to rotation around the C11-C12 double bond (Abrahamson and Japar, 1972).

A distinguishing feature of any photopigment is the wavelength at which it absorbs light maximally, termed the λ_{max}. The λ_{max} of retinal itself is in the ultraviolet around 380 nm, which corresponds to the absorbance of the π–electron shell of the whole conjugated system (Rodieck, 1973). However, the covalent binding of retinal to opsin, via a protonated Schiff's base linkage produces a long-wavelength shift of absorption to around 440 nm - into the visible region of the spectrum (Kito *et al.*, 1968). A shift in the λ_{max} to longer wavelengths such as this is referred to as a bathochromic shift, whereas a shift to shorter wavelengths is known as a hypsochromic shift.

To create further spectral shifts requires interactions between the opsin protein and the retinal chromophore, yielding the specific absorption characteristics of each photopigment type. A bathochromic shift requires interactions with the opsin to further delocalise the π–electron shell, and decrease the energy gap between the ground and excited states, thus enabling photoisomerisation by longer wavelengths, with correspondingly lower photon energies. Conversely, to produce a hypsochromic shift requires a stabilization of the π-electron shell, increasing the energy gap. In this manner photopigments are tuned to specific wavelengths by the amino acid residues of the opsin molecule, in particular the charged amino acids surrounding the retinal binding pocket (Applebury and Hargrave, 1986, Applebury, 1991 and Bowmaker and Hunt, 1999).

As well as being limited by the available light, the absorbance of photopigments is also photochemically limited between a range of around 300 nm in the ultraviolet up to 850 nm in the infrared. Photons below about 300 nm possess sufficiently high quantal energy to be destructive to proteins, whereas those much above 800 nm possess

insufficient energy to cause photoisomerisation of organic molecules (Knowles and Dartnall, 1977).

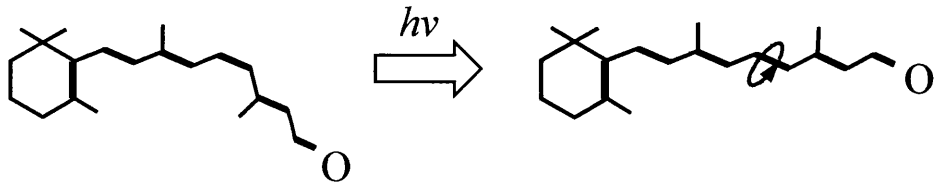


FIGURE 3.1. The photoisomerisation of retinal. Absorption of a photon of light (*hv*) by 11-*cis* retinal (left) leads to rotation around the C11-C12 bond to form the all-*trans* configuration (right).

A) VISUAL PIGMENT TEMPLATES

In 1953 Dartnall noted that if the absorbance spectra of a series of A_1–based visual pigments were plotted on a wave frequency basis rather than the conventional wavelength scale, the shape of the curves were almost identical (Dartnall, 1953). Given that all such curves have the same basic shape, the absorbance spectrum of any rhodopsin can be completely characterized by just a single parameter, the λ_{max} (Knowles and Dartnall, 1977).

A visual pigment template (or nomogram) based on the absorption of rhodopsin at a specified wavelength can be fitted to the absorbance spectra obtained from experiment, and the accuracy of the match may be calculated, providing an accurate means of analysing spectral data.

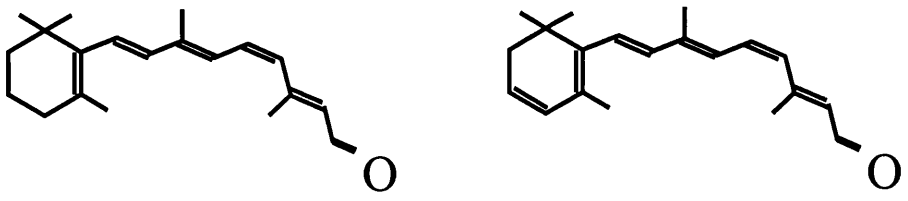
Based upon rhodopsin extracts, Dartnall's original template was shown to only be appropriate for pigments with λ_{max} around 500 nm, and pigments with λ_{max} at longer or shorter wavelengths demonstrated systematic deviations from the template. Furthermore, this template was also not appropriate for A_2-based photopigments. Given the practical value of photopigment templates in the interpretation of spectral data, extensive work has been conducted to both refine the accuracy of these templates, and

to attempt to determine the underlying physics behind the relationship between molecular structure and absorbance characteristics (see for example Partridge and De Grip, 1991, Stavenga *et al.*, 1993 and Govardovskii *et al.*, 2000).

B) PORPHYROPSINS

Porphyropsins, photopigments based on a 3-dehydroretinal chromophore, are found in a variety of vertebrate species. First identified in freshwater teleosts, they have since been demonstrated in amphibians and some reptilian species (Knowles and Dartnall, 1977 and Bridges, 1972).

3-dehydroretinal differs from retinal only in the presence of an extra C=C bond in the terminal ring, adding to the conjugated bond system of the molecule (see figure 3.2). As a consequence of this extra bond, photopigments containing 3-dehydroretinal (porphyropsins) require smaller excitation energies and consequently absorb light of longer wavelengths. The A_2 chromophore also produces a change in the shape of the absorbance spectrum, which is broader than that of the rhodopsin template when plotted on a frequency basis. As such Dartnall's nomogram does not hold true for porphyropsins, and a second corrected template was subsequently published (Bridges, 1967 and Munz and Schwanzara, 1967). An example of the effects of replacing retinal with 3-dehydroretinal are shown for a rhodopsin of λ_{max} 500 nm, which as a porphyropsin possesses a broader absorbance spectrum which is red-shifted to 522 nm (see figure 3.3).



11-cis retinal *11-cis 3-dehydroretinal*

FIGURE 3.2. Structure of 11-*cis* retinal and 3-dehydroretinal, demonstrating the extra double bond in the terminal ring of 3-dehydroretinal.

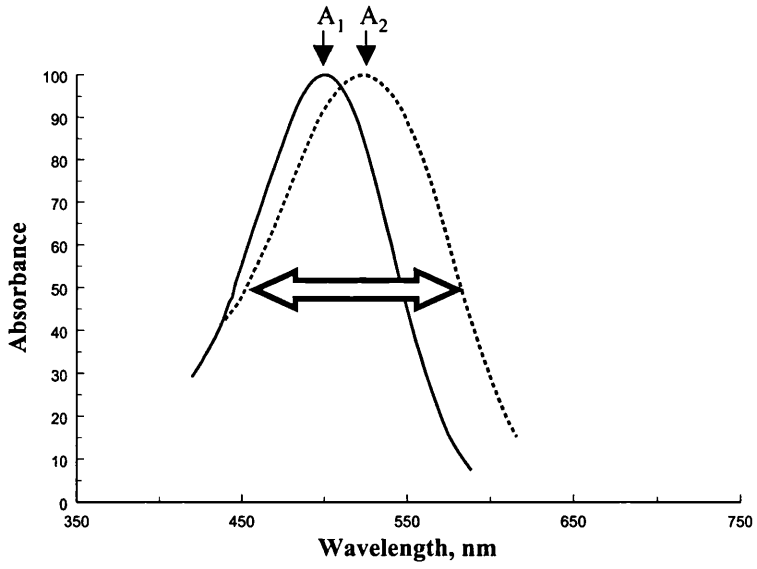


FIGURE 3.3. A_1 compared with A_2 nomogram. A rhodopsin of λ_{max} 500 nm is red-shifted to become a porphyropsin of λ_{max} 523 nm when retinal is substituted for 3-dehydroretinal. The arrow indicates the half-band width, which also increases proportionally in porphyropsins.

3.3. OPSIN

The light-sensitive retinal chromophore is situated within a binding pocket of a membrane-embedded protein, known as opsin. Opsin belongs to a family of transmembrane receptors all of which function through activation of G-proteins which bind guanosine diphosphate (GDP).

Opsins consist of a single polypeptide chain made up of between 340 and 500 amino acids that form seven membrane-spanning hydrophobic regions (Khorana, 1992). The transmembrane regions of the opsin molecule take the form of α-helices consisting of approximately 26 amino acids each, of which only the central 18 are actually located in the membrane (Schertler *et al.*, 1993). The remainder of the non-helical opsin molecule takes the form of straight chain extra-membrane loops, which may be intra or extra-cellular (Hargrave *et al.*, 1984). The two-dimensional structure of a typical vertebrate opsin is shown in figure 3.4, and the three-dimensional folding and retinal binding is illustrated in figure 3.5.

The protein structure of opsin has been studied in great detail, allowing predictions as to which amino acids are involved in the primary events of vision (see Schertler *et al.*, 1993 and Schertler, 1998) and the spectral tuning of the photopigment molecule (for examples, see Lin and Sakmar, 1998 and Bowmaker and Hunt, 1999).

A recent study by Palczewski *et al.*, (2000) has illustrated the molecular structure of bovine rod opsin at even greater resolution, allowing still greater understanding of the interactions of amino acid residues and the retinal chromophore that result in the spectral tuning of the photopigment molecule.

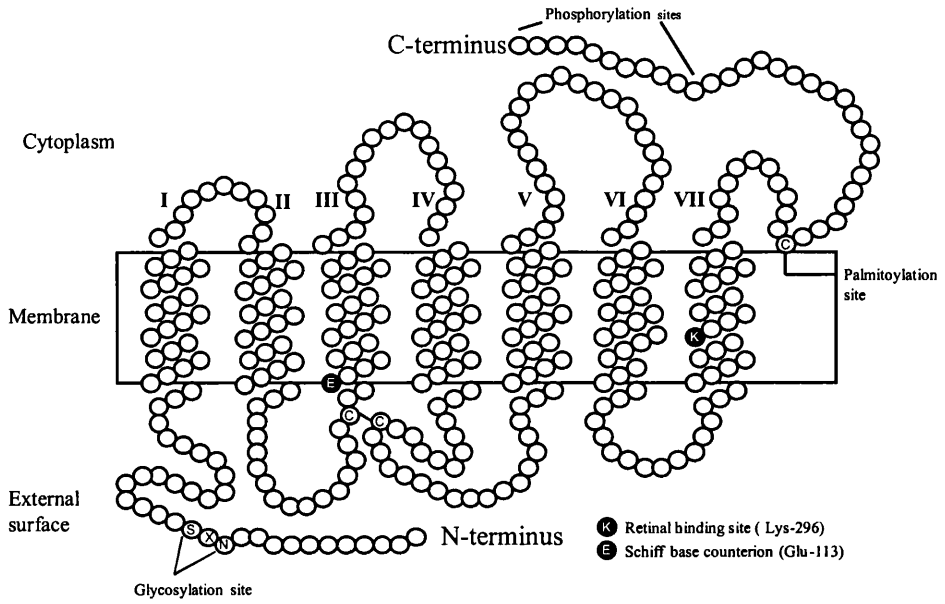


FIGURE 3.4. The structure of a generic vertebrate opsin, based on bovine rhodopsin, showing sites of functional interest (see text for details).

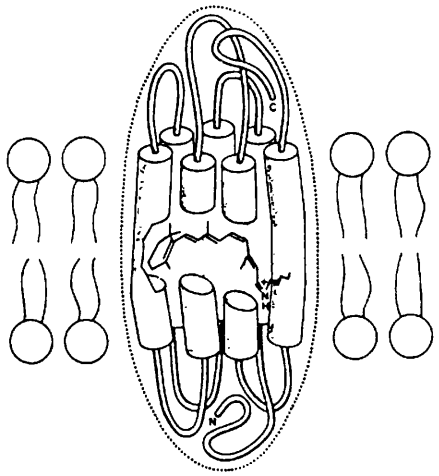


FIGURE 3.5. Diagram illustrating how retinal is bound within the opsin protein, situated within the membrane of the photoreceptor outer segment (Hargrave *et al.*, 1984).

A) Structural Features of Opsins

Studies using a variety of approaches, such as site-directed mutagenesis have demonstrated several amino acids that are of key interest in the function of the protein. These sites of functional interest include the following (numbering based upon bovine rod opsin):

1. The retinal chromophore is covalently bound by a protonated Schiff base linkage to lysine 296 on helix VII (Bownds, 1967).
2. A glutamic acid at site 113 on helix III forms the counterion to the Schiff base linkage (Sakmar *et al.*, 1989).
3. A disulphide bridge is formed between cysteines 110 and 187, connecting the first and second extracellular loops (Karnik *et al.*, 1988 and Karnik and Khorana, 1990).
4. One or more serine residues are present located on the cytosolic C-terminal. These are thought to be the site of light-dependent phosphorylation by rhodopsin kinase (Applebury and Hargrave, 1986 and Baylor and Burns, 1998).
5. The carboxyl terminus typically contains two palmitoylated cysteines (though many cone opsins contain just one), serving to anchor the C-terminal to the membrane to form a fourth cytoplasmic loop (Ovchinnikov *et al.*, 1988).
6. One or two asparagines residues occur near the N-terminal, forming glycosylation sites (Hargrave *et al.*, 1984 and Applebury and Hargrave, 1986).
7. Despite inter-species differences the transmembrane helices are remarkably conserved. The helical amino acids that project into the retinal binding pocket may interact with the chromophore, influencing the spectral tuning of the photopigment (Bowmaker and Hunt, 1999).
8. The cytoplasmic loops contain several charged amino acids, thought to be involved in the activation of the heterotrimeric G-protein transducin (Franke *et al.*, 1992).

B) VISUAL ECOLOGY

The theory that visual pigments have evolved to maximise the detection and recognition of stimuli within their specific light environment is perhaps the central tenet of visual ecology. In its simplest formulation, this theory would therefore predict that the λ_{max} of a photopigment would be tuned to the wavelength of maximum photon flux (Lythgoe, 1979 and Lythgoe, 1984). And in many cases, a correlation between the environmental light and photopigment sensitivity does exist. For example, the λ_{max} of rod opsins from species inhabiting deep-water environments are tuned to around 480 nm, the region in which the majority of the available light is centred (Knowles and Dartnall, 1977).

However, in addition to providing maximum sensitivity photopigments are also tuned to specific wavelengths optimum for the photic tasks they mediate. An example of such tuning occurs in colour vision, where the presence of differing cone opsin complements in animals inhabiting the same photic environment suggests that the visual tasks critical for the survival and reproductive success of each are different (Lythgoe and Partridge, 1989).

However, a classical problem of visual ecology is presented by the rod opsins of most terrestrial vertebrates. Several adaptational theories have been proposed to explain the clustering of the λ_{max} of these photopigments to around 500 nm, despite the fact that the maximum available light during both day and night is centred at longer wavelengths. These theories can be summarised as follows: 1) Rod opsins may have evolved for maximum sensitivity at twilight, a time of day when the available spectrum is blue-shifted, particularly in the aquatic environment (see section 2.2A). 2) Vision in dim light may have evolved in environments dominated by reflections from green plants. 3) Scotopic vision is particularly sensitive to dark noise, caused by spontaneous isomerisation of retinal by chance thermal events. Long-wavelength pigments are more prone to such dark noise, which becomes a major problem when absolute sensitivity is critical (Lythgoe, 1984 and Goldsmith, 1990).

As well as tuning to the light environment and photic task, photopigments may be incidentally tuned due to the effects of amino acids serving other functions, not totally independent of one another. Such a form of evolutionary constraint may arise due to

the necessary conservation of regions of the opsin molecule important for intracellular interactions, such as transducin activation (Goldsmith, 1990).

C) OPSIN EVOLUTION

Many studies have addressed the question of how vertebrate visual pigments have evolved, especially with regard to colour vision. The fundamental requirements for the evolution of colour vision have been summarized in four steps (Nathans, 1987):

1. Duplication of a primordial visual pigment gene
2. Accumulation of sequence changes in one of the duplicated genes that alter the spectral absorbance of the encoded pigment.
3. Accumulation of sequence changes that lead to expression of one of the duplicated genes in a set of photoreceptor cells different from that set in which the other gene is expressed.
4. Development of second order neurons sensitive to differences in the extents of excitation of the two photoreceptor sets.

Based upon such a model, sequence comparisons of vertebrate opsins enable the generation of phylogenetic trees of visual pigments based upon gene duplication and subsequent sequence divergence. From studies such as these it has been determined that the ancestral vertebrate visual pigment appears to have been cone-like with a λ_{max} around 500-520 nm (see Bowmaker and Hunt, 1999 and references therein).

An early gene duplication of this ancestral opsin is thought to have lead to the separation of a long wavelength, anion-sensitive group (LWS) and a short-wavelength, anion-insensitive group. Two further gene duplications within the short wavelength group led to three opsin classes, giving the UVS, SWS and MWS cone opsins. A fourth gene duplication within the MWS class is thought to have led to the rod opsins. A simplified phylogenetic tree illustrating this scheme of visual pigment evolution is shown in figure 3.1, overleaf. These opsin classes demonstrate around 40% amino acid identity with one another, whereas within an opsin class identity is better than 80% (Bowmaker, 1998).

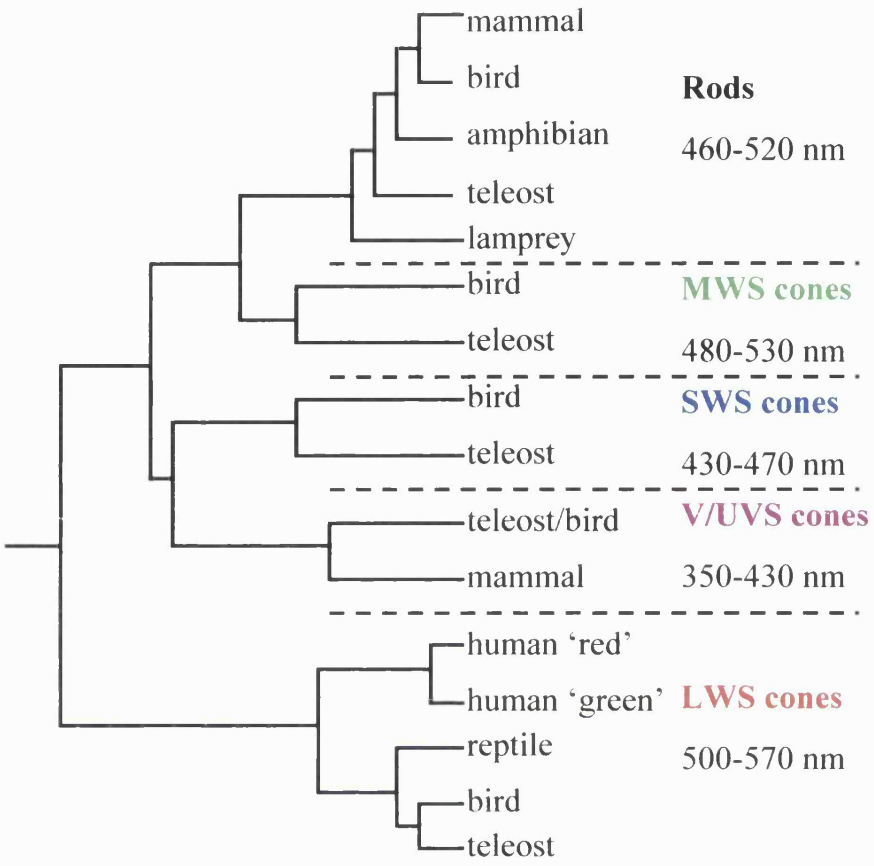


FIGURE 3.6. A highly simplified phylogenetic tree illustrating the evolution and classification of vertebrate opsins, redrawn from (Bowmaker, 1998). Gene duplication led to LWS and shorter wavelength opsin groups. Subsequent duplications of the shorter wavelength group have resulted in SWS, UVS and MWS cone subgroups. A duplication of the MWS cone opsin led to the evolution of rod opsins. Scheme based on Hisatomi *et al.*, 1994.

3.4. PHOTOTRANSDUCTION

Phototransduction is the process whereby a photon of light absorbed by the photopigment produces the hyperpolarisation of the photoreceptor cell membrane, via a cascade of intracellular reactions. The hyperpolarisation of the photoreceptor cell leads to an inhibited release of the neurotransmitter glutamate. Glutamate may inhibit or excite the second order neurons (bipolar cells), which in turn modulate the output of the ganglion cells providing the signal to the brain via the optic nerve (Wu, 1994).

The last decade or so has seen an explosion in our knowledge of the molecular details of the phototransduction cascade, reflected in the number of reviews upon this subject area. General reviews include Koutalos and Yau, 1993, Pugh, 1990, Kaupp and Koch, 1992, Yau, 1994, Polans *et al.*, 1996 and Pepe, 1999, of which the following provides a brief summary.

A) THE RESTING STATE

In their resting state photoreceptor cells are partially depolarised. This is due to cGMP-sensitive ion channels present in the cell membrane of the photoreceptor, which are selectively permeable to Na^+ and Ca^{2+}. In darkness these ion channels are open and allow Na^+ and Ca^{2+} ions to flow into the outer segment down an electrochemical gradient. Meanwhile, an ion-exchange pump selectively extrudes K and Ca^{2+} ions from the cell, creating a high intracellular concentration of Na^+. The selectivity of this ion exchange creates a 'dark current' partially depolarising the photoreceptor, a resulting in release of neurotransmitter from the synaptic terminal.

B) PHOTOISOMERISATION

The photoisomerisation of opsin-bound retinal by a photon of light leads to the rearrangement of the surrounding opsin protein, producing a series of intermediate forms (indicated in parenthesis) that initiate the phototransduction cascade. The nomenclature of opsin metastates given here is taken from Shichida (1998) based on those originally proposed by Wald (see Wald, 1968).

Photoisomerisation of retinal occurs extremely rapidly - in the order of 200 fs (resulting in *photorhodopsin* - see Mathies, 1998). This isomerisation results in a subsequent

rearrangement of the surrounding amino acids, leading to a conformational change in the protein structure (going from *bathorhodopsin* → *lumirhodopsin* → *metarhodopsin Ia*), exposing previously concealed sites to allow G-protein binding (*metarhodopsin Ia*) and activation (*metarhodopsin II*). Following transducin activation, the all-*trans* retinal dissociates from the opsin, and is eventually regenerated in the RPE (Saari, 2000).

C) Activation of the Phototransduction Cascade

The G-protein transducin is comprised of an α-subunit (to which GDP is bound) and a βγ-subunit. Binding of photoactivated rhodopsin (metarhodopsin II) to transducin leads to the exchange of GDP for GTP on the α-subunit, leading to the dissociation of the α-GTP and βγ-subunits. The released subunits remove inhibition from phosphodiesterase (PDE), which catalyses the conversion of cGMP into 5'GMP, thus decreasing the intracellular concentration of cGMP. This in turn leads to a closure of the cGMP sensitive cation channels in the cell membrane, resulting in hyperpolarisation of the cell to around –80 mV, and a subsequent reduction in transmitter release.

At each step of this chain of events the response is amplified – a single molecule of rhodopsin can activate numerous transducin molecules (in turn affecting many more molecules of PDE and cGMP) resulting in an effect on even more ion channels. It is this cascade of effects that potentially enables a single photon of light to be detected.

D) Inactivation of the Phototransduction Cascade

Photoactivated opsin is deactivated by phosphorylation by rhodopsin kinase and binding of arrestin. Transducin is in turn deactivated due to the hydrolysis of GTP to GDP, which no longer disinhibits PDE.

Following closure of the cGMP-gated channels the ion exchange pumps continue to function lowering the intracellular concentration of Ca^{2+}. The reduction in Ca^{2+} stimulates retinal guanylate cyclase (RetGC) through the guanylate cyclase activating protein (GCAP). RetGC synthesizes cGMP from GTP producing a rise in the intracellular levels of cGMP leading to reopening of the cGMP-gated cation channels and a restoration of the resting state dark current.

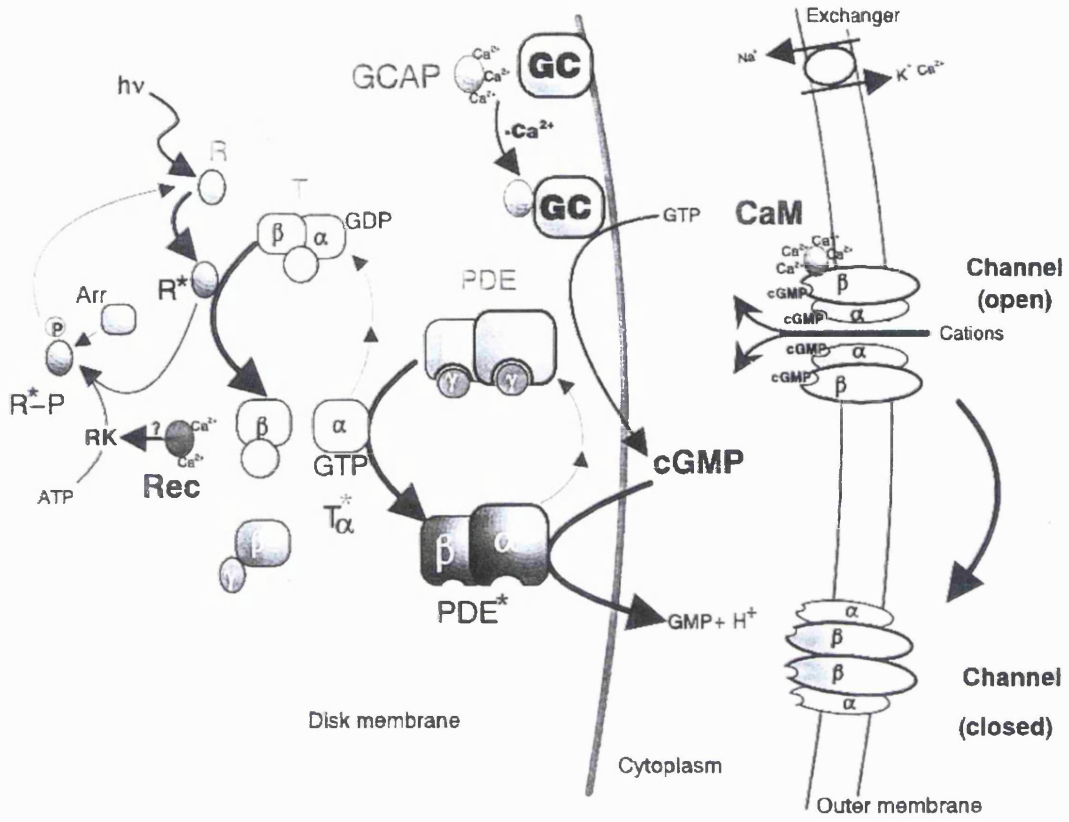


FIGURE 3.7. Summary of the phototransduction cascade. See text for details. Abbreviations as follows: PDE (phosphodiesterase), Tα-GTP (Transducin α-subunit with bound GTP), Tα-GDP (Transducin α-subunit with bound GDP), Tβγ (Transducin βγ-subunit), RK (Rhodopsin kinase), ARR (Arrestin). Diagram taken from Baehr and Palczewski (1996).

3.5. NON-VISUAL OPSINS

Studies within the course of the last decade have lead to the isolation and characterization of a number of novel retinal and extra-retinal opsin-like proteins. The main factor linking these opsins is that unlike the visual pigments previously isolated, none of these proteins actually appears to be involved in any form of image formation. These opsins are either localized to extra-ocular tissues, or expressed outside the photoreceptor cell layer of the retina. This recent explosion of non-visual opsins is summarized in table 3.2, with the species in which they have been identified, their localization and λ_{max} where known. An independent analysis of these opsins is beyond the scope of this study, and would only lead to a repetition of the original articles to which the reader is referred (see also Kojima and Fukada, 1998 and von Schantz *et al.*, 2000).

Of particular interest to the study of pineal photosensitivity are pinopsin and exo-rhodopsin, although both parapinopsin and VA opsin have also been localized to the pineal. Pinopsin is known to be the primary photopigment of the photosensory chicken pineal, and has also been isolated from the pigeon and American chameleon. Pinopsin has been the subject of several biochemical studies, demonstrating that it is capable of activating transducin in a similar manner to the visual pigments (see Max *et al.*, 1998, Nakamura *et al.*, 1999 and Okano and Fukada, 1997). Exo-rhodopsin (also termed ER rod-like opsin) is a recently isolated opsin similar to the retinal rod opsin, localized specifically within the teleost pineal. As such, it appears that different vertebrates have adopted alternative photopigments during the course of the evolution of pineal photosensitivity. Due to the relevance of exo-rhodopsin to this work, this opsin is discussed in more detail in section 5.1.

The isolation of non-visual opsins provides several candidates for the photopigments mediating extra-retinal photoreception (as discussed in section 1.2), and as such may provide an indication as to the nature of the photopigments responsible for the regulation of the vertebrate circadian system.

Opsin	Species	Location	λ_{max} (nm)	References
PINOPSIN[1]	*G. gallus* *C. livia* *A. carolinensis* *X. laevis*	Pineal organ, deep brain	470	(Okano *et al.*, 1994), (Max *et al.*, 1995), (Kawamura and Yokoyama, 1996), (Kawamura and Yokoyama, 1997)
VA OPSIN	*S. salar* *D. rerio*	Retina, pineal, deep-brain	466 500	(Soni, 1997), (Soni *et al.*, 1998), (Philp *et al.*, 2000b), (Kojima *et al.*, 2000)
PARAPINOPSIN	*I. punctatus*	Parapineal and pineal	Unknown	(Blackshaw and Snyder, 1997)
MELANOPSIN	*X. laevis*	Dermal melanophores, Retinal ganglion cells	Unknown	(Provencio, 1998), (Provencio *et al.*, 2000)
PEROPSIN	*H.sapiens*	RPE	Unknown	(Sun *et al.*, 1997)
RGR	*B. Taurus*	RPE	Unknown	(Hao and Fong, 1996), (Hao and Fong, 1999)
ENCEPHALOPSIN	*M. musculus*	Hypothalamus	Unknown	(Blackshaw and Snyder, 1999)
EXO-RHODOPSIN[3]	*D. rerio* *A.anguilla* *O.latipes* *S. salar* *T.ribrupes* *C.carpio*	Pineal	Unknown	(Mano *et al.*, 1999) (Philp *et al.*, 2000a)

TABLE 3.1. Summary of the non-visual opsins identified to date. Footnotes as follows: 1) also known as P-opsin, 2) λ_{max} for VAL opsin and 3) also known as ER rod-like opsin.

CHAPTER 4

THE TELEOST PINEAL

The pineal organ in teleosts is a photosensory organ, containing photoreceptor cells exhibiting many similarities to those of the retina. The teleost pineal is thought to convey information to the rest of the organism by means of a dual neural and hormonal output, and in many species has also been demonstrated to contain a circadian oscillator.

The teleost pineal has been subject of numerous anatomical and neurophysiological studies, yet many fundamental questions remain unanswered as to the precise role this organ plays. A review of the extensive literature relating to the teleost pineal has been provided by Ekstrom and Meissl, (1997) to which the reader is also referred.

4.1. ANATOMY OF THE TELEOST PINEAL

In teleosts the pineal consists of an end-vesicle attached to the brain via the pineal stalk (see figure 4.1). The stalk arises caudal to the habenular commisure and rostral to the optic tectum, and the end-vesicle is usually located either directly below or within the cranium (Hafeez, 1971). In some species this region of the cranium lacks melanophores, forming a depigmented 'pineal window' (see section 4.1B, below). In certain teleosts the pineal end vesicle is very large and may cover the whole telencephalon, notable examples including the pike and trout - two of the most closely studied species (possibly for this very reason).

The pineal end-vesicle consists of a hollow sac with a central lumen, which is continuous with the third ventricle (Holmgren, 1965, Hafeez, 1971 and Flight, 1979), though in some species an open ventricular connection does not exist (Oksche, 1965). The outer segments of the photoreceptor cells project into this central lumen (Hafeez, 1971). The pineal is well vascularised, and is located outside of the blood-brain barrier (Omura *et al.*, 1985). The blood vessels around the pineal end-vesicle do not penetrate the pineal parenchyma, and instead remain outside the basal lamina. Consequently, the pineal photoreceptors are exposed apically to the CSF environment of the lumen, and

basally to the haemal environment of the capillaries (Omura *et al.*, 1985 and Ekstrom and Meissl, 1997).

Several studies have addressed inter-species differences in the morphology of the teleost pineal (for example, see Hafeez, 1971). A comprehensive survey and summary of these studies is provided by (Ekstrom and Meissl, 1997).

The pineal epithelium is typically extensively folded presenting no clearly organized cellular layers such as those occurring in the retina (Flight, 1979). This folding of the pineal epithelium multiplies the cellular density at the expense of the pineal lumen, and the teleost pineal may therefore be considered a 'folded retina' (Vigh *et al.*, 1998). This folding, coupled with the lack of any dioptric apparatus, indicates that the teleost pineal is incapable of image formation (Meissl and Yanez, 1994).

A) ONTOGENY

In teleosts the pineal complex develops as a two medial invagination of the dorsal diencephalon (Holmgren, 1965 and Oksche, 1965). The pineal organ develops from the more posterior of these invaginations, whereas the more anterior invagination, the parapineal organ, reduces in size, and is rudimentary or absent in the adult fish (Ekstrom *et al.*, 1983b).

The pineal develops dorsally as a hollow outgrowth, with the pineal stalk later constricting to form the more familiar pineal end vesicle. In many ways the development of the pineal lumen mirrors the development of the retinal lumen, but whereas the retinal lumen almost vanishes (except for a thin extracellular space between the inner and outer layers of the eye cup) the pineal lumen is found to be more prominent (Flight, 1979).

The photoreceptors of the teleost pineal appear to develop before those of the retina, although the neurons develop simultaneously in the pineal and retina (Ostholm *et al.*, 1988).

B) LIGHT TRANSMISSION OF THE SKULL AND OVERLYING TISSUE

Due to the intra-cranial position of the teleost pineal the spectral composition of the light reaching the pineal end-vesicle is considerably more restricted than that reaching the retina. In some species the parietal bones form a pineal window – a region of reduced pigmentation, facilitating the penetration of light through the skull. The attenuation of light by the overlying tissues is in the order of 1-2 log units (Dodt and Meissl, 1982 and Meissl and Brandstatter, 1992).

The transmission characteristics of the tissues overlying the pineal must be taken into account when considering the intensity and spectral composition of light available for detection by intracranial photoreceptors. Studies of tissue transmission by Hartwig and van Veen, 1979 suggest that the transmission of the cranium is primarily determined by three factors:

1. Short wavelength light is scattered to a greater degree than longer wavelengths, thus allowing longer wavelengths to penetrate more deeply.
2. The presence of the pigment melanin, which absorbs maximally at shorter wavelengths.
3. The absorption spectrum of haemoglobin. Haemoglobin, like melanin, absorbs shorter wavelengths, having a soret band with an absorption peak around 415 nm. The haemoglobin pigment also has α and β absorption peaks which occur around 576 and 541 respectively (Prosser and Brown, 1962).

As such longer wavelengths (from 700-750 nm) penetrate some 100-1000 times more effectively into the level of the diencephalon than shorter wavelengths (from 400-450 nm). A small plateau occurs in the transmission spectra around 500-530 nm, which is believed to be due to the haemoglobin transmission maxima (which is of course inversely proportional to the absorption maxima). This spectral window may be expected to be of significance when considering the spectral tuning of intracranial photosensitivity.

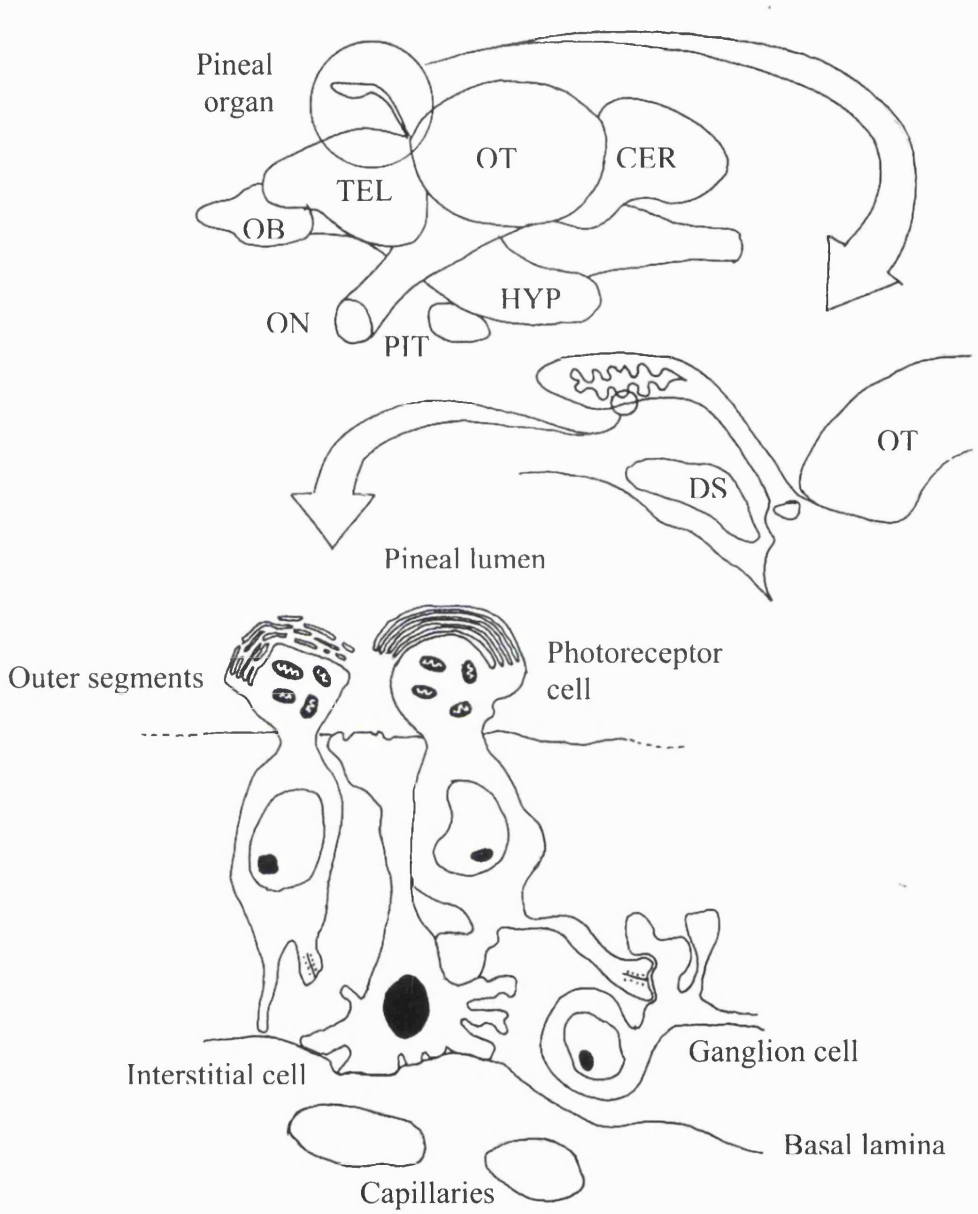


FIGURE 4.1. Schematic diagram demonstrating the structure and location of a typical teleost pineal organ. Top picture shows position of the pineal along with the major subdivisions of the teleost brain. Middle picture shows the pineal in relation to the habenula. The lower picture shows a schematic diagram of the arrangement of photoreceptors, interstitial cells and neurons within the pineal epithelium. Abbreviations as follows: CER (cerebellum), DS (dorsal sac), HYP (hypothalamus), OB (olfactory bulb), ON (optic nerve), OT (optic tectum), PIT (pituitary), TEL (telencephalon). Redrawn from Ekstrom and Meissl, (1997).

4.2. CELLULAR CONSTITUENTS OF THE TELEOST PINEAL

Light and electron microscopic studies have identified four main cell types within the teleost pineal. These are the photoreceptor cells, interstitial cells, neurons and macrophages.

A) PHOTORECEPTOR CELLS

The photoreceptor or sensory cells of the teleost pineal may take a variety of forms, but unlike the retina apparently possess no clearly defined morphological subtypes. Pineal photoreceptors can be divided into a basal and apical pole, with the basal pole consisting of the axon and terminal pedicle, and the apical pole consisting of the outer and the inner segments (Ekstrom and Meissl, 1997).

The outer segment consists of a stack of membrane lamellae containing the photopigment, connected to the inner segment by a stereocilium of the 9 x 2 + 0 type, in a manner similar to the photoreceptors of the retina. These lamellae usually number in the region of 20-70 per photoreceptor, and although they may resemble retinal cones, are more usually dome or cup shaped. The inner segment usually contains numerous mitochondria and cytoskeletal elements.

The axons of the pineal photoreceptors are usually short, contacting either postsynaptic pineal neurons or the basal lamina surrounding the pineal parenchyma. The axon terminals usually contain 'synaptic ribbons' in a similar manner to retinal photoreceptors, which are indicative of tonically active synapses ensuring uninterrupted neurotransmitter release (Wagner, 1997). A certain population of photoreceptors may possess long axons projecting directly to the brain (Ekstrom, 1987b).

A number of studies have demonstrated that the photoreceptor cells contain elements of the phototransduction cascade, similar to retinal photoreceptors. Immunoreactivity has been demonstrated to rod opsin, and in some cases cone opsins (see 5.1B), the α-subunit of transducin, arrestin, guanylate cyclase and recoverin (see Vigh Teichmann *et al.*, 1980, Vigh and Vigh-Teichmann, 1981, Vigh-Teichmann *et al.*, 1982, Vigh Teichmann *et al.*, 1983, Collin *et al.*, 1986, van Veen *et al.*, 1986, Ekstrom *et al.*, 1987a, Ostholm *et al.*, 1988, Ekstrom and Meissl, 1990, Vigh-Teichmann *et al.*, 1992, Vigh *et al.*, 1998, Hisatomi *et al.*, 1999 and Ekstrom and Meissl, 1997 for comprehensive

reference list). As such, it would appear that pineal phototransduction bears many similarities to the well-characterised retinal cascade.

In addition to elements of the phototransduction cascade, the enzymes of the melatonin biosynthetic pathway have also been localized within teleost pineal photoreceptors (see section 4.4A).

B) Interstitial Cells

Another major constituent of the pineal parenchyma are the interstitial or supportive cells. These are located between the photoreceptor cells, attached by tight junctions to form a diffusion barrier from the pineal lumen. The interstitial cells demonstrate many ultrastructural features associated with high levels of synthetic activity, although immunohistochemical studies have demonstrated that they do not contain elements of either the phototransduction cascade or melatonin biosynthesis (Ekstrom and Meissl, 1997).

The interstitial cells fulfil criteria for both ependymal and glial elements, and may be involved in supportive functions, analogous to the Muller cells of the retina (Flight, 1979).

C) Neurons

Pineal neurons take the form of both second order neurons and output cells of the pineal, apparently combining the roles of the bipolar and ganglion cells of the retina. The responses of these cells are discussed in more detail in section 4.3B. In addition to this role, a smaller population of pineal neurons may act as interneurons, although the neural circuitry of the pineal is poorly understood when compared to that of the retina (Meissl and Brandstatter, 1992).

A large proportion of pineal neurons, along with some photoreceptors, send projections to the brain forming of the pineal tract. The majority of these projections are unmyelinated, but myelination does occur in small fraction. The number of axons making up the pineal tract varies between species, with estimates ranging from nearly 4000 in the rainbow trout, down to a few hundred in most species (Ekstrom and Meissl, 1997).

The pineal tract has been shown to give rise to bilateral projections to pretectum, dorsal and ventral thalamic nuclei, hypothalamus, tegmentum, habenular nucleus and preoptic region. The central axonal projections of the pineal tract partly overlap with those of the optic nerve, a feature that is particularly evident in the pretectal and thalamic nuclei. (Ekstrom and van Veen, 1983a, Ekstrom, 1984, Jimenez *et al.*, 1995 and Ekstrom and Meissl, 1997). The broad range of these projections suggests a role in the modulation of a variety of neural systems in response to light.

D) MACROPHAGES

Macrophages have been reported in the pineal organs of several species, and may be involved in outer segment breakdown and renewal. Macrophages have also been observed in the pineal epithelium and perivascular space, where they have been suggested to be involved in the breakdown of substances penetrating from the bloodstream (Omura *et al.*, 1985 and Ekstrom and Meissl, 1997).

4.3. NEUROPHYSIOLOGY

The first direct evidence of the photosensory nature of the teleost pineal came from the work of (Dodt, 1963), who demonstrated that the pineal organ of the rainbow trout responded to light with a maximum sensitivity around 505 nm. Other workers have since identified pineal responses to light by a variety of electrophysiological techniques, including recordings from whole pineal organs as an electropinealogram or EPG (similar in most respects to the more familiar electroretinogram), as well as using intracellular recordings from individual photoreceptors and output neurons.

A) PHOTORECEPTOR RESPONSES TO LIGHT

Intracellular recordings from the pineal photoreceptors of several species indicate many similarities to the response kinetics of retinal photoreceptors. In their resting (dark-adapted) state the membrane potentials of pineal photoreceptors are around -20 to -30 mV, similar to that of retinal photoreceptors (Meissl and Ekstrom, 1988b). Flashes of light produce a hyperpolarisation to around -60 mV. This light-induced hyperpolarisation is graded with light intensity, that is the photoresponse increases in

proportion to the light intensity, ranging from threshold photoresponses evoked by dim light, up to the saturating responses evoked by bright light. The amplitude-intensity relationship of teleost pineal photoreceptors is fundamentally similar to retinal rods and cones (Meissl and Yanez, 1994).

Upon illumination trout pineal photoreceptors demonstrated a decrease in cGMP of 30-40%. This response to light was attenuated in the presence of pertussis toxin, known to inhibit α-transducin activity, and also by phosphodiesterase inhibitors, which prevent the hydrolysis of cGMP. Similar effects are observed in retinal photoreceptors (Falcon *et al.*, 1992, Falcon and Gaildrat, 1997 and Falcon, 1999).

As such the hyperpolarizing response demonstrated by pineal photoreceptors is fundamentally similar to that of retinal photoreceptors, and appears to involve the same phototransduction cascade, although subtle differences may remain as yet undiscovered.

i) Time course

Despite the many similarities between retinal and pineal photoreceptor responses, a difference is apparent in the time course of pineal photoreceptors. Pineal photoresponses are typically slower than their retinal counterparts, evident in both the latency between stimuli and response as well as the recovery duration. The latency of pineal photoresponses in the trout is around 1200 ms for stimuli near threshold, decreasing to around 300 ms with saturating stimuli (Meissl and Ekstrom, 1988b). In comparison the latency of threshold responses in retinal rods is around 350 ms, which decreases to about 50 ms for saturating stimuli (Cervetto *et al.*, 1977). Following saturating stimuli the time taken for pineal photoreceptors to recover to the dark potential is exceptionally long. Response durations of up to 60 s were observed in both trout and goldfish (Meissl *et al.*, 1986, Meissl and Ekstrom, 1988a and Meissl and Ekstrom, 1988b). This slower time course may be accounted for by differences in the interactions with elements of the phototransduction cascade when compared with retinal photoreceptors. These differences may occur at the level of the photopigment molecule itself or the downstream elements of the cascade.

The slower time course of pineal photoresponses suggests that teleost pineal photoreceptors are incapable of discriminating between rapidly changing stimuli, as

would be required for any form of spatial representation of the external world. However, these slower photoresponses do suggest that the teleost pineal is instead specialized for detecting gradual changes in the light environment, consistent with the theory that the pineal may be involved in monitoring the overall level of irradiance for the regulation of temporal physiology.

ii) Adaptation

Teleost pineal photoreceptors demonstrate dark adaptation in a similar manner to the photoreceptors of the retina (Meissl and Yanez, 1994 and Meissl and Brandstatter, 1992). Light adaptation is also apparent, with the time required to reach peak response becoming progressively shorter as light intensity increases. However, the time-dependent response decay of retinal photoreceptors is not observed, and the light-induced hyperpolarisation is maintained at a stable membrane potential which is dependent upon the level of ambient illumination. The sensitivity to flashes of light is depressed relative to the background light intensity. As such, pineal photoreceptors do not undergo sensitivity changes except those occurring at the onset of illumination. This maintenance of a light intensity-related membrane potential during steady illumination may reflect the role of these cells in the voltage-dependent synthesis of melatonin in relation to the ambient photoperiod (see section 4.4, and Meissl and Ekstrom, 1988a, Meissl and Ekstrom, 1988b, Koch, 1992, Meissl and Brandstatter, 1992, Marchiafava and Kusmic, 1993 and Meissl and Yanez, 1994).

B) NEURAL OUTPUT

In darkness the second order neurons that form the pineal tract are spontaneously active. Light stimulation of all wavelengths produces an inhibition of this activity in the majority of pineal cells. These cells are consequently known as luminance or *achromatic* units, and have a sensitivity threshold in the same order as the light threshold of the retinal rods (Meissl and Yanez, 1994). This sensitivity was originally thought to originate from a convergence of photoreceptors onto second order neurons. However, similar values have been obtained from intracellular recordings of pineal photoreceptors, suggesting that this is in fact a property of the pineal photoreceptor cell

(Meissl and Ekstrom, 1988a). This sensitivity is remarkable considering the rudimentary morphology of pineal photoreceptors when compared to retinal rods (Meissl and Brandstatter, 1992 and Ekstrom and Meissl, 1997).

A small proportion of neurons exist in some species of teleost that are capable of a *chromatic* response. These chromatic units are inhibited by short wavelength light and excited by longer wavelengths. These opposing effects interact, with the net output depending upon the balance of excitation and inhibition in response to the spectral composition of the ambient light (see Dodt and Meissl, 1982). The basis of this mechanism remains poorly understood, but probably involves multiple photoreceptor populations. Multiple photoreceptor populations have been suggested in both the trout and the pike (Kusmic *et al.*, 1993 and Falcon and Meissl, 1981). An alternative theory suggests the interconversion of a bistable photopigment, in which retinal is isomerised by long wavelength light and re-isomerised by short wavelengths (Eldred and Nolte, 1978).

Whatever the basis of the chromatic response in these species such a mechanism may be of value in detecting the changes in spectral composition of the light environment. This may be of value in detecting the twilight transition between light and dark, which is thought to play an important role in the regulation of circadian phenomena (see section 2.2A).

4.4. MELATONIN SYNTHESIS

Like the pineal of all vertebrates studied to date, the teleost pineal rhythmically synthesizes the photoperiod-mediating hormone melatonin. Melatonin production by the teleost pineal is regulated by the ambient photoperiod, and as in other vertebrates is high during the scotophase and low during the photophase. The melatonin synthesis profile of the pineal, but not the retina, is reflected in the plasma melatonin profile, indicating that pineal melatonin is the primary source of circulating melatonin in teleosts (Falcon, 1999).

A) Melatonin Synthesis by Pineal Photoreceptors

The cells responsible for the synthesis of melatonin are the photoreceptor cells themselves. Suggestive evidence for this comes from the demonstration that pineal photoreceptors contain and are capable of metabolising indoles, including serotonin and melatonin (Falcon, 1984, van Veen *et al.*, 1984, Ekstrom and Meissl, 1990, and Falcon *et al.*, 1992). Expression of HIOMT, the final enzyme in the melatonin biosynthetic pathway, has been localized exclusively to the photoreceptors in the pike pineal (Falcon *et al.*, 1994). Furthermore, when pike pineal photoreceptors are isolated from other cellular components, these isolated photoreceptor cells are capable of melatonin synthesis, indicating that they are the source of pineal melatonin synthesis (Bolliet *et al.*, 1996b).

B) An Intra-pineal Oscillator Controls Melatonin Synthesis

Several studies have demonstrated that the teleost pineal is capable of maintaining the rhythmic synthesis of melatonin even in the absence of external light cues. This melatonin output rhythm is entrained to the external photoperiod, even when the pineal is isolated from the rest of the rest of the animal in culture. These studies demonstrate that a circadian oscillator is present within the pineal of these teleost species, and that the photoreceptor responsible for entraining this oscillator is also present within the pineal (Bolliet *et al.*, 1996a, Bolliet *et al.*, 1996b, Cahill, 1996 and Iigo *et al.*, 1991).

The endogenous rhythm of melatonin synthesis persists in culture for several days, although the exact duration varies between species. This rhythm appear to be damped in the absence of photoperiodic cues, suggesting a population of uncoupled oscillators, or alternatively, the accumulation of an endogenous compound affecting melatonin production – possibly melatonin itself (see Falcon, 1999). The Salmonids provide a notable exception to this rule, and do not appear to possess an intra-pineal oscillator. As such the melatonin profile in these species simply reflects the prevailing light conditions (Gern *et al.*, 1992).

In summary, the teleost pineal organ, and possibly each individual photoreceptor cell, comprises a full photoneuroendocrine system, containing photoreceptor, oscillator and output components (see Korf *et al.*, 1998 and Falcon, 1999).

C) REGULATION OF MELATONIN SYNTHESIS

The rhythmic synthesis of melatonin is regulated at a number of levels, including the acute suppression of melatonin synthesis by light, regulation by the intra-pineal oscillator, as well as modulation by temperature and neurotransmitters.

i) Photoperiodic Regulation

The phototransduction cascade of teleost pineal photoreceptors appears to be similar to that of retinal photoreceptors, as demonstrated by intracellular recordings, pharmacological studies and immunocytochemical demonstration of numerous elements of the retinal cascade within pineal photoreceptors. As such how may the phototransduction cascade be linked to the acute suppression of melatonin synthesis produced by light?

Firstly, increases in cGMP levels apparently have only a marginal influence on melatonin synthesis within the teleost pineal (Thibault *et al.*, 1993). In contrast, increases in intracellular cAMP do produce an increase in NAT activity, and a consequent increase in melatonin production. However, cAMP does not accumulate in the pineal in darkness under natural conditions, although this may represent increases in cAMP utilization or metabolism.

In contrast, Ca^{2+} appears to play in important role in the regulation of melatonin synthesis. Increases in intracellular Ca^{2+} produce an increase in melatonin synthesis, and blocking Ca^{2+} channels blocks this increase (Begay *et al.*, 1994a and Meissl *et al.*, 1996). The effects of Ca^{2+} appear to be mediated through Ca^{2+} binding proteins, and appear to act through modulation of cAMP. However, cAMP and melatonin synthesis respond differently to inhibitors of Ca^{2+} binding proteins, and as such it is thought that Ca^{2+} exerts its effects through cAMP interactions and unknown cAMP-independent mechanisms (Begay *et al.*, 1994b).

Given the similarities noted between phototransduction in pineal and retinal photoreceptors it may be assumed that pineal photoreceptors generate a dark-current in a similar manner to their retinal counterparts. The cGMP-gated channels open in darkness will allow an influx of cations, including Ca^{2+}, thus increasing intracellular Ca^{2+}. In addition to this, L-type voltage-gated Ca^{2+} channels have been demonstrated to occur in teleost pineal photoreceptors. These channels are open when the cell membrane is depolarised, and close upon membrane hyperpolarisation. As such, the dark state, in which the photoreceptor is depolarised, favours Ca^{2+} entry into the cell (Begay *et al.*, 1994b and Kroeber *et al.*, 2000). Based upon this model hyperpolarisation would induce a decrease in intracellular Ca^{2+}, resulting in a light-induced suppression of melatonin synthesis.

This voltage-dependent synthesis model may also relate to the observation that pineal photoreceptors maintain a fixed degree of hyperpolarisation in relation to the ambient light level (see section 4.3Aii). In this way, the maintenance of a fixed relation between membrane potential and environmental light will be reflected by the intracellular Ca^{2+} concentration and consequently melatonin synthesis. Evidence for a direct link between electrophysiological and biochemical responses are currently lacking due to complications produced by the differences in the time scale of the responses (Meissl and Brandstatter, 1992).

Much of the information available regarding the intracellular transduction of light signal to melatonin synthesis originates from work conducted in either the trout or pike pineal. This work demonstrates many similarities to studies conducted on the photosensitive pineal organs of other species, notably the chicken pineal which has been the most extensively studied (see Zatz, 1996, Zatz *et al.*, 2000 and Okano and Fukada, 1997). Parallels may be drawn between the transduction mechanisms of these two systems, although caution must be exercised when doing so.

Reviews of the current understanding of the intracellular regulation of melatonin synthesis in the photosensory pineal are provided by Korf *et al.* (1998) and Falcon (1999).

ii) Regulation by the Intra-pineal Oscillator

As discussed previously the pineal organ of most teleost species contains a circadian oscillator capable of producing rhythmic melatonin synthesis in constant conditions. Further studies have localized this oscillator to the photoreceptor cells. The oscillator appears to regulate melatonin synthesis through NAT, as the expression of NAT mRNA demonstrates rhythmic variations between day and night (Falcon, 1999). Little is known as to the mechanisms by which the oscillator produces this rhythmic variation in NAT expression, although one proposed mechanism is through the modulation of cAMP levels.

The light-induced entrainment of the oscillator is also poorly understood. However, in the chicken pineal the acute light-induced suppression of melatonin and photoentrainment of the oscillator appear to involve different signal transduction pathways. The suppression of melatonin synthesis involves mechanisms sensitive to pertussis-toxin, suggestive of α-transducin, whereas the photoentrainment pathway involves pertussis toxin-insensitive mechanisms (Okano and Fukada, 1997).

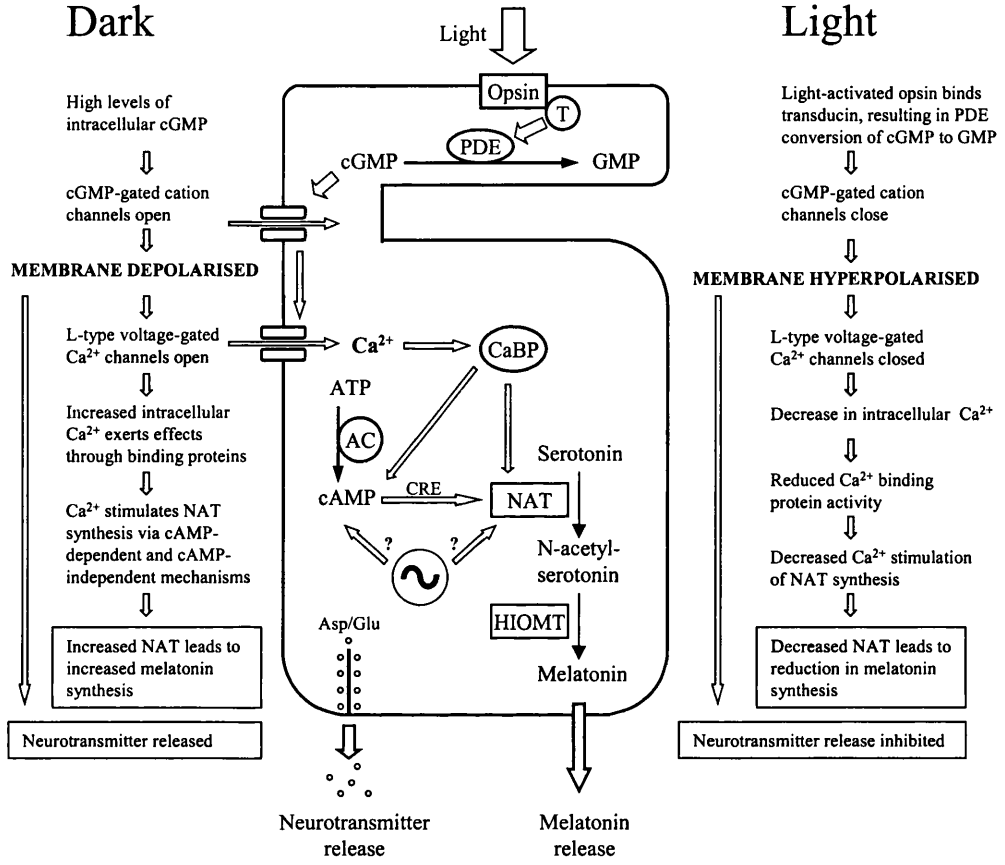


FIGURE 4.2. Intracellular mechanisms regulating neural output and melatonin synthesis in teleost pineal photoreceptors. See text for details. Modified from Falcon (1999).

4.5. PHYSIOLOGICAL ROLE

The teleost pineal has been suggested to be involved in a wide range of physiological roles, including the regulation of circadian locomotor rhythms, behavioural thermoregulation, metabolic processes, reproduction, growth, pigmentation as well as a developmental role (see Zachmann *et al.*, 1992 and Ekstrom and Meissl, 1997).

The teleost pineal is thought to function primarily as a luminance detector, transducing information regarding the level of environmental irradiance into a dual neural and hormonal output (Meissl and Yanez, 1994 and Ekstrom and Meissl, 1997). The hormonal output of melatonin implies an important role in the regulation of circadian physiology, especially in the entrainment of the multiple oscillators of the teleost circadian axis. The neural output of the teleost pineal may be involved with providing precise signals regarding both the amount and composition of the environmental light, as well as modulating non-circadian responses to light such as behavioural thermoregulation and negative phototaxis (see Shand and Foster, 1999 and Ekstrom and Meissl, 1997).

Several studies have demonstrated that the photoreceptors of the pineal develop before those of the retina, although the neurons of both retina and pineal develop simultaneously. This early development may reflect a role in circadian organization in early ontogeny, when the melatonin-producing pineal photoreceptors are functional, even though the neural connections of the pineal remain undeveloped (Ostholm *et al.*, 1988 and van Veen *et al.*, 1984).

Other studies have implicated the pineal in a wide range of biological functions, but this work, typically involving pinealectomy or administration of exogenous melatonin, has been prone to a variety of errors, including incomplete pinealectomy and the timing and dosage of melatonin administration. As such, the results of these studies must be considered inconclusive. For a review and critique of these studies see Ekstrom and Meissl (1997) and Mayer *et al.* (1997).

CHAPTER 5

THE PHOTOPIGMENT CONTENT OF THE TELEOST PINEAL

5.1. PREVIOUS STUDIES

What information is available concerning the spectral sensitivity of teleost pineal photoreceptors originates primarily from three sources – electrophysiological studies, labelling studies and the recent isolation of non-visual opsins.

A) ELECTROPHYSIOLOGY

Electrophysiological studies have provided a great deal of information regarding the response kinetics of the photoreceptors and the resulting output signals to the brain (see section 4.3). Table 5.1 provides a summary of the electrophysiological studies conducted to date on the spectral sensitivity of teleost pineal photoreceptors. As a means of determining an exact λ_{max} value, and providing information regarding the molecular nature of the pineal photopigments these studies have been suggestive though inexact.

Most of these studies suggest a primary luminance response from teleost pineal photoreceptors, with λ_{max} values similar to those of the respective rod opsin. However, in the trout and pike there is evidence of multiple photoreceptor populations, suggested by the presence of a chromatic response (see section 4.3B). In both species a small population of neurons has been shown to exhibit inhibitory responses to short wavelengths and excitatory responses to longer wavelengths, suggestive of multiple photopigment populations.

It should be noted that the majority of intracellular recordings from the trout pineal are likely to be from a single photoreceptor population, with the λ_{max} differences around 500-530 nm probably occurring due to chromophore variations occurring between the various studies (trout rod opsin pigment pair λ_{max} = A_1 505 nm/A_2 527 nm). Such chromophore variations are particularly problematical when the λ_{max} is obtained from a

limited number of spectral points, as in the case of electrophysiology. Chromophore differences are also likely to be the source of the two values noted in the pineal of *A.fasciatus* (see 5.2B).

SPECIES	METHOD	λ_{MAX}	REFERENCES
Rainbow trout *Salmo gairdneri*	EPG	505	1
	Intracellular	500/533	2
	Intracellular	520–530	3-4
	Intracellular	495/521	5
	MSP	463/561	6
Pike *Esox lucius*	Intracellular	380/620 530/620	7
Minnow *Phoxinus phoxinus*	Intracellular	530	8
Goldfish *Carassius auratus*	Intracellular	530	9
Mexican tetra *Astyanax fasciatus*	Intracellular	494/525	10

TABLE 5.1. Summary of the studies of the spectral sensitivity of teleost pineal photoreceptors conducted to date. References as follows: 1: Dodt (1963), 2: Dodt, (1973), 3: Meissl and Ekstrom (1988a), 4: Meissl and Ekstrom (1988b), 5: Marchiafava and Kusmic (1993), 6: Kusmic *et al.*, (1993), 7: Falcon and Meissl (1981), 8: Nakamura (1986), 9: Meissl *et al.* (1986), 10: Tabata, (1982).

B) LABELLING STUDIES

Immunocytochemical studies have demonstrated the presence of opsin-based photopigments within the teleost pineal, utilising antibodies raised to frog rod opsin (Vigh and Vigh-Teichmann, 1981 and Vigh Teichmann *et al.*, 1980) and bovine opsin (Vigh Teichmann *et al.*, 1983 and Vigh-Teichmann *et al.*, 1992). Immunocytochemical studies have demonstrated only a weak immunoreaction to pinopsin in the teleost pineal, and seem suggestive of a cross-reaction (Vigh *et al.*, 1998).

Data from *in situ* hybridisation in the developing goldfish pineal suggests that opsin expression in the pineal precedes that of the retina. Robust expression of rod opsin and LWS cone opsin was observed, with less consistent MWS cone opsin hybridisation (Stenkamp and Raymond, 1995). Similarly, expression of the LWS cone opsin has been reported in zebrafish pineal (Kojima and Fukada, 1998).

In conclusion, these labelling studies suggest that teleost pineal photoreceptors predominantly contain an opsin-based photopigment, certainly similar to retinal rod opsin at the molecular level. The suggestion of cone opsin expression (particularly LWS cone opsin) in a subpopulation of pinealocytes has also been made in some species.

C) NON-VISUAL OPSINS

Of the non-visual opsins discovered to date, several have been localized to the teleost pineal, and consequently represent good candidates for the basis of pineal photosensitivity. VA opsin, parapinopsin and exo-rhodopsin have so far been localized to the teleost pineal organ.

VA opsin, originally isolated in the retina of the Atlantic salmon (*Salmo salar*), has also been demonstrated in the Salmonid pineal (Philp *et al.*, 2000b). Expression of VA opsin in the zebrafish (*Danio rerio*), however, appears to be confined to horizontal cells of the retina and around the third ventricle in the thalamus (Kojima *et al.*, 2000). Parapinopsin has been demonstrated in the parapinealocytes of channel catfish (*Ictalarus punctatus*), as well as in a subset of pineal photoreceptors. Evidence for parapinopsin in other teleost species is currently lacking (Blackshaw and Snyder, 1997).

i) Exo-rod opsin

During the course of this work, two papers have been published describing a pineal-specific rod-like opsin in teleosts. The first study, by Mano *et al.,* named this opsin exo-rhodopsin (for ExtraOcular), the second by Philp *et al.,* ERrod-like (for ExtraRetinal rod-like). As noted by Philp *et al.,* rhodopsin is a term used to define photopigments containing the 11-*cis* retinal chromophore, as opposed to porphyropsins containing 11-*cis* 3-dehydroretinal (see section 3.2A). As both rhodopsins and porphyropsins may occur in teleosts, the name exo-rhodopsin is therefore misleading. Given the phylogenetic grouping of this opsin with the retinal rod opsins, rather than utilising an alternative name and risking inevitable confusion, in this work it will be henceforth be simply referred to as exo-rod opsin (which can be abbreviated to ER opsin as a compromise if so desired).

These studies demonstrate the presence of exo-rod opsin in the pineal organs of six species in total – Zebrafish (*Danio rerio*), Medakafish (*Oryzias latipes*), European eel (*Anguilla anguilla*), Atlantic salmon (*Salmo salar*), Common carp (*Cyprinus carpio*) and Pufferfish (*Takifugu rubripes*). Both reports demonstrate that exo-rod opsin is expressed solely in the pineal and not in the retina, concluding that this opsin is the primary photopigment responsible for pineal photosensitivity in teleosts.

The discovery of exo-rod opsin is consistent with both electrophysiological and labelling findings, and immunoreaction to rod opsin probes is highly likely given the sequence similarity of the rod and exo-rod opsin proteins.

5.2. SPECIES STUDIED

The goldfish, *Carassius auratus*, was the central model used in this study. A limited comparative study was also conducted on the pineal organs of two other teleost species – the Golden orfe (*Leuciscus idus*) and the Mexican tetra (*Astyanax fasciatus*).

A) GOLDFISH, *CARASSIUS AURATUS*

The goldfish is a member of the cyprinid family, which consists of carp and minnows. Originating from central Asia, China and Japan, the goldfish is now found worldwide. The goldfish is primarily prized as an ornamental fish, with over a hundred different

varieties giving a huge range of colours and forms. They may grow up to 45 cm in length and attain a weight of up to 2 kg. In the wild goldfish inhabit the stagnant or slow-moving waters of lakes, ponds, ditches and rivers. They are remarkably hardy, tolerating temperatures ranging from freezing to over 40°C, although preferring cold water. Goldfish are omnivorous, feeding on a wide range of food, typically consisting of plants, crustaceans, insects and detritus (Froese and Pauly, 2000).

The goldfish was chosen as an experimental model for pineal photosensitivity for a number of reasons. Primarily, goldfish are readily obtainable, cheap and easy to keep, and as such the goldfish has become a common experimental model, especially in visual science. As such there exists an extensive literature on goldfish sensory physiology, including both anatomical and electrophysiological studies of the pineal (see below).

i) Visual Pigments of the Goldfish

The visual system of the goldfish has been extensively studied. The goldfish visual pigments are porphyropsin dominated, and have been classically regarded as pure A_2 based. However, it has been shown that this is not the case, and that the visual pigments may adapt to the prevailing photothermal regime (Tsin and Beatty, 1978 and Tsin *et al.*, 1981).

Goldfish are tetrachromatic, possessing rod photoreceptors and four distinct cone classes (see Table 5.2). The rod, SWS, LWS and two forms of MWS opsin have been sequenced and published (Johnson *et al.*, 1993).

ii) The Goldfish Pineal

The morphology of the goldfish pineal has been studied in detail at both the light and electron microscope levels (Takahashi, 1969 and McNulty, 1981), and contains numerous comparatively large photoreceptors. The goldfish pineal is much the same in cellular make-up as the pineal of most teleost species, containing approximately 33,000 photoreceptor cells, with a similar number of interstitial cells. The photoreceptor cells are typically dumbbell shaped, with outer segments that project into the cell lumen, sometimes forming a cup over the inner segments. It has been estimated that the goldfish pineal contains over 500 nerve cells, and calculations estimate that between 65

and 107 photoreceptor cells are related to each nerve cell in the end vesicle. This high level of convergence precludes any form of spatial representation, and seems indicative of a role as a sensitive luminance detector.

The pineal tract of the goldfish projects to numerous regions of the CNS, mirroring the connections of the teleost pineal as noted in section 4.2C. There is no evidence in the goldfish for any form of central pineal innervation (Jimenez *et al.*, 1995), such as that occurring in higher vertebrates (see Korf *et al.*, 1998 for review).

As noted above, goldfish pineal photoreceptors have been shown to respond to light with the graded hyperpolarisation typical of the teleost pineal. Although based upon a small data set, the λ_{max} of this response has been shown to be around 530 nm (Meissl *et al.*, 1986).

PHOTORECEPTOR CLASS	λ_{max} Native A_2	λ_{max} 11-cis A_1	λ_{max} 9-cis A_1	λ_{max} SPE
Rods	522	503	490	-
UVS cones	365	370	374	356
SWS cones	460	447	427	447
MWS cones	537	516	493	537
LWS cones	625	566	526	623

TABLE 5.2. Photoreceptor complement of the goldfish retina. Native photopigments are predominantly porphyropsins, whereas 11-*cis* and 9-*cis* data are based upon reconstitution with exogenous retinoids (Parry and Bowmaker, 2000). λ_{max} SPE is based upon intracellular recordings made with suction pipette electrode (Palacios *et al.*, 1998).

PHOTORECEPTOR CLASS	*Astyanax fasciatus* λ_{max} (nm)	*Leuciscus idus* λ_{max} (nm)
Rods	519	518
UVS cone	407	366
SWS cone	450	405
MWS cone	535	498
LWS cone	546 580	575

TABLE 5.3. The visual pigments of the Mexican tetra (*Astyanax fasciatus*) and the Golden orfe (*Leuciscus idus*). Data from (Campbell, 2000) and Parry (unpublished data). See text for details.

B) MEXICAN TETRA, *ASTYANAX FASCIATUS*

The Mexican tetra, *Astyanax fasciatus,* is a characid found in habitats throughout Central and South America. The *Astyanax* genus is rich in species and subspecies, and *A. fasciatus*, the species used in this study, are found in a diverse variety of habitats, but are most abundant in the clear running waters of rivers and lakes with gravel bottoms. *A. fasciatus* are seldom found at depth, preferring shallow waters. Like most tetras, *A. fasciatus* is carnivorous, feeding predominantly on small aquatic insects and the larvae of terrestrial insects, although they will also eat small fish. The Mexican tetra is an aggressive schooling species, and wherever they are found they are generally the dominant fish (Froese and Pauly, 2000).

Troglobitic forms of *A. fasciatus* inhabit a series of caves in Mexico, in three hydrologically isolated areas. These troglobitic forms are fully interfertile with the surface populations, and hybrid forms are known to occur naturally as well as in the lab. The individuals used in this study were from a hybrid population kindly provided by Horst Wilkens. Eye development and visual capability were variable but typically degenerate in these hybrids. Although the use of such hybrids introduces a further element of variability, it is of interest to investigate pineal photosensitivity in a species in which visual function, whilst not wholly absent, is certainly compromised.

The visual pigments of this hybrid population have been measured and the photoreceptor complement is shown in table 5.3. The chromophore content of these photoreceptors was found to be variable, but a ratio of around 70% A_1/30% A_2 usually fitted the data most closely. Unusually, these animals also appear to possess two LWS cone opsins (Parry, unpublished data).

The pineal of *A.fasciatus* appears much like that of other teleosts, especially in the surface population. In cave populations an age-dependent, gradual regression of the pineal photoreceptor outer segments occurs, possibly in response to environmental light conditions (Herwig, 1976). Electrophysiological recordings from the pineal of *A.fasciatus* indicate two response maxima, one with a λ_{max} at 494 nm and the other at 525 nm (Tabata, 1982). However, these values are based on a very limited number of data points, and may represent variations in chromophore content rather than spectrally distinct photoreceptor populations.

c) Golden Orfe, *Leuciscus idus*

The orfe or Ide, is a cyprinid similar in appearance to the roach (*Rutilus rutilus*). Orfe are found wild in clear pools of medium to large rivers, ponds and lakes throughout Europe and Asia, although they have also become a popular ornamental pond fish. Orfe spawn in spring, usually migrating to clear upstream waters or the shallows of lakes. Upon hatching, the fry migrate downstream or to the deeper regions of the lake. The small fish demonstrate remarkable schooling behaviour. Fully mature at around five years, orfe may live for up to twenty, and grow up to one meter in length. Orfe are primarily insectivorous, feeding on invertebrates such as mayflies and stoneflies at the waters surface, as well as water snails, freshwater clams and shrimps. The young feed on organic detritus, algae or smaller invertebrates, whereas larger individuals may feed on other fish (Froese and Pauly, 2000).

The Orfe was chosen as a comparative species for several reasons. Firstly, the orfe provides an interesting wild cyprinid counterpoint to domestic goldfish. Although no published data exists on the orfe visual system, work conducted in the lab by Rob Campbell (2000) has characterized the retinal photoreceptors of this species by MSP, illustrating the typical tetrachromatic cyprinid photoreceptor complement of four spectral classes of cone and single rod class (see table 5.3). Unlike the goldfish, the retina was found to predominantly possess A_1 photopigments, although traces of A_2 pigments were noted, especially in the rods. The retinoid content may be influenced by photothermal regime in a similar manner to other teleost species (see Bridges and Yoshikami, 1970, Bridges, 1972 and Whitmore and Bowmaker, 1989).

Goldfish

(*Carassius auratus*)

Cave tetra

(*Astyanax fasciatus*)

Golden orfe

(*Leuciscus idus*)

FIGURE 5.1. Species in which the spectral sensitivity of pineal photoreceptors was assessed by MSP in this study. *C.auratus* and *L. idus* are both members of the family Cyprinidae, whereas *A.fasciatus* belongs to the Characidae. Pictures taken from Froese and Pauly, 2000.

5.3. AIMS OF STUDY

A) QUESTIONS TO BE ADDRESSED

This aim of this study is to answer some of the following outstanding questions relating to the photopigment content of the photosensory pineal of teleosts.

- *What is the spectral sensitivity of teleost pineal photoreceptors?*
- *Does spectral tuning to the intracranial light environment occur?*
- *Are pineal photopigments the same as the visual opsins of the retina?*
- *If different to retinal opsins, do these pigments represent novel non-visual opsins?*
- *How do non-visual opsins differ from visual opsins at the molecular level?*
- *Can any molecular differences account for the differences between visual and non-visual photoreception?*
- *Do multiple photoreceptor populations exist within the teleost pineal?*
- *How does pineal photosensitivity differ between teleost species?*

B) HYPOTHESIS

Based upon the current understanding of teleost pineal photosensitivity and the ecology of vertebrate visual photopigments, the following hypothesis is proposed with regard to the photopigment content of the teleost pineal:

Photosensitivity of the teleost pineal organ is likely to be due to the presence of non-visual opsins, rather than the extra-retinal expression of visual opsins. These non-visual opsins may be expected to differ from their retinal counterparts due to the photosensory tasks with which they are involved. Differences in amino acid residues implicated in G-protein interactions, light-dependent phosphorylation and spectral tuning are expected. It is also expected that the λ_{max} values of any photopigments present in the pineal organ will reflect the transmission characteristics of the overlying tissues, and as such may be expected to lie within the region of 490-530 nm.

5.4. STUDY PROPOSALS

The study can be broken down in to two different, though complementary, approaches – microspectrophotometry (MSP) and molecular genetics. The photoreceptor population of the pineal was initially investigated using MSP, the results of this work then serving to direct the molecular investigation of the photopigments present in the pineal. This dual approach is reflected throughout this work, and the conclusions of the study rely upon a synthesis of both spectral and molecular data.

A) MICROSPECTROPHOTOMETRY

Microspectrophotometry is a technique used to measure the absorbance of the photopigment contained within isolated photoreceptor outer segments (see section 6.2). MSP has been widely applied to the study of the visual pigments of the retina, with work conducted on the photoreceptor complement of a wide variety of vertebrate eyes, including fishes, amphibians, reptiles, birds and mammals (see Liebmann, 1972 and Bowmaker, 1984 for reviews). MSP data allow accurate absorbance spectra to be obtained from individual photoreceptors, enabling identification of the photopigment content by its distinctive λ_{max}.

In contrast to the number of MSP studies conducted on retinal photoreceptors the only published MSP data from pineal photoreceptors in any vertebrate species has been the work of Hartwig and Baumann (1974) on the frog end-organ, and a study by Kusmic *et al.*, (1993) on the pineal organ of the rainbow trout (see table 5.1). This scarcity of pineal MSP data clearly illustrates the need for further studies to improve our understanding of the photopigments mediating non-visual photoreception.

B) MOLECULAR GENETICS

To determine the molecular basis of teleost pineal photosensitivity a variety of molecular biological techniques were utilized. Due to time constraints imposed upon this study the molecular side of this project was confined to an investigation of the goldfish pineal alone.

Messenger RNA was first isolated from the goldfish pineal, enabling the synthesis of pineal-specific cDNA. This cDNA was then used as a template for screening for the

presence of the retinal opsins using PCR. By using degenerate primers designed to conserved regions of the opsin molecule, the presence of novel opsin sequences was also investigated. The opsins thus isolated were then characterised by sequence analysis.

MATERIALS AND METHODS

CHAPTER 6

MICROSPECTROPHOTOMETRY AND HISTOLOGY

6.1. SUBJECTS

Goldfish (5-7 cm body length) were kept in tanks under a 12:12 light:dark cycle, fitted with daylight simulation fluorescent lamps. The temperature was regulated to around 21-22°C. Animals were kept in these conditions for at least a week before any experiments were conducted, to allow them to adapt to the photoperiod, which may influence the rhodopsin/porphyropsin ratio within photoreceptors (Bridges and Yoshikami, 1970 and Bridges, 1972).

A) PINEAL DISSECTION

Fish were sacrificed during the dark phase by cervical transection, and the dorsal surface of the skull removed with dissecting scissors. Using a binocular dissecting microscope the pineal end vesicle was located lying anterior to the cartilaginous rib. Excess fat was removed from the around the pineal using fine forceps, before the end vesicle was carefully detached, and placed into a drop of 5% dextran made up in cyprinid saline (135 mM NaCl, 1.9 mM KCl, 10 mM $NaHCO_3$ and 22 mM glucose) on a coverslip. Using forceps the end vesicle was gently teased apart, then a tissue squash was made using a second coverslip. Excess dextran was removed with filter paper before the tissue squash was sealed with histological wax. The slide was then transferred to the stage of the microspectrophotometer. All procedures were conducted under dim red light to minimise photopigment bleaching (Kodak Safelight No. 2)

6.2. MICROSPECTROPHOTOMETRY

MSP depends upon a comparison of the transmission of two light beams, the first of which is passed through the outer segment of the photoreceptor (sample beam), and the second of which is passed through a clear section of the tissue preparation (reference beam). The dual-beam MSP utilises a single beam of monochromatic light, which is 'chopped' to produce sequentially both the sample and reference

beam. These beams are passed through an objective condenser lens to match them to the size of the photoreceptor outer segment.

During a single recording scan, the MSP scans through the spectrum (between 350 – 750 nm), measuring the transmission at each wavelength, allowing the absorption to be calculated. Differences in the sample and recording beams occur due to the origin of the beams from different points on the lamp filament, the differences in the optical pathways the beams traverse, and the points at which the beams strike the photocathode. The reference beam provides feedback to the photomultiplier, to allow for any variations in beam quality produced by fluctuations in the light source. A baseline recording is also made by passing both beams through a region devoid of tissue to account for background absorbance.

Due to the small size of photoreceptor outer segments and the consequently low concentration of photopigment, MSP recordings necessitate a high light exposure to minimise noise fluctuations to obtain a good resolution. However, the more light the photoreceptor is exposed to, the more likely the photopigment contained therein will be bleached, thus distorting the absorbance spectrum measured. As such there exists a fine line between using a high enough light intensity to yield a high signal-to-noise ratio, yet low enough to minimise photopigment bleaching (Liebmann, 1972).

A) PINEAL MSP MEASUREMENTS

Microspectrophotometry measurements were made using a modified Liebmann dual-beam microspectrophotometer under computer control. A measuring beam of approximately 2 μm^2 cross-section was aligned to pass transversely through the photoreceptor outer segment using an infrared converter, whereas the reference beam passed through a clear region of the preparation. The MSP was programmed to step from 750 to 350 nm in 2 nm steps, taking recordings at even-numbered wavelengths, then returning from 351 to 749 nm, taking recordings at odd-numbered wavelengths. To minimise bleaching each cell was recorded only once, although two baseline measurements were made to limit the effects of background absorption. To verify the presence of a photolabile pigment the photoreceptor was then bleached by exposure to white light for three minutes, then re-measured to provide a bleach spectrum. Individual records were stored to disk for analysis.

Native pineal MSP measurements were made from 7 individual animals, amounting to a total of 79 individual cell recordings.

6.3. Analysis of Photopigment Spectra

Photoreceptor absorbance spectra were analysed using a standardised computer program that compared a visual pigment template of a specified wavelength to the MSP data. Firstly the two measurements from each cell (with different baselines) were averaged, and the absorbance values at pairs of adjacent wavelengths were averaged to obtain a mean curve (to account for any differences produced by bleaching between outward and return measurements).

The λ_{max} of a photoreceptor was estimated by two methods. Firstly, each of the 20 absorbance values on the long-wavelength limb of the curve was referred to a standard template curve to obtain an estimation of the λ_{max} (termed λ_{maxR}). A second estimation was obtained by comparing the 25 absorbance values at either side of the top of the absorbance curve, 50 points in total, to a standard template curve (termed λ_{maxT}).

λ_{maxR} is typically used for the determination of visual pigment λ_{max} as the long-wavelength, or right-hand, limb contains the steepest portion of the absorbance spectrum. As such, a small change in wavelength corresponds to a large change in absorbance. In addition, the short-wavelength limb is often subject to distortion by wavelength-dependent scattering and the presence of photoproducts (Bowmaker *et al.*, 1991).

The A_1 template used was either the Dartnall standard curve for rhodopsin, corrected for the presence of cone opsins (Wyszecki and Stiles, 1982) and the A_2 template used was that of (Munz and Schwanzara, 1967). The standard deviation of the data from the template was also calculated, along with the difference between λ_{maxR} and λ_{maxT}, to show discrepancies between these two estimates. Values for short-wavelength absorbance (%), mean absorbance of the top 7 points, the baseline absorbance and true absorbance of the record were also determined.

A) Selection Criteria

Selection criteria were used when analysing individual photoreceptor cells to eliminate records that were inaccurate due to operator error, background noise, optical problems or absorbance by other pigments present in the prep.

The standard selection criteria used in this study were as follows:

$\lambda_{max}R$ standard deviation	< 5 nm
λ_{maxT}-λ_{maxR}	< 5 nm
Short-wave absorbance	< 70%
True absorbance	> 0.01

B) Mixed Templates

By combining the A_1 and A_2 visual pigment templates, it was also possible to produce templates for pigments in which there is a mixture of A_1 and A_2 photopigments. Although this is based upon the assumption that an A_1/A_2 mixture behaves in a predictable mathematical manner, in practice this does appear to be the case. However, it should be noted that the visual pigment nomograms are based on purely empirical observations, and extreme care must be exercised when attempting to use these templates as a predictive tool to estimate the relative content of a rhodopsin/porphyropsin mixture.

6.4. Reconstitution with 9-*cis* Retinal

To overcome problems encountered with variability in pineal chromophore content, the pineal was bleached of its exogenous chromophore and reconstituted with a single artificial chromophore, 9-*cis* retinal, using the protocol described by Parry and Bowmaker (2000).

The cranial roof was removed and the pineal left attached to the skull with as much fat or connective tissue removed as possible. The skull was then placed in cyprinid saline, and exposed to bright white light for at least 5 minutes to bleach any photopigment present. The skull, with pineal still attached, was then placed in a phosphatidycholine vesicle preparation containing 9-*cis* retinal and incubated at 4°C for at minimum of 3 hours. Following this procedure, the skull was removed from

the vesicle preparation (in darkness) and the pineal dissected away from the skull and prepared for MSP as described above.

9-*cis* retinal reconstitution was conducted on 6 individual animals, amounting to a total of 73 individual cellular recordings. Records were analysed as described above.

6.5. Measurement of Skull Transmission

Transmission of the skull was measured in two animals by means of spectrophotometric measurements. The dorsal portion of the skull (to which the pineal end vesicle is attached) was removed as described above, and then placed into a 10mm black-walled cuvette filled with cyprinid saline. The tissue was positioned so as the recording beam passed through region of the skull just anterior to the cartilaginous rib, where the pineal end vesicle is located. All measurements were made using a Perkin Elmer Lambda 2 spectrophotometer.

6.6. Histology

Given the extensive studies of the goldfish pineal conducted by Takahashi (1969) and McNulty (1981), a further investigation of the histology of the pineal was regarded as unnecessary. However, photographs were taken from pineal MSP preps to illustrate the appearance of the pineal photoreceptors measured. For this procedure the coverslips were separated, and the tissue was lightly fixed in 1% glutaraldehyde before staining with a basic nissl stain.

6.7. Electron Microscopy

A single pineal organ was isolated and prepared for electron microscopy by Dr. P. Munroe in the E.M. unit of the Institute of Ophthalmology according to the following procedure. The isolated pineal end vesicle was first fixed overnight in 3% gluteraldehyde/1% paraformaldehyde. The tissue was then rinsed in 0.1 M Na^+ cacodylate before staining in 1% $OsO_{4(aq)}$, before the excess was rinsed away and the sample was dehydrated using a graded concentration series of ethanol. Ethanol was then removed by two washes with propylene oxide. The stained tissue was infiltrated with resin for 4-6 hours before embedding in araldite and cured overnight

at 60°C. Ultrathin (80nm) sections were cut using a diamond blade on a Leica Ultracut microtome. Sections were placed on copper grids (100 mesh), stained with lead citrate and viewed on a Jeol 1010 transmission electron microscope at 80 kV. Photographs were taken using Kodak electron microscope film 4489.

CHAPTER 7

MOLECULAR GENETICS

7.1. EXTRACTION OF PINEAL MRNA

Extraction of mRNA from pineal tissue was performed using the 'Quickprep Micro mRNA Purification Kit' (27-9255-01: Pharmacia P-L Biochemicals).

To provide sufficient mRNA for subsequent reactions it was necessary to use a minimum of 6 individual pineal organs. For most of the mRNA extractions conducted 8 animals were used. Fish were killed at ZT ~ 3 (just after subjective dawn), the time at which retinal rod opsin mRNA levels have been shown to be at their highest (Korenbrot and Fernald, 1989). The pineal organs were dissected away from the skull in the light using sterile forceps. A stereoscopic zoom microscope was used for this procedure, with light field background to make the end vesicle more visible against the translucent skull. As much excess tissue and fat were removed as was possible.

Following dissection the isolated pineals were placed into 0.4 ml of extraction buffer on ice. The tissue was then homogenised to a uniform suspension using a 1 ml syringe with a 21G then 25 G needle. 0.8 ml of elution buffer was then added, and the suspension briefly homogenised again. The extracted sample and a 1 ml aliquot of Oligo (dT)-cellulose were then centrifuged in parallel at full speed (13, 000 rpm) using a bench-top microcentrifuge (Sanyo). The supernatant of the extract was then added to the Oligo (dT)-cellulose pellet and resuspended by manual inversion to allow binding of the Oligo (dT)-cellulose to the tissue mRNA. After a brief centrifugation (10 seconds) the supernatant was discarded. The remaining pellet then underwent 5 washes with 1 ml aliquots of High-salt buffer, after each of which the solution was resuspended and then spun down briefly for 10 seconds. Following this two washes with Low-salt buffer were performed in a similar manner. Following the final wash, the pellet was resuspended in 0.3 ml of Low-salt buffer and transferred into a Microspin column placed in a clean microcentrifuge tube. The bound mRNA was washed 3 more times with 0.5 ml aliquots of Low-salt buffer, centrifuging between washes at full speed for five seconds. To extract the bound polyadenylated mRNA from the Microspin column, the column was first placed in a

clean microcentrifuge tube, then subjected to two applications of 0.2 ml of pre-warmed elution buffer, centrifuging again for five seconds between applications. The eluted mRNA was then placed on ice.

To precipitate the mRNA for cDNA synthesis 10 µl of Glycogen solution and 40 µl of K Acetate solution were added to the 400 µl of eluted mRNA. 1 ml of 95% ethanol (chilled to –20°C) was then added to the sample, which was then placed at –80°C for a minimum of 30 minutes. The precipitated mRNA was pelleted by centrifugation at 13,000 rpm for 5 minutes at 4°C. The supernatant was decanted from the pelleted mRNA, and excess liquid was removed by inverting the tube over a clean paper towel. The resulting mRNA pellet was finally resuspended in 10 µl of elution buffer or DEPC treated water, and stored at –20°C.

7.2. SYNTHESIS OF CDNA

To synthesise first strand cDNA from poly (A)+ RNA the 3'/5' RACE (Rapid Amplification of cDNA Ends) kit (1734792: Boehringer Mannheim) was used. Table 1 shows the components of this cDNA synthesis reaction, which were pipetted into a sterile microcentrifuge tube on ice.

TABLE 7.1. CDNA SYNTHESIS REACTION

Component	Amount (µl)
cDNA synthesis buffer	4
dNTP mix (10 mM each)	2
Oligo-dT anchor primer	1
RNasin (40 u/µl)	0.5
Poly $(A)^+$ mRNA	5
AMV Reverse Transcriptase (20 u/µl)	1
DEPC-treated water	6.5
TOTAL	20 µl

This reaction mixture was briefly spun down and then incubated at 55°C for 60 minutes. A final step of 65°C for 10 minutes was then applied to deactivate the

reverse transcriptase. The newly synthesised cDNA was then briefly spun down again and stored at –20°C.

A) β-ACTIN CONTROL

To test that viable cDNA was synthesised a control PCR (see below) was conducted using primers designed to the goldfish β-actin gene (accession AB039726). β-actin is a ubiquitous cytoskeletal protein, and presence in the cDNA indicated that cDNA had been successfully synthesised.

7.3. PCR AMPLIFICATION

The polymerase chain reaction (PCR) is a standard tool of molecular biology, allowing the amplification of DNA sequences from as little as one starting template molecule. PCR was utilised to screen pineal cDNA for opsins, based upon the published sequences of the goldfish retinal opsins (Johnson *et al.*, 1993). An assortment of primers was used in an attempt to amplify fragments the various retinal opsins.

The PCR conditions shown in table 7.2 (overleaf) were used for most reactions, due to the exact sequence being unknown. These reaction conditions were refined once a sequence had been isolated (see 'Optimisation' below).

A master-mix preparation containing all the reagents for several reactions, always including a negative control (containing no DNA), to ensure that all reactions contained equal reagent concentrations. The PCRs were performed using either Hybaid or Techne thermal cyclers. Cycling conditions are shown in table 7.3.

For the majority of PCR reactions used BioTaq DNA Polymerase (Bioline). However, to increase PCR specificity and fidelity, HotStarTaq (Qiagen) was also used in some reactions.

When the target sequence is not known, amplification of primer mismatches is actually desired. As such higher concentrations of dNTPs, primers and enzyme are useful.

PCR PROTOCOL

TABLE 7.2. DEGENERATE PCR REACTION

Component	Amount (µl)	Final Conc. (µM)
NH_4 Reaction Buffer (x 10)	5	x 1
$MgCl_2$ (50 mM)	3	3 mM
dNTP mix (4 mM each)	2.5	200 µM each
Forward primer (25 µM)	1	0.5 µM (25 pmol)
Reverse primer (25 µM)	1	0.5 µM (25 pmol)
BioTaq DNA Polymerase (5u/µl)	0.5	2.5 u
SDW (Sterile Distilled Water)	36	-
Template DNA	1	-
TOTAL	50 µl	

TABLE 7.3. CYCLING CONDITIONS

	Temperature (°C)	Duration
Initial denaturation	96	1 min
35 Cycles of PCR: Denaturation	96	30 s
Primer annealing	58*	30 s
Primer extension	72	1 min
Final Extension	72	3 mins
Terminate	4	

** Annealing temperature (T_m) was varied according to the primers used and the specificity required (see below).*

A) OPTIMISATION

Many factors can influence the yield, specificity and fidelity of PCR (Innis and Gelfand, 1990). As such it was often necessary to vary reagent concentrations and cycling conditions to provide the optimum PCR performance. The most common optimisation is the modification of annealing temperature (T_m), which was varied according to the primers used and the specificity desired.

Once pineal opsin sequences had been identified and specific primers had been designed to these sequences, the PCR protocol was modified to increase specificity and reduce mis-priming at non-target sites (which was previously desired). An example of a sequence-specific PCR protocol is shown in table 7.4.

Magnesium concentration can affect primer annealing, strand dissociation (of both template and product), primer-dimer formation and enzyme activity and fidelity. A standard concentration of 3 mM $MgCl_2$ was initially used, but in reactions demonstrating poor amplification a magnesium titration from 0.5 mM to 4 mM (in 0.5 mM increments) was conducted.

High concentrations of enzyme, dNTPs and primers can all lead to extension of non-target sequences, and consequently these parameters were modified according to the precise nature of the PCR.

TABLE 7.4. SEQUENCE-SPECIFIC PCR PROTOCOL

Component	Amount (µl)	Final Conc. (µM)
NH_4 Reaction Buffer (x 10)	5	x 1
$MgCl_2$ (50 mM)	3	3 mM
dNTP mix (4 mM each)	1	80 µM each
Forward primer (25 µM)	0.4	0.2 µM (10 pmol)
Reverse primer (25 µM)	0.4	0.2 µM (10 pmol)
BioTaq DNA Polymerase (5u/µl)	0.2	1 u
SDW (Sterile Distilled Water)	39	-
Template DNA	1	-
TOTAL	50 µl	

For specific PCR of a known target sequence a lower concentration of dNTPs, primer and DNA polymerase help prevent mispriming and amplification of non-specific background products.

B) PRIMER DESIGN

The primers used for PCR were designed along certain parameters to provide optimum performance. The melting temperature (T_m) of the primers used was roughly determined by the simple formula: 2°C for each A or T base, and 4°C for each G or C base. The primers used in this study were designed by Dr. K.S. Dulai (fish rod opsin degenerate primers) and S.N. Peirson, and synthesised by Sigma-Genosys Ltd.

- A primer length of 18-30 bases was used, a sequence of 18 bases representing a unique DNA sequence amongst $4^{18} = 7 \times 10^{10}$ nucleotides, and as such should hybridise to only one position in most eukaryotic genomes (which consist of 10^9 or 10^{10} bases). Shorter primer sequences increase the probability of non-specific amplification.
- The primer sequence should contain between 40-60% GC content. A lower GC content may reduce strength of annealing, whereas a higher content will increase the strength of non-specific annealing.
- The 3'-terminal sequence of the primer is critical for PCR specificity and sensitivity. It is from this end that the primer is extended, so binding is critical. Runs of three or more G or C bases may stabilise non-specific binding, whereas a T at the 3' end was avoided as this base is more prone to mis-priming than other nucleotides.
- Primer sequences were checked for self-complementarity to avoid secondary structure formation, such as hairpin loops.
- Primer pairs were also checked for complementarity to avoid primer-dimer formation. Creation and subsequent amplification of these primer-dimers reduces the availability of the primer for template binding.
- When degenerate primers were used, degeneracy at the 3' end was avoided. Mis-matching in this region greatly reduces PCR efficiency as mentioned above.

- Degenerate primers were also designed so that the last base of the 3' end terminated on the first base of a codon. As the second, and particularly the third base of a codon are more likely to vary between species/individuals ('silent' substitutions), termination on the first base allowed for minor sequence differences without affecting PCR performance.

These primer design guidelines are based on those suggested by Innis and Gelfand, 1990.

PRIMER LIST

TABLE 7.5. DEGENERATE TELEOST ROD OPSIN PRIMERS

Primer	Sequence	F/R	GC%	T_m (°C)*	Target Seq.
FRHO 0$^+$	WWW WWA TGA ACG GVA CRG AGG	Fwd	38	58	Fish rod opsin 0 – 16
FRHO B$^+$	CTT CCC YRT CAA CTT CCT CAC	Fwd	48	62	Fish rod opsin 153 – 173
FRHO B$^-$	TGC TTG TTC AWG CAG ATG TAG	Rev	43	60	Fish rod opsin 935 – 915
FRHO J$^-$	AGT GVM MGA NRA ACA TGT AGA TGA C	Rev	32	66	Fish rod opsin 634 – 610
RHOE +	CCC TAY GCS RGY GYR GCC TGG T	Fwd	55	68	Fish rod opsin 753 – 773
RHOF+	CAG GAG TCN GAG ACY ACC CAG AG	Fwd	57	72	Fish rod opsin 722 – 744

* Annealing temperature based upon GC x 4, + AT x 2 (°C), or on T_m determined by manufacturer when available.

TABLE 7.6. GOLDFISH RETINAL CONE OPSIN PRIMERS

Primer	Sequence	F/R	GC%	T_m (°C)*	Target Seq.
GF UV+	CAG CCN CTC AAC TAC ATC YTG GTS AA	Fwd	42	74	GF UVS 250-275
GF UV-	CCN ACC ATC ACM ACM ACC AT	Red	45	58	GF UVS 818-799
GF BL+	AAR CTC MGW TCY CAC CTT AAY TA	Fwd	30	60	GF SWS 211-233
GF BL-	CAC CAT CAC CAC CAC CAT CTT	Rev	52	64	GF SWS 785-765
GF GR+	ATC TMG CTG AAC CAT GGC AG	Fwd	50	60	GF MWS 95-104
GF GR-	GCT GAG TCC TGC TGT TGA GC	Rev	60	64	GF MWS 729-710
GF RD+	GCA ATC TTC GCA GCT AGG C	Fwd	58	65	GF LWS 103-121
GF RD-	TGT CCG GGG TCC TCA CTT CCG	Rev	67	75	GF LWS 719-699

* Annealing temperature based upon GC x 4, + AT x 2 (°C), or on T_m determined by manufacturer when available.

TABLE 7.7. EXO-ROD OPSIN SPECIFIC PRIMERS

Primer	Sequence	F/R	GC%	T_m (°C)*	Target Seq.
EXO 38+	AGC ACA AGA AGC TGC GCA CG	Fwd	60	71	GF exo-rod
EXO 70+	ATC CTG CTC AAT CTG GCC GTG G	Fwd	59	73	GF exo-rod
EXO 399-	CAC TGC ATT CCC TCT GGA ATA TAC	Rev	46	64	GF exo-rod
EXO 444-	TTG TTG ATC TCA GGC TTG GG	Rev	50	65	GF exo-rod
EXO 710-	ATC ACT ATA GGA CCA AAC TCC GC	Rev	48	64	GF exo-rod
EXO 828-	CGG GTT CTT TCC ACA GCG G	Rev	63	70	GF exo-rod
EXO 226+	TCT CTG GTG GTC TTG GCT G	Fwd	58	64	GF exo-rod
EXO 463+	TAC ATG TTC ATC CTG CAC TTC TC	Fwd	43	63	GF exo-rod
EXO 124-	AGG GCG GTG TAG AGG GTG AC	Fwd	65	68	GF exo-rod Walking PCR
EXO 164-	TCG ATG TTG CAG CCG GTG AC	Rev	60	72	GF exo-rod Walking PCR
Exostart	**GAA TTC** ATG AAT GGA ACG GAA GGT CCG	Fwd	52	64	GF exo-rod Expression
ExostopA	**GTC GAC** GGC GGG AGC CAC CTG ACT	Rev	67	60	GF exo-rod Expression

* Annealing temperature based upon GC x 4, + AT x 2 (°C), or on T_m determined by manufacturer when available.

Bases noted in blue are *SalI* restriction digest sites, whereas those noted in red are *EcoRI* restriction digest sites.

C) VISUALISATION OF PCR PRODUCT

Following thermal cycling, PCR products were visualised by gel electrophoresis. 8 µl of PCR product, plus 2 µl of Blue/Orange loading dye (diluted 1:10) were loaded onto a 1% agarose gel (containing 1/100,000 parts ethidium bromide) and electrophoresed in 1 x TAE buffer (see Appendix B) at 90-100 V. A 1-kilobase ladder was loaded alongside these samples to provide a marker to size the amplified fragments (markers at 250, 500 and 750 bp, then 1 kb, 1.5 kb, 2 kb etc;). Gels were visualised using a UV transilluminator, and pictures taken for future records.

D) BAND ELUTION

Following visualisation, any bands that appeared to be close to the expected size were excised using a sterile scalpel blade. Gels were viewed on the UV transilluminator for as short a time as possible, to prevent damage to the double-stranded DNA. The excised band was placed in a Wizard microspin column (Promega), which was placed in a sterile 1.5 ml microcentrifuge tube, and centrifuged at 13,000 rpm for 2 minutes using a benchtop microcentrifuge (Sanyo). The eluate was then transferred to a clean microcentrifuge tube for use in either ligation or direct sequencing reactions.

E) QUANTIFICATION OF DNA

As a quick means of quantifying the concentration of DNA in an eluted band, a sample of template was run out on a 1% agarose gel (usually around 5 µl). 5 µl of Hyperladder I (Bioline) was used as a molecular weight marker, allowing a quick visual determination of DNA concentration.

F) CONTAMINATION CONTROL

The very sensitivity that makes the PCR such a valuable tool is also potentially a drawback. Contamination of the PCR reaction by exogenous DNA may lead to false positives, a major problem when working on very small tissue samples, which is further compounded when working in a busy lab. To prevent such contamination, the following protocols (suggested by Kwok and Higuchi, 1989 and Victor *et al.*, 1993) were strictly adhered to.

- To prevent carry-over of amplicons (amplified sequence fragments) all PCR products were physically isolated from the PCR set-up area. All samples were prepared in a separate room not used for other DNA-based procedures.
- A separate set of pipettes was used for PCR set-up, using disposable filter tips.
- Whenever possible, all reagents were divided into aliquots, to minimise the amount of repeated pipetting.
- Disposable gloves were used at all times, and were changed regularly, especially after handling any samples containing DNA, when re-entering PCR area or when spillages occurred.
- DNA was always added to the amplification vessel last, to prevent the inadvertent transfer of DNA to other reaction vessels.
- Samples were spun down to the bottom of tubes prior to opening to prevent splashes and glove contamination.
- When positive controls (based on gDNA) were used, the DNA was substantially diluted to reduce the chance of carry-over.
- Negative controls (containing no DNA) were *always* included, and if indicating contamination the PCR was repeated.

7.4. CLONING PROTOCOL

Fragments of DNA amplified by PCR were typically cloned to provide a purified stock for further manipulations.

A) LIGATION

The ligation and subsequent cloning of PCR-amplified fragments was conducted using the pGEM®-T Easy Vector System (Promega). The reaction shown in table 7.8 was set up and incubated for 1 hour at room temperature.

Optimisation of the ligation reaction is often necessary, with the optimum template:vector ratio varying considerably. If initial ligations were unsuccessful, ratio optimisation was conducted, with the optimum template:vector ratio usually being within the range of 3:1 to 1:3 (8-75 ng of DNA). Obviously the optimisation of this ratio is limited by the DNA concentration of the PCR fragment.

TABLE 7.8. LIGATION OF PCR FRAGMENT

Component	Amount (µl)
2 x T4 DNA ligase buffer	5
pGEM®-T Easy Vector (25 ng)	0.5
T4 DNA Ligase (3u/µl)	0.5
PCR-amplified fragment	4
TOTAL	10 µl

B) TRANSFORMATION

Following ligation JM109 competent cells were then used to express the pGEM®-T vector containing the desired gene fragment. The JM109 cells were thawed on ice for 5 minutes, before adding 25 µl of cells to 2 µl of the above ligation mix, flicking to mix and then leaving on ice for 20 minutes. Following this incubation, the cells were heat-shocked at 42°C for 45-50 seconds then placed immediately onto ice for 2-3 minutes. 200 µl of SOC medium was then added to each transformation reaction, before incubating at 37°C in a rotary shaker incubator for 1-1.5 hours.

Prior to the transformation, LB agar plates were prepared containing the antibiotic Ampicillin (50mg/ml) as well as 100 µl IPTG (24mg/ml) and 50 µl X-Gal (10mg/ml) for blue/white screening. 100 µl of the transformation mix was spread evenly across each plate. These plates were then incubated inverted overnight at 37°C.

C) COLONY REPLATING AND SCREENING

Following incubation, the plates were kept at 4°C. Successfully transformed colonies were then identified by their white coloration. To ensure the correct-sized insert was present, these white colonies were screened. A sterile pipette tip was used to remove a colony and then this colony was dabbed into either an eppendorf tube or multi-well plate (depending on the number of colonies to be screened). The pipette tip was also dabbed onto another Ampicillin treated plate and incubated again overnight to provide a permanent stock for further procedures.

A standard PCR was then conducted using pTag primers (3.2 pmol), priming to a region adjacent to the insert site (approximately 100 bp to either side, 5' and 3'). When run out on an agarose gel non-recombinants gave a band of around 200bp, whereas successful recombinants appeared as bands of the insert size plus 200bp.

D) MINIPREP PLASMID PURIFICATION

A permanent stock of the plasmid DNA was made in many cases where further analysis was to be conducted (using QIAprep Miniprep kit). Successful recombinants were added to 5 ml of LB broth (containing 50 μg/ml of ampicillin) and incubated at 37°C in a rotary shaker overnight. Following this incubation the culture was spun down at 13, 000 rpm (refrigerated centrifuge) for 10 minutes. The pelleted cells were then resuspended in 250 μl of Buffer P1, and transferred to a microcentrifuge tube. 250 μl of Buffer P2 was then added and the tube gently inverted 4-6 times to mix. This step produces cell lysis, and was not allowed to proceed for more than 5 minutes before 350 μl of Buffer N3 was added, and the tube again inverted to mix the solution. The samples were then centrifuged for 10 minutes, and the supernatant applied to a QIAprep spin column in a 2 ml collection tube. The supernatant was then spun through the column (30-60 seconds) and the flow-through discarded. A 0.5 ml aliquot of Buffer PB was then applied to the spin column and spun through to wash the bound vector, and remove trace nuclease activity. Another wash using 0.75 ml of Buffer PE was applied to the spin column, and the flow-through again discarded, before centrifuging for 1 minute to remove all residual wash buffer. The spin column was then placed into a clean 1.5 ml microcentrifuge tube, before the DNA was finally eluted by addition of 50 μl of Buffer EB (or SDW) to the centre of the spin column, leaving for 1 minute, then centrifuging for 1 minute.

7.5. SEQUENCING

Sequencing reactions were conducted using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) with AmpliTaq® DNA Polymerase, FS. AmpliTaq® is a variant of Taq DNA polymerase, with point mutations resulting in less discrimination against nucleotides, and elimination of 5'→3' nuclease activity. The BigDye™ terminator ready reaction mix contains Amplitaq® and also BigDye™ terminators labelled with novel, high-sensitivity dyes. These contain a fluorescein donor dye linked to a dichlorohodamine acceptor dye. The donor dye has an excitation spectra corresponding to the argon ion laser of the ABI sequencer. However, the 4-dideoxynucleotide terminators are each labelled with a slightly differing acceptor dye, with corresponding different emission spectra, allowing differentiation between nucleotides. The terminators are labelled as follows:

Terminator	**Acceptor Dye**
A-Dye	dichloro [R6G]
G-Dye	dichloro [R110]
C-Dye	dichloro [ROX]
T-Dye	dichloro [TAMRA]

The BigDye™ ready reaction mix also contains deoxynucleoside triphosphates (dATP, dCTP, dITP, dUTP), $MgCl_2$ and Tris-HCl buffer (pH 9.0).

A) CYCLE SEQUENCING

This protocol was used to prepare a successfully cloned fragment for sequencing.

The following were added to a 0.2 µl microcentrifuge tube:

TABLE 7.9. BIGDYE™ LABELLING REACTION

Component	Amount
ABI BigDye terminator ready reaction mix	4 µl
PTag primer	1.6 pmol
Cloned DNA template	2-3 µg
SDW	To 10 µl

The reaction was then mixed, and the following cycling conditions used (using a Perkin Elmer Cetus systems 9600 or 2400 thermal cycler): 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes, with a final holding step of 4°C.

B) DIRECT SEQUENCING

In cases where a single clean band was obtained by PCR, the band was eluted was described above and directly sequenced without cloning. In this case, the cycle sequencing reaction was set up as described for cloned templates, typically using 4 µl of BigDye, 0.5 µl of primer and 5.5 µl of eluted PCR product as the template.

C) PURIFICATION OF EXTENSION PRODUCTS

Following the labelling reaction, the labelled template was cleaned prior to sequencing. Firstly, the labelling mix was transferred to a 0.5 ml eppendorf, 30 µl of SDW and 60 µl of 100% ethanol were then added (for a final ethanol content of 60 ± 3 % ethanol), and this mix was incubated at room temperature for 15 minutes. Following this incubation, a 20 minute centrifugation (13, 000 rpm) was conducted using a benchtop microcentrifuge, following which the supernatant was removed by aspiration, leaving a pellet of labelled DNA. 200 µl of 70% ethanol was then added to this pellet, which was again centrifuged (13,000 rpm) for 10 minutes. Finally, all the ethanol was removed by aspiration, and the tubes of labelled product were then left to dry on the benchtop for 1-2 hours.

D) PREPARATION OF SEQUENCING GELS

Automated sequencing was conducted using a 373a DNA Sequencer (ABI) using 6% acrylamide gels. The glass gel plates were thoroughly cleaned with tap water then rinsed with de-ionised water prior to assembly with 1mm spacers. The gel mix consisted of 40 ml of Sequagel 6^{TM} sequencing gel solution with 6 ml of Sequagel^{TM} complete buffer reagent. 0.04 g of APS was then added and dissolved to induce gel polymerisation. The gel mixture was then injected between the glass plates using a 50 ml syringe, with tapping of the plates to encourage gel movement if slow, and a flat comb was placed at the gel front. The gel was left to polymerise for at least one hour before the plates were washed again with tap- then de-ionised water, and assembled into the sequencer for a pre-scan. The rest of the components of the sequencer were then assembled according to the manufacturer's specifications, and 1 x TBE buffer was added to both top and bottom tanks. Following a pre-run of 15 – 60 minutes, a 48 or 64 well comb was inserted into the gel, the wells were then washed out to remove any traces of urea and then 1-1.5 µl of labelled sequencing sample was loaded per lane. The 373a DNA sequencer was run in accordance with the manufacturers instructions, typically electrophoresing for 12 hours and yielding roughly 300-700 bp read, depending on sample purity and structure.

E) ANALYSIS OF SEQUENCING DATA

Data from the sequencer was stored in raw form as an electropherogram, showing the dye emissions as peaks, and then in a translated form as the actual sequence read by the operating program. Electropherograms were based upon automatic tracking of the sample, but it was often necessary to track each lane by eye then re-extract the data to optimise the sequencing read. When clean unambiguous sequence was produced base-calling was usually accurate allowing analysis of the translated data. However, in some cases, particularly when faced with difficult templates, when reads extended beyond 400 bases (when longer extension products are scarce) or after runs of identical bases, analysis of the electropherogram to confirm the translated sequence was necessary.

7.6. RAPID AMPLIFICATION OF CDNA ENDS (RACE)

To obtain the 3' end of the opsin coding sequence the 5'/3' RACE kit (1734792: Boehringer Mannheim) was used. The first step of 3' RACE is the synthesis of complementary DNA as described previously. Then a PCR anchor primer that binds to the 3' region of the cDNA, and a gene-specific primer can be utilised to selectively amplify the 3' end of the cDNA.

TABLE 7.10. 3' RACE PROTOCOL

Component	Amount (μl)
Reaction Buffer x 10	5
CDNA	1
PCR Anchor Primer (12.5 μM)	1
Gene-specific Primer (12.5 μM)	1
dNTP mix (4 mM)	2.5
Taq DNA Polymerase	0.5
SDW	39
TOTAL	50 μl

7.7. WALKING PCR

To obtain the 5' region of the opsin coding sequence Walking PCR (also known as 'Unpredictably-primed PCR') was used (Dominguez and Lopez-Larrea, 1994). Walking PCR utilises two universal sense primers, which will prime to recurring sequences within genomic DNA. By use of two gene-specific α-sense primers (referred to as 'inner' and 'outer'), the PCR can then selectively amplify unknown upstream regions, allowing 'walks' along the genomic DNA into the unknown 5' region. Walking PCR consists of two rounds of PCR as described below.

A) FIRST ROUND

The reaction mix shown in table 7.11 was prepared. The reaction mix was then split into two 23 µl aliquots, and placed in a thermal cycler with the following cycling conditions: 1 min initial denaturation at 94°C (pause), 30s at 80°C, 2 min at 15°C, 10 min at 25°C, 1 min at 72°C, 1 min at 90°C (pause), followed by 35 cycles of 10s at 94°C (denaturation), 45s at 65°C (annealing) and 1 min at 72°C (extension). Final extension for 2 min at 72°C.

TABLE 7.11. WALKING PCR – FIRST ROUND

Component	Amount (µl)
Template (gDNA)	2
PCR Reaction Buffer x10	5
Primer UNI 33 (12.5 µM)	2
dNTP mix (5 mM each)	2
$MgCl_2$ (25 mM)	3
SDW	32
TOTAL	46 µl

At the first pause 1 µl of Taq (diluted 1/10) was added to each reaction. At the second pause 1 µl of outer gene-specific primer was added to each reaction. A sample of the reaction mix was run out on a 1% agarose gel. Usually a smear was apparent, but sometimes a clear band was present at this stage.

B) SECOND ROUND

1 µl of the first round reaction was diluted 1:1000 to use as a template for a second round. The second round reaction mix is shown in table 7.12.

TABLE 7.12. WALKING PCR – SECOND ROUND

Component	Amount (µl)
Template DNA	2
PCR Reaction Buffer x10	5
Primer UNI 17 (12.5 µM)	2
Inner gene-specific primer (12.5 µM)	2
dNTP mix (5 mM each)	2
$MgCl_2$ (25 mM)	3
SDW	32
TOTAL	48 µl

Again the reaction mix was split into two aliquots of 24 µl each. The reactions were then placed in a thermal cycler under the following cycling conditions: initial denaturation at 94°C for 1 min (hot-start), followed by 35 cycles of 5s at 94°C (denaturation), 5s at 58°C (annealing) and 30s at 72°C (extension). Final extension for 5 mins at 72°C.

After the hot-start 1 µl of Taq (diluted 1/10) was added to each reaction.

7.8. RESTRICTION DIGESTION

Restriction digestion was used at various experimental stages – to check for correct-sized inserts when cloning, to test for the presence of sequence-specific restriction sites and to excise labelled PCR products for expression (as described below). Whatever the process, the same basic restriction digestion protocol was used, and the sample incubated at 37°C for 1-2 hours.

TABLE 7.13. RESTRICTION DIGESTION REACTION

Component	Amount (µl)
One-Phor-All Buffer x 5	2
Template DNA	2.5
Restriction enzyme (10 u/µl)	0.5
SDW	5
TOTAL	10 µl

The sample was then visualised on an agarose gel as described previously.

RESULTS AND DISCUSSION

CHAPTER 8

HISTOLOGY AND MICROSPECTROPHOTOMETRY

8.1. HISTOLOGY

Given the comprehensive histological studies of the goldfish pineal conducted by McNulty (1981) and Takahashi (1969), any further investigations are largely superfluous. As such the following section provides just a few relevant observations regarding the pineal photoreceptors of the goldfish, and especially the photopigment-containing outer segments.

A) GOLDFISH PINEAL MSP TISSUE PREPARATION

Although of poor histological quality figure 8.1 demonstrates the basic morphology of the goldfish pineal tissue, illustrating the characteristics of pineal photoreceptors as seen under the MSP. Despite many similarities to retinal photoreceptors, the morphology of pineal photoreceptor outer segments is quite different, appearing more dome or cup-shaped (see Ekstrom and Meissl, 1997 and Kusmic and Gualtieri, 2000).

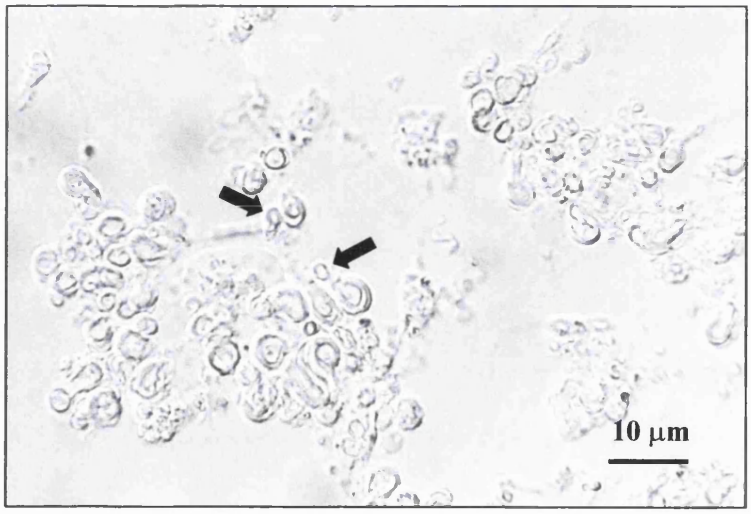


FIGURE 8.1. Goldfish pineal preparation, showing pineal photoreceptor outer segments as recorded by MSP. Photoreceptor outer segments indicated by arrows.

B) ELECTRON MICROSCOPY

Electron micrographs of pineal tissue are shown in figures 8.2. Figure 8.2A shows pineal photoreceptor outer segments (labelled with black arrows), demonstrating the membrane whorls commonly found projecting into the pineal lumen (compare with McNulty, 1981). The outer segments are formed from invaginations of the cell membrane, and are comparable to retinal cones. However, the dome- or cup-shaped formation of these outer segments are particularly characteristic of pineal photoreceptors (Ekstrom and Meissl, 1997).

Figure 8.2B shows a presynaptic body or "ribbon" synapse. Ribbon synapses are characteristic of cells which release neurotransmitter tonically, providing a binding site for synaptic vesicles (involving cytoskeletal proteins) allowing an uninterrupted flow of neurotransmitter into the synapse. Ribbon synapses are commonly found in retinal photoreceptors and bipolar cells which tonically release neurotransmitter. As such the presence of ribbon synapses in pineal photoreceptors suggests that these cells function in a similar manner to retinal photoreceptors, releasing glutamate at tonically active synapses (Wagner, 1997). The presence of such synapses in the goldfish pineal has previously been reported by McNulty, and concurs with the data surrounding the mode of action of pineal photoreceptors in this species (Meissl *et al.*, 1986).

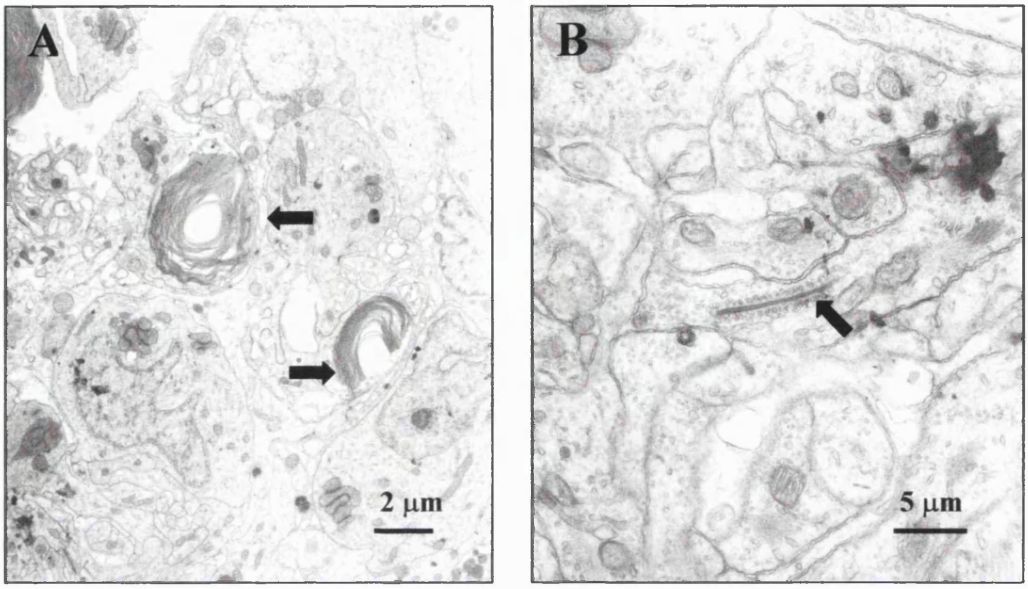


FIGURE 8.2. Electron micrographs of goldfish pineal end-vesicle, sectioned at 1 μm. **A)** Pineal photoreceptor outer segments, indicated by arrows, demonstrating membrane lamellae forming a typical dome- or cup-shape. **B)** Pineal photoreceptor synaptic terminal. Arrow indicates 'ribbon' synapse, characteristically found in retinal photoreceptors, and indicative of tonic neurotransmitter release.

8.2. MSP OF GOLDFISH PINEAL PHOTORECEPTORS

A) ABSORBANCE SPECTRUM

Microspectrophotometry was conducted on a total of 79 photoreceptor cells from 7 individual goldfish. Of these measurements 54 cells met the selection criteria (see section 6.3A for rationale and details of criteria). The absorbance spectra of these 54 cells were averaged to produce a mean absorbance spectrum, and a corresponding bleach spectrum, which is shown in figure 8.3A.

The analysis of this native data was based upon the λ_{max} derived from the top of the curve (λ_{maxT}), as opposed to fitting the nomogram to the right hand limb (λ_{maxR}), as is usually conducted. This procedure was applied due to the unknown chromophore content of the photoreceptors, and the fact that variability in A_1/A_2 content was apparent between individual cells. Fitting a nomogram to the right-hand limb would bias the λ_{max} to longer wavelengths, whereas the top of the curve remains more consistent, even with variations in nomogram composition, and is certainly a more accurate guide to the true λ_{max} of the photopigment.

The template most closely describing the data is a nomogram of 50% A_1/50% A_2 of λ_{max} 509.97 nm (SD 1.43). This suggests that the chromophore content of the pineal photoreceptors is not predominantly A_2 as is the case in the goldfish retina.

B) DIFFERENCE SPECTRUM

Following subtraction of the bleach recordings from the absorbance spectra the absorbance of the photopigment can be determined correcting for the presence of any other absorbing pigments which may be present. However, due to the accumulation of free-retinal photoproduct produced by the bleaching process, the bleach record often demonstrates an increased absorbance in the UV region, around 380 nm, corresponding to the main absorbance band of retinal (Knowles and Dartnall, 1977). As such the difference spectrum may often exhibit a slight long-wave shift.

As in the case of the absorbance spectrum, due to the unknown and potentially variable chromophore content of pineal photoreceptors the top of the curve was used to determine λ_{max}. The difference spectrum of the goldfish pineal photoreceptors is most closely fitted by a 50% A_1/ 50% A_2 template of λ_{max} 515.28 nm (SD 4.62), as

shown in figure 8.3B. In this case the long-wave shift produced by bleached photoproduct is 5 nm. This discrepancy is not surprising when the bleach spectrum in figure 8.1a is considered, as a quite noticeable rise in the spectrum is apparent, up to an absorbance peak of around 0.075 above the baseline at 380 nm. This rise most likely corresponds to both retinal photoproduct and an increased scattering of shorter wavelengths. In this respect the difference spectrum is in this case a less reliable indication of the true λ_{max} of the photopigment.

	λ_{maxR}	SD	λ_{maxT}	SD	O.D.	Template
Absorbance spectrum	510.60	1.06	509.97	1.43	0.0162	50% A_1/A_2
Difference spectrum	512.97	1.15	515.28	4.62	0.0135	50% A_1/A_2

TABLE 8.1. Summary of native goldfish pineal MSP data. Results based on averaged spectra from 54 cells.

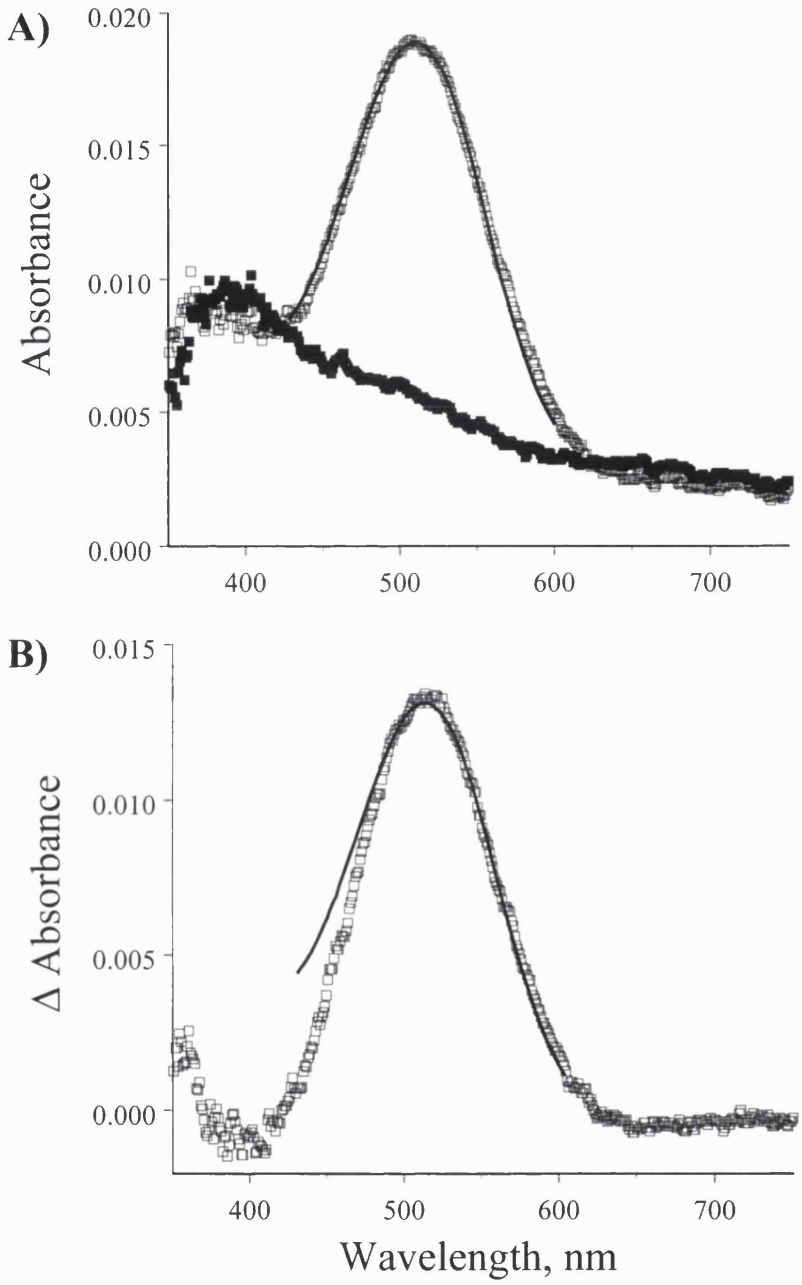


FIGURE 8.3. Absorbance spectra based on averaged data from 54 pineal photoreceptors from the goldfish. A) Absorbance spectrum fitted with 50% A_1/50% A_2 nomogram of λ_{max} 510 nm. Filled symbols show bleach recording following a 3 minute exposure to white light. B) Difference spectrum fitted with 50% A_1/50% A_2 nomogram of λ_{max} 515 nm. Poor fit on the short-wave limb is due to accumulation of photoproduct (see text).

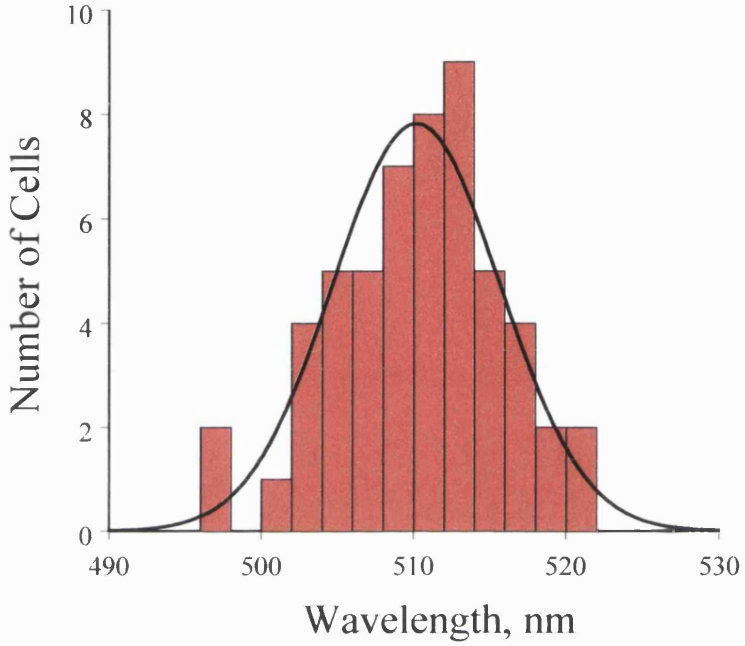


FIGURE 8.4. Histogram demonstrating distribution of λ_{max} values from individual photoreceptor cells in the goldfish pineal. Data from 54 individual cells sorted into 2 nm bins based upon λ_{maxT}. Solid line indicates a normal distribution.

C) VARIABILITY BETWEEN INDIVIDUAL PHOTORECEPTORS

To determine whether the goldfish pineal organ contained just a single photoreceptor population, or distinct spectral subtypes, the λ_{max} of the individual records were plotted as a histogram. The records were fitted into 2 nm intervals, and a normal distribution was plotted as a point of comparison.

Each record was analysed based upon λ_{maxT}, determined by fitting a template to the top of the curve to reduce errors introduced through chromophore variability (see section 8.2A). Individual cell λ_{max} values ranged from 496.1 nm to 520.4 nm. The mean λ_{max} calculated from individual records was 510.2 nm with a standard deviation of 5.5 nm (see table 8.3).

As can be seen from figure 8.4, only a single photoreceptor population is apparent from the native goldfish pineal data, which corresponds closely with a normal distribution, although the peak is slightly skewed to longer wavelengths. Based upon this data there is therefore no evidence for multiple, spectrally distinct photoreceptor populations in the goldfish pineal, unless the λ_{max} values of these populations are extremely similar and thus indistinguishable due to chromophore variations.

8.3. RECONSTITUTION OF PINEAL WITH 9-*CIS* RETINAL

To eliminate variations in chromophore content, pineal photoreceptors were bleached of their endogenous retinal content, and reconstituted with a single artificial retinal chromophore, 9-*cis* retinal. If pineal photoreceptors in the goldfish contain the retinal rod opsin, the λ_{max} of these reconstituted photoreceptors should match the value of 489 nm reported for the goldfish rod isorhodopsin by Parry and Bowmaker (2000).

A) ABSORBANCE SPECTRUM

Following measurement of 73 reconstituted pineal photoreceptors from six individual animals, 44 cells fell within the selection criteria. These cells formed a single photoreceptor population, most closely fitting an A_1 template of λ_{max} 484.22 nm (SD 0.38) as shown in figure 8.5A overleaf. The close fit of the A_1 template to

this data is suggestive of a single chromophore. As such, the right-hand limb was used to determine λ_{max}, although the difference between λ_{maxR} and λ_{maxT} is only 1 nm (see table 8.2 below).

B) DIFFERENCE SPECTRUM

The difference spectrum of the reconstituted photoreceptors is shown in figure 8.5B, and most closely fitted an A_1 template of λ_{max} 488.14 nm (SD 0.72). The long-wavelength shift demonstrated by this difference spectrum is probably due to the presence of both photoproducts, as well as free 9-*cis* retinal from the reconstitution process. Furthermore, it was noted that 9-*cis* retinal does not appear to bleach with the same efficiency as the endogenous retinoids, and following a 3 minute exposure to white light a visible peak is still evident in the bleach record (see figure 8.5A). Again these factors make the difference spectrum a less reliable indication of the λ_{max} of the pineal photopigment.

	λ_{maxR}	SD	λ_{maxT}	SD	O.D.	Template
Absorbance spectrum	484.22	0.38	483.26	1.43	0.0228	A_1
Difference spectrum	488.14*	0.72	490.59*	4.06	0.0146	A_1

TABLE 8.2. Summary of 9-*cis* reconstituted pineal MSP data. Data based on 44 cells. Asterisks denote long-wavelength shift produced by photoproducts (see text).

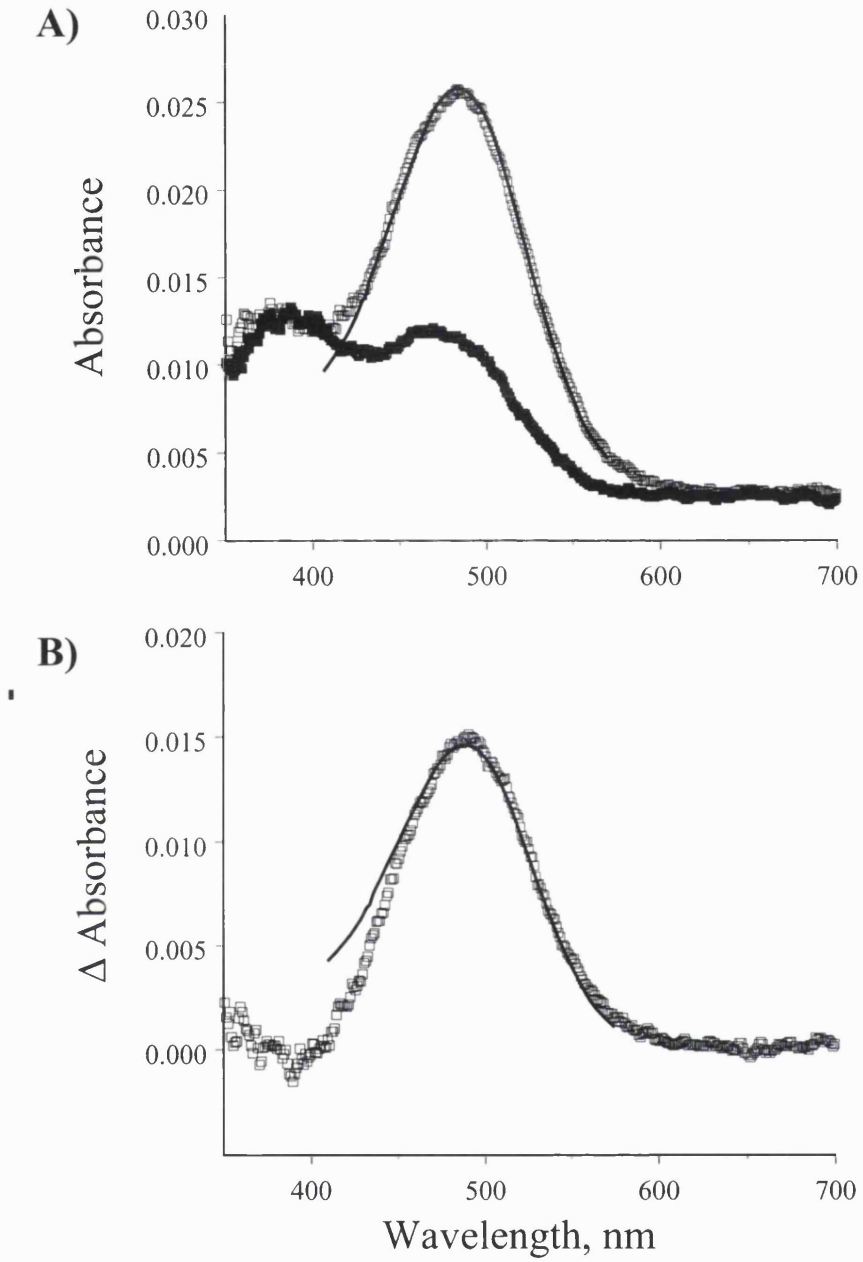


FIGURE 8.5. Absorbance and difference spectra of goldfish pineal photopigment regenerated with 9-*cis* retinal. A) Absorbance spectrum of regenerated pineal photopigment, based on averaged spectra from 44 photoreceptors. Solid line shows A_1 nomogram of λ_{max} 484 nm. Filled symbols show bleached record following 3 min exposure to white light. B) Difference spectrum, with A_1 nomogram of λ_{max} 488 nm, demonstrating long-wave shift due to presence of photoproduct.

C) Variability Between Individual Reconstituted Photoreceptors

As in the case of the native data, records were analysed individually and the individual λ_{maxT} values were plotted as a histogram in 2 nm intervals. The results of this are shown in figure 8.6, along with the native data to highlight the hypsochromic shift produced by reconstitution with 9-*cis* retinal. The individual cell records ranged from 475.9 – 490.0 nm. The mean λ_{max} of the individual records was 483.2 nm, with a standard deviation of 2.8 nm.

Again the results provide a clear indication of a single photoreceptor population, with no evidence of bimodality suggestive of multiple photoreceptor subtypes.

D) Summary of Goldfish Pineal MSP Data

Table 8.3 provides a summary of pineal photoreceptor and retinal rod photoreceptor λ_{max} values, derived from averaged absorbance spectra and analysis of individual records. As can be seen, the goldfish pineal photopigment is 12-13 nm short-wave shifted in its native state (depending upon mode of analysis), although this difference could be explained by differences in A_1/A_2 chromophore content of retina and pineal. However, following removal of potential chromophore variability by reconstitution with 9-*cis* retinal, pineal photoreceptors do NOT exhibit the same λ_{max} value as the retinal rod opsin. This suggests that goldfish pineal photoreceptors contain a rod-like opsin, 5-7 nm short-wavelength shifted from the rod isorhodopsin.

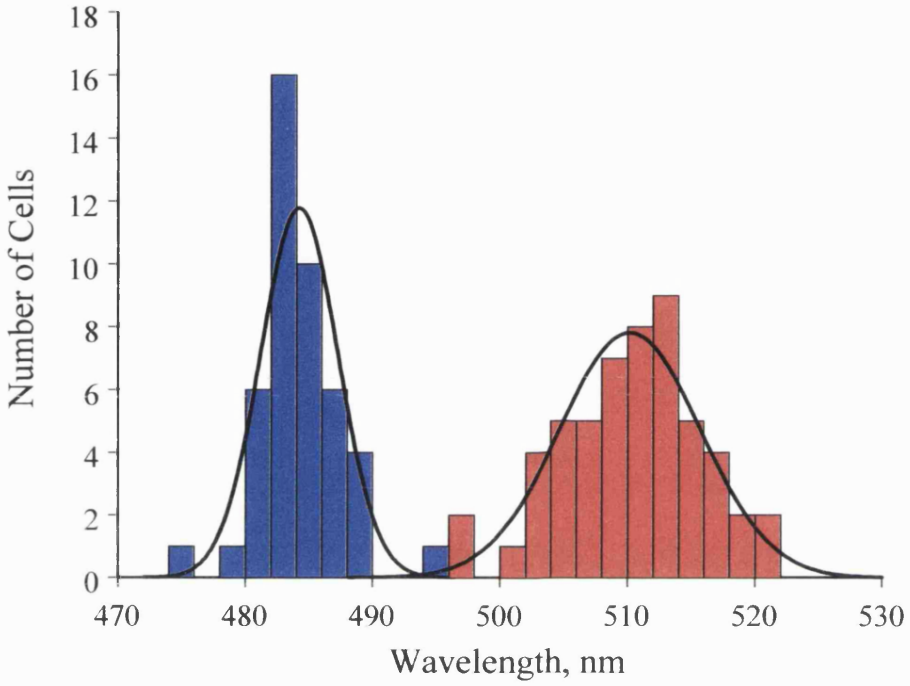


FIGURE 8.6. Distribution of λ_{max} values of individual regenerated pineal photoreceptor recordings, compared with native distribution. Data in red shows native photoreceptor λ_{max} distribution (mean λ_{max} 510.2 nm). Data in blue indicates hypsochromic shift produced by reconstitution with 9-*cis* retinal (mean λ_{max} 483.2 nm). Solid lines illustrate normal distributions based on data.

	λ_{MAX} (nm)	MEAN λ_{MAX}
NATIVE		
Pineal (50% A_1/A_2)	510.0 ± 1.4	510.2 ± 5.5
Rod (A_2)	522.2 ± 0.7	523.2 ± 3.6
9-*CIS* RETINAL		
Pineal	484.2 ± 0.4	483.2 ± 2.8
Rod	489.6 ± 0.9	490.4 ± 3.7

TABLE 8.3. Summary of native and 9-*cis* reconstituted goldfish pineal MSP results, with comparison to respective rod opsin retinal data (from Parry and Bowmaker, 2000). λ_{max} value based upon template fitting to the averaged absorbance spectrum of all records. Standard deviation of template fit is shown in each case. Mean λ_{max} value based upon the analysis of individual records. Standard deviation of individual records from mean is shown in each case. Reconstituted rod data were re-analysed to conform to criteria of this study (data courtesy of J.W. Parry).

8.4. TRANSMISSION OF GOLDFISH SKULL

To test whether pineal photoreceptors in the goldfish are tuned to their intra-cranial light environment the transmission of the overlying skull was also measured. The averaged spectra from two animals are shown in figure 8.7. The transmission spectrum is shown in figure 8.7A between 0.4 and 0.6 (with 1 being 100% transmission of incident light) to highlight the features of the spectrum, which otherwise appears as a more-or-less straight line, with transmission increasing at longer wavelengths.

A) INDIVIDUAL COMPONENTS AFFECTING TRANSMISSION

The absorbance spectrum from the skull is shown in figure 8.7B, obviously showing an inverse correlation to the transmission. The absorbance spectra recorded from three inert pigments found to be present within goldfish pineal MSP preparations are also shown. These pigments are haemoglobin, melanin and the carotenoid pigment responsible for the distinctive colour of goldfish.

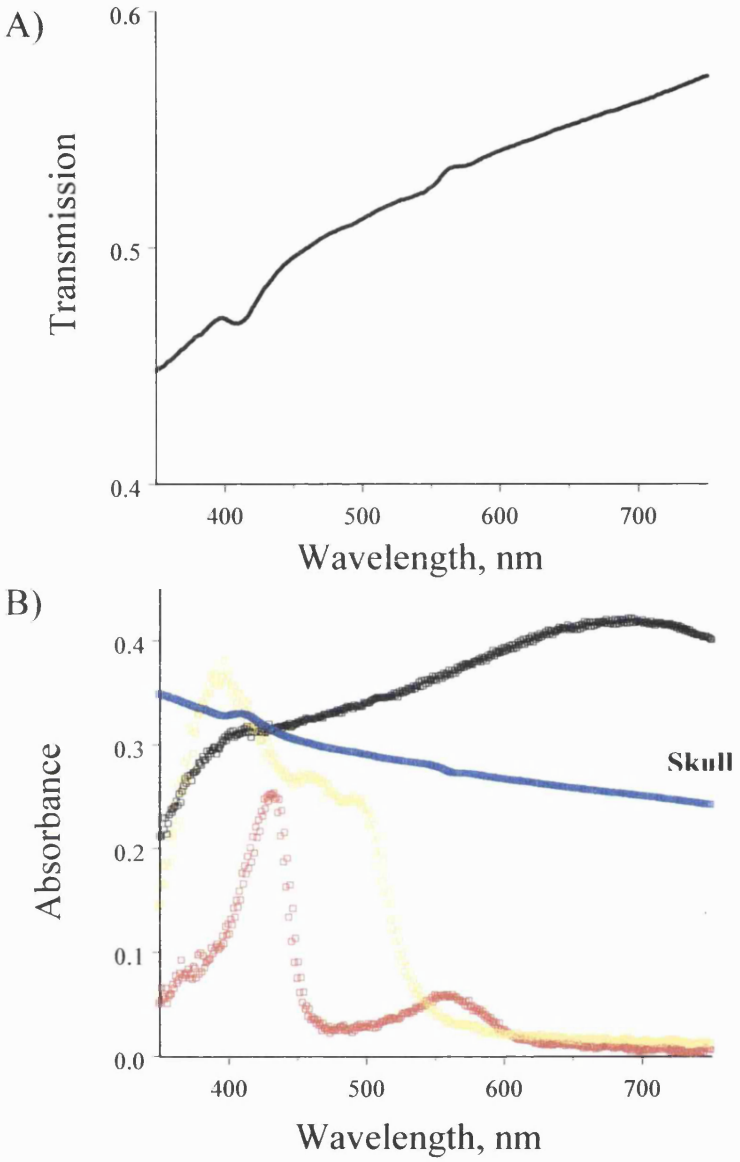


FIGURE 8.7. Transmission characteristics of goldfish skull overlying pineal end vesicle. A) Averaged skull transmission taken from two animals. Scale magnified to demonstrate transmission characteristics. B) Skull transmission (Blue) plotted as absorbance to compare with absorbance spectra of pigments present in pineal tissue. Pigments include haemoglobin (red), carotenoid mixtures (yellow) and melanin granules (black).

8.5. COMPARATIVE TELEOST PINEAL PHOTOSENSITIVITY

As a comparative study MSP was also conducted on the pineal photoreceptors of the Mexican tetra, *Astyanax fasciatus*, and of the Golden orfe, *Leuciscus idus*.

A) *ASTYANAX FASCIATUS*

i) Absorbance spectrum

Following MSP analysis 25 photoreceptors were measured from the pineal organs of five individual *A. fasciatus*, of which 17 cells met the selection criteria. Again only a single photoreceptor population was apparent, with a λ_{max} of 509.60 nm (SD 2.60, see figure 8.8A). As with the goldfish, the absorbance spectrum most closely fitted a template comprised of 50% A_1/50% A_2. λ_{maxT} was again used as the chromophore content of the photoreceptors was unknown, and potentially contained both A_1 and A_2 chromophores.

i) Difference spectrum

Upon construction of a difference spectrum, a bathochromic shift of nearly 5 nm was observed due to the presence of photoproducts. In this case the λ_{max} was shifted to 514.46 nm (SD 7.05, figure 8.8B).

	λ_{max}R	SD	λ_{max}T	SD	O.D.	Template
Absorbance spectrum	509.62	0.88	509.60	2.60	0.0198	50% A_1/A_2
Difference spectrum	510.67	1.18	514.46	7.05	0.0167	50% A_1/A_2

TABLE 8.4. Summary of *Astyanax* pineal MSP data. Data based on 17 cells.

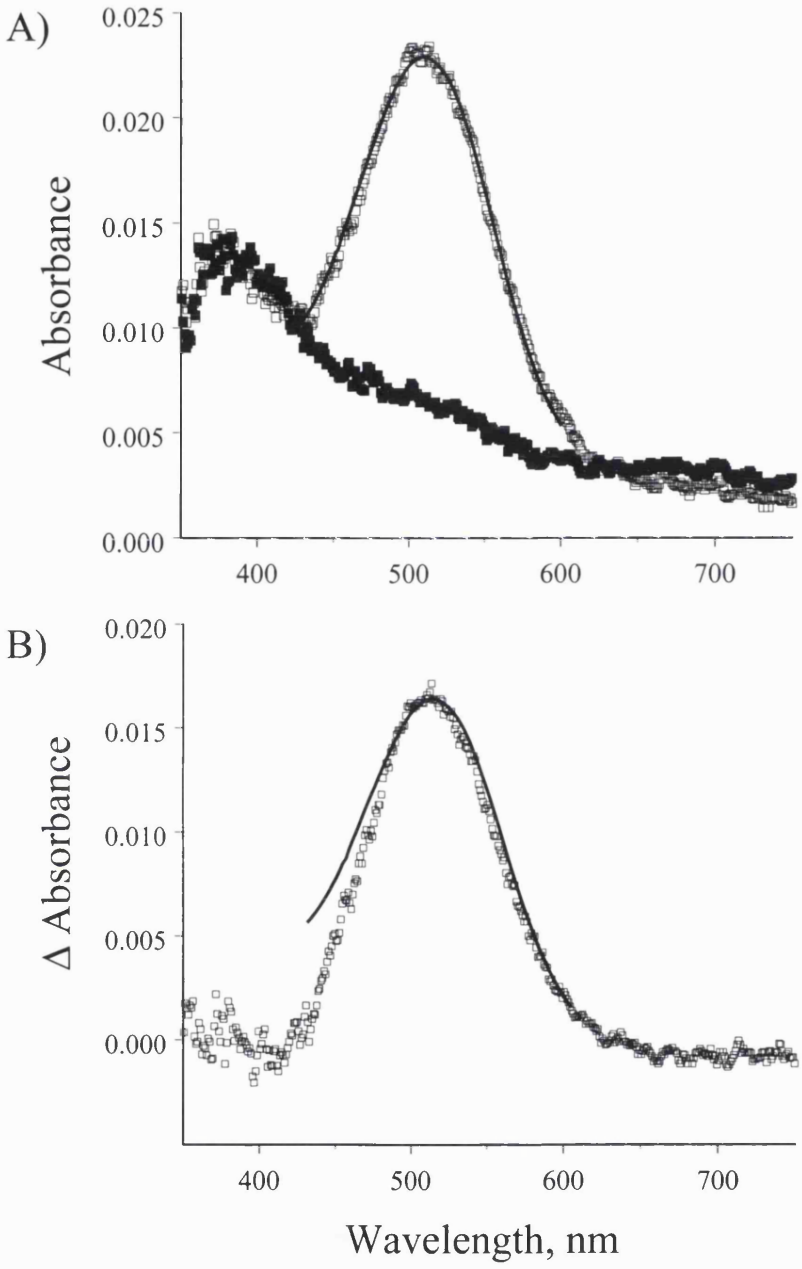


FIGURE 8.8. Absorbance spectra of pineal photoreceptors in *Astyanax fasciatus*. A) Absorbance spectrum fitted with 50% A_1/50% A_2 template of λ_{max} 510 nm. Filled symbols indicate rerecording following 3 min bleach. B) Difference spectrum fitted with 50% A_1/50% A_2 template of λ_{max} 514 nm.

B) *LEUCISCUS IDUS*

MSP analysis of pineal photoreceptors in *L.idus* was complicated by a number of factors. The major problem encountered was the difficulty in finding outer segments in pineal preparations. Both the number of pineal photoreceptors and the size of the outer segments are apparently reduced in the orfe pineal when compared to those of the goldfish and *A. fasciatus*, as demonstrated by the lower optical density of these photoreceptors (see table 8.5 below). As such, the selection criteria previously used were not applied to the 10 cells recorded, or the data set would be grossly limited.

i) Absorbance spectrum

The averaged absorbance spectrum of the orfe pineal most closely correlated to an A_2 template of λ_{max} 528.60 (SD 5.43). λ_{maxT} was again used as the chromophore content of the photoreceptors was unknown. Both the small data set and the quality of the records makes any attempt to determine the chromophore content based on the shape of the spectrum prone to error. The increased bandwidth observed may actually just reflect the low absorbance and poor quality of the data.

Again, there was no evidence for multiple photoreceptor populations, although given the small data set attempting to identify any pattern in the distribution of λ_{max} values is difficult and prone to inaccuracies.

ii) Difference spectrum

The difference spectrum of the orfe pineal photoreceptors most accurately fitted an A_2 template of λ_{max} of 533.02 nm (SD 9.67). A bathochromic shift was of around 4 nm was observed.

	$\lambda_{max}R$	SD	$\lambda_{max}T$	SD	O.D.	Template
Absorbance spectrum	531.09	2.10	528.60	5.43	0.0087	A_2
Difference spectrum	530.31	3.91	533.02	9.67	0.0069	A_2

TABLE 8.5. Summary of golden orfe pineal MSP data. Data based on 10 cells.

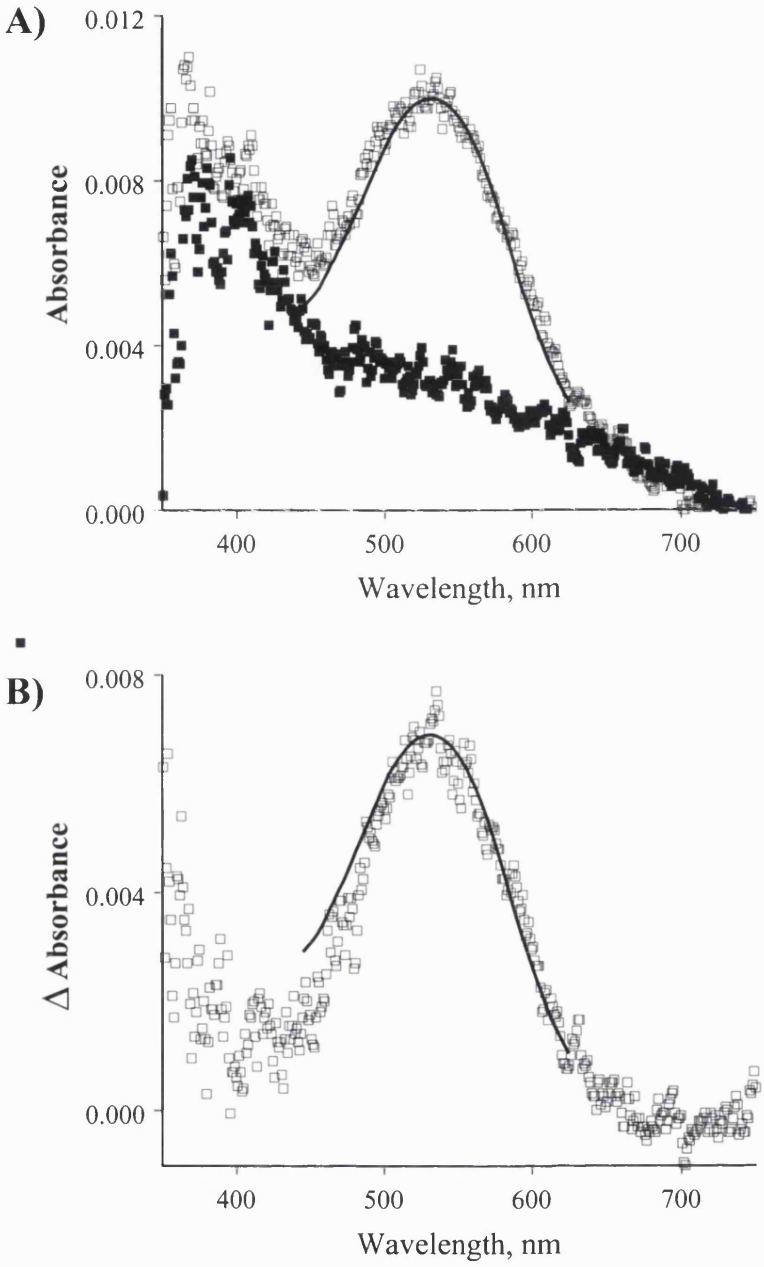


FIGURE 8.9. Absorbance spectra of pineal photoreceptors in the golden orfe, *Leuciscus idus*. A) Absorbance spectrum, most closely fitting A_2 nomogram of λ_{max} 529 nm. Filled symbols indicate rerecording following 3 min bleach. B) Difference spectrum, fitting A_2 nomogram of λ_{max} 533 nm.

SUMMARY OF PINEAL MSP DATA

SPECIES	PINEAL PHOTOPIGMENT λ_{MAX} (nm)	RETINAL ROD OPSIN λ_{MAX} (nm)
Native Goldfish, *Carassius auratus*	511	522
9-*cis* Goldfish, *Carassius auratus*	484	490
Mexican tetra, *Astyanax fasciatus*	510	519
Golden orfe, *Leuciscus idus*	531	518

TABLE 8.6. Summary of MSP data from the pineal photoreceptors of three teleost species. The respective rod opsin λ_{max} value is provided for comparison. λ_{max} values are noted to the nearest nm.

MOLECULAR GENETICS

8.6. ROD OPSIN EXPRESSION IN GOLDFISH PINEAL

Following screening of pineal cDNA using degenerate primers B+ and B-, based upon teleost rod opsin sequences, a single band of 783 bp was obtained, and following sequencing were found to correspond to the retinal rod opsin. Rod opsin was detected in pineal cDNA prepared from three separate groups of fish, and as such, unless contamination occurred with an alarming regularity, cannot be considered an artefact (especially given the attention paid to mRNA extraction and PCR protocols – see section 7.1 and 7.3F).

The rod opsin was simultaneously amplified from goldfish retinal cDNA as well as genomic DNA for comparison to the pineal rod opsin sequence. The rod opsin sequences isolated from pineal, retinal and genomic DNA were all found to be identical to the goldfish rod opsin sequence reported by (Johnson *et al.*, 1993).

As goldfish are tetraploid, a second rod opsin sequence has been postulated, due to the possible accumulation of mutations in the multiple copies of these genes. Two different retinal rod opsin genes have been isolated in the closely related carp, with a 97.2 % identity at the nucleotide level, 98.6% at the amino acid level (Lim *et al.*, 1997). However, during the course of this work no evidence was obtained to indicate a second rod opsin sequence in the goldfish, possibly indicating that the extra chromosomes imparted by tetraploidy in this species possess identical rod opsin genes.

8.7. ISOLATION OF GOLDFISH EXO-ROD OPSIN

Using degenerate primers B+ and J- (see 7.3), a 462 bp fragment was isolated from goldfish pineal cDNA. This band was cloned, and following sequencing of individual colonies the majority yielded a sequence corresponding to the goldfish rod opsin. However, a second rod opsin-like sequence differing from the retinal rod was also detected. Primers were then designed to points within this sequence that differed from the retinal rod opsin, and 3' RACE and Walking PCR were used to obtain a full-length coding sequence (see figure 8.10).

A) SEQUENCE

Thus novel goldfish rod opsin sequence consists of 1064 bp, coding for a 354 amino acid protein, of identical sequence length to the goldfish rod. However, differences occurring at the nucleotide level do lead to significant changes in the coding sequence. At the nucleotide level the sequence displayed 73% identity to the goldfish rod opsin, with a 74% identity at the amino acid level.

i) Opsin characteristics

Examination of the amino acid sequence of goldfish exo-rod opsin demonstrated the presence of all the key characteristics of a functional opsin protein (see section 3.3a). These amino acids are highlighted in figure 8.10, and include:

- Retinal binding site at Lys-296
- Schiff base counterion at Glu-113
- Cys-110 and Cys-187 forming extra-cellular disulphide bridge
- Cys-322 and Cys-323 forming the fourth intracellular loop
- Asp-2 and Asp-15, location of N-linked oligosaccharides
- Multiple Ser and Thr residues on the C-terminus involved in inactivation via light-dependent phosphorylation by rhodopsin kinase
- Glu-122 was also evident in the goldfish exo-rod opsin, a site thought to be involved in generating the different response kinetics of rod and cone opsins (Shichida and Imai, 1998).

```
ATGAATGGAA CGGAAGGTCC GAACTTCTAC GTGCCCATGT CCAACAAGAC       50
M  N  G  T   E  G  P   N  F  Y   V  P  M  S   N  K  T       17
   ▲ N2                                       ▲ N15

GGGTGTGGTG CGGAGCCCGT TTGAGGAGCC GCAGTACTAT CTGGCAGAGC      100
 G  V  V   R  S  P  F   E  E  P   Q  Y  Y   L  A  E  P      34

CGTGGAAGTA CTCGCTCCTG GCGGCGTACA TGCTCTTCCT CATCATCACT      150
  W  K  Y   S  L  L   A  A  Y  M   L  F  L   I  I  T       50

TCGTTCCCCG TCAACTCCCT CACGCTCTAC GTCACCGTGC AGCACAAGAA      200
S  F  P  V   N  S  L   T  L  Y   V  T  V  Q   H  K  K      67

GCTGCGCACG CCGCTCAACT ACATCCTACT CAATCTGGCC GTGGCCGACC      250
 L  R  T   P  L  N  Y   I  L  L   N  L  A   V  A  D  L     84

TGTTCATGGT GGTGGGTGGC TTCACCGTCA CCCTCTACAC CGCCCTGCAC      300
  F  M  V   V  G  G   F  T  V  T   L  Y  T   A  L  H       100

GGGTACTTCG TCCTGGGGGT CACCGGCTGC AACATCGAAG GCTTCTTCGC      350
G  Y  F  V   L  G  V   T  G  C   N  I  E  G   F  F  A      117
                          ▲ C110    ▲ E113

CACGCTGGGA GGGGAGATCG CGCTGTGGTC TCTGGTGGTC TTGGCTGTCG      400
 T  L  G   G  E  I  A   L  W  S   L  V  V   L  A  V  E     134
             ▲ E122

AGCGTTATAT TGTGGTGTGT AAGCCAATGA CAACGTTTCG TTTCGGAGAG      450
  R  Y  I   V  V  C   K  P  M  T   T  F  R   F  G  E       150

AAGCACGCCA TCGTGGGAGT GGGCTTCACC TGGGTCATGG CCCTCACCTG      500
K  H  A  I   V  G  V   G  F  T   W  V  M  A   L  T  C      167

CTGCATGCCG CCCCTGCTGG GCTGGTCCAG GTATATTCCA GAGGGAATGC      550
 C  M  P   P  L  L  G   W  S  R   Y  I  P   E  G  M  Q     184

AGTGCTCCTG TGGGATAGAT TACTACACTC CCAAACCTGA GATCAACAAC      600
  C  S  C   G  I  D   Y  Y  T  P   K  P  E   I  N  N       200
        ▲ C187

ACCTCGTTTG TCATCTACAT GTTCATCCTG CACTTCTCCA TCCCTCTGCT      650
T  S  F  V   I  Y  M   F  I  L   H  F  S  I   P  L  L      217

CATCATCTTC TTCTGCTACA GTCGCCTTCT CTGCACTGTC CGTGCGGCCG      700
 I  I  F   F  C  Y  S   R  L  L   C  T  V   R  A  A  A     234

CTGCCCAGCA GCAGGAGTCA GAGACGACCC AGAGGGCCGA ACGGGAAGTG      750
  A  Q  Q   Q  E  S   E  T  T  Q   R  A  E   R  E  V       250
```

```
ACACGTATGG TGGTTGTCAT GGTGGTCTCG TTCTTAGTGT GCTGGGTGCC      800
T  R  M  V   V  V  M   V  V  S   F  L  V  C   W  V  P      267

GTATGCCAGC GTGGCCTGGT ACATCTTTGC TAATCAGGGG GCGGAGTTTG      850
 Y  A  S   V  A  W  Y   I  F  A   N  Q  G   A  E  F  G      284

GTCCTATAGT GATGACGATA CCTGCTTTCT TTGCTAAGAG TGCAGCGCTT      900
  P  I  V   M  T  I   P  A  F  F   A  K  S   A  A  L      300
                                    ▲ K296

TACAACCCTG TCATCTACAT CATACTCAAC AGACAGTTCA GGAACTGCAT      950
Y  N  P  V   I  Y  I   I  L  N   R  Q  F  R   N  C  M      317

GCTCACTACG GTCTGCTGTG GAAAGAACCC GTTTGGTGAG GAGGAGACCA     1000
 L  T  T   V  C  C  G   K  N  P   F  G  E   E  E  T  S     334
            ▲  ▲ C322-323

GCATGATGGC CTCCAGTAAG ACTCAGTCCT CGGTGGTCTC GTCCAGTCAG     1050
  M  M  A   S  S  K   T  Q  S  S   V  V  S   S  S  Q      350

GTGGCTCCCG CC                                             1062
V  A  P  A                                                354
```

FIGURE 8.10. Full nucleotide coding sequence of goldfish exo-rod opsin. Corresponding amino acid sequence is shown, with key amino acids involved in opsin function indicated as follows: N2, N-linked glycosylation site; N15, N-linked glycosylation site; C110, disulphide bridge with C187; E113, Schiff base counterion; E122, glutamic acid important in rod/cone differentiation; C187, disulphide bridge with C110; K296, Schiff base linkage with retinal; C322, palmitoylation site to form fourth intracellular loop; C323, palmitoylation site to form fourth intracellular loop.

ii) Opsin classification

When compared to a selection of visual and non-visual opsins it was immediately apparent that this goldfish sequence most closely resembled exo-rhodopsin or ER rod-like opsin, demonstrating a 91% identity at the amino acid level with zebrafish exo-rhodopsin, and 87% and 89% identity with salmon and fugu ER rod-like opsins, respectively (see table 8.7, overleaf).

Amino acid identities with goldfish cone opsins and other non-visual opsins were all below 50%, with the exception of the MWS cone opsin, which is thought to be the evolutionary precursor of the rod opsins (see Okano *et al.*, 1992 and Register *et al.*, 1994).

BLAST analysis of GF exo-rod opsin shows a slightly higher nucleotide identity with other vertebrate rod opsins, rather than with the teleost rod opsins as would be expected. This difference is slight, and may just reflect the limited number of sequences chosen for comparison.

Opsin	Species	Identity (%)	GENBANK
Goldfish Opsins			
Rod	*Carassius auratus*	74	AAA49191
MWS1	*Carassius auratus*	68	AAA49168
LWS	*Carassius auratus*	42	AAA49190
SWS	*Carassius auratus*	47	AAA49164
UVS	*Carassius auratus*	44	BAA12889
Teleost Rod Opsins			
Zebrafish	*Danio rerio*	75	AAD24751
Carp	*Cyprinus carpio*	74	AAB06368
Catfish	*Ictalarus punctatus*	71	AAB84052
Atlantic salmon	*Salmo salar*	75	AAF44620
Pufferfish	*Takifugu ribrupes*	73	AAF44621
Mexican tetra	*Astyanax fasciatus*	73	AAA74422
Vertebrate Rod Opsins			
Human rod	*Homo sapiens*	77	AAC31763
Mouse rod	*Mus musculus*	76	AAA39861
Chicken rod	*Gallus gallus*	77	BAA00610
Chameleon rod	*Anolis carolinensis*	75	AAA62614
Toad rod	*Bufo bufo*	76	AAB93704
Coelacanth rod	*Latimeria chalumnae*	77	AAD30519
Lamprey rod	*Lampetra japonica*	73	AAA49342
Non-Visual Opsins			
Exo-rhodopsin	*Danio rerio*	91	BAA88958
ER rod-like	*Salmo salar*	87	AAF44619
	Takifugu ribrupes	89	AAF44622
Pinopsin	*Gallus gallus*	47	AAA64223
Parapinopsin	*Ictalarus punctatus*	40	AAB84050
VA opsin	*Salmo salar*	38	AAC60124
Encephalopsin	*Mus musculus*	32	AAD32670
Melanopsin	*Xenopus laevis*	31	AAC41235

TABLE 8.7. Sequence amino acid identity between goldfish exo-rod opsin and a selection of visual and non-visual opsins, illustrating highest percentage identity with zebrafish exo-rhodopsin and salmon and fugu ER rod-like opsins.

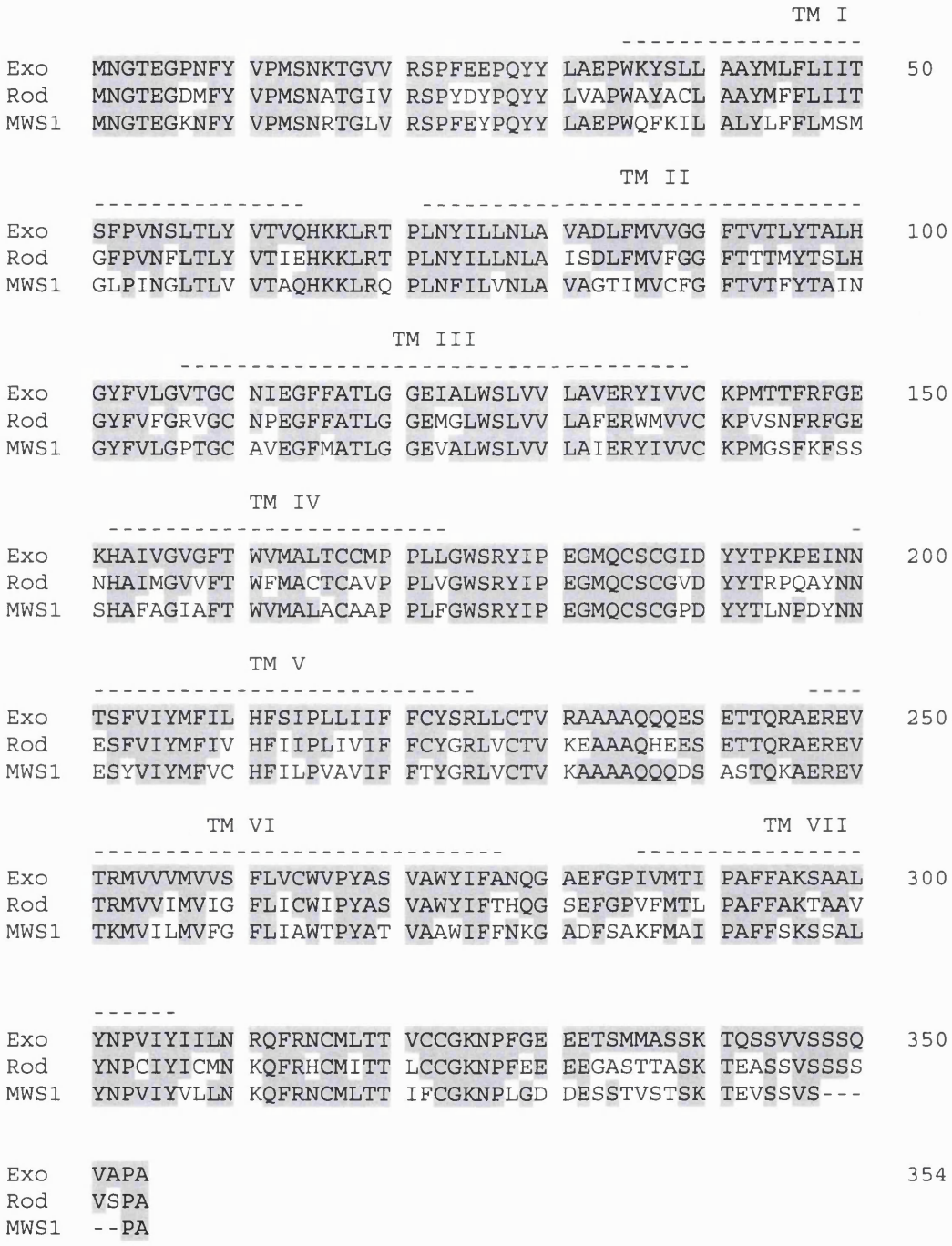


FIGURE 8.11. Sequence alignment of goldfish exo-rod opsin with retinal rod opsin and MWS cone opsin sequences. Regions of amino acid identity are shaded. Positions of transmembrane helices indicated (based upon Palczewski *et al.*, 2000).

iii) Sequence alignment

The goldfish exo-rod opsin polypeptide sequence was aligned with the goldfish retinal rod opsin and MWS cone opsin sequences to illustrate regions of identity and the location of amino acid substitutions (as shown in figure 8.11). Only the MWS cone opsin was used, due to the considerable sequence differences exhibited by the LWS, SWS and UVS cone opsins (all <50% polypeptide identity). The goldfish retina contains two highly similar MWS cone opsins (91% identity), of which only one (MWS1) was used for alignments.

Goldfish exo-rod opsin was also aligned with the other published exo-rod opsin sequences - zebrafish exo-rhodopsin and *Salmo* and *Fugu* ER rod-like opsins, to illustrate homologies and potential differences between these photopigment molecules (figure 8.12).

FIGURE 8.12. (*Overleaf*) Sequence alignment of goldfish exo-rod opsin with zebrafish, salmon and Fugu sequences. Regions of amino acid identity are shaded. Position of transmembrane helices indicated (based on Palczewski *et al.*, 2000).

```
                                                          TM I
                                                 -----------------
GFexo MNGTEGPNFY VPMSNKTGVV RSPFEEPQYY LAEPWKYSLL AAYMLFLIIT   50
ZF    MNGTEGPNFY VPMSNRTGLV RSPFEEPQYY LAEPWQFSLL AAYMLFLILG
Fugu  MNGTEGPNFY IPMSNKTGVV RSPFEYPQYY LAEPWKYSLV AAYMLFLIIT
Salmo MNGTEGPNFY VPMSNKTGVV RSPFEHPQYY LAAPWKYSLL AAYMIFLIIT

                                              TM II
      ---------------          --------------------------------
GFexo SFPVNSLTLY VTVQHKKLRT PLNYILLNLA VADLFMVVGG FTVTLYTALH   100
ZF    SFPINALTLY VTVQHKKLRT PLNYILLNLA VADLFMVLGG FTVTLYTALH
Fugu  AFPVNFLTLF VTVKHKKLRT PLNYVLLNLA VADLFMVIGG FTVTLYTALH
Salmo AFPVNFLTLY VTVQHKKLRT PLNYILLNLA VADLFMVVGG FTVTLATALQ

                           TM III
            ------------------------------------
GFexo GYFVLGVTGC NIEGFFATLG GEIALWSLVV LAVERYIVVC KPMTTFRFGE   150
ZF    GYFLLGVTGC NIEGFFATLG GEIALWSLVV LAIERYIVVC KPMSTFRFGE
Fugu  AYFVLGVTGC NIEGFFATLG GEIALWSLVV LAVERYIVVC KPMTNFRFGE
Salmo GYFFLGVTGC NVEGFFATMG GEIALWSLVV LAIERYIVVC KPMTNFRFNE

                  TM IV
       ------------------------                            -
GFexo KHAIVGVGFT WVMALTCCMP PLLGWSRYIP EGMQCSCGID YYTPKPEINN   200
ZF    KHAIIGVGFT WVMALTCAVP PLLGWSRYIP EGMQCSCGID YYTPKPEVHN
Fugu  KHAIAGLVFT WIMALTCATP PLLGWSRYIP EGMQCSCGID YYTPKPEINN
Salmo RHAIVGVAFT WIMSLTCALP PLCGWSRYIP EGMQCSCGID YYTPTPELGN

                  TM V
      ---------------------------                      ----
GFexo TSFVIYMFIL HFSIPLLIIF FCYSRLLCTV RAAAAQQQES ETTQRAEREV   250
ZF    TSFVIYMFIL HFSIPLLIIF FCYSRLLCTV RAAAAQQQES ETTQRAEREV
Fugu  TSFVIYMFIL HFSIPLAIIF FCYSRLLCTV RAAAALQQES ETTQRAEKEV
Salmo TSFVIYMFTL HFSIPLVIIG FCYGRLLCTV RAAAALQQES ETTQRAEKEV

              TM VI                                  TM VII
      ----------------------------       ----------------
GFexo TRMVVVMVVS FLVCWVPYAS VAWYIFANQG AEFGPIVMTI PAFFAKSAAL   300
ZF    TRMVVVMVIA FLVCWVPYAS VAWYIFANQG AEFGPVFMTV PAFFAKSAAL
Fugu  TRMVIVMVIS FLVCWVPYAS VAWYIFANQG TEFGPVFMTA PAFFAKSAAL
Salmo TRMVIVMVIS YLVCWMPYAT VAWYIFANQG TNFGPVMMTI PAFFAKSAAL

      ------
GFexo YNPVIYIILN RQFRNCMLTT VCCGKNPFGE EETSMMASSK TQSSVVSSSQ   350
ZF    YNPVIYIMLN RQFRNCMLST VCCGKNPLAE DESSSAVSSK TQSSVVSSAQ
Fugu  YNPVIYIILN RQFRNCMITT VCCGKNPFGD DDAATTVS-K TQSSSVSSSQ
Salmo YNPIIYILLN RQFRNCMLTI VCCGKNPFGE EETST-ASSK TQASSISASQ


GFexo VAPA                                                   354
ZF    VSPA
Fugu  VAPA
Salmo VAPA
```

B) EXO-ROD OPSIN HYDROPATHY PROFILE

As a simple means of determining the two-dimensional structure of the exo-rod opsin polypeptide chain a Kyte-Doolittle hydrophobicity plot was constructed using GeneWorks™ sequence analysis software (figure 8.13).

The Kyte-Doolittle hydrophobicity plot for goldfish exo-rod opsin demonstrates the seven hydrophobic transmembrane regions (upper region of plot) characteristic of heptahelical receptors, to which receptor family the opsin protein belongs. This hydropathy profile illustrates the interconnecting hydrophilic regions (lower region of plot) corresponding to the intra- and extra-cellular loops. The intracellular loops are indicated.

The values taken from this plot were used to determine which regions of the opsin molecule were hydrophobic, thus forming the transmembrane helices. This data yields a good correlation with models of the transmembrane structure of vertebrate rod opsins (Schertler *et al.*, 1993, Schertler, 1998 and Palczewski *et al.*, 2000).

Based on this hydrophobicity plot and the existing models of opsin structure a schematic diagram of the transmembrane position of the opsin polypeptide chain was constructed to illustrate the location of the various amino acids with respect to their membrane environment, one another and the retinal molecule (see figure 8.14).

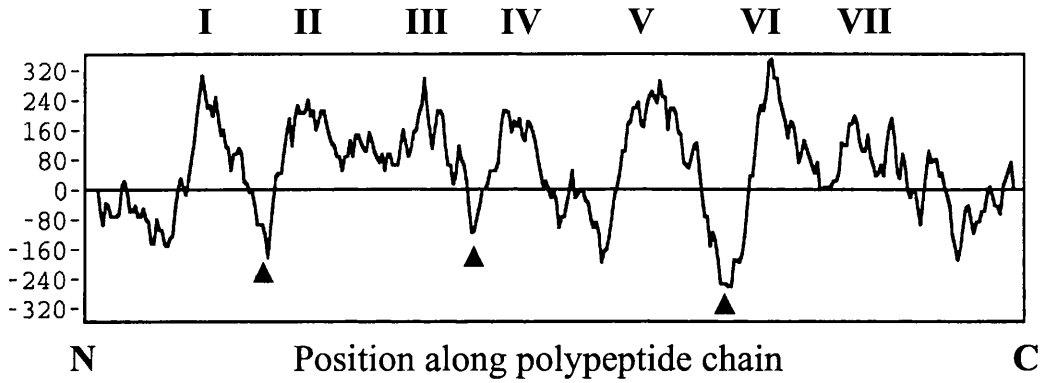


FIGURE 8.13. Kyte-Doolittle hydrophobicity plot for goldfish exo-rod opsin, illustrating the alternating hydrophobic and hydrophilic regions along the polypeptide chain, from N to C terminus. Arrowheads denote the three cytoplasmic loops.

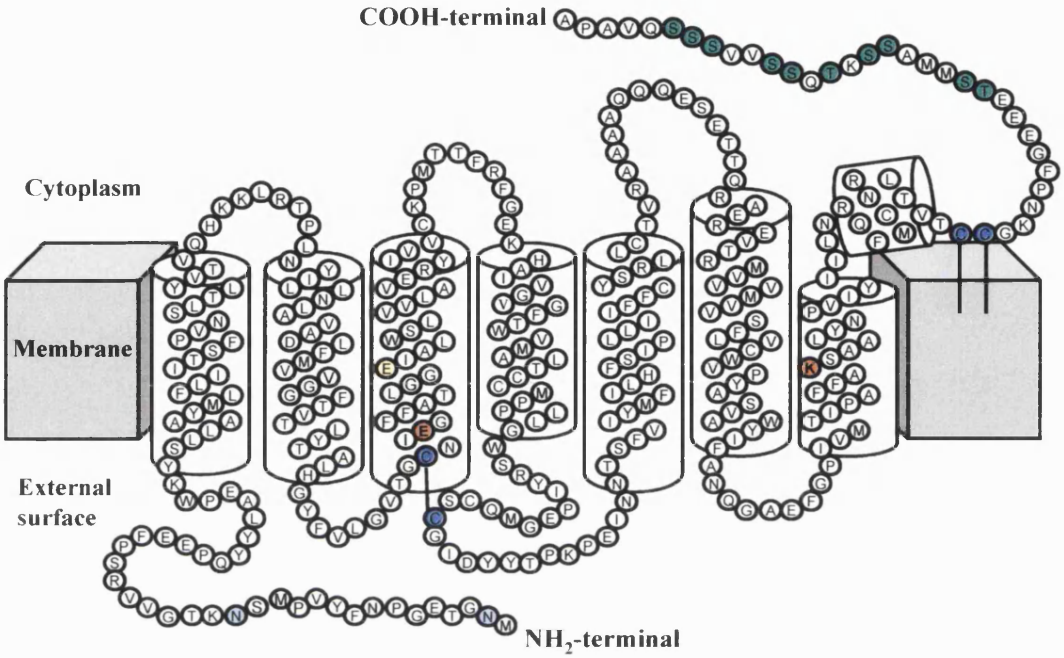


FIGURE 8.14. Two-dimensional model of goldfish exo-rod opsin, illustrating key opsin characteristics. Retinal binding site (Lys-296) and counterion (Glu-113) are highlighted in red. Disulphide bridge (Cys110/Cys187) and palmitoylation site (Cys-322/Cys-323) are noted in blue. Pale blue indicates N-linked glycosylation sites (N2/N15). Potential phosphorylation sites are highlighted in green. Glu-122 is highlighted in yellow. Modified from Palczewski *et al.*, 2000.

C) SPECTRAL TUNING OF GOLDFISH EXO-ROD OPSIN

i) Comparison with retinal rod opsin

Table 8.8 shows all the amino acid substitutions that occur within the helical regions of goldfish exo-rod opsin when compared to the goldfish retinal rod opsin. The nature of the substitution is noted, based upon the difference of the amino acid side chain in the exo-rod opsin.

ii) Comparison with known exo-rod opsins

Table 8.9 illustrates some of the key differences between helical amino acids within the known exo-rod opsin sequences, which may potentially be involved in producing different spectral tuning between these non-visual opsins.

As noted by Philp *et al.* (2000) the A164S and T261F substitutions observed in the salmon exo-rod suggests a long-wavelength shift in this opsin in comparison to the other exo-rod opsins.

Helix I

Amino Acid	Position in Helix	Exo	Rod	Difference in Side-Chain
38	1	S	A	Addition of OH group
39	2	L	C	Loss of cysteine[1]
45	8	L	F	Loss of aromatic group
51	14	S	G	Addition of OH group
56	19	S	F	Addition of OH group

Helix II

Amino Acid	Position in Helix	Exo	Rod	Difference in Side-Chain
81	12	V	I	*Conserved substitution*
82	13	A	S	Loss of OH group
88	19	V	F	Loss of aromatic group
93	24	V	T	Loss of OH group
95	26	L	M	*Conserved substitution*

Helix III

Amino Acid	Position in Helix	Exo	Rod	Difference in Side-Chain
112	2	I	P	Loss of proline[2]
123	13	I	M	*Conserved substitution*
124	14	A	G	*Conserved substitution*
133	23	V	F	Loss of aromatic group
136	26	Y	W	Addition of OH group

Helix IV

Amino Acid	Position in Helix	Exo	Rod	Difference in Side-Chain
155	5	V	M	*Conserved substitution*
158	8	G	V	*Conserved substitution*
162	12	V	F	Loss of aromatic group
165	15	L	C	Loss of cysteine[1]
168	18	C	A	Addition of cysteine[1]
169	19	M	V	*Conserved substitution*

HELIX V

AMINO ACID	POSITION IN HELIX	EXO	ROD	DIFFERENCE IN SIDE-CHAIN
210	**9**	L	V	*Conserved substitution*
213	**12**	S	I	Addition of OH group
217	**16**	L	I	*Conserved substitution*
218	**17**	I	V	*Conserved substitution*
224	**23**	S	G	Addition of OH group

HELIX VI

AMINO ACID	POSITION IN HELIX	EXO	ROD	DIFFERENCE IN SIDE-CHAIN
256	**7**	V	I	*Conserved substitution*
259	**10**	V	I	*Conserved substitution*
260	**11**	S	G	Addition of OH group
263	**14**	V	I	*Conserved substitution*
266	**17**	V	I	*Conserved substitution*

HELIX VII

AMINO ACID	POSITION IN HELIX	EXO	ROD	DIFFERENCE IN SIDE-CHAIN
286	**1**	I	V	*Conserved substitution*
287	**2**	V	F	Loss of aromatic group
297	**12**	S	T	*Conserved substitution*
300	**15**	L	V	*Conserved substitution*
304	**19**	V	C	Loss of cysteine[1]
308	**23**	I	C	Loss of cysteine[1]
309	**24**	L	M	*Conserved substitution*
311	**26**	R	K	*Conserved substitution*

TABLE 8.8. Helical amino acid substitutions between goldfish rod and exo-rod opsin. Amino acids facing the retinal-binding pocket are shaded. The difference in the nature of the amino acid side-chain in exo-rod opsin is noted in each case. Footnotes: 1. Cysteine residues are involved in the formation of disulphide bridges, 2. Proline residues induce kinks in the polypeptide chain.

HELIX	AA	GF	ZF	FUGU	SALMO	FUNCTIONAL DIFFERENCES
I	51*	S	S	A	A	Addition of OH group in GF and ZF
	56	S	A	F	F	Addition of OH group in GF
	60	Y	Y	F	Y	Loss of OH group in Fugu
IV	164*	A	A	A	S	Addition of OH group in Salmo
	168*	C	A	A	A	Cysteine only in GF exo
	169	M	V	T	L	Addition of OH group in Fugu
V	209	I	I	I	T	Addition of OH group in Salmo
	224	S	S	S	G	Loss of OH group in Salmo
VI	260*	S	A	S	S	Addition of OH group GF, Fugu and Salmo
	261*	F	F	F	Y	Addition of OH group in Salmo

TABLE 8.9. Non-conserved amino acid differences between known exo-rod opsin sequences at potential spectral tuning sites. Asterisks indicate sites in the vicinity of the retinal binding pocket. Shading indicates sites previously implicated in spectral tuning (Bowmaker and Hunt, 1999).

D) CYTOPLASMIC DOMAINS

The cytoplasmic loops and C-terminus of the opsin molecule are involved in the intracellular interactions involved in the initiation of the phototransduction cascade (see section 3.4). Differences in amino acids in these regions may lead to altered interactions with the elements of the cascade.

i) G-Protein interactions

Alignments of the intracellular regions of the goldfish exo-rod opsin sequence with other teleost exo-rod and rod opsin sequences, along with goldfish cone opsin sequences are illustrated in figure 8.15. Furthermore, chicken pinopsin is also shown as it represents the most intensively studied pineal-specific opsin to date, particularly with regard to its intracellular interactions with transducin (see Max *et al.*, 1998, Nakamura *et al.*, 1999 and Matsushita *et al.*, 2000).

The number of charged amino acids, thought to be important in the interactions between opsin and transducin, are shown within each loop. Asterisks denote substitutions of potential interest (see section 9.2Bi).

ii) Deactivation by rhodopsin kinase

Differences between the potential phosphorylation sites of rod opsins and exo-rod opsins are noted in figure 8.16. Due to the substantial differences occurring in these regions between different opsin groups only the rod opsins and exo-rod opsins are aligned. Asterisks denote residues thought to be most important in light-dependent phosphorylation (see section 9.2Bii).

LOOP 1

Amino Acids 61-72

	70	Charged
GF Exo	VTVQHKKLRT PL	4
ZF Exo	VTVQHKKLRT PL	4
Fugu ER	VTVKHKKLRT PL	5
Salmo ER	VTVQHKKLRT PL	4
	*	
GF Rod	VTIEHKKLRT PL	5
ZF Rod	VTIEHKKLRT PL	5
Fugu Rod	VTIEHKKLRT PL	5
Salmo Rod	VTIEHKKLRT AL	5
GF MWS	VTAQHKKLRQ PL	4
GF LWS	ATAKFKKLRH PL	5
GF SWS	CTIQFKKLRS HL	4
GF UVS	VTMKYKKLRQ PL	4
Pinopsin	VSICYKKLRS PL	3

LOOP 2

Amino Acids 134-154

	140 150	Charged
GF Exo	ERYIVVC KPMTTFRFGE KHAI	7
ZF Exo	ERYIVVC KPMSTFRFGE KHAI	7
Fugu ER	ERYIVVC KPMTNFRFGE KHAI	7
Salmo ER	ERYIVVC KPMTNFRFNE RHAI	7
	* *	
GF Rod	ERWMVVC KPVSNFRFGE NHAI	6
ZF Rod	ERWMVVC KPVSNFRFGE NHAI	6
Fugu Rod	ERWVVVC KPISNFRFGE NHAI	6
Salmo Rod	ERWLVVC KPISNFRFSE THAI	6
GF MWS	ERYIVVC KPMGSFKFSS SHAF	5
GF LWS	ERWVVVC KPFGNVKFDA KWAS	6
GF SWS	ERWLVIC KPLGNFTFKT PHAI	5
GF UVS	ERYVVIC KPFGSFKFGQ SQAL	4
Pinopsin	ERYVVVC RPLGDFQFQR RHAV	7

LOOP 3

Amino Acids 226-254

	230	240	250	Charged	Ser/Thr
GF Exo	LLCTV	RAAAAQQQES	ETTQRAEREV TRMV	8	5
ZF Exo	LLCTV	RAAAAQQQES	ETTQRAEREV TRMV	8	5
Fugu ER	LLCTV	RAAAALQQES	ETTQRAEKEV TRMV	8	5
Salmo ER	LLCTV	RAAAALQQES	ETTQRAEKEV TRMV	8	5
		** ** ↑			
GF Rod	LVCTV	KEAAAQHEES	ETTQRAEREV TRMV	11	5
ZF Rod	LVCTV	KEAAAQQQES	ETTQRAEREV TRMV	9	5
Fugu Rod	LLCAV	KEAAAAQQES	ETTQRAEREV TRMV	9	4
Salmo Rod	LLCAV	KAAAAAQQES	ETTQRAEREV TRMV	9	4
GF MWS	LVCTV	KAAAAQQQDS	ASTQKAEREV TKMV	7	5
GF LWS	VWLAI	RTVAQQQKDS	ESTQKAEKEV SRMV	9	5
GF SWS	LLITL	KLAAKAQADS	ASTQKAEREV TKMV	8	5
GF UVS	LLGAL	RAVAAQQAES	ASTQKAEKEV SRMV	7	4
Pinopsin	LLLTL	RAAAAQQKFA	DTTQRAEREV TRMV	8	4

LOOP 4

Amino Acids 307-323

	310	320	Charged
GF Exo	IILN	RQFRNCMLTT VCC	2
ZF Exo	IMLN	RQFRNCMLST VCC	2
Fugu ER	IILN	RQFRNCMITT VCC	2
Salmo ER	ILLN	RQFRNCMLTI VCC	2
		* *	
GF Rod	ICMN	KQFRHCMITT LCC	3
ZF Rod	ICMN	KQFRHCMITT LCC	3
Fugu Rod	ICMN	KQFRHCMITT LCC	3
Salmo Rod	VLMN	KQFRHCMITT LCC	3
GF MWS	VLLN	KQFRNCMLTT IFC	2
GF LWS	VFMN	RQFRVCIMQL FGK	3
GF SWS	VLMN	KQFRSCMMKM VCG	3
GF UVS	AFMN	KQFNACIMET VFG	2
Pinopsin	VFMN	KQFQSCLLEM LCC	2

FIGURE 8.15. Intracellular loop regions involved in G-protein interaction of goldfish exo-rod opsin. Also included are the known exo-rod opsin sequences and the respective rod opsins, the goldfish cone opsins and chicken pineal photopigment pinopsin. Asterisks denote differences in charged amino acids important in G-protein interactions. Those in red denote differences peculiar to goldfish alone. Arrow denotes S240, a site involved in light-dependent phosphorylation.

CARBOXYL TERMINAL

```
                  330         340         350        Ser/Thr
GF Exo      VCCGKNPFGE EETSMMASSK TQSSVVSSSQ VAPA      10
ZF Exo      VCCGKNPLAE DESSSAVSSK TQSSVVSSAQ VAPA      10
Fugu ER     VCCGKNPFGD DDAATTVS-K TQSSSVSSSQ VAPA      10
Salmo ER    VCCGKNPFGE EETST-ASSK TQASSISASQ VAPA      10
                       *   *     *
GF Rod      LCCGKNPFEE EEGASTTASK TEASSVSSSS VSPA      12
ZF Rod      LCCGKNPFEE EEGASTTASK TEASSVSSSS VSPA      12
Fugu Rod    LCCGKNPFEE EEGASTT-SK TEASSVSSSS VSPA      12
Salmo Rod   LCCGKNPFEE EEGASTTASK TEASSVSSSS VAPA      11
```

FIGURE 8.16. Potential phosphorylation sites within the carboxyl terminal region of goldfish exo-rod opsin. Other known exo-rod opsin sequences and the respective rod opsins are also shown for comparison. The total number of serine and threonine residues are noted in each case. Asterisks denote those sites thought to be most important in light-dependent phosphorylation by rhodopsin kinase.

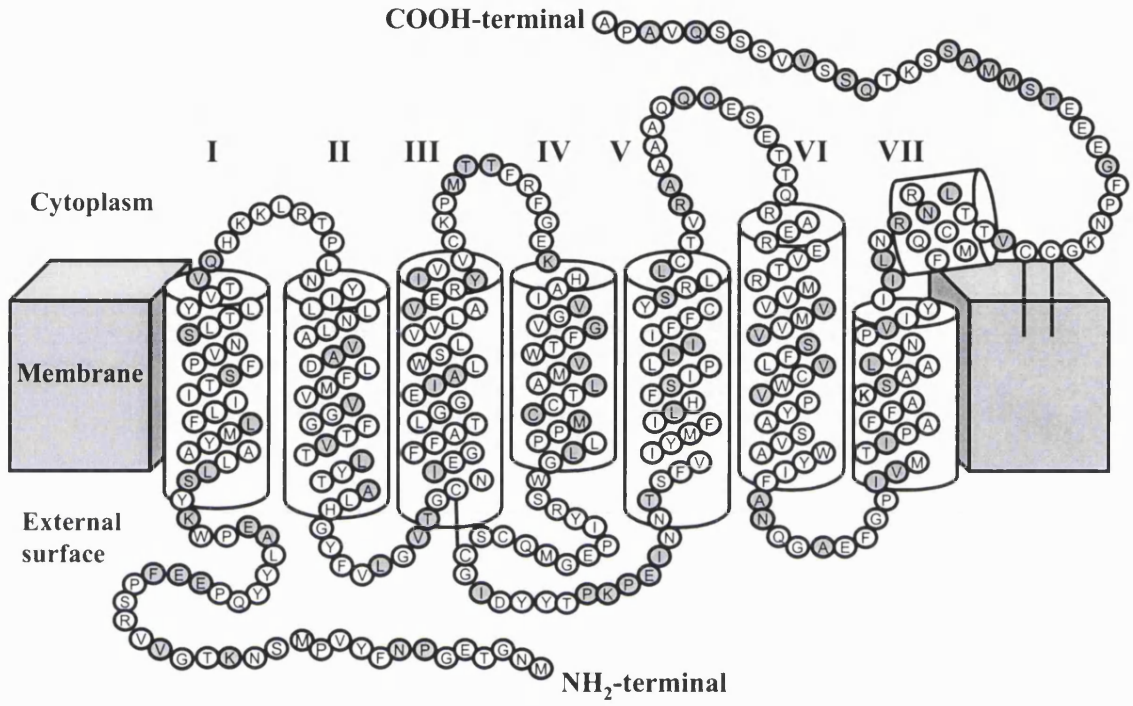


FIGURE 8.17. Schematic diagram of goldfish exo-rod opsin. Amino acid substitutions from the retinal rod opsin are shaded. Modified from Palczewski *et al.*, 2000.

E) GENE STRUCTURE

Upon PCR amplification of goldfish exo-rod opsin from genomic DNA it became evident that in contrast to the teleost retinal rod opsins, the goldfish exo-rod opsin gene contains 4 introns, breaking the coding sequence down in to 5 exons. Figure 8.19 shows the intron/exon boundaries of goldfish exo-rod opsin, which are located in the same positions as those of other vertebrate opsins. Bovine rod opsin is shown as a point of comparison (Nathans and Hogness, 1983, accession number AAA30674).

The teleost rod opsins are unique among vertebrate opsins in that they contain no introns (Fitzgibbon *et al.*, 1995). The functional significance of this difference is discussed in the next chapter.

```
                                                    -------------
Exo   MNGTEGPNFY VPMSNKTGVV RSPFEEPQYY LAEPWKYSLL AAYMLFLIIT   50
Bos   MNGTEGPNFY VPFSNKTGVV RSPFEAPQYY LAEPWQFSML AAYMFLLIML

      --------------          ---------------------------
Exo   SFPVNSLTLY VTVQHKKLRT PLNYILLNLA VADLFMVVGG FTVTLYTALH   100
Bos   GFPINFLTLY VTVQHKKLRT PLNYILLNLA VADLFMVFGG FTTTLYTSLH

                     INTRON A
                   -------▼--------------
Exo   GYFVLGVTGC NIEGFFATLG GEIALWSLVV LAVERYIVVC KPMTTFRFGE   150
Bos   GYFVFGPTGC NLEGFFATLG GEIALWSLVV LAIERYVVVC KPMSNFRFGE

                              INTRON B
        ------------------------   ▼
Exo   KHAIVGVGFT WVMALTCCMP PLLGWSR YIP EGMQCSCGID YYTPKPEINN  200
Bos   NHAIMGVAFT WVMALACAAP PLVGWSR YIP EGMQCSCGID YYTPHEETNN

                                      INTRON C
       ------------------------         ▼
Exo   TSFVIYMFIL HFSIPLLIIF FCYSRLLCTV RA AAAQQQES ETTQRAEREV  250
Bos   ESFVIYMFVV HFIIPLIVIF FCYGQLVFTV KE AAAQQQES ATTQKAEKEV

       ----------------------------       ------------------
Exo   TRMVVVMVVS FLVCWVPYAS VAWYIFANQG AEFGPIVMTI PAFFAKSAAL   300
Bos   TRMVIIMVIA FLICWLPYAG VAFYIFTHQG SDFGPIFMTI PAFFAKTSAV

                INTRON D
      ---------   ▼
Exo   YNPVIYIILN RQ FRNCMLTT VCCGKNPFGE EETSMMASSK TQSSVVSSSQ  350
Bos   YNPVIYIMMN KQ FRNCMVTT LCCGKNPLGD DEASTTV-SK TET-----SQ


Exo   VAPA                                                     354
Bos   VAPA
```

FIGURE 8.18. Gene structure of goldfish exo-rod opsin, aligned against the known intron structure of bovine rod opsin (*Bos taurus*).

8.8. PHYLOGENETIC ANALYSIS OF EXO-ROD OPSIN

Constructing phylogenetic trees based upon the nucleotide sequence of the goldfish exo-rod opsins and a selection of other vertebrate opsins, it is evident that teleost exo-rod opsin evolved by gene duplication of the ancestral rod opsin, as described by Mano et al., 1999 and Philp et al. 2000.

Figure 8.19 shows a phylogenetic cladogram constructed by maximum parsimony using the PAUP™ software. The goldfish exo-rod opsin is shown to clade with the vertebrate rod opsins, diverging from the teleost retinal rod opsins following their divergence from the other vertebrates. Due to the presence of introns in goldfish exo-rod opsin, the gene duplication of the teleost rod opsin must have occurred before the ancestral gene lost its introns.

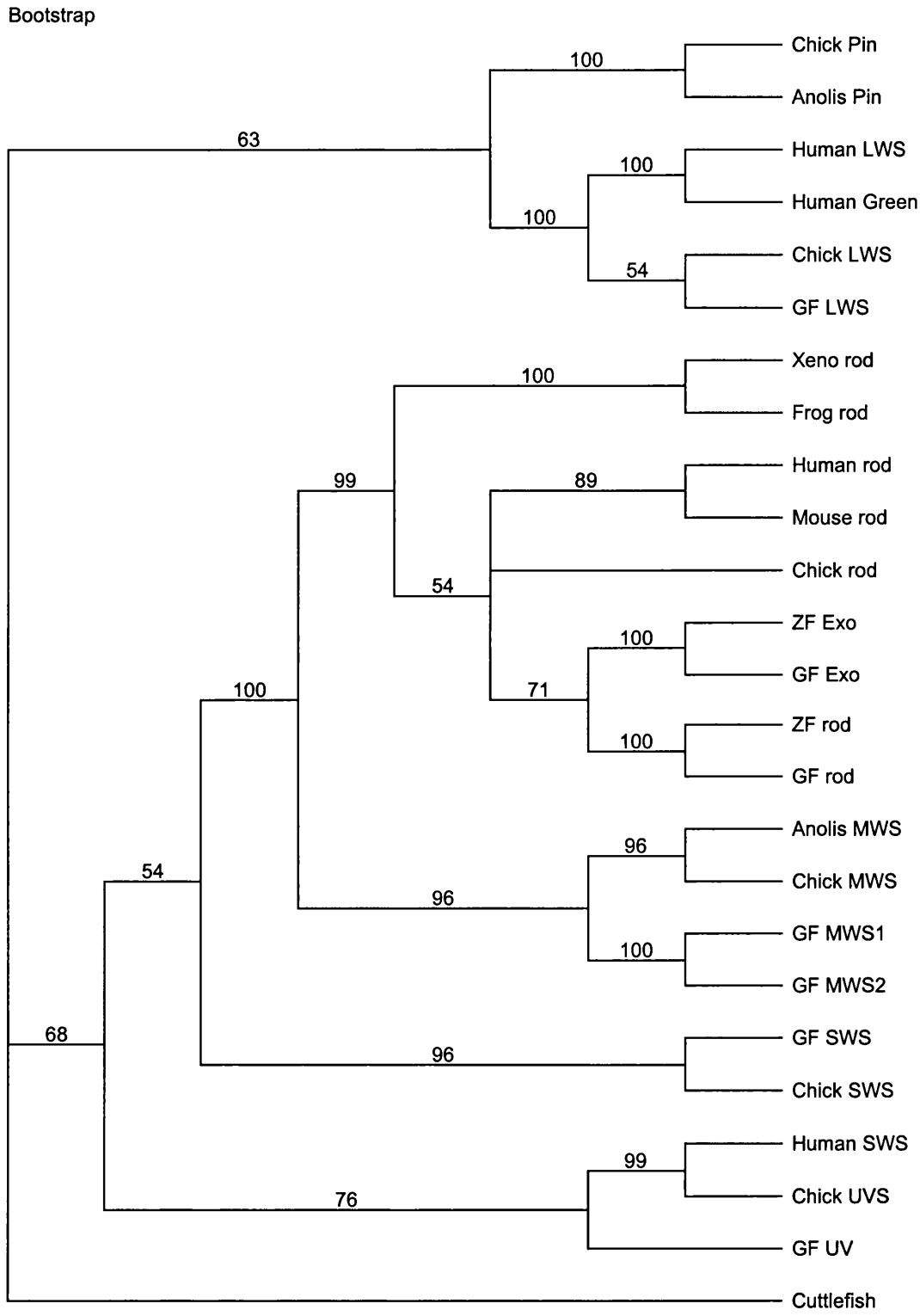


FIGURE 8.19. Phylogenetic cladogram showing position of goldfish (GF) exo-rod opsin in comparison with other vertebrate opsins. Constructed using maximum parsimony. Numbers indicate bootstrap values for each node.

8.9. Retinal Opsin Expression in Goldfish Pineal

Pineal and retinal cDNA were screened for the visual opsins present in the goldfish retina by PCR. The amplification of fragments of each opsin demonstrated that most of the visual pigments were in fact expressed in the pineal, with the apparent exception of the SWS cone opsin (see figure 8.20).

The possibility of these results being the product of contamination of pineal cDNA with retinal cDNA was an initial concern. However, despite retinal and pineal mRNA being extracted from the same animals, pineal organs and retinae were kept physically isolated at all stages, including throughout the cDNA synthesis procedure. Extreme care was also exercised whenever cDNA was utilised.

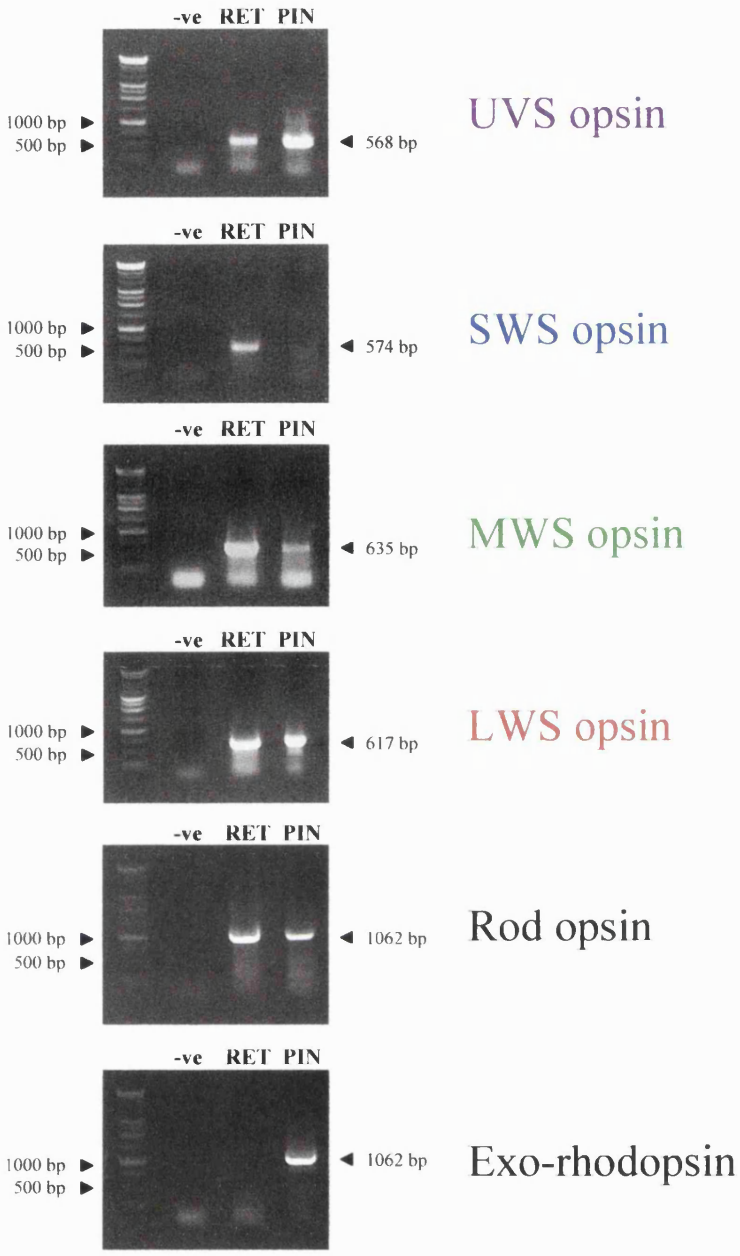


FIGURE 8.20. Expression of retinal cone opsins in goldfish retinal and pineal cDNA, assayed by tissue-specific PCR using gene-specific primer pairs. First lane in all pictures is 1 kb ladder with 1 kb and 500 bp bands indicated. Second lane shows negative control reaction containing no DNA.

CHAPTER 9

DISCUSSION

9.1. PHOTORECEPTOR COMPLEMENT OF THE GOLDFISH PINEAL

Photoreceptors were identified in the goldfish pineal and recorded under the MSP as tissue preparations as shown in figure 8.1. Individual photoreceptors showed differences in morphology, which though not evident under the MSP, could be observed from electron microscopy (see figure 8.2).

A) NATIVE GOLDFISH PINEAL PHOTOPIGMENT

MSP analysis of native goldfish pineal indicated the presence of a single photoreceptor population, with a λ_{max} of 511 nm, most closely fitting a nomogram of 50% A_1/50% A_2 (figure 8.3A). Given that the native retinal rod photoreceptors containing porphyropsins (pure A_2) demonstrate a λ_{max} of 522 nm, and goldfish rhodopsin (pure A_1) from rod photoreceptors has been shown to have a λ_{max} of 503 nm, a 50% A_1/A_2 chromophore mix would be expected to have a λ_{max} around 512.5 nm. As such, it would initially appear that the pineal photoreceptors most probably contain the rod opsin, but with a different chromophore content to that of the retina. The native pineal photoreceptors demonstrated quite a degree of variability in their individual λ_{max} values, which varied over a 24 nm range from 496 - 520 nm. This variation was attributed to differences in the A_1/A_2 chromophore content between cells (see figure 8.4). Despite this variability between photoreceptors the distribution of the data does conform to a normal distribution, indicating only a single photoreceptor population occurs in the goldfish pineal.

Electrophysiological studies by Meissl *et al.*, (1986), demonstrated that goldfish pineal photoreceptors demonstrated intracellular responses with a maximum sensitivity around 530 nm. Obviously, this data is long-wave shifted from the MSP findings of this study, but given the inaccuracy of the electrophysiological action spectra (the template fitted to just 12 data points), it appears likely that the λ_{max} reported in this study represents the same photoreceptor population. Furthermore,

differences between the populations and individual animals used may have resulted in different A_1/A_2 chromophore content between studies, particularly if the animals were kept under different photothermal regimes that may influence the chromophore content as it does in the retina (Bridges, 1972).

B) CHROMOPHORE VARIABILITY IN THE GOLDFISH PINEAL

Following the detection of considerable variability in the chromophore content of pineal photoreceptors in the goldfish, it is of interest to consider the mechanisms by which the pineal photoreceptors may regulate their retinoid content. Furthermore, the variability in λ_{max} produced by this chromophore regulation in the native pineal has implications as to the spectral tuning of the pineal photopigment in this species.

i) Chromophore regulation in the pineal

Both retinal and 3-dehydroretinal have been isolated from the pineal of the rainbow trout, in comparable ratios to the retina but at much lower concentrations (Tabata, 1985). The same authors also reported that the chromophore content of the sturgeon pineal mirrored that of the retina, containing just 3-dehydroretinal. Although obviously not a teleost, (Provencio and Foster, 1993) report similar findings from the pineal of the lizard *Anolis carolinensis*, which possesses visual pigments based upon the A_2 chromophore. As such it was suggested that the pineal may passively utilise whatever retinoids have been adaptively selected by the visual system.

The chromophore content of retinal photoreceptors is primarily regulated by the retinal pigment epithelium (RPE). However, in the absence of RPE cones of the frog retina have been shown to be capable of visual pigment regeneration, indicating that other pathways capable of the required retinoid metabolism are present elsewhere in the retinal tissues. The detection of two of the proteins involved in retinoid metabolism - cellular retinol-binding protein (CRBP) and cellular retinaldehyde-binding protein (CRALBP) in the Muller cells of the retina may indicate that these supportive cells also play a role in the provision of retinal for the regeneration of visual pigments (Saari, 2000).

The absence of any homologue to the RPE in the photosensory teleost pineal thus provides a comparable scenario. Pineal photoreceptors must utilise RPE-independent processes for the regeneration of their photopigment, and this

difference in the provision of chromophore may account for the A_1/A_2 variability apparent in the pineal photoreceptors of the goldfish. Whether the chromophore content therefore reflects the levels of circulating blood retinoids or retinoid metabolism by pineal photoreceptors or the (relatively unstudied) interstitial cells remains unknown. However, the teleost pineal could provide a useful system for the investigation of the mechanisms involved in the RPE-independent regulation of retinoid content by the photoreceptors or supporting cells.

ii) Chromophore variability and pineal function

Given the variability in chromophore content, and hence λ_{max}, observed in the native pineal photoreceptors of the goldfish, precise spectral tuning appears not to be an important aspect of pineal photosensitivity in this species. The λ_{max} of pineal photoreceptors was observed to vary over a range of around 24 nm, which is not consistent with a photoreceptor system evolved to precisely discriminate between wavelengths. However, variability in the λ_{max} of individual photoreceptors would not be critical if the goldfish pineal primarily serves as a luminance detector, as has been suggested.

C) SPECTRAL TUNING TO THE INTRACRANIAL LIGHT ENVIRONMENT

From the recordings of the transmission of the goldfish skull (illustrated in figure 8.7) two factors are immediately apparent. Firstly, the amount of light penetrating the skull is considerable. Of the incident light, between 40-60% is transmitted. Secondly, longer wavelengths appear to be transmitted more effectively than shorter wavelengths. However, over the spectral range measured (350-750 nm), the increase in transmission is only in the order of 30% more at longer wavelengths. This increase in transmission most likely reflects scattering of light by bone, which occurs to a greater degree at shorter wavelengths. As such it is obvious that the goldfish skull does not provide a particularly limiting factor when considering the wavelengths of light that can reach, and thus stimulate, the pineal photoreceptors in this species.

Intra-cranial factors that may influence the spectral tuning of pineal photopigments are the transmission window of haemoglobin, which has a maximum transmission between 450 and 530 nm, and the carotenoids of the characteristic goldfish

pigmentation, which absorb strongly at wavelengths below 520 nm. In contrast, melanin has a slightly increased absorption at longer wavelengths, but otherwise acts as a neutral density filter. The influence of these pigments on the actual transmission spectrum of the goldfish skull appears negligible (see 8.7B). However, the blood vessels of the pineal are located around the end-vesicle, and are interposed between the incident light and the photoreceptors. In the native state the goldfish pineal photoreceptors possess a λ_{max} of 511 nm, within the haemoglobin transmission window. As such, haemoglobin may potentially have influenced the adaptation of pineal photoreceptor sensitivity to the intracranial light environment. The high transmission of light through the goldfish skull provides a light environment exerting less selective pressure on the tuning of intracranial photoreceptors than would occur in species with thicker skulls or heavily pigmented overlying tissues. In this manner the age (and thus size) of the animal may also be important factors. For instance, the amount of incident light reaching the pineal in species such as the trout is as little as 10% in young animals, decreasing down to 1.4% in older specimens (Dodt, 1973).

The λ_{max} of pineal photoreceptors in the goldfish may also be adapted to this region of the spectrum due to the irradiance detection tasks they mediate. Although not tuned to the wavelength of maximum photon flux, the high sensitivity of pineal photoreceptors (see section 4.3B) may suggest that dark noise produced by thermal isomerisation could be a factor in the tuning of pineal photopigments. Adaptation for increased sensitivity at twilight, and the molecular constraints imposed by the evolutionary history of the photopigment molecule may also be contributing factors in the tuning to this region of the spectrum.

D) GOLDFISH PINEAL ISORHODOPSIN

To eliminate variability in the chromophore ratio, the pineal photoreceptors were bleached and regenerated as isorhodopsins with a single artificial chromophore, 9-*cis* retinal. The reconstituted photoreceptors had a λ_{max} of 484 nm, fitting an A_1 nomogram (figure 8.5). Again only a single photoreceptor population was evident, as demonstrated by the accurate fit of the data to a normal distribution (figure 8.6).

As shown in figure 8.6 the range of the data was reduced down to 14 nm (476 - 490 nm), demonstrating that the differences in individual λ_{max} values occurring between native pineal photoreceptors primarily originate from A_1/A_2 chromophore variability as suggested.

Parry and Bowmaker (2000) in this lab have recently measured porphyropsins, rhodopsins and isorhodopsins from the retinae of goldfish. Moreover, this data came from the same population of goldfish as the pineal MSP data, thus eliminating any potential variations produced by population differences, allowing a more controlled retinal/pineal comparison. These retinal recordings demonstrate that the goldfish rod isorhodopsin has a λ_{max} of 490 nm. As such, the goldfish pineal isorhodopsin, with a λ_{max} of 483 nm, is 5-7 nm short-wavelength shifted with respect to the retinal rod opsin.

i) Calibration of isorhodopsin data

Based on the equations from Parry and Bowmaker, an isorhodopsin of λ_{max} 484 nm should have a rhodopsin equivalent of 507 nm and a porphyropsin of 527 nm. These values are obviously incorrect, as the retinal rod opsin λ_{max} values are 490, 503 and 522 nm for isorhodopsin, rhodopsin and porphyropsin respectively. This inaccuracy is probably due to the inclusion of both rod and cone opsin data in the equations. The relationship between the λ_{max} of pigments formed from the different isomers of retinal is non-linear, and at the extremes of the visible spectrum (below approximately 450 nm and above 550 nm) functions used to describe this relationship typically break down. Due to the presence of opsins with λ_{max} values of 370 nm to nearly 620 nm in the aforementioned study, any equation that encompasses the extremes of this scale unfortunately may become less accurate.

To get a more realistic indication of the rhodopsin and porphyropsin λ_{max} values, simple regression coefficients were determined based upon the rod opsin λ_{max} values alone, using the data from Parry and Bowmaker and also from an independent study by (Makino *et al.*, 1999).

The following equations were generated:

EQUATION 1. ***9-cis retinal → 11-cis retinal***

$$Y = 1.167X - 68.5$$

EQUATION 2. ***9-cis retinal → 11-cis 3-dehydroretinal***

$$Z = 2X - 457$$

EQUATION 3. ***11-cis retinal → 11-cis 3-dehydroretinal***

$$Y = 1.714Z - 339.4$$

Where: X = 9-*cis* retinal λ_{max}

Y = 11-*cis* retinal λ_{max}

Z = 11-*cis* 3-dehydroretinal λ_{max}

It should be noted that due to the available experimental data these equations are based only on a very limited data set, and will only provide accurate estimations for opsins with λ_{max} values around those of the rod opsins (i.e. from around 480 – 530 nm).

Based upon these coefficients, the goldfish pineal isorhodopsin with a λ_{max} of 484 nm is predicted to have an equivalent rhodopsin of λ_{max} 496 nm and a porphyropsin of λ_{max} 511 nm.

E) SUMMARY OF MSP FINDINGS

The goldfish pineal contains a single photoreceptor population, with a λ_{max} of 511 nm in the native population, compared to 522 nm for the retinal rods. The native pineal appears to exhibit considerable variation in chromophore content, making a direct comparison with the predominantly A_2 retina difficult.

The spectral tuning of the goldfish pineal photopigment does not appear to directly correlate with the transmission characteristics of the skull. However, the pineal photoreceptors are tuned to within the haemoglobin transmission window, which may be important in the determination of the intracranial light environment. In addition, it may be that in the goldfish the intracranial light environment is not restricted enough to impose a high selective pressure upon the tuning of pineal photopigments.

Removal of chromophore variability by reconstitution with 9-*cis* retinal yields a pineal isorhodopsin with a λ_{max} of 484 nm, 5-7 nm shorter than the retinal rod isorhodopsin. This data therefore suggests that goldfish pineal photoreceptors contain a novel photopigment spectrally similar to the retinal rod opsin, but slightly short-wave shifted. The isolation of exo-rod opsin from the goldfish pineal provides a very strong candidate for the photopigment responsible for this difference between retinal and pineal spectral sensitivity.

9.2. GOLDFISH EXO-ROD OPSIN

With the isolation of goldfish exo-rod opsin from goldfish pineal cDNA, it appears likely that this non-visual opsin will play a role in pineal photosensitivity in this species. It is therefore of interest to determine if the amino acid differences between the goldfish rod and exo-rod opsin sequences can account for the 5-7 nm hypsochromic shift suggested by the MSP data. Furthermore, analysis of the substitutions within the intracellular regions of the opsin may provide an indication as to whether altered interactions with elements of the phototransduction cascade occur, reflecting differences in visual and non-visual photoreception.

A) SPECTRAL TUNING

Although the sequence differences between exo-rod opsin and rod opsin suggest a number of amino acid substitutions, there appear to be no significant differences at sites close to the chromophore. A comparison of the 27 amino acids within 4.5 Å of the retinal molecule in bovine rod opsin, 26 are identical in both the goldfish rod opsin and exo-rod opsin sequences. The only difference that does occur is the conserved substitution at site 189 of valine in the goldfish retinal rod for isoleucine in the pineal exo-rod opsin.

This suggests that exo-rod opsin will possess a λ_{max} value very similar to that of the retinal rod opsin, which from the MSP data certainly does appear to be the case. However, could any of the substitutions that do occur potentially account for the relatively small hypsochromic shift suggested by the MSP data? This difference is 5-7 nm in the respective isorhodopsins, and though more difficult to predict in the native state due to chromophore variations, may be expected to be around 12-13 nm.

Of the helical differences that occur between the rod opsin and exo-rod opsin sequences, the majority are conserved substitutions between non-polar amino acids (see table 8.8). Discounting these changes, a number of substitutions remain which could potentially account for altered interactions between opsin and retinal, leading to a difference in the λ_{max} of the two photopigments (see table 9.1). These substitutions may be broken down into four groups – sites previously implicated with spectral tuning, sites at which changes in polarity occur (previously unconnected with spectral tuning), substitutions producing potential structural

differences and finally those involving steric effects. The helical positions of the most likely candidates for spectral tuning are illustrated with respect to the retinal chromophore in figure 9.1.

i) Substitutions at previously identified tuning sites

Differences between the goldfish rod and exo-rod opsin sequences occur at three sites previously implicated in spectral tuning – G124A, I217L and G224S (Rod→Exo). Of these, I217L is conserved, but the differences at site 124 and 224 bear closer inspection.

Site 124 is close to the glutamic acid counterion at site 113, and has been implicated in short-wavelength shifts (see Chang *et al.*, 1995 and Bowmaker and Hunt, 1999). This site is typically occupied by non-polar residues (alanine or glycine) in long-wavelength opsins and by polar residues in short-wave opsins (serine or threonine). As such the substitution of glycine for alanine appears unlikely to account for the hypsochromic shift observed between the goldfish retina and pineal.

Site 224 has been regarded as a weak candidate for short-wavelength shifts, as it lies on the outer face of transmembrane helix V, facing away from the chromophore-binding pocket. However, this site is occupied by a polar serine or threonine in all the UVS opsins, but by a glycine in the SWS, MWS and rod opsins (Bowmaker and Hunt, 1999). As such, despite its position, the substitution of glycine for serine in goldfish exo-rod opsin forms a strong candidate for the short-wavelength shift observed.

ii) Substitutions involving changes in polarity

A number of substitutions involve changes in polar amino acids. Of particular interest are substitutions involving hydroxyl-bearing side-chains, which may affect the charge distribution of the π-electron system of retinal, altering the band-gap between ground and excited states, and producing spectral shifts.

Two substitutions, S82A and T93V, lead to a loss of a hydroxyl group in exo-rod opsin. Both of these substitutions occur in helix II and face the retinal-binding pocket. Both are in the vicinity of the Glu-113 counterion (especially site 93), and could potentially affect the stability of the Schiff base, altering the λ_{max} of exo-rod opsin with respect to the retinal rod opsin.

In contrast, six substitutions lead to additional hydroxyl groups in exo-rod opsin when compared to the retinal rod opsin. Of these, only three face the retinal-binding pocket, G51S, W136Y and G260S. Site 136 is located near the cytoplasmic side of helix III, at the opposite end of the helix from the counterion, and consequently appears unlikely to directly influence spectral tuning. Sites 51 and 260 are located mid-way through the helices I and VI respectively. Site 51 projects directly into the binding pocket, and thus provides a good candidate for tuning. This site is occupied by a glycine residue in most vertebrate opsins sequences, with the exception of the LWS cone opsins which all possess a polar serine. As such, this substitution is therefore perhaps more suggestive of a long-wavelength shift.

Site 260 is in the vicinity of the binding pocket, but does not project as clearly as site 51 (see figure 9.1). However, the neighbouring site 261 has been implicated as a tuning site within the rod opsins. In addition, most UVS cone opsins possess a polar serine residue at this site. Consequently, the addition of a hydroxyl-bearing residue at site 260 provides a potential candidate for the hypsochromic shift in goldfish exo-rod opsin.

The sites facing away from the retinal binding pocket (A38S, F56S and I213S) should not be excluded, but given the lack of other evidence to support a tuning role, they do appear less likely candidates. The phenyalanine at site 56 is conserved within the rod opsins. It is perhaps interesting to note that amongst the only opsins bearing a polar residue at this site are those of the SWS cone class. Site 213 is also typically non-polar in the majority of opsins, though a polar threonine is present in both human and dolphin rod opsin sequences, the latter of which is significantly short-wave shifted (Fasick et al., 1998).

iii) Substitutions involving structural differences

Upon analysis of the helical amino acids a number of substitutions were noted which involved amino acids bearing side-chains with special structural properties. Such substitutions could potentially lead to alterations in the secondary structure of the opsin, affecting the position of the residues interacting with the retinal molecule.

The substitution of proline for isoleucine at site 112 is of particular interest, as this site neighbours the Glu-113 counterion. Proline produces kinks in the polypeptide chain, and may as such affect the position of the counterion in the goldfish rod

opsin, in turn influencing its stability. The loss of the proline residue at this site may thus produce a tuning effect between the goldfish rod and exo-rod opsins.

A number of substitutions between the rod and exo-rod sequences involve cysteine residues, which may form disulphide bridges and as such influence the secondary structure of the molecule. The addition of cysteine occurs in the exo-rod opsin at site 168, whereas cysteine residues are replaced at sites 39, 165, 304 and 308 with non-polar residues. The effects of such substitutions are difficult to predict, and may be inconsequential. The addition of cysteine at site 168 in the goldfish exo-rod replaces a non-polar alanine, and projects into the retinal binding pocket, potentially providing another means of tuning. None of the other substitutions are in the vicinity of the retinal molecule. However, these substitutions have the potential to alter the structure of the opsin molecule and thus affect which residues are capable of interacting with the chromophore to influence the λ_{max} of the photopigment.

iv) Substitutions affecting steric hindrance

A number of substitutions occur that involve amino acids bearing aromatic side-chains. Although these differences may be less critical in the spectral tuning of photopigments than those residues bearing charged or polar side-chains, they cannot be overlooked as potential tuning sites, as the bulky aromatic side-chains may induce steric hindrance of the retinal chromophore.

There are five sites in at which aromatic residues that occur in the rod opsin are substituted for smaller side-chains in the exo-rod opsin – 45, 88, 133, 162 and 287. However, none of these substitutions project into the retinal-binding pocket, and as such they may be precluded from having any major influence upon the spectral tuning of these photopigments.

v) Summary

In conclusion, seven substitutions provide the best candidates for the short-wavelength tuning observed by MSP (see figure 9.1). Firstly, the glycine to serine substitution at site 224 has been previously implicated in short-wavelength tuning, despite facing away from the retinal-binding pocket. Secondly, four substitutions projecting into the retinal binding pocket involve the loss or addition of hydroxyl-bearing residues (sites 51, 82, 93 and 260), which could potentially produce tuning

effects. The addition of a cysteine at site 168 facing the retinal-binding pocket provides another candidate tuning site. Finally, the substitution of the proline that occurs at site 112 in the rod opsin could lead to structural differences in the vicinity of the counterion, thus affecting the λ_{max} of the opsin.

The presence of two additional polar residues in exo-rod opsin at site 56 and 213 also provide potential tuning sites, but given that these residues face away from the retinal-binding pocket, they must be considered weaker candidates.

In the absence of *in vitro* expression studies and subsequent site directed mutagenesis, the participation of these individual sites in the spectral tuning of the two photopigments is impossible to establish.

SUBSTITUTION	AA	ROD	EXO	FUNCTIONAL EFFECTS
i) Tuning sites				
	124*	G	A	Conserved
	217	I	L	Conserved
	224	G	S	Polar S or T in UVS opsins
ii) Polar Residues				
	82*	S	A	Loss of OH group
	93*	T	V	Loss of OH group
	38	A	S	Addition of OH group
	51*	G	S	Addition of OH group
	56	F	S	Addition of OH group
	136*	W	Y	Addition of OH group
	213	I	S	Addition of OH group
	260*	G	S	Addition of OH group
iii) Structural effects				
	112	P	I	Loss of proline
	168*	A	C	Addition of cysteine
	39	C	L	Loss of cysteine
	165	C	L	Loss of cysteine
	304	C	V	Loss of cysteine
	308	C	I	Loss of cysteine
iv) Steric effects				
	45	F	L	Loss of aromatic side-chain
	88	F	V	Loss of aromatic side-chain
	133	F	V	Loss of aromatic side-chain
	162	F	V	Loss of aromatic side-chain
	287	F	V	Loss of aromatic side-chain

TABLE 9.1. Helical substitutions occurring between goldfish rod and exo-rod opsins. Asterisks denote amino acids facing the retinal binding pocket. The strongest candidates for potential tuning sites are indicated in blue. See text for details.

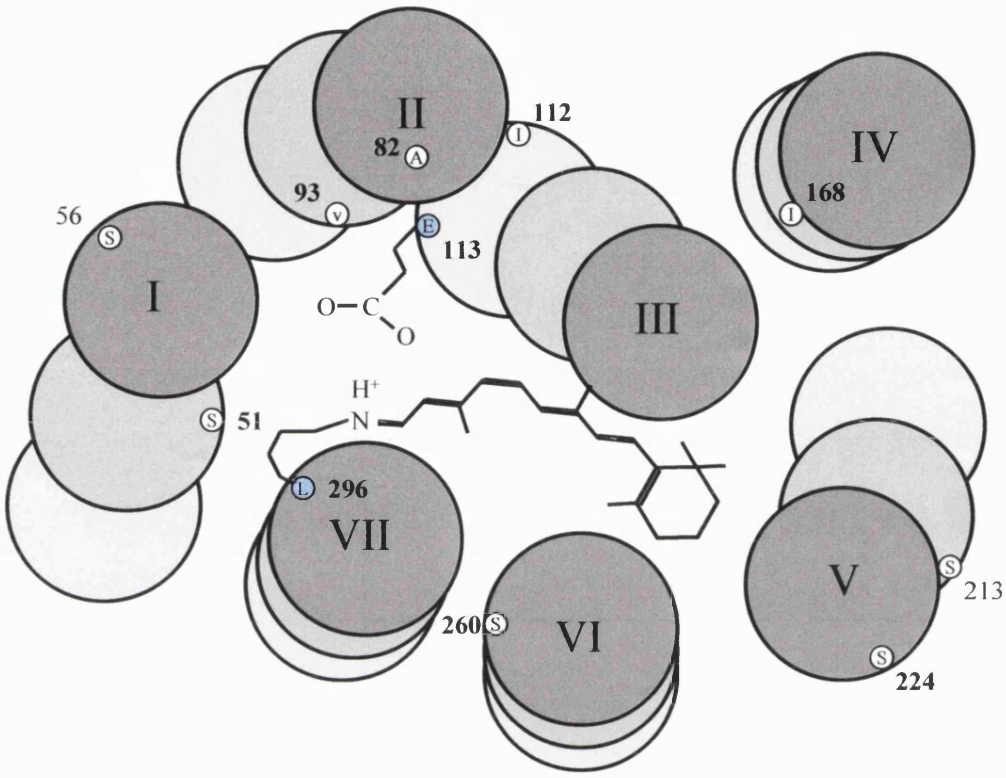


FIGURE 9.1. Schematic helical arrangement demonstrating the location of potential spectral tuning sites within the transmembrane helices of goldfish exo-rod opsin. The strongest candidates for the hypsochromic shift are the loss or addition of hydroxyl-bearing residues at sites 51, 82, 93, 224 and 260. Sites 112 and 168 may also be involved (see text). Sites 56 and 213 provide weaker candidates. The retinal binding site (L296) and glutamate counterion (G113) are also shown. Diagram courtesy of D. Hunt, helical positions based upon Bowmaker and Hunt (1999).

B) INTRACELLULAR INTERACTIONS

Given the sequence similarities between goldfish exo-rod opsin and the many known retinal opsins it appears likely that this opsin is capable of interacting with transducin, to initiate the phototransduction cascade in a similar manner as occurs in retinal photoreceptors. A similar mode of inactivation via light-dependent phosphorylation may also be expected.

However, slight differences between goldfish rod opsin and exo-rod opsin within the intracellular loops may result in subtly altered G-protein interactions, potentially accounting for the differences between retinal and pineal photoresponses (see section 4.2).

i) Transducin interactions

From the alignments shown in figure 8.16 it is evident that certain substitutions appear to occur between the goldfish rod opsin and exo-rod opsin within the intracellular domains. Of particular interest are the differences in the distribution and identity of charged amino acids within the intracellular loops, which are known to be involved in transducin activation.

Of particular importance are the second and third cytoplasmic loops, of which several regions have been shown to be important for transducin binding and activation. Chief amongst these regions are the ERY motif occurring at the intracellular end of helix III (amino acids 134-136) and the QRAEKE motif occurring at the intracellular end of helix VI (amino acids 244-249). Deletions or non-conserved substitutions within these regions result in abolished transducin activation. Another region of the third cytoplasmic loop known to be important in transducin interactions are amino acids 236-239, corresponding to the sequence QQQE in bovine rhodopsin. Deletion of this region results in a 50% decrease in transducin activation (Franke et al., 1992).

In goldfish exo-rod opsin the three aforementioned regions of the second and third loops are conserved. However, when the goldfish rod opsin is compared, there is a notable addition of two charged residues within the QQQE region, namely histidine 237 and glutamic acid 238, which are typically uncharged glutamine residues in goldfish exo-rod opsin and bovine rod opsin. The significance of these substitutions

is difficult to ascertain, but the addition of charged amino acids into a region known to be involved in transducin activation may be indicative of a difference in G-protein interactions. This addition of charged residues in this region only occurs in the goldfish rod opsin and is not a general feature of teleost rod opsins.

Alignments of the other known exo-rod opsin sequences illustrate that many of the differences between goldfish rod opsin and exo-rod opsin are mirrored by the rod opsins and exo-rod opsins of other species (see table 9.2). The presence of identical substitutions between species is more suggestive of a functional difference between these opsin groups.

Substitutions of E64Q, E232A and H315N (rod→exo) all lead to the loss of a charged residue from the intracellular domains of the exo-rod opsin. In contrast, the substitution of N151K results in an additional charged residue in the second intracellular loop of the exo-rod opsin. Differences between charged amino acids also occurs at K231R and K311R, although these substitutions are conserved and less likely to reflect major functional differences. As such, it appears that differences in the distribution and identity of charged amino acids do occur between the retinal rod opsins and pineal exo-rod opsins of teleosts.

What is the functional significance of these intracellular differences? It could be that these substitutions lead to a reduced rate of transducin activation consistent with the increased latency of pineal photoreceptor responses, or alternatively activation of G-proteins other than transducin, resulting in interactions with other intracellular signalling pathways. However, lacking the evidence of detailed biochemical studies, whether any or all of these intracellular differences are actually significant and will lead to alterations in intracellular responses is currently just speculation.

Finally, as mentioned in section 9.2A, differences also occur in the number and distribution of cysteine residues within the transmembrane helices of the rod and exo-rod opsins. Such substitutions may be indicative of differences in the secondary structure of these proteins. Given the rearrangement of the helices that occurs following the photoisomerisation of retinal, these differences could also produce effects upon the interactions between opsin and transducin.

INTRACELLULAR LOOP	AMINO ACID	ROD	EXO	FUNCTIONAL DIFFERENCE
1	64	**E**	Q	Loss of charge
2	136	W	Y	ERY motif
	151	N	**K**	Addition of charge
3	231	**K**	**R**	Conserved substitution
	232	**E**	A	Loss of charge
	237	**H**	Q	QQQE motif. GF only
	238	**E**	Q	QQQE motif. GF only
4	311	**K**	R	Conserved substitution
	315	**H**	N	Loss of charge

TABLE 9.2. Cytoplasmic loop substitutions between rod and exo-rod opsins potentially involved in G-protein interactions. Substitutions occur in all known exo-rod opsins unless otherwise noted. Charged amino acids indicated in bold.

ii) Light-dependent phosphorylation

Deactivation of photoexcited opsin is achieved through phosphorylation of serine and threonine residues on the carboxyl terminus and third cytoplasmic loop by rhodopsin kinase (RK). Following this arrestin (also known as 48K protein or S-antigen) binds to the phosphorylated opsin, sterically blocking transducin activation (see section 3.4).

Three serine residues in the C-terminus of bovine rod opsin have been implicated as the preferred sites for RK phosphorylation – S334, S338 and S343 (Baylor and Burns, 1998). These residues are all present in goldfish exo-rod opsin, suggesting that it is inactivated in a similar manner. In the teleost rod opsins, assuming a similar preference for phosphorylation sites, it appears that these residues are displaced by one amino acid toward the C-terminus. Although the teleost rod opsins do appear to contain slightly more serine and threonine residues in their C-termini (2 extra in the goldfish), it should be noted that the presence of a serine or threonine residue does not automatically imply a phosphorylation site (Shichida and Imai, 1998), and there is currently no evidence to suggest that these extra residues play any functional role in the deactivation of the photoexcited opsin.

Serine 240 in the third intracellular loop has also been demonstrated to form a substrate for RK (Applebury and Hargrave, 1986). This residue is also conserved in goldfish exo-rod opsin, and in all the teleost exo-rod and rod sequences investigated.

In conclusion, it would appear that exo-rod opsin is inactivated by light-dependent phosphorylation by RK in a similar manner to the retinal rod opsins.

iii) Potential differences in phototransduction

Given the potential of phototransduction-induced hyperpolarisation to inhibit NAT synthesis (and thus melatonin production) indirectly through lowering intracellular calcium levels, are there really any special adaptations necessary for a pineal photopigment?

Given the broad similarities within the regions involved in spectral tuning, transducin binding and inactivation demonstrated by the goldfish exo-rod opsin molecule, no major differences in molecular function are apparent. Instead, the differences between the retinal rod and exo-rod opsins are suggestive of subtle molecular adaptations to different photosensory tasks. Despite this, the same basic

photopigment plan, interacting with a similar phototransduction cascade appears to exist in both retina and pineal. It is perhaps an indication of the importance of the neural output of the teleost pineal that the hyperpolarizing response characteristic of retinal photoreception remains so well conserved in pineal photoreceptors.

As in retinal rods and cones, differences in the constituents of the phototransduction cascade may also occur within the pineal. Such factors could account for the differences observed between retinal and pineal photoreceptors at the electrophysiological level, such as the slower onset of hyperpolarisation, slower rate of recovery and differences in light adaptation.

As the opsin molecule is an exquisitely adapted light-detector, capable of efficiently transducing photon-absorption into an intracellular second messenger cascade, it seems probable that elements of the cascade would have had more scope for divergence, rather than the functionally-constrained photopigment. As such it would seem more likely that differences in the elements of the cascade such as transducin, GCAP, RetGC, Arrestin and RK, as well as the intracellular messengers such as Ca^{2+}, cGMP and cAMP, would be the factors that differ between visual and non-visual photoreceptors. In addition, other elements may have evolved in non-visual photoreceptors, adapting the existing phototransduction cascade to produce modified responses and outputs to suit their differing role.

Evidence for such differences between visual and non-visual photoreception does exist - examples include the antagonistic cGMP pathway in the lizard parietal eye (Xiong *et al.*, 1998), evidence for a transducin-independent pathway responsible for photoentrainment of melatonin rhythms in chicken pineal photoreceptors (Okano and Fukada, 1997), and the presence of voltage-sensitive Ca^{2+} channels in teleost pineal photoreceptors (Falcon, 1999, and see section 4.4).

As such, it should not be overlooked that these intracellular factors are likely to account for many of the differences exhibited by non-visual photoreceptors rather than extensive differences between the photopigment molecules themselves.

9.3. Evolution of Teleost Rod Opsins

Unlike all other vertebrate opsin genes, the rod opsin of teleost fishes lacks introns. This intronless rod opsin may have arisen via a gene duplication of the ancestral rod opsin gene followed by a reverse transcription event leading to incorporation of an intronless rod opsin cDNA into the genome by homologous recombination. It has been suggested that the ancestral gene was either lost or assumed an alternative function (Fitzgibbon *et al.*, 1995). Exo-rod opsin thus provides an ideal candidate for this ancestral rod opsin, having been adopted for a non-visual role in the pineal organ.

In this manner, it would follow that it is the retinal rod opsin of teleosts rather than the exo-rod opsin that represents the more recently diverged and thus more 'novel' opsin. The rod opsin appears to have arisen from the duplication and subsequent divergence of the ancestral rod opsin, having uniquely amongst opsins, lost its introns *en route*.

A) The Divergence of the Teleost Rod Opsins

It has been suggested that the duplication of the ancestral teleost rod opsin must have occurred early in the lineage of the Actinopterygii (ray-finned fishes) after their divergence from the Sarcopterygii (lobe-finned fishes) such as the coelacanth. Based upon the rate of sequence divergence the time of this gene duplication has been placed at around 205 million years ago in the early Jurassic period (Philp *et al.*, 2000a).

The subsequent evolution of the two teleost rod opsins may be thought of as having occurred along similar lines to the evolution of colour vision (as described in section 3.3B), with the initial gene duplication followed by a subsequent sequence divergence and specialisation to differing photic tasks.

The original gene duplication event must have included the upstream regulatory elements of the ancestral rod opsin, or a continuation of photoreceptor-specific expression is unlikely to have been maintained. The subsequent sequence changes occurring in these regulatory regions will lead to different expression patterns of rod opsin and exo-rod opsins, but given their common ancestry some similarities in expression pattern may occur if as long as they do not yield a selective disadvantage.

The evolution of such tissue-specific regulation may therefore vary between species, depending upon the different selective pressures they have been exposed to (see section 9.5).

The reason that the novel intronless rod opsin was preferentially expressed in the retina rather than the intron-containing rod opsins of other vertebrate species remains a mystery. It could be that one of the ancestral rod opsins acquired a mutation leading to a coding change providing a selective visual advantage in the ancestral light environment, and was thus selected for retinal expression. Alternatively, there may be an advantage inherently associated with the lack of introns, such as reduced post-transcriptional modification allowing either a conservation of energy or increased opsin turnover (Gentles and Karlin, 1999).

9.4. Significance of Retinal Opsin Expression

The detection of retinal opsin expression in addition to the non-visual exo-rod opsin raises many questions regarding the function of these photopigments in the pineal, particularly given the discovery of just a single population of pineal photoreceptors by MSP.

a) Possible Explanations for Presence of Retinal Opsins

The presence of visual opsins may be explained in a number of ways, which when placed in the context of this study and previous work conducted on pineal photosensitivity, may appear more or less plausible.

i) Low incidence

The essential question raised by the presence of the retinal opsins in the goldfish pineal is that of whether these opsins are expressed in a different photoreceptor population. If this is the case, these non-rod-like populations must occur with an extremely low frequency, given that a total number of 152 photoreceptors were recorded from the goldfish pineal (including both native and regenerated records) and no suggestion of any other spectral class of photoreceptor was detected.

Obviously, given a low incidence of cone opsin-containing photoreceptors, random sampling by MSP (even given large sample sizes) is an unreliable means of stating categorically that such a photoreceptor population does not exist. A study utilising *in situ* hybridisation or monoclonal immunocytochemistry could provide a clearer answer to this question. Alternatively, the relative level of opsin expression could be assessed using quantitative PCR techniques.

ii) Different photoreceptor types

A second explanation is that these opsins are expressed in a morphologically different photoreceptor class, and as such they have been overlooked when sampling by MSP. However, the presence of a photoreceptor population so wholly different to all retinal and other pineal photoreceptors so as to be unnoticed seems unlikely. This alternative also appears unlikely given extensive morphological studies of the goldfish pineal providing no suggestions of morphologically different photoreceptor cell types (Takahashi, 1969, McNulty, 1984 and McNulty, 1984).

iii) Non-functional expression

Another possibility is the production of these cone photopigments but their absence from any morphologically distinct outer segment. The photopigment could be localised within the cell body of the photoreceptor, or in modified pinealocyte outer segments, such as those found in some higher vertebrates. In both cases the concentration of these visual photopigments in structures lacking the precise arrangement and density of photopigment found in outer segments would produce a much lower absorbance. Due to the practical limit imposed by the sensitivity threshold of the photomultiplier used on the MSP, true absorbance values below 0.002-0.004 would be too low to detect and thus effectively 'invisible', camouflaged by the background noise of the apparatus.

iv) Untranslated mRNA

The visual opsins may be actually expressed but may be regulated at the level of post-transcriptional modification, and the RNA may consequently never actually be translated into protein. An alternative, yet similar scenario is translation but regulation at the protein level (through degradation or trafficking), as such never forming a functional photopigment.

v) Co-activation by common transcription factors

A final alternative is that the transcription factors regulating the expression of photoreceptor-specific proteins in the pineal organ may also co-activate transcription of retinal opsins. Given the multiplicity of transcriptional factors responsible for correct gene expression in the retina (see Chen *et al.*, 1997, Freund *et al.*, 1997 and Furukawa *et al.*, 1997 for references), the absence of an equally stringent regulatory mechanism in the pineal could account for co-activation of similar genes, as long as their level of expression did not seriously compromise the function of the photoreceptor cell. The minor effects on the absorbance spectrum produced by a low percentage of visual opsin co-expression within a photoreceptor involved in luminance detection would not be detrimental to the effective functioning of such a cell, and would not be possible to detect by MSP. Several recent papers have demonstrated co-expression of visual opsins in retinal photoreceptors, providing evidence that opsin expression is not as mutually exclusive as is usually implied

(Rohlich *et al.*, 1994, Makino and Dodd, 1996, Xiao and Hendrickson, 2000 and Applebury *et al.*, 2000).

B) ARE RETINAL OPSINS FUNCTIONALLY EXPRESSED IN THE PINEAL?

i) Cone opsins

MSP data from this study suggest a single photoreceptor population, with no evidence of cone opsin photoreceptor subtypes. This would correlate with the finding that the goldfish pineal demonstrates a luminance response, with no evidence for spectral discrimination (Meissl *et al.*, 1986).

However, results from Stenkamp and Raymond (1995), using *in situ* hybridisation suggest that the MWS and LWS cone opsins may be expressed in the developing goldfish pineal, although the MWS cone opsin signals appeared less consistent. Kojima and Fukada (1998) also suggest that the LWS opsin may also be expressed in a small subset of pineal photoreceptors in the zebrafish. These studies both suggest a subpopulation of LWS photoreceptors in the pineal organ of cyprinids.

Little evidence exists for the presence of UVS and MWS cone opsins in the goldfish pineal, and as such the detection of these opsins by PCR in this study appears likely to be due to co-activation at background levels. However, the evidence for LWS cone opsin requires further consideration. The LWS opsin of the goldfish is particularly long-wave sensitive with a λ_{max} of 618 nm, primarily due to the bathochromic shift produced by the A_2 chromophore. However, even in the A_1 form, the λ_{max} is around 566 nm. This long-wave sensitivity introduces a technical problem with MSP, as tissue is prepared under dim-red light. In retinal MSP this problem may be reduced by rapid dissection using the bare minimum of light. However, due to the size of the pineal organ dissection takes longer and requires significantly more light, the result of which is that if such a LWS cone opsin population does occur in the goldfish pineal, light exposure during tissue preparation will result in it being bleached. This problem was circumvented by reconstitution with 9-*cis* retinal, yielding a LWS opsin of λ_{max} 526 nm, low enough to rule out any significant bleaching. However, it does mean that the sample population potentially containing unbleached LWS photoreceptors is smaller than that of other opsins.

In conclusion, the results of this study do not preclude the presence of a LWS cone opsin population, but if such a population does occur it must account for only a small fraction of the pineal photoreceptors in this species. Assuming the goldfish pineal is functioning primarily as a luminance detector, the expression of the LWS opsin would broaden the spectral operating range. Although the presence of at least two photopigments in the goldfish pineal would potentially allow spectral discrimination, given the absence of a chromatic response in this species (Meissl *et al.*, 1986), and the lack of any evidence for the neural mechanisms necessary to compare the relative outputs of the photoreceptors (Meissl and Brandstatter, 1992), spectral discrimination in the goldfish pineal appears unlikely.

ii) Rod opsin

The presence of a rod opsin-containing photoreceptor population is more difficult to rule out due to the predicted similarity in λ_{max} between rod opsin and exo-rod opsin. The distribution of the λ_{max} of 9-*cis* retinal reconstituted photoreceptors provides no evidence of bimodality, so although both the rod opsin and exo-rod opsin are expressed in the goldfish pineal, they are not localised to separate photoreceptor populations. The retinal rod opsin may actually be present in the goldfish pineal, but co-expressed with exo-rod opsin in pineal photoreceptors. In this case the λ_{max} observed in the pineal could represent a mixture of rod and exo-rod opsins, and the true λ_{max} of the exo-rod opsin alone may be further short-wave shifted. Such a mixture of opsins may be expected to produce a broadening of the absorbance spectrum. However, if the λ_{max} values of the two pigments are close together (within 10-20 nm) distortion of the absorbance spectra is only apparent when the proportions of the two pigments are nearly equal (Knowles and Dartnall, 1977). Consequently, the observed goldfish pineal isorhodopsin λ_{max} could potentially be produced by co-expression of the rod opsin and an exo-rod opsin with a further short-wave shifted λ_{max}. In the absence of double-labelling studies, such a theory is difficult to prove or refute. However, as mentioned above, co-expression of opsins does appear to occur far more frequently than previously thought.

In the zebrafish, Mano *et al.* have demonstrated via *in situ* hybridisation that exo-rod opsin is not expressed in the retina, whereas the retinal rod opsin is not expressed in

the pineal. The demonstration of rod opsin expression in the goldfish pineal reported in this study could be down to several explanations. Firstly, the sensitivity of the probes used in *in situ* studies must be considered, as if expression levels are low a visible hybridisation signal will not be evident. In contrast, due to the extremely sensitive nature of PCR and the exponential amplification it involves, even a very low level of rod opsin mRNA will allow considerable amplification, yielding a visible product.

However, Philp *et al.*, 2000 have demonstrated using tissue-specific PCR that the retinal rod opsin of salmon is not expressed in the pineal organ of this species. In the light of this work it would therefore appear that differences in the mechanisms regulating retinal and extra-retinal opsin expression may occur between species, depending upon the exact role served by the pineal. In this respect, the Salmonid pineal is of interest, for as well as lacking an intrapineal oscillator, the trout pineal is one of the few species in which a chromatic response has been demonstrated (see section 4.4B).

iii) Regulation of tissue-specific opsin expression

Assuming the retinal rod opsin arose as a duplication of an ancestral rod opsin that in turn gave rise to exo-rod opsin, the two genes would be expected to share certain transcription factors. The recruitment of the intronless rod opsin for retinal expression must have led to the development of differing transcriptional regulation in the eye and pineal. Given the stringent regulatory mechanisms involved in opsin expression to specific photoreceptor subtypes that occur in the retina (see for example, Wang *et al.*, 1999), this could account for the absence of exo-rod opsin in the retinal tissues. In contrast, a less-stringent mechanism regulating opsin expression in the pineal could plausibly lead to expression of the retinal rod opsin.

The presence of complex mechanisms regulating the expression of photoreceptor-specific genes is also evident from the development of the complex morphology of the retina such as the photoreceptor mosaic. The development of the retinal photoreceptor mosaic demonstrates highly ordered spatial and temporal patterns of opsin expression, thought to involve cell-cell interactions (Stenkamp *et al.,* 1996, Stenkamp *et al.*, 1997, Schmitt *et al.*, 1999 and Cook and Chalupa, 2000). Although

the pineal undoubtedly exhibits mechanisms to regulate the expression of opsin, it has no requirement for the precise spatio-temporal expression patterns of the retina. In this respect, a less-stringent regulation of opsin expression appears more plausible. Furthermore, the presence of elements of the phototransduction cascade does not necessarily imply functional photosensitivity, as demonstrated in the mammalian pineal (see Korf, 1994 and Foster and Provencio, 1999).
Given that the primary role of the pineal appears to be in irradiance detection, the impact of visual opsin expression within pineal photoreceptors would not be expected to produce a selective disadvantage. In fact, co-expression of opsins with differing λ_{max} values may actually serve to broaden the spectral sensitivity of the photoreceptor, potentially providing an advantage in luminance detection.

Obviously, these arguments are rather speculative given the lack of knowledge relating to the mechanisms regulating opsin expression in both eye and pineal. However, they provide a tentative theory for the apparently contradictory results observed within this study. Further work is obviously required.

9.5. Inter-species Differences in Pineal Photosensitivity

Following the identification of exo-rod opsin in a growing number of teleost species, it was also of interest to address the question as to how pineal photosensitivity may differ between species.

a) Comparative Pineal MSP

Following MSP of pineal photoreceptors in the Mexican tetra (*Astyanax fasciatus)* and the golden orfe (*Leuciscus idus)*, again only single photoreceptor populations were apparent. The results from both species are broadly similar to the respective retinal rod opsins, although the exact λ_{max} values observed were subtly shifted.

i) Astyanax fasciatus

The pineal photoreceptors of *Astyanax* demonstrated a λ_{max} of 510 nm, most closely fitting a 50% A_1/50% A_2 template. The rods of the *Astyanax* retina have a λ_{max} of 519 nm, and typically contain around 70% A_1/30% A_2 chromophore. As such, the pineal photoreceptors demonstrate a broadly similar chromophore content to the retina, suggesting that the pineal photopigment is short-wave shifted with respect to the retinal rod opsin. Given the rod-like nature of this pineal photopigment, it appears likely that it represents the *Astyanax* exo-rod opsin.

ii) Leuciscus idus

As noted previously the pineal photoreceptors of the golden orfe were fewer in number and noticeably smaller than those of either *Astyanax* or the goldfish. However, a photopigment with λ_{max} 529 nm was measured (A_2), and although the data is not as accurate at in the other species, a significant bathochromic appears to occur between the pineal pigment and the retinal rod, which has a λ_{max} of 518 nm (A_1). However, given that the retinal rods predominantly contain A_1-based photopigments, this shift could be accounted for by a difference in the chromophore content of the pineal in this species.

B) INTER-SPECIES DIFFERENCES IN EXO-ROD OPSIN

Given the rod-like nature of the photopigments measured from the pineal of *Astyanax* and the orfe, it would appear likely that the pineal photopigment of these species is also an exo-rod opsin.

Based upon current evidence it would therefore appear that exo-rod opsin is the predominant photopigment of the pineal organ of teleosts. The demonstration of exo-rod opsin in zebrafish, medaka, eel, salmon, fugu, carp and now goldfish, along with MSP evidence of a rod-like opsin in the pineal of *Astyanax* and orfe, all support this hypothesis. Furthermore, these findings correlate with previous electrophysiological and labelling studies in a wide variety of teleost species (see section 5.1A).

i) Spectral tuning of exo-rod opsins

The data from this study and previous electrophysiological work both suggest that the spectral sensitivity of these pineal exo-rod opsins may vary between species, in a similar manner to the variations in λ_{max} observed between the retinal rod opsins of teleosts (see Bowmaker, 1995).

A closer inspection of the known exo-rod opsin sequences provides some suggestions as to the basis of different spectral tuning between these photopigments. As shown in table 8.9, a number of substitutions occur between the known exo-rod opsin sequences. Of these differences, three are apparent at recognised tuning sites – sites 164, 224 and 261. Substitutions at these sites only occur in the exo-rod opsin of the salmon, and are suggestive of a long-wavelength shift in pineal sensitivity in this species, as previously suggested by Philp *et al.*, 2000.

It is also interesting that the serine at position 224, suggested to be of importance in the hypsochromic shift in the goldfish pineal, also occurs in both zebrafish and fugu exo-rod opsins. If this site is involved in the short-wave tuning of exo-rod opsin, then the pineal photopigments of these species would also be expected to demonstrate a short-wave shift in sensitivity, as is the case in the goldfish.

Sites 51, 168 and 260 also exhibit differences between the known exo-rod opsins in the vicinity of the retinal-binding pocket. As noted in section 9.2A, these three substitutions all provide candidates for spectral tuning, and as such it would appear likely that the exo-rod opsins of these species will exhibit different λ_{max} values.

Further MSP studies on the pineal photoreceptors of these species could provide evidence to support the involvement of these substitutions in the spectral tuning of the exo-rod opsins.

ii) Reasons for differential tuning

These variations in pineal photosensitivity may reflect the not only the habitat and behaviour of the species, but also the transmission of the tissues overlying the pineal, as well as the role the pineal plays within the circadian organisation of the species.

The circadian axis of teleosts like any other species would be expected to adapt to the environment in which the animal must survive and propagate. Consequently, given the broad range of ecological niches in which teleosts are found, differences in the function of the pineal, and therefore its spectral sensitivity and photopigment complement are to be expected.

9.6. Further Studies

As is ever the case, the results of this study raise many more questions than they answer. Although recent advances in the field of circadian biology and non-visual photoreception have shed much light on the many and varied photopigments expressed in both retinal and extra-retinal tissues, functional evidence for these pigments being involved in specific physiological roles is often lacking. Questions relating to the evolution of these pigments, as well as how they may act at the molecular and cellular level to regulate temporal physiology all provide an opportunity for further work.

a) Questions Raised

The following are just a selection of questions raised over the course of this work.

1. Are the retinal rod and exo-rod opsin co-expressed in the pineal photoreceptors of the goldfish, or are they expressed in separate photoreceptor populations?
2. Does a small subpopulation of LWS pineal photoreceptors exist within the goldfish pineal, and if so with what frequency?
3. Are homologues of VA opsin and parapinopsin expression in goldfish pineal?.
4. Is exo-rod opsin expression confined to the pineal alone?
5. Do differences occur between rod and exo-rod opsin G-protein interactions? Can these differences be correlated to any of the amino acid substitutions that occur?
6. Are the elements of the phototransduction cascade in pineal photoreceptors rod or cone-like, or do alternative pineal isoforms occur?
7. How are exo-rod opsins tuned in individual species? Can behaviour, habitat and/or the transmission of the overlying tissues be related to the tuning of pineal photopigments?
8. How is the transcription of opsins regulated in the teleost pineal? What transcription factors and regulatory sequences are common to both pineal and retinal photoreceptors?
9. What mechanisms are involved in the photic entrainment of the pineal oscillator regulating melatonin synthesis? Which photopigment mediates this entrainment pathway?

10. Are homologues of the vertebrate clock genes found in teleosts, and more specifically what is their localisation and expression profile in the pineal?
11. What other G-proteins are expressed in teleost pineal photoreceptors?
12. How is the retinoid content of pineal photoreceptors regulated? Are similar mechanisms involved in photopigment regeneration in the retina in the absence of the RPE?
13. What is the temporal and spatial pattern of non-visual photopigment expression? Are different opsins expressed in juvenile and adult stages? Does a bathochromic shift in photosensitivity or a regression of pineal photosensitivity occur with age as the overlying tissues reduce transmission?
14. In species exhibiting chromatic responses, is secondary photoreceptor population present? Or, alternatively does the photopigment utilise a invertebrate-like pathway for photopigment regeneration or a cGMP–based antagonistic mechanism as in case of reptilian parietal eye?

B) STUDIES PROPOSED

To determine whether separate rod, exo-rod and LWS cone opsin photoreceptor populations occur within the goldfish pineal studies utilising *in situ* hybridisation or monoclonal immunocytochemistry are required. Such studies should utilise double-labelling to determine if opsin expression is confined to separate and distinct photoreceptor populations or whether co-expression of opsins occurs. Such a study should be conducted in juvenile and adult individuals, to determine if the opsin content of the teleost pineal varies with age. A potential alternative, though not providing the spatial resolution of labelling studies, is quantitative PCR to determine the relative levels of expression of both visual and non-visual opsins within the pineal.

Furthermore, screening for other known non-visual opsins should be conducted on the goldfish pineal, particularly homologues of VA opsin and parapinopsin.

Expression of goldfish rod and exo-rod opsins as functional photopigments is necessary to determine if the hypsochromic shift of exo-rod opsin suggested by MSP of pineal photoreceptors occurs in the purified opsin. If this hypothesis is supported,

then site-directed mutagenesis may enable the determination of the amino acid(s) responsible for this short-wave tuning.

Analysis of the upstream 5' regions of the rod and exo-rod opsins may allow determination of the presence of any similar transcription factor binding sites. Use of techniques such as DNA footprinting or band-shift assays may allow differences in transcription factor binding profiles to be determined.

Biochemical investigations of the kinetics of rod and exo-rod opsin interactions with intracellular signal-transduction pathways may enable differences between retinal and pineal transduction to be elucidated. Potential differences between relative binding and activation of rod/cone transducins and phosphorylation by rhodopsin kinase may prove informative. Furthermore, screening pineal cDNA for homologues of the retinal phototransduction cascade and alternative G-proteins may suggest potential differences between visual and non-visual photoreception.

9.7. EVALUATION OF STUDY

A) ANSWERS TO QUESTIONS POSED

The following section is intended to address the questions proposed at the outset of this study, and to determine what insights this work has actually provided regarding the nature of the photopigment content of the teleost pineal.

i) What is the spectral sensitivity of teleost pineal photoreceptors?

In the goldfish the λ_{max} of the pineal photoreceptors is 511 nm. Considerable differences occur between individual cells, primarily due to chromophore variations. Removal of chromophore variability was therefore conducted by reconstitution with 9-*cis* retinal, the results of which suggest that the pineal photopigment of goldfish is short-wave shifted with respect to the retinal rod opsin.

ii) Does spectral matching to the intracranial light environment occur?

Transmission recordings of the goldfish skull demonstrate that a considerable amount of light of a broad spectral range is able to penetrate to the level of the pineal end vesicle. Although transmission increases with wavelength, pineal photoreceptors do not appear to be tuned to this long-wavelength region of maximum transmission. The λ_{max} of goldfish pineal photoreceptors does however fall within the transmission window of haemoglobin (490 – 530 nm), which may be an important factor due to the blood vessels surrounding the pineal end-vesicle.

iii) Do multiple photoreceptor populations exist within the teleost pineal?

Only a single photoreceptor population spectrally was detected within the pineal of the goldfish by MSP. Other studies have suggested the presence of other photoreceptor populations, notably the LWS cone opsin. Using tissue-specific PCR the retinal rod and cone opsins were detected in the goldfish pineal. The failure to detect other photoreceptor populations by MSP could be due to a number of possible reasons, including the absence of separate spectrally-distinct photoreceptor populations in the pineal, that a cone opsin-containing photoreceptor population occurs only as a tiny percentage of the total pineal photoreceptor population, or that

the opsin expression detected by PCR may reflect incidental co-activation of opsin expression by similar transcription factors.

iv) Are pineal photopigments the same as the visual opsins of the retina?

Expression of retinal opsin mRNA does appear to occur within the pineal of the goldfish. Whether this reflects the presence of small populations of functional spectrally distinct photoreceptors or co-activation by similar transcription factors remains unclear.

v) If different, do these pigments represent novel non-visual opsins?

Based upon spectral data from MSP and sequence isolation, the predominant photopigment in the goldfish pineal appears to be exo-rod opsin. Exo-rod opsin is a non-visual photopigment that occurs uniquely in the pineal of teleosts and not in the retina.

vi) How do non-visual opsins differ from visual opsins at the molecular level?

Exo-rod opsin bears a striking sequence homology to the retinal rod opsin (74%), and is likely to have arisen as a gene duplication of the ancestral rod opsin. The two teleost rod opsins are then expected to have diverged and specialised to mediate different photosensory tasks.

vii) Can any molecular differences account for the differences between visual and non-visual photoreception?

Due to the lineage of exo-rod opsin as part of the rod opsin group many of the molecular characteristics of rod opsins are maintained. Analysis of the goldfish exo-rod opsin sequence suggests that a number of substitutions, either alone or in concert, could account for the slight short-wavelength shift suggested by MSP recordings from pineal and retinal photoreceptors. Differences in the number and identity of charged amino acids also exist within the intracellular domains of the exo-rod opsin, and these substitutions may provide potential effects on transducin binding and activation.

viii) How does pineal photosensitivity differ between teleost species?

Comparative data from *Astyanax* suggest a similar pineal photoreceptor sensitivity as occurs in the goldfish (λ_{max} 510 nm), again with just a single photoreceptor population. Pineal photoreceptors in *Astyanax* appear similarly developed to those of the goldfish. Studies of the golden orfe illustrate smaller and more degenerate photoreceptors, and as such the quality of the data is not as high. Again a single photoreceptor population appears to exist, although the spectral sensitivity of these cells is red-shifted in comparison to the pineal photoreceptors of the goldfish and *Astyanax*, with a λ_{max} of 531 nm.

B) NEW HYPOTHESES

In the light of the data obtained in this work the initial hypothesis proposed, although correct in certain respects, must be rejected. From the insights gained from this work, the following hypotheses concerning the photopigment content of the teleost pineal is proposed.

1. *The predominant photopigment of the teleost pineal organ is the recently identified non-visual opsin, exo-rod opsin. Exo-rod opsin belongs to the rod opsin gene family, and has evolved as a gene duplication of the ancestral rod opsin, prior to the reverse transcription event that has led to intron loss from the retinal rod opsins.*
2. *The spectral sensitivity of exo-rod opsin is similar to that of the retinal rod opsin. However, the λ_{max} may be slightly short or long-wave shifted respective to the rod opsin, depending on species. This may be expected to reflect the habitat and behaviour of the species, as well as the transmission of overlying tissues (if limiting), along with the role mediated by pineal photoreception in each species.*
3. *As a consequence of their ancestry the retinal rod and exo-rod opsin genes may share certain transcription factors regulating their expression. Due to the complex visual tasks mediated by the retina, photoreceptor-specific gene expression may be more stringently regulated. Differences in the mechanisms regulating tissue-specific gene expression may account for the presence of visual opsins detected in the pineal organ in this study.*

CHAPTER 10

CONCLUSIONS

MSP was conducted on the pineal photoreceptors of the goldfish (*Carassius auratus*), identifying a single photoreceptor population with λ_{max} 510 nm (50% A_1/50% A_2). This value most closely resembled that of the retinal rods, which demonstrate a λ_{max} of 522 nm (A_2). Individual variations between photoreceptor λ_{max}, whilst not indicative of multiple photoreceptor subtypes, were suggestive of A_1/A_2 chromophoric variation.

To remove this variability, pineal photoreceptors were bleached and the photopigment contained therein was reconstituted with a single artificial chromophore, 9-*cis* retinal. This again revealed a single photoreceptor population, with an isorhodopsin λ_{max} of 484 nm (A_1). Data from the goldfish retina demonstrate that the retinal rod isorhodopsin has a λ_{max} of 490 nm (A_1), demonstrating that although spectrally similar, the pineal photopigment is 5-7 nm short-wave shifted from the retinal rod opsin. The variation observed between individual photoreceptor λ_{max} was also reduced following reconstitution, confirming the theory that these variations in the native state are produced by differences in chromophore content.

Spectrophotometry of the goldfish skull demonstrated significant transmission of all wavelengths, with a slight attenuation at shorter-wavelengths. The λ_{max} of the pineal photopigment lies within the transmission window of haemoglobin, which may be influential in the spectral tuning of pineal photopigments. However, as the transmission of the skull is not particularly limiting in the goldfish, tuning to the intracranial light environment is difficult to ascertain.

Molecular screening for opsins in the goldfish pineal revealed that the retinal rod opsin was in fact expressed in the pineal, along with a goldfish homologue of the pineal rod-like opsin described by Mano *et al.* (1999) and Philp *et al.* (2000). This goldfish exo-rod opsin shares 74% amino acid identity with the retinal rod opsin, and possesses all the key characteristics of a functional opsin. Goldfish exo-rod opsin contains several substitutions that provide candidates for spectral tuning, that could potentially account for the hypsochromic shift observed by MSP. Several

substitutions were also observed in the intracellular domains, suggesting potential differences in G-protein interactions.

From phylogenetic analysis it appears that exo-rod opsin originated from a gene duplication of the ancestral rod opsin early in the teleost lineage, which must have occurred prior to the intron loss event described by Fitzgibbon *et al.*, as the gene structure of goldfish exo-rod opsin is identical to that of other vertebrate opsins.

Tissue-specific PCR illustrated that in addition to the retinal rod opsin, UVS, MWS and LWS cone opsins were also expressed in the goldfish pineal. The presence of these opsins does not necessarily imply a functional cone opsin photoreceptor population, for as well as the MSP evidence of this study, labelling and electrophysiological studies provide little evidence for a separate cone opsin pineal photoreceptor population in the goldfish, except in the case of the LWS cone opsin. In the light of recent studies, it could be that these visual opsins are be co-expressed in the same photoreceptors as exo-rod opsin, particularly in the case of the retinal rod opsin, due to its common ancestry with exo-rod opsin. However, the presence of a separate LWS cone-opsin photoreceptor population accounting for a small fraction of the total photoreceptor content cannot be ruled out.

MSP of the pineal photoreceptors of the Mexican tetra (*Astyanax fasciatus*) and the golden orfe (*Leuciscus idus*) was also conducted. The pineal of *Astyanax* contains a single photoreceptor population, with λ_{max} 511 nm (50%A_1/50% A_2), compared with the retinal rod opsin λ_{max} of 519 nm. The pineal of the orfe also contains a single photoreceptor population with λ_{max} 531 nm (A_2), in contrast to the retinal rod opsin λ_{max} of 518 nm (A_1).

The similarity in pineal photosensitivity between these species and the goldfish suggests the presence of an exo-rod opsin homologue. However, the spectral tuning of these photopigments appears to differ from that of the goldfish exo-rod opsin. Such differences in spectral tuning have been extensively characterised in the retinal rod opsins, and also appear to occur in the exo-rod opsins sequenced to date. These differences in non-visual tuning may reflect habitat, behaviour, light transmission of the overlying tissues and the role mediated by the pineal in each species.

APPENDIX A

AMINO ACID PROPERTIES

AMINO ACID	SINGLE LETTER	THREE LETTER	CHARGE	NATURE OF SIDE CHAIN
Alanine	A	Ala	0	Non-polar
Cysteine	C	Cys	0	Polar uncharged, Sulf
Aspartic Acid	D	Asp	-	Charged:acidic, OH
Glutamic Acid	E	Glu	-	Charged: acidic, OH
Phenylalanine	F	Phe	0	Non-polar, aromatic
Glycine	G	Gly	0	Non-polar
Histidine	H	His	+	Charged: basic
Isoleucine	I	Ile	0	Non-polar
Lysine	K	Lys	+	Charged: basic
Leucine	L	Leu	0	Non-polar
Methionine	M	Met	0	Non-polar, Sulf
Asparagine	N	Asn	0	Polar uncharged
Proline	P	Pro	0	Non-polar, Kinked
Glutamine	Q	Glu	0	Polar uncharged
Arginine	R	Arg	+	Charged: basic
Serine	S	Ser	0	Polar uncharged, OH
Threonine	T	Thr	0	Polar uncharged,
Valine	V	Val	0	Non-polar
Tryptophan	W	Try	0	Non-polar, aromatic
Tyrosine	Y	Tyr	0	Polar uncharged, aromatic, OH

APPENDIX B

BUFFERS AND OTHER SOLUTIONS

Ampicillin

A 50 mg/ml stock solution was made in dd H_20 and filter sterilised through a 0.22 micron filter. This stock solution was stored at –20°C.

Denaturing Solution

43.83 g	sodium chloride (1.5 M)
10 g	sodium hydroxide (0.5 M)

Made up to 500ml in dd H_20.

IPTG (100 mM) Stock Solution

A 24 mg/ml stock solution of IPTG was made up in dd H_20, and filter sterilised through a 0.22 micron filter. This stock solution was stored at –20°C.

LB & Ampicillin

100 μg/ml of ampicillin was added to cooled (approximately 50°C) autoclaved LB.

Loading Buffer for Agarose Gels

For 30 ml of 10 x buffer:

7.6 g	ficoll (Mr 400 kD)
0.75 g	Orange G, 0.25 M.

Loading Buffer for Sequencing Reactions

For 39 ml 10 x buffer:

12 ml	glycerol
0.06 g	bromophenol blue
0.06 g	xylene cyanol FF
6 ml	50 x TAE

Luria Bertani (LB) Medium

For 1 litre of LB broth:

10 g	tryptone
10 g	sodium chloride
5 g	yeast extract

Made up to 1 litre with dd H_20.

NE Buffer

150 mM	sodium chloride
25 mM	EDTA

Neutralising Solution

43.83 g	sodium chloride
30.28 g	Tris pH 7.2 (0.5 M)
186 g	EDTA (0.001 M)

Made up to 500 ml in dd H_20.

TAE Buffer

For 500 ml 50 x buffer:

121 g	Tris (0.04 M)
28.6 ml	glacial acetic acid
9.3 g	EDTA pH 8.0 (0.001 M)

Made up to 500 ml in dd H_20.

TBE Buffer

For 1 litre of 10 x buffer:

108 g	Tris (0.045 M)
55g	boric acid
9.5 g	EDTA (0.001 M)

Made up to 1 litre in dd H_20.

TE Buffer

For 1 litre of 10 x buffer:

12.11 g Tris (0.01 M)

3.7 g EDTA (0.001 M)

Made up to 1 litre in dd H_20.

X-Gal (5%) Stock Solution

A 50 mg/ml stock solution of X-Gal was made in N, N' dimethyl-formamide in a sterile glass bottle covered with silver foil and stored at –20°C.

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