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Distribution and the circadian regulation of Gq/11α proteins in the vertebrate retina

by

Shima Kassirian

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmacology
University of Toronto

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Abstract

Recent studies have demonstrated that the retina of mammals contain an intrinsic circadian clock. The molecular components of this machinery have not been entirely identified. The expression of some signal transduction proteins have been shown to oscillate over a 24 hour light-dark cycle and as such they may play an important role in circadian regulatory processes. I have used this system to explore the cellular distribution and circadian regulation of Gq/11α in rat retina.

Gq/11α was found to be expressed in the inner and outer plexiform layers of the retina. Quantitation of Gq/11α and Gtα concentrations during light-dark cycles indicate that their concentrations change in a cyclic manner. The regulation of Gq/11α and Gtα were determined to be circadian in nature by quantitating G protein concentrations under constant dark and light conditions. My results also suggest that Gtα and Gq/11α concentrations may be regulated through different mechanisms. These findings may help us understand circadian regulatory mechanisms within the retina and their role in maintaining the health of the retina.
ACKNOWLEDGMENTS

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I would also like to thank my parents for their love and support.
# TABLE OF CONTENTS

**ABSTRACT**

**ACKNOWLEDGMENTS**

**TABLE OF CONTENTS**

**LIST OF FIGURES**

**LIST OF ABBREVIATIONS**

**CHAPTER 1 INTRODUCTION**

1.1 Structure of vertebrate retina

1.2 Phototransduction in the vertebrate retina
   1.2.1 Phototransduction turn-off
   1.2.2 Ca$^{2+}$ dependent mechanism of adaptation

1.3 Neurotransmitters and neuromodulators of the vertebrate retina
   1.3.1 Glutamate and Aspartate
   1.3.2 Gamma-Aminobutyric acid (GABA)
   1.3.3 Glycine
   1.3.4 Acetylcholine
   1.3.5 Dopamine
   1.3.6 Melatonin
   1.3.7 Serotonin
   1.3.8 Epinephrine and Norepinephrine
   1.3.9 Neuropeptides

1.4 G proteins
   1.4.1 G proteins
   1.4.2 Expression of G proteins in the retina

1.5 Regulatory processes of the retina
   1.5.1 Light evoked and circadian regulation
      1.5.1.1 Assembly and turnover of photoreceptor outer segments
      1.5.1.2 Retinomotor movements
      1.5.1.3 Expression of visual transduction genes
      1.5.1.4 Expression and role of neuromodulators in retinal cycles
1.6 Rational and hypothesis 38
1.7 Specific objectives of the present study 39

CHAPTER 2 METHODS 40

2.1 Materials 40
2.2 Animals 40
2.3 Immunohistochemistry 41
2.4 Protein extraction 42
2.5 Protein assay 43
2.6 Quantitative immunoblotting 44
   2.6.1 SDS-Page and western blot 44
   2.6.2 Quantitation 45
2.7 Purification of G protein standards 46
2.8 Antibody specificity 46
2.9 Statistical analysis 47

CHAPTER 3 RESULTS 48

3.1 Antibody specificity 48
3.2 Study 1: Immunohistochemistry of rat retina 48
   3.2.1 Distribution of Gq/11α and Gtα proteins in rat retina 48
   3.2.2 Conclusions from study 1 51

3.3 Study 2: Regulation of Gq/11α and Gtα proteins in rat retina 51
   3.3.1 Expression of Gq/11α proteins during light/dark cycles 51
   3.3.2 Expression of Gtα proteins during a light/dark cycle 56
   3.3.3 The effects of constant light and dark conditions on the level of expression of Gq/11α and Gtα proteins 60
   3.3.4 Histology of rat retina 67
   3.3.5 Conclusions from study 2 69
3.4 Overall conclusions 69
CHAPTER 4 DISCUSSION

4.1 Distribution of Gq/11α in rat retina

4.2 Regulation of Gq/11α and Gtα protein concentrations during a light-dark cycle
   4.2.1 Gq/11α and Gtα protein concentrations during a light/dark cycle
   4.2.2 Circadian regulation of Gq/11α and Gtα protein levels in rat retina

4.3 Possible circadian regulators of Gq/11α and Gtα

4.4 The possible role of Gq/11α in the vertebrate retina

4.5 Conclusion

CHAPTER 5 REFERENCES
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Structure of the vertebrate retina.</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Structure of rods and cones.</td>
<td>4</td>
</tr>
<tr>
<td>Figure 3</td>
<td>G protein subtypes and homologies.</td>
<td>22</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Light-dark cycling of opsin, Gtα, Gtβ and 48 kDa protein (aresstin) mRNA levels in adult mouse.</td>
<td>33</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Gq/11α and Gtα antibody specificity.</td>
<td>49</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Localization of Gq/11α and Gtα in rat retina.</td>
<td>50</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Quantitation of Gq/11α expression levels.</td>
<td>53</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Expression of Gq/11α proteins during light-dark cycles.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 9</td>
<td>The maximum and minimum levels of expression of Gq/11α during a light-dark cycle.</td>
<td>55</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Expression of Gq/11α in spleen.</td>
<td>57</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Quantitation of Gtα expression levels.</td>
<td>58</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Expression of Gtα proteins during light-dark cycles.</td>
<td>59</td>
</tr>
<tr>
<td>Figure 13</td>
<td>The maximum and minimum levels of expression of Gtα during a light-dark cycle.</td>
<td>62</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Gq/11α expression in constant dark conditions.</td>
<td>63</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Gq/11α expression in constant light conditions.</td>
<td>64</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Gtα expression in constant dark conditions.</td>
<td>65</td>
</tr>
</tbody>
</table>
Figure 17. Gtα expression in constant light conditions.

Figure 18. Comparison of the effect of various light conditions on retinal histology in rats.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>Avidine biotine complex</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>1,2-diacylglycerol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>G_{44}</td>
<td>Squid eye (44kDa) G protein</td>
</tr>
<tr>
<td>G_{q/11\alpha}</td>
<td>q or 11 α subunit</td>
</tr>
<tr>
<td>G_{\alpha}</td>
<td>Transducin α subunit</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>GCL</td>
<td>Ganglion cell layer</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HC</td>
<td>Horizontal cell</td>
</tr>
<tr>
<td>HIOMT</td>
<td>Hydroxyindol-O-methyltransferase</td>
</tr>
<tr>
<td>IP_3</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IPL</td>
<td>Inner plexiform layer</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyltransferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>OPL</td>
<td>Outer plexiform layer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline plus triton</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>Pre I</td>
<td>Pre-immune</td>
</tr>
<tr>
<td>ROS</td>
<td>Rod outer segments</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>SCN</td>
<td>Superchiasmic nucleus</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS- polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Structure of vertebrate Retina

Eyes are highly specialized organs of photoreception. The image sensing component of the eye, the retina, consists of the non-neural pigment epithelium and the tightly apposed neural retina. The neural retina is a peripheral extension of the forebrain with which it has common embryological origins. The cellular structure of the retina has been well studied and is shown in figure 1. The outer most layer of the retina is the retinal pigment epithelium (Besharse, 1982). Embedded in the pigment epithelium are the tips (outer segments) of the photoreceptive rods and cones. The nuclei of the rods and cones are present in the inner segments of these cells and their cell bodies are located in the outer nuclear layer of the retina. In the next layer, known as the outer plexiform layer, the axons of rods and cones synapse with dendrites of second order neurons. The two types of neurons in this layer are bipolar cells that relay impulses to third order neurons and horizontal cells that interconnect photoreceptors laterally. The inner nuclear layer that comes next contains cell bodies of the bipolar cells and connecting amacrine cells. This layer also contains cell bodies of Muller cells, the chief glial cells of the retina which span almost its full thickness. In the next layer, the inner plexiform layer, bipolar cells directly synapse with ganglion cells, and amacrine cells connect each bipolar cell with several ganglion cells. The final inner most
Figure 1. Structure of the vertebrate retina. Obtained from Berman, 1991.
layer, termed the ganglion cell layer, contains the cell bodies of the ganglion cells, who’s axons lead to the optic nerve. (figure 1)

Operationally and functionally, the retina is usually considered as two regions; the outer (light detecting) photoreceptor cells and the inner (signal processing) layers. Two types of photoreceptor cells are found in the retina rods and cones. The rod photoreceptor cells are located in the peripheral areas of the retina in humans, rats, and other nocturnal animals. The rod cells are receptors for dim light (i.e. night vision) and do not distinguish colours. Rod photoreceptor cells are thought to provide scotopic visual activity. Cone photoreceptors, which provide photopic vision, are shorter than rods and usually cone shaped. The cones are specialized for colour vision and require higher light intensities than rods (Stryer, 1986). The proportion of rods to cones varies considerably among species. The human eye contains 100 to 120 million rods and about 6 million cones. Most rodents have rod dominated retinas, for instance, rods make up approximately 98% of photoreceptor cells in rats (Farber et al, 1981).

1.2 Phototransduction in the vertebrate retina

The vertebrate photoreceptors are highly specialized structures for phototransduction (figure 2). The rod outer segments contain a stack of densely packed disks. It is estimated that each ROS can contain up to 2000 disks (Stryer,
Figure 2. Structure of rods and cones. Obtained from Berman, 1991.
1986). Such membrane structures provide a large surface area on which the molecular interactions of phototransduction occur.

Rhodopsins, the actual photoreceptor molecules are embedded in the disk membrane structure. It is estimated that each disk contains 800,000 rhodopsin molecules which make up 95% of the protein content of the disk (Dratz and Hargrave, 1983). Rhodopsin (39 kDa) has a maximal absorption of green light at around 500 nm maximal, therefore its colour is red when observed in the dark. Rhodopsin consists of a protein moiety (opsin) and a chromophore (11-cis retinal). Opsins belong to the seven-transmembrane G-protein coupled receptor superfamily (Jackson, 1991). Retinal, the light sensitive chromophore, is linked to a lysine residue located at the transmembrane portion of the rhodopsin by a protonated Schiff base linkage (Nathans, 1990).

Upon light absorption, the 11-cis retinal isomerizes to an all-trans conformation. This photoisomerization triggers rhodopsin to undergo a series of conformational changes, which result in the conversion of rhodopsin from metarhodopsin I to the metarhodopsin II state which is the biochemically activated form of rhodopsin. In the final stage of rhodopsin bleaching, Metarhodopsin II is converted to opsin and all-trans retinal directly, as well as indirectly through metarhodopsin III. The all-trans retinal formed during bleaching is reduced to vitamin A (all-trans-retinol) and transported to the pigment epithelium, where it is
rapidly esterified and isomerized to 11-cis retinol. The visual cycle is completed when this stereoisomeric form of vitamin A is returned to the photoreceptors for rhodopsin regeneration (Berman, 1991).

Once activated by light, metarhodopsin II couples to transducin (Gt). Transducin is a heterotrimeric G-protein composed of an $\alpha$ (40 kDa), a $\beta$ (35 kDa), and a $\gamma$ (5 kDa) subunit. The $\alpha$ subunit is the site of guanine nucleotide binding and metarhodopsin II catalyses the exchange of GDP for GTP and converts the transducin molecule to its active form (GTP-bound). The light signal becomes highly amplified at this stage since every metarhodopsin II can catalyze the guanine nucleotide exchange of approximately 500 Gt$\alpha$ subunits (Fung and Stryer, 1980).

The GTP-bound Gt$\alpha$ activates cGMP phosphodiesterase (PDE) by removing two inhibitory $\gamma$ subunits from the $\alpha$ and $\beta$ catalytic subunits of PDE (Piriev et al., 1993). Activated PDE rapidly hydrolyses cGMP and the subsequent decrease in intracellular cGMP level results in the closure of cGMP-gated sodium and calcium channels located at the ROS membrane (Yau and Nakatani, 1985). The light induced blockage of sodium and calcium ion entry into the ROS results in the hyperpolarization of the cell membrane, which in turn slows glutamate release at synapses with bipolar and horizontal cells (Copenhagen, 1991).

The neurons of the outer retina are highly specialized in that they do not possess regenerative sodium conductance and so do not generate action potentials.
Instead, they respond to light with graded potential changes. Bipolar and horizontal cells all pose a linear range of response proportional to light intensity. The visual system is subdivided into ON- and OFF- pathways in the retina by two functionally distinct classes of bipolar cells. ON-center bipolar cells which depolarize and OFF-center bipolar cells which hyperpolarize in response to central illumination of their receptive field (Kaneko, 1971). This difference in response to the decrease in glutamate release from photoreceptor cells is achieved by different glutamate receptors expressed in ON and OFF bipolar cells. OFF bipolar cells have ionotropic glutamate receptors which are directly linked to cation channels. Closure of these channels occurs when glutamate levels fall in response to light, resulting in hyperpolarizing responses. ON-bipolar cells on the other hand possess a novel class of glutamate metabotropic receptors (mGluR6), which is indirectly linked via a cGMP cascade to the closure of cation channels (Shiells and Falk, 1990). The fall in glutamate release with light is thus transduced by ON-bipolar cells into a rise in intracellular cGMP, which opens cGMP-gated cation channels, generating depolarizing responses. This glutamate cGMP cascade functions to amplify small signals in response to a few absorbed photons in rods by about a 100 fold (Shields and Falk, 1990). It is now known that mammalian rod bipolar cells are predominantly the ON-type (Daw et al, 1990), and so this synaptic amplification is probably essential for the high sensitivity of the rod visual system.
Bipolar cells send axons to the retinal inner plexiform layer where they synapse with amacrine and ganglion cells. There is some controversy as to which neurotransmitter is released at this synapse. The substances proposed to be the neurotransmitter of bipolar cells are glutamate, aspartate, homocysteic acid and serotonin. The first three of these substances are amino acids that interact with glutamate receptors, whereas the latter interacts with serotonin receptors. The pattern of connectivity in the inner plexiform layer (IPL) is again highly selective. Axons of ON- and OFF-center bipolar cells are confined to distinct areas of this layer and synaptic contacts are further restricted to specific ganglion cell types. Thus, at the IPL level, information continues to be processed in synaptically specific pathways. The excitatory neurotransmitter released from bipolar cells will couple to receptors on the ganglion cell surface and lead to the opening of cation channels. If there is sufficient depolarization an action potential will be produced in the ganglion cells and the action potential will propagate down the ganglion cell axons into the optic nerve and eventually to the visual cortex in the occipital lobe of the brain for visual perception (Stryer, 1991).

Photoreceptor cells also synapse with a second class of neurons called the horizontal cells within the outer plexiform layer. The excitatory neurotransmitter glutamate will couple to either ionotropic glutamate receptors for direct activation of ion channels or mGluRs that are linked to phosphatidylinositol metabolism for modulatory responses. Coupling of glutamate to ionotropic glutamate receptors on
the surface of horizontal cells will lead to the opening of cationic channels and the depolarization of these cells which leads to the release of the inhibitory neurotransmitter GABA from these cells. Horizontal cells transmit lateral inhibitory signals within the OPL to other photoreceptor cells and/or bipolar cells. The role of these cells is to provide a signal representing the mean luminance, averaged over a broad retinal area. This inhibitory signal is subtracted from a specially localized input of photoreceptors to bipolar cells to yield a signal coding for local contrast (Yazulla et al., 1985).

1.2.1 Phototransduction turn-off

Most of the activated components in the phototransduction cascade are inactivated after termination of the light stimulus. Photoactivated rhodopsin is inactivated by phosphorylation of multiple serine and threonine residues in its carboxy-terminal region by rhodopsin kinase. Phosphorylation of metarhodopsin II decreases its ability to catalyze guanine nucleotide exchange on transducin (Palczewski and Benovic, 1991). Phosphorylated metarhodopsin II is further deactivated by the binding of arrestin. Arrestin binding prevents dephosphorylation of metarhodopsin II by phosphatase 2A (Palczewski et al., 1989) and blocks the binding of transducin to phosphorylated metarhodopsin II to prevent further PDE activation. Thus hydrolysis of cGMP is decreased and its concentration recovers to
the original dark levels by guanylate cyclase an enzyme found in the photoreceptor cell membranes (Kawamura and Murakami, 1989).

1.2.2 Ca\(^{2+}\) dependent mechanism of adaptation

The vertebrate retina can respond to a large range of light intensities. The relation between light intensities and rod photoresponse can approximately be expressed in terms of the Michaelis-Menten equation (Stryer, 1991). Under dark-adapted conditions rods can respond to light intensities in the range of 3 log units. However, when a photoreceptor is light adapted this range shifts by another 3 log units. Thus during light-adaptation rod photoreceptors are sensitive over a range of 6 log units of light intensities. According to the Michaelis-Menten theory, enzyme reactions saturate with substrate concentrations over 3 log units. The factor believed to be responsible for widening the response range of rods, is a light induced decrease in Ca\(^{2+}\) concentration (Yau and Nakatani, 1985b).

The mechanism involved in light-induced decrease in Ca\(^{2+}\) concentration has been established (Crevetto et al., 1989). In the dark, Ca\(^{2+}\) enters the photoreceptor cells through cGMP-gated channels. The entering calcium is pumped out by a Na\(^{+}/Ca^{2+}, K^{+}\) exchanger situated in the plasma membrane of the rod outer segments. This entry and extrusion of calcium is balanced, thus cytoplasmic calcium levels are maintained at a steady state level. The exchanger operates continuously, irrespective of light-dark conditions. In the light the cGMP-
gated channels close and Ca\(^{2+}\) entry into the cell is blocked. However, the extrusion of calcium through the exchanger continues and leads to a decrease in cytoplasmic Ca\(^{2+}\) levels.

Attempts have been made to explain how this light induced decrease in calcium concentrations leads to photoreceptor cell adaptation. Studies have shown that guanylate cyclase (the enzyme that synthesizes cGMP) activity is enhanced at low physiological Ca\(^{2+}\) concentrations, whereas high calcium levels inhibit its activity (Koch and Stryer, 1988; Kawamura and Murakami, 1989b). The enhanced activity of guanylate cyclase due to low calcium concentrations allows some recovery of cGMP levels though not to the same extent as the dark levels. The recovery of cGMP levels is also thought to be enhanced by desensitization of phosphodiesterase under low calcium concentrations, through a sensitivity modulating protein known as S-Modulin (Kawamura and Murakami, 1993).

Another effect of the low calcium concentrations in the rod photoreceptor cells has been described by Hsu and Molday, 1993. They showed that at high physiological calcium concentrations, Ca\(^{2+}\) couples to calmodulin found in the rods and this complex binds to cGMP-gated channels and decreases the channels sensitivity to cGMP. Thus at low calcium concentrations the cGMP-gated channels are sensitized and can still respond to low cGMP concentrations found during continuous light stimulation (light adaptation).
When continuous light is presented to rods, the photoresponse is initially saturated due to the hydrolysis of cGMP. However, the partial recovery of cGMP levels due to effects of reduced calcium levels on guanylate cyclase and phosphodiesterase enzymes in combination with the sensitization of cGMP-gated channels allows the rod photoreceptor cells to remain active over a wider range of light intensities.

1.3 Neurotransmitters and neuromodulators of the vertebrate Retina

Although the primary input to photoreceptor cells is light and the control of many of the functions of photoreceptor cells are self contained within the visual cell or the photoreceptor-pigment epithelial complex, aspects of photoreceptor metabolism and regulation are subject to modulation by neurotransmitters or neuromodulators that are found in the neuronal layers of the retina downstream from the photoreceptor cell layer.

Neurotransmitters and neuromodulators in general are chemical messengers that mediate intracellular communication in neural tissue. Neurotransmitters are defined as endogenous substances that elicit rapid electrophysiological responses of short duration. Neuromodulators are also endogenous substances that are released from neurons, but their effects are distinct from those of neurotransmitters. Neuromodulators are thought to act postsynaptically by activating intracellular enzyme systems that control a variety of cellular functions.
The action of most neuromodulators are relatively long lived (Iuvone, 1986). At least 15 putative neurotransmitters and neuromodulators have been described in the retina. Here I will briefly highlight the localization of some of the better documented transmitters and modulators of the retina.

1.3.1 Glutamate and Aspartate

Both of these acidic amino acids are present in high concentrations in the retina. The highest concentration of these amino acids are within the inner and outer plexiform layers of the retina (Iuvone, 1986). Glutamate is the neurotransmitter used by both photoreceptors and bipolar cells. It is released on to bipolar and horizontal cells in the OPL and onto ganglion cells and amacrine cells in the IPL (Tachibana and Okada, 1991). Until recently, most physiological studies of glutamatergic neurotransmission have focused on ionotropic receptors. Increasing evidence, however supports the hypothesis that metabotropic glutamate receptors play an important role in retinal synaptic transmission. For instance, as outlined above, mGluR6 is the major glutamate receptor expressed in rod bipolar cells and is thought to mediate synaptic transmission from rod photoreceptors to rod bipolar cells (Hartveit et al., 1995). Recently the expression of all of the mGluRs have been investigated and with the exception of mGluR3 all other mGluRs has been detected in rat retina. Metabotropic glutamate receptors 1 and 5 which couple to PLC activation through a G-protein, have been localized to the
inner plexiform and ganglion cell layer and are thought to be expressed by ganglion cells and some amacrine cells. mGluR2, 4, and 7 which couple to adenylyl cyclase inhibition through a G-protein are mostly found in the ganglion cell layer (GCL). mGluR6 which also couples to adenylyl cyclase inhibition is found in the inner nuclear layer of the rat retina (Hartveit et al., 1995).

1.3.2 Gamma -Aminobutyric acid (GABA)

GABA is not only one the most important inhibitory transmitters as the main neurotransmitter released from horizontal cells in the vertebrate retina, but it is also one of the earliest transmitters expressed during the development of the retina (Redburn and Madtes, 1987). Two classes of GABAergic neurons (amacrine cells and horizontal cells) have been identified and there is also some evidence suggesting a role for GABA as a neurotransmitter in ganglion cells in rabbits (Yu et al., 1988). Histochemical studies have shown GABAergic amacrine and horizontal cells and have demonstrated the presence of GABAergic synaptic sites within the IPL. Adult retina contain high levels of GABA; high affinity uptake as well as the GABA synthesizing enzyme, glutamic acid decarboxylase, have been detected in horizontal cells and in amacrine cells of many species (Iuvone, 1986). Thus, there is strong evidence implicating GABA as a neurotransmitter in horizontal cells and some amacrine cells.
1.3.3 Glycine

Glycine is present in the retina and found in greatest concentrations within the inner nuclear layer and the inner plexiform layer. Glycine is accumulated by amacrine and displaced amacrine cells (Iuvone, 1986). However, the role of this transmitter in the retina is not established yet.

1.3.4 Acetylcholine

Acetylcholine was one of the first neuroactive substances identified in the retina, and is now established as a major transmitter in the vertebrate retina. The cholinergic neurons of the rabbit retina consist of both amacrine and displaced amacrine cells, the latter comprising about one third or more of the ganglion cell neurons (Masland and Tauchi, 1986). The cholinergic system receives an inhibitory input from GABA amacrine cells, and co-localization of GABA and acetylcholine in amacrine cells of the rabbit retina demonstrated by immunohistochemical staining establishes that cholinergic cells are a major subpopulation of GABA immunoreactive amacrine and displaced amacrine cells (Brecha et al., 1988). The presence and release of both an inhibitory and an excitatory transmitter from the same cell has been suggested to be a mechanism for the formation of complex receptor field properties of the ganglion cells (Brecha et al., 1988).
1.3.5 Dopamine

Dopamine is a neurotransmitter synthesized and released from amacrine cells of the inner retina. In every species studied to date, highest levels of dopamine occur during the light phase of a light-dark cycle (Iuvone, 1986). An extensive literature now documents effects of dopamine on many aspects of photoreceptor metabolism. Photoreceptor cells of mammalian and non-mammalian species are known to contain dopamine receptors. These receptors have been identified as the D2 and D4 subtypes (Wagner and Behrens, 1993), and the mRNA of these receptors have been localized to photoreceptor cells by in situ hybridization (Dearry, et al., 1991; Cohen et al., 1992). Stimulation of D2/D4 appears to reduce cAMP formation in chick photoreceptor cells (Iuvone, 1986). Dopamine is thought to be a major neuromodulator in the retina and this aspect of Dopamine action will be dealt with in more detail in section 1.5.1.4.

1.3.6 Melatonin

Melatonin is another important neuromodulator of the retina. Melatonin, locally synthesized in the retina by photoreceptor cells in darkness, regulates photoreceptor metabolism. All of the enzymes necessary for melatonin biosynthesis are present in the retina. The formation of melatonin from the amino acid precursor L-tryptophan involves consecutive action of four enzymes, tryptophan hydroxylase, and aromatic aminoacid decarboxylase produce serotonin
and then, serotonin N-acetyltransferase (NAT), and hydroxyindole-O-methyltransferase (HOMT) synthesize melatonin from serotonin (Cahill et al., 1991). Radioligand binding studies indicate that melatonin receptors are localized primarily to the inner nuclear and plexiform layers of the neural retina and in some retinal pigment epithelial cells (Laitinen and Saavedra, 1990). In human retina the melatonin receptor has been characterized as the Melatonin 1b receptor a subtype exclusively expressed in retina, and coupled to cAMP inhibition through a G-protein (Reppert and Weaver, 1995). Again the neuromodulatory role of Melatonin will be discussed in more detail in section 1.5.1.4.

1.3.7 Serotonin

Serotonin accumulating amacrine cells have been observed in the retina of virtually every species; however, there is considerable species variation in the level of expression of this transmitter (see review by Massey and Redburn, 1987). Immunohistochemical studies have indicated the presence of serotonin in the inner nuclear and ganglion cell layers of the retina. The site of synaptic transmission is thought to be within the inner plexiform layer; however, it is not clear which serotonin receptors are involved in this synaptic transmission (Ehimger and Floren, 1996). Enzymes necessary for serotonin synthesis appear to be present indigenously within the retina (Thomas and Iuvone, 1989).
1.3.8 Epinephrine and Norepinephrine

The catecholamines, epinephrine and norepinephrine, are present in rat and bovine retinas, and they appear to be localized in a subclass of amacrine cells. Binding sites for labeled norepinephrine have been reported, and photic stimulation causes a transient increase in epinephrine levels in rat retina (Iuvone, 1986). However a role for these two catecholamines as transmitters, or possibly neuromodulators, in the retina remains to be established.

1.3.9 Neuropeptides

Thirty or more peptides that appear to function as neurotransmitters or neuromodulators have been characterized in brain and other neural tissues and at least eight of these have been identified in the retina. Their localization in the retina, mainly in amacrine and displaced amacrine cells, have been deduced using immunohistochemical studies (for review see Massey and Redburn, 1987). Little is known about the function of neuropeptides in the retina. In general their physiological effects are thought to be long term or of the slow acting type (for review see Iuvone, 1986).

1.4 G Proteins

Many biological processes are mediated through receptors that are coupled to guanine nucleotide binding proteins (G proteins). External signals such as light,
odorants, as well as chemical messengers such as hormones, neurotransmitters, and growth factors serve as ligands for these receptors. The stimulation of these receptors results in the activation of effector proteins (e.g. enzymes or ion channels), which produce chemical second messengers. A cascade of molecular reactions is triggered by the second messengers leading to changes in cellular behaviour. In most organisms, a family of proteins called G proteins play an essential role in linking these receptors to effector proteins.

In the last two decades there has been tremendous interest in the involvement of G proteins in regulatory processes within the retina. The majority of this work has focused on the phototransduction pathway within the photoreceptor cell outer segments and thus has revealed the well characterized rhodopsin-\( \text{Gt}_\alpha \)-PDE signaling system as described in detail above. Much less attention has been paid to the down stream processes in the secondary neuronal cells within the retina. Summarized below are some of the findings in regards to G protein expression and their possible roles within the retina, but first I will introduce some of the members of the G protein superfamily.

1.4.1 G proteins

G proteins are heterotrimeric proteins composed of three distinct subunits: \( \alpha \), \( \beta \), and \( \gamma \). The \( \beta \) and \( \gamma \) subunits exist as a tightly associated complex that functions as a unit. G protein \( \alpha \) subunits are believed to be the major signaling
component because they bind and hydrolyze GTP in effect making them the catalytic subunits of G proteins. Duplication and divergence of genes during the course of evolution have created structural diversity in each component of the G protein heterotrimer. In mammals, four families of G protein α subunits have been defined and encoded by at least 17 genes.

The first G protein isolated was Gt or transducin (Godchaux and Zimmerman, 1979). Subsequently several other G proteins including Gs, Gi, and Go were also purified in several laboratories (Northup et al., 1980; Bokoch et al., 1983; Codina et al., 1983; Stemweis and Robishaw, 1984). Later cloning and sequencing techniques were used to identify many other G proteins. The G proteins are now classified into four major groups based on amino acid sequence homology: Gs, Gi, Gq, and G12. As well, each of these groups can be subdivided into subfamilies of G protein α subunits. (figure 3).

Gs family members include Gs and Golf (Jones and Reed, 1989) which interact with hormones and odorant receptors respectively to stimulate adenylyl cyclase, and thus enhance the rate of cyclic AMP (cAMP) synthesis. Gs is expressed ubiquitously as four distinct polypeptides due to alternative splicing of a single precursor mRNA. These splice variants have been characterized and shown to have similar functions. Golf shows 88% amino acid sequence homology with Gs. Golf expression is restricted to specific neural tissues and is enriched in neurons of the olfactory epithelium (Jones and Reed, 1989).
The Gi family is composed of Gi, Go, Gt, G\text{gust} and Gz. The Gia group is subdivided into three subtypes: Gi₁, Gi₂, and Gi₃ (41 kDa). These subtypes are encoded by three different genes but all function to inhibit adenylyl cyclase and thus inhibit cAMP production (Suki et al., 1987).

Goα has two splice variants Goαₐ and Goαₐ (39 kDa). The variants are the result of alternative splicing at the carboxy terminal end of the amino acid sequence and thus might have different effector and receptor affinities. Although Goα is ubiquitously distributed throughout the central nervous system in large quantities its function remains to be determined. Goα has been implicated as a link between calcium channels and receptors (Ewald et al., 1989).

Gt or transducin is a 40 kDa protein that is exclusively expressed in the retinal photoreceptor membranes. There are two forms of transducin: Gt₁α and Gt₂α, which differ in approximately 20% of the encoded amino acid residues. Gt₁ is expressed in rods where as Gt₂ is found in cones (Lerea et al., 1986). However both forms are linked to phosphodiesterase activation and hydrolysis of cGMP in response to light activation of opsins.

A novel form of a transducin-like G protein named gustducin (G\text{gust}α) has been recently discovered (McLaughlin et al., 1992). It is only expressed in taste buds. The suggested role of gustducin in taste transduction is analogous to that of light transduction by transducin.
Figure 3. G protein subtypes and homologies. Obtained from Hepler and Gilman, 1992)
Gz is primarily expressed in neurons (Hinton et al., 1990). This G protein has an unusual biochemical property in that it shows a very low rate of GTPase activity as compared to other G proteins (Casey et al., 1990). The function of this G protein is not known.

Gq, G11, G14, G15, and G16α are the members of the Gq family, which are known to activate phosphoinositide-specific phospholipase C (PLC). PLC activation results in the hydrolysis of the lipid phosphotidylinositol-4,5-bisphosphate (PIP2) into two second messengers, inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Gqα and G11α are found in most tissues and they are more than 88% homologous in their amino acid sequence. The other three members of the Gq family: G14, G15, and G16α, all show restricted tissue expression. G14 is found in stromal and epithelial cells (Simon et al., 1991). G15 is found in B-lymphocytes whereas G16 is mainly found in T-lymphocytes (Amatruda et al., 1991).

G12 family is composed of G12α and G13α. They are ubiquitously expressed and are known to act as activators of the sodium hydrogen exchangers (Dhanasekaran et al., 1994).

1.4.2 Expression of G proteins in the Retina

Many G protein coupled receptors are found in the retina which are either involved in the primary phototransduction pathway or other modulatory pathways
within the retina. The role of many of these transduction pathways within the retina are not clear; however, there is some evidence as to their function based on the distribution of G proteins and the neuromodulators that might act on receptors that couple to them. Immunohistochemical studies have shown that Gtα is only expressed in the photoreceptor cell outer segments. This is where Gt couples to rhodopsin or opsin and transduces the light signal by a series of complex biochemical reactions as described above. Other G proteins are also found in the retina. The distribution of some of these proteins have been deduced.

Gsα is ubiquitously expressed in all cells and as such it is believed to also be present in the retina; however, its distribution has never been studied. It is believed that some neuromodulators that may be present in the retina (e.g. glucagon) couple to Gs in the retina via G protein coupled receptors and stimulated adenylyl cyclase thus increasing cAMP synthesis. cAMP in turn acts as a second messenger and activates a group of enzymes known as cAMP dependent protein kinases which trigger a series of biochemical processes by phosphorylating cellular components that result in physiological changes within the cell (Jones and Reed, 1989).

In addition to transducin, other members of the Gi family are also found in the retina. Gi activation leads to the inhibition of adenylate cyclase and thus causes a decrease in cAMP levels within the cell. All of the Giα subtypes (Gi1, Gi2, and Gi3α) are found in the retina (Ogumi et al., 1996). They are all expressed in the neuronal layers of the retina with no expression in the photoreceptor cell layers.
The level of expression of these proteins differ throughout development. In rats Gi1 and Gi3 first appear at embryonic day 15 in the inner layers of the neural retina, while Gi2 is found in both outer and inner layers of the neural retina. In adult rats Gi1 and Gi3 are expressed in the inner plexiform layers whereas Gi2 is expressed in the outer plexiform layer (Oguni et al., 1996). This differential distribution during development suggests that each of these G proteins plays a unique role in the developing retina. One suggested role is that these proteins might be involved in regulation of axonal growth within the retina (Bates and Meyer, 1996).

Another member of the Gi family, Goa has also been implicated in the regulation of axonal growth (Bates and Meyer, 1996). The Goa protein as mentioned before is widely distributed in the central nervous system; however, despite extensive investigations no function has been exclusively assigned to this G protein. In bovine retina its distribution has been localized to the inner and outer plexiform layers as well as the ganglion cell layer (Lad et al., 1987). In rats it is also expressed in the inner and outer plexiform layers but is sparsely expressed in the ganglion cell layer (Terashima et al., 1987). It has been suggested that because of the selective enrichment of Go in the synaptic zones of the retina it may play a major role in neuronal transmission within the retina but this role remains to be determined.
The only other members of the G protein superfamily that have been investigated within the retina are the G proteins belonging to the Gq family. These studies have been carried out in the invertebrate retina, because a Gq/11 like protein has been purified and cloned in squid (Pottinger et al., 1991) and Drosophila (Lee et al., 1990) photoreceptors, and is thought to be involved in phototransduction analogous to transducin in the vertebrate retina. Following light activation of invertebrate rhodopsin into metarhodopsin the activated metarhodopsin couples to Gq and catalyzes GTP binding to Gqα (Pottinger et al., 1991). The activated Gqα subunit interacts with PLC which leads to the hydrolysis of phosphatidyl-inositol bisphosphate into IP3 and DAG (Mitchell et al. 1995). Light induced IP3 synthesis causes release of calcium from intracellular storage sites (Rayer et al., 1990). The increase in intracellular calcium levels opens cation channels on photoreceptor membranes (Minke and Selinger, 1992) and sodium influx across the plasma membrane leads to depolarization with subsequent release of the neurotransmitter histamine from the photoreceptor cells (Sarthy, 1991). Note that in the invertebrate phototransduction pathway the light signal leads to the depolarization of photoreceptor cells instead of the hyperpolarization observed in the vertebrate phototransduction.

1.5 Regulatory processes of the retina

Regulatory processes allow the retina to process visual stimuli over a large range of light intensities. Much of this regulation occurs on a biochemical level
within the outer segments, and occurs rapidly in response to luminance. Other aspects of regulation occur more slowly as diurnal or circadian rhythms. These latter aspects of regulation involve inner retinal neurons as well as photoreceptor cells, and may occur as changes in synaptic strength, coupling and uncoupling of neurons through gap junctions, and release of neurotransmitters or neuromodulators that have been shown to influence the assembly and metabolism of photoreceptor cells which are essential for the normal functioning of the retina (Besharse et al., 1988; Dowling, 1989).

1.5.1 Light evoked and circadian regulation

Many important aspects of retinal cell biology and metabolism are controlled by light, photoperiod or circadian clocks. Some processes are acutely affected by light exposure. Others are temporally regulated as rhythmic events. These rhythms are diurnal or circadian in nature. Diurnal rhythms are defined as day-night rhythms that require the presence of a light-dark cycle for expression. In contrast circadian rhythms are defined as daily rhythms controlled by endogenous oscillators or clocks that are entrained by the light-dark cycle but that persist in constant darkness (Cahill et al., 1991). In contrast to diurnal rhythms, circadian mechanisms have the unique capacity to anticipate the transition between light and darkness, and can prepare a cell, tissue, or organism for the next phase of the photoperiod. Circadian clocks have been localized in both invertebrate and
vertebrate retinas (for review see Cahill et al., 1991). Even though the existence of such an oscillator has been long suspected in the mammalian retina, experimental difficulties have until recently limited definitive evidence of their existence. The mammalian retinal clock has been shown to be an intrinsic oscillator that can function independently of superchiasmic nucleus (an area in the hypothalamus that is believed to be the site of the circadian clock that regulates most behavioural and endocrine rhythms in mammals) or the pineal gland (Tosini and Menaker, 1996). The major neuromodulators involved in the retinal clock, like that of the SCN, are thought to be melatonin and dopamine (Tosini and Menaker, 1996). The molecular components of this machinery have not been fully identified and require further investigation. This clock plays an important role in many regulatory processes in the retina some of which I will summarize in the following sections.

1.5.1.1 Assembly and turnover of photoreceptor outer segments

Photoreceptor cells are terminally differentiated cells, that once formed do not undergo differentiation or cell division. However they do undergo a process of renewal. During this renewal process most of the cells contents, namely the disc membranes, are dynamically replaced by synthesis and turnover (Young, 1976). This renewal process is termed disc shedding and is most dramatic in rod photoreceptor cells. During this process newly synthesized proteins in the inner segment are transported to the distal inner segment membrane near the connecting
cilium and then assembled into membranous discs at the base of the outer segment. These discs are gradually displaced distally by other newly forming discs. They ultimately become detached or shed from the tips of the outer segments and are phagocytosed by pigment epithelium cells (RPE) (Young, 1976). Disc shedding is thought to involve active processes by both photoreceptor cells and RPE cells, because it does not appear to occur in the absence of RPE. Photoreceptor outer segment tips are surrounded by RPE processes that participate in the mechanism of detachment and phagocytosis. Fragments of the outer segments that are to be shed are engulfed by pseudopods of RPE cells, subsequently these outer segment fragments are detached into the RPE cytoplasm as phagosomes, and are transported into the cell body where they are digested by lysosomes (for review see Besharse, 1982). The process of shedding and phagocytosis is believed to be mediated by receptor ligand interaction and second messenger systems in the RPE cells (Heth and Schmit, 1991). This hypothesis has been based on the observation that the phagocytosis of outer segment fragments are accompanied by changes in protein phosphorylation in RPE cells. Several second messengers have been implicated in this process. Cyclic AMP analogues and phosphodiesterase inhibitors have been shown to inhibit disc shedding and also phagocytosis by RPE cells (Besharse et al., 1982). Low calcium concentration in the retina has also been shown to inhibit disc shedding (Greenberger and Besharse, 1983). Phospholipase C activation has been shown to be involved in the phagocytosis of disc membranes.
in cultured RPE cells; however the mechanism involved in this is not clear (Heth and Marescalchi, 1994).

Disc shedding and phagocytosis are temporally regulated processes, and their relation to the photoperiod differs for rods and cones. Rod disc shedding occurs just after light onset whereas cone disc shedding occurs at night just after light offset. Rod outer segment disc shedding in rats has been shown to be primarily circadian in nature. In rats that have been entrained to a light-dark cycle and then kept in constant darkness, the morning shedding persists in similar magnitude to that seen in cyclic light and dark. However rod disc shedding is dramatically suppressed in constant light. In these animals a short duration of exposure to darkness will initiate shedding, but does so most effectively near the subjective time of light onset. Like other circadian rhythms, the circadian disc shedding can be phase shifted by changing the light-dark cycle to which rats are exposed (Goldman et al., 1980). Even though light per se is not required for the mechanical process of shedding and phagocytosis, it serves as an initiating stimulus (Besharse et al., 1988). This circadian disc shedding is thought to be under control of an intrinsic oscillator since it persists following the removal of the SCN (Terman et al., 1980).

1.5.1.2 Retinomotor movements

Photoreceptor cells in some species have the capacity to elongate or contract via actin-based and microtubule based cytoskeletal systems in the inner
segment region of the cell (for review see Burnside and Dearry, 1986). In some animals (e.g. fish) kept in a daily light-dark cycle, cones contract in the light and elongate during darkness, where as rods contract in darkness and elongate during light. The role of these movement are not clear, but it has been suggested that they are morphological components of light and dark adaptation (Burnside and Dearry, 1986). A study of cone movement in fish has shown this process to be circadian in nature since it persists in constant darkness, and also this elongation precedes the onset of light during the light-dark cycle, presumably preparing the retina for light onset (Kohler et al., 1990).

During the dark, in some species, it has been noted that pigments migrate from vesicles of the RPE cells to their cell bodies where they aggregate. This pigment migration process which is thought to be part of the dark adaptation of the retina is believed to be regulated by the light dark cycle (Burnside and Dearry, 1986).

Second messenger systems are also believed to be involved in the retinomotor movement processes. The major second messenger implicated is cAMP (Burnside and Dearry, 1986). Cyclic AMP promotes dark adaptive rod and cone movements and RPE pigment aggregation. In rods, cAMP and calcium act together to promote dark adaptive contraction. Cyclic AMP is believed to be the key mediator in dark adaptive responses of the photoreceptor-pigment epithelial complex (Besharse, 1988).
1.5.1.3 Expression of visual transduction genes

Expression of the mRNAs for several of the proteins in the visual transduction cascade is regulated as a function of the light dark cycle (fig 4). Diurnal rhythms of opsin (rhodopsin apoprotein) mRNA abundance have been observed in mouse, fish, and toad (Bowes et al., 1988; Korenbrot and Fernald, 1989; McGinnis et al., 1992). Opsin mRNA levels peak in early morning, near the time of light onset, and decreases thereafter to reach a minimum in the early to mid-dark phase. The retinal opsin mRNA levels may be controlled by a circadian oscillator since the rhythms persist in constant darkness in toad.

The mRNA of transducin is also regulated as a function of the light dark cycle (Bowes et al., 1988). Transducin mRNA levels in mouse and rat retinas fluctuate in a diurnal fashion that resembles the rhythm of opsin mRNA levels (fig 4). However, to date it has not been distinguished whether these changes in mRNA levels of transducin are circadian or light driven, and if they reflect transcriptional activation or a change in mRNA stability.

Arrestin (S-antigen, 48 kDa protein) mRNA levels in mouse and rat retinas display robust diurnal regulation with peak expression in the middle or late light phase (Bowes et al., 1988; McGinnis et al., 1992) (fig 4). As with transducin, the role of circadian oscillators in the regulation of arrestin mRNA levels is unclear.
Figure 4. Light-Dark cycling of opsin, Gtα, Gtβ and 48kDa protein (arrestin) mRNA levels in adult mouse. Obtained from Bowes et. al., 1988
McGinnis et al. (1992) has concluded that the diurnal fluctuations in mRNA levels are due to changes in transcriptional activity and not to changes in mRNA stability based on kinetic arguments related to rate of change of arrestin mRNA levels and its half life.

1.5.1.4 Expression and role of neuromodulators in retinal cycles

**Melatonin**

Melatonin is a putative neuromodulator that influences many aspects of dark adapted and rhythmic retinal physiology (Besharse et al., 1988). Melatonin is synthesized locally in the retina, primarily by photoreceptor cells, in a diurnal or circadian manner (reviewed by Cahill et al., 1991). Day-night differences in retinal melatonin content or release have been described in a number of species. In almost all of the vertebrate and invertebrate animals studied to date, highest melatonin levels occur in the dark while the lowest levels occur in light, during a 24 hour light-dark cycle (Reppert and Sagar, 1983; Tosini and Menaker, 1996). Light suppresses this nocturnal increase in retinal melatonin content (Hamm and Menaker, 1980). Melatonin levels in retinas that were kept under constant dark conditions, increased during the subjective night and was suppressed during the subjective day (Tosini and Menaker, 1996). This rhythmicity is believed to be produced through acute suppression of melatonin synthesis by light as well as nocturnal stimulation of synthesis by circadian oscillators (Hamm and Menaker, 1980).
This regulation has clear functional importance within the retina, where melatonin can act as a signal for darkness (Besharse et al., 1988). In the retina melatonin mimics darkness in causing aggregation of melanin pigments in the retinal pigment epithelium (Burnside and Darry, 1986), activation of disc shedding in rod photoreceptors, inhibition of retinal dopamine release (see below), and elongation of cone photoreceptor cells (Besharse et al., 1988). All of these processes are highly regulated and necessary for the normal functioning of the retina.

To a large extent, the circadian regulation of retinal melatonin is thought to result from the regulation of serotonin N-acetyltransferase (NAT) which is the rate limiting enzyme in the pathway of melatonin synthesis (Hamm and Menaker, 1980). Retinal NAT activity in most of the species that have been studied is high at night, low during the day, and suppressed by light (Besharse and Iuvone, 1983). Furthermore the circadian rhythmicity of NAT persists in constant darkness (Hamm and Menaker, 1980). It is not clear what molecular and cellular mechanisms are involved in the regulation of NAT. However, there is some evidence that suggests the involvement of cAMP and calcium in this mechanism. Cyclic AMP has been shown to induce the activity of NAT in cultured photoreceptor cells (Iuvone, 1990). As well, the nocturnal increase in NAT has been shown to be a calcium dependent process (Zawilska and Nowak, 1990).
Other neuromodulators are also involved in these regulatory mechanisms (see below).

**Dopamine**

Dopamine is a neurotransmitter synthesized and released from amacrine cells of the inner retina (Iuvone, 1986). A light adaptive role for retinal dopamine in a variety of species is supported by several studies indicating that retinal dopamine synthesis, release and turnover are stimulated by light (Besharse et al., 1988). The activity of retinal tyrosine hydroxylase, the rate limiting enzyme in dopamine synthesis, is stimulated by light and exhibits a diurnal rhythm with higher activity during the day (Parkinson and Rando, 1983). An extensive literature now supports the hypothesis that dopamine is a neuromodulator responsible for light adaptive responses observed in the retina. This neuromodulation is mediated through D2/D4 receptors, which couple to cAMP inhibition through Gi and are localized to the inner neuronal layers of the retina (Iuvone, 1986; Darry et al., 1991). Dopamine regulates photoreceptor retinomovement by eliciting light adaptive cone contraction (Burnside et al., 1993). Dopamine also mimics the effects of light on photopigment movements in the photoreceptor cells (Stenkamp et al., 1994). These observations suggest a functional role for dopamine in light evoked and circadian retinomotor movements.
Dopamine and D2 receptor agonists inhibit rod outer segment disc shedding, thus dopamine is believed to be a mediator of daytime or light mediated suppression of shedding (Besharse et al., 1988). As further evidence for the above hypothesis it has been shown that drugs that stimulate the release and synthesis of dopamine reduce the rhythm of disc shedding in rat retina (Reme et al., 1991).

Dopamine and D2 receptor agonists mimic the effect of light exposure, namely the inhibition of NAT activity and melatonin biosynthesis (Iuvone, 1986). Activation of dopamine receptors at night also shift the phase of the circadian clock that regulates melatonin biosynthesis, with a phase response relationship similar to that produced by light (Cahill and Besharse, 1991). These inhibitory effects of dopamine have been shown to be mediated by D2 and/or D4 like dopamine receptors which are coupled to cAMP inhibition (Iuvone, 1986). It should also be noted that the calcium dependent release of dopamine is dramatically suppressed by melatonin through a receptor mediated mechanism (Dubocovich, 1988). Although the mechanism of these processes are not clear it is thought that melatonin and dopamine act as antagonistic neuromodulators within the retina to modulate its regulatory processes (Cahill et al., 1991).

Serotonin

Serotonin is the immediate precursor to melatonin in its biosynthetic pathway. Even though it is widely accepted that NAT is the rate limiting enzyme
for melatonin synthesis, the synthesis of melatonin is significantly limited by the availability of the precursor serotonin. There have been some reports of diurnal variation in serotonin content of chicken retina, indicating elevated levels at night (Ehinger and Rose, 1988). There is very little known about the regulation of serotonin and its possible role in circadian regulations within the retina, thus it warrant further investigation.

**Glutamate**

The neurotransmitters glutamate as well as aspartate, stimulate rod disc shedding in a light independent manner (Greenberger and Besharse, 1985). Glutamate also produces an apparent increase in adhesion between RPE and photoreceptor cells (Matsumoto et al., 1987). Although the mechanism involved is not clear it appears to be receptor mediated (Greenberger and Besharse, 1985).

In terms of circadian or diurnal regulation there is very little known about this neurotransmitter. However, it has been speculated that since many metabotropic glutamate receptors are expressed in the retina and that their expression are localized to inner neuronal layers, glutamate may act as a neuromodulator within the retina (Hartveit et al., 1995).

**1.6 Rational and hypothesis**

Many regulatory processes in the retina are controlled by diurnal or circadian rhythms. These processes are mediated through neuromodulators and
neurotransmitters, many of which act through $G$ protein coupled receptors. As outlined above, many of these neuromodulators act on receptors coupled to Gi or Gq/11α proteins that mediate regulation of cAMP or Ca$^{2+}$. The goal of my work was to determine the expression of Gq/11α proteins in the retina and whether this protein is regulated by the light dark cycle. The hypothesis to be tested is as follows:

*Gq/11α is expressed in the vertebrate retina and its expression is regulated as a function of the light-dark cycle in a circadian manner.*

1.7 Specific objectives of the present study

1. To localize Gq/11α in rat retina.

2. To quantitate the levels of Gq/11α proteins present in the retina during the normal light dark cycle and compare this to changed in the Gtα protein levels.

3. To determine if any regulation of Gq/11α and Gtα are circadian or diurnal in nature.
2. Methods

2.1 Materials

Antibody raised against the C-terminal decapeptide of Gq/11α was purchased from Dupont NEN, Missisauga, ON. Horseradish peroxidase-linked goat anti-rabbit IgG and ECL (detection reagents) were purchased from Amersham Life Science, Arlington Heights, IL. A polyclonal antibody raised against purified Gtα was a generous gift from Dr. John K Northup, National Institute Of Mental Health, Bethesda, MD. Bovine Serum Albumin (BSA) was purchased from Sigma chemical company, St. Louis, MO. Ultra-sensitive ABC Peroxidase staining kit was obtained from Pierce, Rockford, IL USA Frozen Squid eyes were purchased from Calamari Inc., Woods Hole, MA. Bovine eyes were obtained from St. Helen's Meat Packers, Toronto, ON.

2.2 Animals

Adult male Wistar rats weighing approximately 250-300 grams were purchased from Charles River Laboratories, Montreal, Quebec. They were housed in pairs and fed with standard rat chow and water. Control animals were kept under 12 hour light 12 hour dark conditions for one week, other animals were kept either in constant dark or constant light conditions for four days before experiments. To utilize animals during the dark phase some animals where adapted
to reverse light dark cycles i.e. 12 hour dark 12 hour light conditions for one week before further experimentation.

2.3 Immunohistochemistry

Rat eyes were prepared for immunohistochemistry as follows. All steps were carried out under fluorescent laboratory light, except for animals kept in constant dark conditions in which dim light (same intensity as the original dark conditions) was used. Rats were sacrificed in a carbon dioxide chamber, the eyes were dissected out and placed in a 4% formaldehyde solution freshly dissolved in PBS, pH 7.3, for 4 days. Subsequently the eyes were passed through a gradient of 5%, 15%, and 25% sucrose dissolved in PBS for a period of 24 hours in each solution. The eyes were then enucleated (lens and aqueous humor removed), embedded in OCT embedding medium, (Tissue-Tek Miles Inc, USA) and frozen in isopropyl alcohol over liquid nitrogen and stored at -70 C until needed.

Gelatin coated slides were prepared to ensure adherence of tissue sections to standard microscope slides. The slides were coated with a solution containing 1% gelatin + 0.05% chromium potassium sulfate in water and oven dried at 60 C for 2 hours. The slides were then cooled to room temperature before use.

14 μm serial sections were cut using a cryostat (Jung CM3000) and placed on gelatin coated slides. After drying over night sections were preincubated for 30 min with PBS pH 7.3 with 1% BSA (to reduce background). Next they were
incubated with either anti-Gq/11α (diluted 1:1000 in PBS + BSA) or anti-Gtα (diluted 1:1000 in PBS + BSA) antibodies. Control sections were incubated with preimmune rabbit serum (Pre I, diluted 1:1000 in PBS). Then the sections were incubated with a biotinylated secondary anti-rabbit IgG (provided in the ABC kit) for 30 min and finally in ultra sensitive ABC peroxidase reagent for another 30 min (also provided in the ABC Kit). To visualize antibody binding the sections were incubated with 0.1% diamino-benzidine for 5 minutes which resulted in the formation of a brown oxidation reaction product. All washes were performed using PBS pH 7.3. For cytological assessment sections were counterstained with hemotaxyline.

In experiments which compared the effect of various light conditions on retinal histology, sections were prepared from animals kept in constant dark or constant light conditions and were compared to sections from animals kept in 12 hour light 12 hour dark cycles. These sections were stained with hemotaxyline alone.

The sections were examined using a Diastar (Reichert) microscope and photographed with Kodac technical pan film at 20X magnification.

2.4 Protein Extraction

To quantitate the total content of specific G protein subunits five rats were sacrificed every 6 hours for a period of 24 hours followed by every 3 hours for the
next 24 hours starting at the beginning of the light cycle. The eyes were harvested, enucleated, and retina was isolated (1 hour from sacrifice to isolation). Total protein was extracted from individual animals by incubating the 2 retinas in 600 µl of extraction buffer (10mM Tris pH 7.5, 10mM NaCl, 1.5mM MgCl, 0.5% Deoxycholate, 1% Tween, -20°C) for 1 hour on ice followed by centrifugation at 15,339g for 20 minutes at 4°C. Extracted proteins in the supernatant fraction were stored at -70°C before assay.

To assess the effect of light cycles on Gq/11α protein expression in a peripheral tissue, samples of spleen from animals at hour 3 and 15 (8 animals at each time point) of a normal 12 hour light 12 hour dark cycle were extracted in the same conditions used for retina.

2.5 Protein assay

Total protein concentrations were determined by the Amido Black protein assay described by Schaffner and Weissman (Schaffner and Weissman, 1973) with some modification. To construct a standard protein curve different amounts of BSA (0-30 µg) were used as standards. Protein extracts and BSA samples were diluted to a final volume of 280 µl with 0.1 M Tris-HCl pH 7.5, 0.2% SDS, 16% TCA to precipitate proteins. Samples were spotted on to 0.45 µm Millipore filters under vacuum. Filters were incubated with 0.25% Amido black in methanol:
acetic acid : distilled water (45 : 10 : 45) for 3 minutes and then destained by washing three times with de-staining solution to remove background dye binding [Methanol : acetic acid : distilled water, (90 : 2 : 8)]. Filters were rehydrated in water, blotted dry and blue protein spots were cut out of the filters and transferred to culture tubes. The blue dye was eluted by addition of 1 ml of elution solution (25mM NaOH, 0.05 mM EDTA and 50% v/v ethanol). The samples were mixed periodically for 10 minutes using a vortex mixer. The absorbance of the eluate was measured at 630 nm. A standard curve was generated by plotting the absorbance values obtained for different amounts of BSA standard against the protein content. The resulting standard curve was used to determine the protein concentrations of unknown samples.

2.6 Quantitative immunoblotting

Quantitative immunoblotting techniques as described by Mitchell and Bansal (Mitchell and Bansal, 1997) were used to quantitate the total amount of specific G protein subunits present in retinal extract preparations.

2.6.1 SDS-PAGE and Western blot

Protein extracts prepared from each retina at 3 different concentrations ranging from 20-40 µg, along with purified G protein subunits were subjected to electrophoresis using SDS-PAGE on 11% gel using Laemmli’s discontinuous
buffer system (Laemmli, 1970). Electrophoresis was performed at room temperature at 40 mA constant current. The separated proteins were transferred to nitrocellulose overnight in transfer buffer (25mM Tris, 190 mM Glycine and 20% methanol) at 4 C at 30 V constant voltage. The nitrocellulose was then blocked by incubation for 1hr in PBS containing 3% BSA and then incubated for 1hr with antiserum raised to specific G protein subunits (Gq/11α or Gtα) diluted 1:5000 in PBS containing 0.2% Triton X-100 (PBST) and 1% BSA. After 3 washes with PBST, blots were incubated for another hour with horseradish peroxidase-linked goat anti-rabbit IgG diluted 1:10,000 in PBST 1% BSA and G protein bands were visualized after 1 minute exposure to ECL reagent on Kodac X-omat autoradiography film.

2.6.2 Quantitation

Protein bands obtained after Western blotting were quantified using a laser densitometer, and the area of each band was determined using Image-Quant software. The amount of G protein present was calculated from a standard curve constructed from the densitometric values obtained with known amounts of purified G protein subunit run on the same gel. Quantities of each G protein subunit from increasing amounts of each retinal extract were plotted using the ENZFIT program. The amount of G protein expressed per milligram of total protein in each sample was calculated from the slope of each line.
The amount of G protein subunit present in protein extracts prepared from spleen was calculated using methods described above.

2.7 Purification of G protein standards

Purified Gtα was prepared from bovine retina by methods previously described (Fung et al., 1981). The Gq/11α standard used in these assays were purified invertebrate G44α extracted from squid eye membranes as described by Mitchell and Basal (Mitchell and Basal, 1997). Gi and Go were purified from bovine brain by methods previously described (Borkoch et al., 1984). All G protein subunits were purified to apparent homogeneity as assessed by SDS-PAGE electrophoresis and quantified by protein assay.

2.8 Antibody specificity

To determine the specificity of Anti-Gq/11α and anti-Gtα various purified G proteins (Gα44, Gt, Gi, and Go) were subject to electrophoresis on SDS-PAGE. Protein bands on the gel were visualized by staining with Coomassie Blue (0.1% Coomassie Blue solution in 40% methanol and 10% acetic acid). The same samples were subjected to electrophoresis, the proteins transferred to nitrocellulose, and probed with anti-Gq/11α or anti Gtα using Western blotting techniques as described above.
2.9 Statistical analysis

The mean values of Gq/11α and Gtα levels were statistically analyzed using unpaired Students t-test as indicated in the text. P values < 0.05 were considered statistically significant.
3. Results

3.1 Antibody specificity.

To determine the specificity of anti-Gq/11α and anti-Gtα that were used in our experiments various purified G proteins; Gα44 (invertebrate Gq), Gt, Gi, Go were subjected to electrophoresis and visualized by commassie blue staining (fig. 5A). The same samples were probed with anti-Gq/11α and anti-Gtα antibodies using SDS-PAGE and western blotting techniques. As shown in figure 5B Gq/11α antibody very specifically recognized only the Gα44 and not the other purified G-proteins. Similarly, anti-Gtα antibody only recognized Gtα and very weakly with the β subunit of Gi but not with Gqα, Giα, Goα (fig 5C).

3.2 Study 1: Immunohistochemistry of rat retina.

3.2.1 Distribution of Gq/11α and Gtα proteins in rat retina.

Having determined the specificity of our two antibodies, I then used immunohistochemical techniques to visualize the distribution of Gq/11α and Gtα proteins in sections of rat retina (fig 6).

As evidenced by a brown reaction product there is abundant expression of Gq/11α proteins in the retina with the majority distributed in the inner and outer
Figure 5. Gq/11α and Gtα antibody specificity. To determine the specificity of Gq/11α and Gtα antibodies several purified G proteins were subjected to SDS-PAGE. Protein bands were visualized by staining with commassie blue. The same samples were subjected to electrophoretic transfer to nitrocellulose, and probed for anti Gq/11α and Gtα using western blotting techniques. A: Commassie stained gel. Lane 1, purified Gα44. Lane 2, Purified Gtα. Lane 3, Purified Gi αβγ protein. Lane 4, Purified Go αβγ protein. B: Western blot with Gq/11α antibody. C: Western blot with Gtα antibody.
Figure 6. Localization of Gq/11α and Gtα in rat retina. 14μm sections of paraformaldehyde fixed rat retina from animals kept in 12 hour light 12 hour dark cycles were probed for anti-Gq/11α and anti-Gtα using immunohistochemical procedures as described in "methods". A: Control section incubated with preimmune rabbit serum. B: Section incubated with Gq/11α antibody. C: Section incubated with Gtα antibody. Each section is representative of 6 experiments. RPE (retinal pigment epithelium), PCL (photoreceptor cell layer), ONL (outer nuclear layer), OPL (outer plexiform layer), INL (inner nuclear layer), IPL (inner plexiform layer), GCL (ganglion cell layer).
plexiform layers. There was no evidence of staining in the photoreceptor outer segments (Fig 6, Panel B).

Gtα is also abundantly expressed in the retina. As anticipated from previous work by many others staining for Gtα was observed in the photoreceptor cell layer with the majority being in the outer segment area of the photoreceptors. No staining of Gtα was observed in any other layer of the retina (Fig 6, panel C). Gtα antibody was shown to react with the β subunit of Gi and Go, however the reactivity was very weak and could not be seen in our sections.

Control sections that were incubated with preimmune rabbit serum did not show any staining (Fig 6 Panel A).

3.2.2 Conclusions from study 1.

The results of these studies indicate that Gq/11α is expressed in the vertebrate retina and its expression is localized to the inner neuronal layers of the retina more specifically the inner and outer plexiform layers. It also confirms that Gtα expression is confined to the outer segments of the photoreceptor cell layer.

3.3 Study 2: Regulation of Gq/11α and Gtα proteins in rat retina.

3.3.1 Expression of Gq/11α proteins during light-dark cycles.

To investigate the expression of Gq/11α proteins in the retina throughout the day night cycle we examined its expression at intervals over a 48 hour period in 12 hour light- 12 hour dark cycles. In our first experiments we assessed the level
of expression every 6 hours for 24 hours and subsequently examined another
group of animals every 3 hours for 24 hours. The steady state levels of expression
of Gq/11α in total retinal extracts were examined using quantitative
immunoblotting techniques as shown in fig 7A and 7B. As shown in figure 3
quantitation of each retinal extract was carried out by immunoblotting three
concentrations of the extract (fig. 7B) and determining the concentrations of
Gq/11α by comparing the densitometric reading from each protein band to a
standard curve of purified Gqα run in the same gel (fig. 7A). Intraassey variability
was tested at some timepoints and were less than 3%.

The level of expression of Gq/11α in retinal extracts taken from animals at
various intervals throughout the 12 hour light 12 hour dark cycles over 48 hours is
shown in figure 8 (each point represents the average concentration of Gq/11α in 5
animals). Gq/11α began to rise near the end of the dark phase of the cycle,
remained high throughout the first half of the light phase and started to drop in the
second half of the light cycle. Protein levels remained low throughout the dark and
began to go up again just before the start of the next light phase. Peak levels of
expression occurred at approximately 6 hours into the light phase (27.8 ± 4.8
pmol/mg, average of 10 animals), while the lowest levels of expression were seen
at approximately the beginning of the dark phase (13.9 ± 3.6 pmol/mg, average of
10 animals) (fig. 9). These data indicate that the level of Gq/11α proteins in the
Figure 7. Quantitation of Gq/11α expression levels. Increasing amounts of purified Gq/11α and retinal membranes were subjected to SDS-PAGE and then immunoblotted using a Gq/11α-specific antipeptide antiserum, as described in “methods”. A: plot of densitometer values vs. amount of purified Gq/11α (blot shown in insert). B: Plot of quantity of Gq/11α derived from densitometer values for each membrane sample vs. amount of membrane protein (blot shown in insert). Results shown are from a representative membrane sample (of approximately 100 animals tested).
Figure 8. Expression of Gq/11α proteins during light-dark cycles. The steady state levels of expression of Gq/11α proteins over a 48 hour period were examined using immunoblotting techniques as outlined in figure 7. Changes in the level of expression Gq/11α over time were plotted. Each time point represents the average level of expression of Gq/11α in 5 animals ± SE. The results shown are representative of three independent experiments.
Figure 9. The maximum and minimum levels of expression of Gq/11α during a light dark cycle. Max: maximum level of expression of Gq/11α occurs at approximately 6 hours into the light phase (average, hour 6 and 30 of figure 8). Min: minimum level of expression of Gq/11α occurs in the beginning of the dark phase (average, hour . Gq/11α levels drop by approximately two thirds within 6 hours. Min values are significantly different from max values P<0.05, Student's t-test (n=10)
retina vary dramatically over the light-dark cycle and can decrease by approximately two thirds within 6 hours.

To assess the effect of the light phase on Gq/11α protein expression in a peripheral tissue, samples of spleen from the same animals used in the retinal study were extracted using the same conditions (at hour 3 and 15 of a normal 12 hour light 12 dark cycle). As shown in figure 10 the level of expression of Gq/11α in spleen did not vary significantly, whereas we had observed a dramatic change in Gq/11α in the retina at the same time points (see fig. 14).

These results indicate that the changes in Gq/11α observed in the retina are specific to this tissue.

3.3.2 Expression of Gα proteins during a light-dark cycle.

Having established that Gq/11α levels are cyclic in nature, we wanted to compare their expression with that of Gα a G protein that is expressed exclusively in the photoreceptor cells of the retina and previously shown to be regulated throughout the light-dark cycle, at least at the mRNA level (see introduction). We examined the expression of Gα every 3 hours over a 24 hour period (12 hour light-dark cycles) using quantitative immunoblotting techniques as shown in fig 11A and 11B. The changes in the level of expression of Gα over a 12 hour light
Figure 10. Expression of Gq/11α in spleen. To assess the effect of the light dark cycle on Gq/11α expression in a peripheral tissue, its expression was examined in spleen. Membrane proteins from animals kept in normal 12 hour light 12 hour dark conditions were prepared and Gq/11α levels were quantitated using immunoblotting techniques. Hour 3: Level of expression of Gq/11α in spleen of 4 animals at 3 hours into the light phase. Hour 15: level of expression of Gq/11α in spleen of 4 animals at 3 hours into the dark cycle. Gq/11α protein levels were not significantly different in the two samples P>0.05, Students t-test (n=4)
Figure 11. Quantitation of Gtα expression levels. Increasing amounts of purified Gtα and retinal membranes were subjected to SDS-PAGE and then immunoblotted using a Gtα-specific antiserum, as described in "methods". A: plot of densitometer values vs. amount of purified Gtα (blot shown in inset). B: Plot of quantity of Gtα derived from densitometer values for each membrane sample vs. amount of membrane protein (blot shown in inset). Results shown are from a representative membrane sample of approximately 50 animals tested.
Figure 12. Expression of Gtα proteins during light-dark cycles. The steady state levels of expression of Gtα proteins over a 24 hour period were examined using immunoblotting techniques as outlined in figure 11. Changes in the level of expression Gtα vs time was plotted. Each time point represents the average level of expression of Gtα in 5 animals ± SE.
12 hour dark cycle are shown in figure 12. Gtα levels started to go up just before the onset of light, they remained high throughout the first half of the light phase and then began to diminish. Gtα levels remained low throughout the hours of darkness cycle and began to go up again just before the start of the light. Gtα levels in the retina were greater than those seen for Gq/11α, and ranged from the highest level of expression of Gtα at the middle of the light phase (532 ± 57 pmol/mg, n = 5) and the lowest level in the dark (266 ± 32 pmol/mg, n = 5) as shown in figure 13. These data indicate that the Gtα protein levels fluctuate dramatically throughout the light-dark cycle, and that they can decrease by approximately one half within 3 to 6 hours.

3.3.3 The effects of constant light and dark conditions on the level of expression of Gq/11α and Gtα proteins.

Having established that both Gq/11α and Gtα protein levels fluctuate in a cyclic manner, we were interested to know whether these cyclic changes are diurnal in nature or are controlled by an intrinsic circadian oscillator. Some of the unique features of circadian rhythms are that they can persist in the absence of stimuli, they can anticipate the next phase of the cycle, and that constant stimuli will abolish their cycling. On the other hand diurnal rhythms require stimulus for
cycling and do not persist in its absence. We therefore examined Gq/11α and Gtα levels in animals that were kept in constant dark (lack of stimuli) or constant light (constant stimuli) for 4 days. As a control we also examined protein levels in another group of animals that were kept under normal conditions (12 hour light 12 hour dark cycles) at the same time. We assessed protein levels at the nominal high point of G protein expression (hour 3 of nominal light, PL) and at the nominal low point for expression (hour 15 of a nominal 24 hour cycle, PD).

As shown in figure 14 the decrease in expression of Gq/11α from light to dark observed in our control animals is maintained in animals that are kept under constant dark conditions. The ratio of PL/PD were 2.2 (average of 5 animals) and 1.9 (average of 10 animals) for control and constant dark respectively (fig 14).

In animals maintained in constant light for 4 days we observed 2 changes relative to controls. First the level of expression of Gq/11α was increased in the constant light animal group, and second, the decrease in Gq/11α levels seen in control animals in the dark phase was abolished in the constant light group. (fig. 15). PL/PD Gq/11α levels were 1.0 (average of 10 animals) for constant light animals.

When the Gtα levels were examined in the constant dark and constant light animals. Gtα PL/PD levels of animal that were kept under constant dark conditions
Figure 13. The maximum and minimum levels of expression of Gtα during a light dark cycle. Max: maximum level of expression of Gtα occurs in the middle of the light cycle (hour 6 of figure 12). Min: minimum level of expression of Gtα occurs at approximately 3 hours into the dark cycle (hour 15 of figure 12). Gtα levels drop by approximately a half within 3-6 hours. Max and min levels are significantly different P<0.05, Student’s t-test (n=5).
Figure 14. Gq/11α expression in constant dark conditions. To determine the effect of constant dark conditions on Gq/11α expression, its levels were quantitated using immunoblotting techniques in animals that were kept in constant dark conditions for 4 days and compared to levels in control animals that were kept in a 12 hour light 12 hour dark cycle. Protein levels were assessed at the nominal high point of G protein expression (hour 3 of nominal light, PL) and at the nominal low point for expression (3 hours into the nominal dark, PD). CO PL, Gq/11α expression in control animals at PL; CO PD, Gq/11α expression in control animals at PD; CD PL, Gq/11α levels in animals kept in constant dark at nominal PL; CD PD, Gq/11α levels in animals kept in constant dark at nominal PD. PL and PD are significantly different in both control and constant dark animals, control animals P<0.05 (n=5), constant dark P<0.05 (n=10), Student’s t-test.
Figure 15. *Gq/11α expression in constant light conditions.* To determine the effect of constant light conditions on *Gq/11α* expression, its levels were quantitated using immunoblotting techniques in animals that were kept in constant light conditions for 4 days and compared to levels in control animals that were kept in a 12 hour light 12 hour dark cycle. Protein levels were assessed at the nominal high point of G protein expression (hour 3 of nominal light, PL) and at the nominal low point for expression (3 hours into the nominal dark, PD). CO PL, Gq/11α expression in control animals at PL; CO PD, Gq/11α expression in control animals at PD; CL PL, Gq/11α levels in animals kept in constant light at nominal PL; CL PD, Gq/11α levels in animals kept in constant light at nominal PD. PL is not significantly different from PD in constant light animals, student's t-test, P>0.05 (n=10).
Figure 16. Gtxα expression in constant dark conditions. To determine the effect of constant dark conditions on Gtxα expression, its levels were quantitated using immunoblotting techniques in animals that were kept in constant dark conditions for 4 days and compared to levels in control animals that were kept in a 12 hour light 12 hour dark cycle. Protein levels were assessed at the nominal high point of G protein expression (hour 3 of nominal light, PL) and at the nominal low point for expression (3 hours into the nominal dark, PD). CO PL, Gtxα expression in control animals at PL; CO PD, Gtxα expression in control animals at PD; CD PL, Gtxα levels in animals kept in constant dark at nominal PL; CD PD, Gtxα levels in animals kept in constant dark at nominal PD. PL and PD levels were significantly different in both constant dark and control animals. Control animals, p<0.05 (n=5), Constant dark animals, P<0.05 (n=10), Student’s t-test.
Figure 17. Gtα expression in constant light conditions. To determine the effect of constant light conditions on Gtα expression, its levels were quantitated using immunoblotting techniques in animals that were kept in constant light conditions and compared to levels in control animals that were kept in a 12 hour light 12 hour dark cycle. Protein levels were assessed at the nominal high point of G- protein expression (hour 3 of nominal light, PL) and at the nominal low point for expression (3 hours in to the nominal dark, PD). CO PL, Gtα expression in control animals at PL; CO PD, Gtα expression in control animals at PD; CL PL, Gtα levels in animals kept in constant light at nominal PL; CL PD, Gtα levels in animals kept in constant light at nominal PD. PL levels are not significantly different from PD levels in constant light animals. P>0.05 (n=10), Student's t-test.
also maintained their difference as compared to control animals (fig. 16). PL/PD Gtα levels were 1.5 and 1.6 for control and constant dark respectively.

The condition of constant light exposure had a somewhat different effect on Gtα expression than that seen for Gq/11α. The absolute value of Gtα levels of animals that were kept under constant light conditions were lower than that of controls and unlike the control animals, the PL and PD levels of these animal did not vary (fig. 17). PL/PD ratio of Gtα levels of constant light animals was 1.3.3.4 Histology of rat retina.

To determine effects of various light conditions on the histology of rat retina, sections were prepared from animals kept in constant dark or constant light conditions and compared to control sections of animals that were kept under normal 12 hour light 12 hour dark cycles by staining with hemotaxyline (fig 18, panel A).

The retina of animals that were kept under constant light conditions appeared normal. However in some instances elongation of the photoreceptor outer segments was observed (fig 18, panel B).

The retina of animals that were kept under constant dark conditions also appeared normal (fig 18 panel C).
Figure 18. Comparison of the effect of various light conditions on retinal histology in rats. Retinal sections were prepared from eyes of rats kept under various light conditions and stained with hemotaxyline for histological observations. A, Control section from animal kept in 12 hour light 12 hour dark cycles; B, Section from animal kept in constant light; C, section from animal kept in constant dark. RPE (retinal pigment epithelium), PCL (photoreceptor cell layer), ONL (outer nuclear layer), OPL (outer plexiform layer), INL (inner nuclear layer), IPL (inner plexiform layer), GCL (ganglion cell layer).
3.3.5 Conclusions from study 2

These results indicate that both Gq/11α and Gtα levels are regulated in the retina differing by a factor of 2-3 folds over a 24 hour light dark cycle. Experiments under conditions of constant light stimulation or constant deprivation of stimulation indicate that these regulations are circadian in nature. The effect of constant light to increase Gq/11α levels but decrease Gtα levels indicate that light has different regulatory effects on the synthesis and turnover of these two G proteins.

3.4 Overall conclusions

1) Gq/11α proteins are expressed in the inner and outer plexiform layers of the retina.

2) Gtα proteins are expressed in the outer segments of the photoreceptor cell layer.

3) Gq/11α protein levels of the retina, are regulated and this regulation is circadian in nature.

4) Gtα protein levels are regulated in a circadian manner
4. Discussion

4.1 Distribution of Gq/11α in rat retina

This study attempted to determine the distribution of Gq/11α proteins in rat retina. My results indicate that Gq/11α is abundantly expressed in the retina and that the majority of this protein is distributed in the inner and outer plexiform layers of the retina which are sites of synaptic connections between different neurons. As described in section 1.1, in the outer plexiform layer the axons of photoreceptor cells synapse with dendrites of bipolar and horizontal cells. The inner plexiform layer is the site in which bipolar cells synapse with ganglion cells and interconnecting amacrine cells. From its distribution pattern, one can speculate that Gq/11α is expressed in either bipolar, horizontal, ganglion, or amacrine cells or indeed in all of these cells. This pattern of Gq/11α distribution is similar to that seen for the Giα proteins.

In adult rats G1 and G3 are distributed within the inner plexiform layers whereas G2 proteins are expressed in the outer plexiform layers. It is not clear which cell types express these proteins within the retina. Goα is also expressed in the outer and inner plexiform layers of the retina. The functional role of any of these Gi family members within the retina is not clear (Terashima et al., 1987). However, we can speculate about the functional role of these G proteins, Gi and
Go and also Gq/11 from the Gq family, to some degree based on receptors expressed in the secondary neurons that are thought to couple to them.

The distribution of some G protein coupled receptors within the retina have been determined. Many of these receptors are expressed in the secondary neuronal layers of the retina. Receptors that are known to couple to cAMP inhibition through the Gic protein and are found in the retina include metabotropic glutamate receptors (mGluR), 2, 4, 7, and 6. Metabotropic glutamate receptors 2, 4, and 7 are expressed in the ganglion cell layer of the retina and mGluR6 is expressed in the inner plexiform layer of the retina. Other receptors that are known to couple to Gic and are found in the retina include dopamine receptors of D2 and D4 subtypes which have been localized to the photoreceptor cell layer. Melatonin receptors which also couple to Gic are found in the inner plexiform layers of the retina. It is not clear which receptors couple to Goalpha; however, one candidate receptor which is expressed in the inner plexiform layers of the retina is the serotonin receptors, function of which is not clear. Metabotropic glutamate receptors 1, and 5 are the only Gq coupled receptors with a known distribution pattern within the retina, and these receptors are distributed within the inner plexiform and ganglion cell layers. The function of many of these receptors within the retina are not clear. However, based on their distribution it is predicted that they may play neuromodulatory roles within the retina.
The distribution pattern of mGluR1 and 5 are consistent with the distribution of Gq/11α observed in my studies and may indicate that Gq/11α is coupled to mGluR1 and 5 within the inner plexiform layers of the retina. This may shed some light into the possible functional role of Gq/11α regulation within the vertebrate retina (for further discussion see section 4.4).

From my studies one segment of the retina where Gq/11α is clearly not expressed is the photoreceptors. Indicating that Gq/11α is likely not involved in the phototransduction process itself. This result is somewhat of a variance with a recent report by Peng et.al. that has shown immunoreactive labeling of bovine photoreceptors with a G11α-specific antibody (Peng et.al., 1997). The difference in our results may come from the different species used in the two studies. However, this seems unlikely as we have not been able to detect Gq/11α in purified bovine photoreceptor preparations. A more likely reason for the contradictory distribution pattern observed by Peng et al. is a possible lack of specificity of the G11α antibodies used in their experiment. The peptide sequence that this antibody was raised against is a mid region sequence of G11α which is 60% homologous to the same region of Gtα as reported by Taylor and Exton, (1991), and Berstein et al., (1992). The specificity of this antibody was apparently not tested against Gtα and it is therefore possible that the observed distribution pattern of G11α by Peng et al. is due to the cross-reactivity of the antibody used
with Gtα due to its abundant expression in the retina and the lack of specificity of the antibody used. The anti-Gq/11α antibody used in my studies clearly does not cross react with Gtα or any other Gα subunit (figure 5). The Gqα distribution in the inner plexiform layer observed by Peng et. al. was similar to our observations using the Gq/11α common antibody.

4.2 Regulation of Gq/11α and Gtα protein levels in rat retina

4.2.1 Gq/11α and Gtα protein concentrations during a light-dark cycle.

In my study I have quantitated the amount of Gq/11α proteins present in the rat retina during several 24 hour light-dark cycles and compared these to the levels of Gtα protein in the eyes of the same animals. Robust changes in the concentration of Gq/11α were observed. High Gq/11α levels occurred in the first half of the light phase whereas low Gq/11α levels were observed in the middle of the dark phase. Gq/11α levels oscillate by approximately two thirds within 6 hours.

In my experiments it was observed that the levels of Gq/11α start to go up following an initiating light signal. Even though Gq/11α levels seem to begin to increase slowly in the middle of the dark cycle a dramatic increase in their levels is not observed until the start of the light cycle.
In order to understand the significance of the changes observed in the level of expression of Gq/11α, it is important to distinguish whether these changes are due to protein degradation, transcription of Gq/11α genes or both. Very little is known about either process for the Gq family of proteins. Studies of Gq/11α turnover in gonadotrophs and Chinese hamster ovary cells indicate that the half life of Gq/11α proteins in these cells are approximately 18-20 hours. This turnover rate for Gq/11α was decreased to 8-10 hours by stimulation through the Gq/11α pathway without altering Gq/11α transcription (Shah et al., 1995; Mitchell et al., 1993). The turnover rate of Gq/11α can be increased by stimulation of pathways that act through this protein. This may account for the dramatic changes observed in Gq/11α concentrations observed in my experiments. My experiments showed that Gq/11α concentrations in the retina dropped 2 to 3 fold within 6 hours. In order for this decrease to be achieved by protein degradation (ie an induction of the turnover rate) the half life of Gq/11α would have to be in the order of 4 hours with no new protein production. A more likely possibility is that both degradation and changes in the level of transcription of Gq/11α genes are responsible for the changes observed in the level of expression of Gq/11α proteins in rat retina. However, further studies that examine Gq/11α mRNA and protein turnover are required to determine if this is the case.
In the current study the level of expression of Gtα during a 24 hour light-dark cycle was also investigated. Gtα levels at all times, are approximately 20 to 30 times higher than that of Gq/11α in rat retina. Gtα levels are high in the first half of the light phase at which point Gtα levels drop by approximately one half within 3 to 6 hours. Gtα levels remain low throughout the dark and begin to go up again just before the start of the next light cycle. The cycling of Gtα concentrations is therefore similar to that of Gq/11α. However, the underlying mechanisms involved in this cycling may be different from that of Gq/11α as discussed later.

These experiments are the first to determine the regulation of Gtα protein in the retina. However, the cyclic nature of Gtα mRNA levels has been demonstrated in both rat and mouse retinas (Brann and Cohen, 1987; Bowes et al., 1988). These studies indicated that the highest levels of Gtα mRNA occur 1.5 hours before the start of the light cycle. The peak of Gtα protein levels is seen at 6 hours into the light cycle indicating that Gtα protein levels follow that of the mRNA levels with a lag time of approximately 7 hours. It is not clear whether these changes observed in Gtα mRNA levels are due to a change in the rate of transcription or the stability of the mRNA and thus warrant further investigations.

4.2.2 Circadian regulation of Gq/11α and Gtα protein levels in rat retina.
To investigate whether the changes observed in Gq/11α levels were circadian or diurnal in nature. The level of this protein at nominal high and low time points were quantitated in the absence of stimulus i.e.: constant darkness. My results indicate that Gq/11α levels continue to cycle in constant darkness similar to that seen in normal 24 hour light-dark conditions. This suggests that the levels of expression of Gq/11α are controlled by a circadian oscillator. It also indicates that light does not stimulate the turnover rate of these proteins as it would be anticipated to do so if the changes observed in my experiments were due to an increase in the turnover rate by light stimulation of the Gq/11α pathway.

To further investigate this possibility the quantity of Gq/11α protein was measured at the nominal high and low time points in animals kept under constant light conditions. Although circadian rhythms can act independently of stimuli (e.g. light), they are abolished by constant light stimuli. Gq/11α cycling was indeed abolished under constant light conditions. These results are further evidence that Gq/11α levels are controlled by a circadian regulator. The high Gq/11α concentrations in these animals indicate that light is not inducing the degradation of these proteins.

Similar experiments were performed to determine the effect of constant light on Gtα protein levels in the same animals. These experiments showed that
Gtα protein levels were also under the control of a circadian oscillator; however, the effect of constant light was to decrease Gtα levels.

These results indicate that although Gq/11α and Gtα proteins oscillate in a similar manner throughout the circadian cycle, they may be regulated by distinct processes. The lack of a dark period in the constant light animals inhibits Gq/11α degradation, thus the Gq/11α concentrations continue to go up. Gtα on the other hand remains at the low levels in these animals. Suggesting that in order to increase Gtα production (either transcription or translation) the photoreceptors need a period of darkness. This result is consistent with previously published data showing that Gtα mRNA levels increase in the dark (Brann and Cohen, 1987; Bowes et al., 1988). Alternatively, there is also the possibility that the constant light conditions have induced retinal damage. This damage generally begins at the rod outer segment area of the retina (site of Gtα expression) and spreads to other areas of the retina at a later stage. Histological examination of the retina from these animals (constant light) did not show any sign of damage, and therefore this is likely not the reason for lower Gtα levels observed in constant light conditions.

4.3 Possible circadian regulators of Gq/11α and Gtα

Many processes in the retina are regulated as rhythmic events some of which are controlled by a circadian oscillator. The mammalian retinal clock is an
intrinsic oscillator, however the molecular components of this machinery have not been fully identified. The major neuromodulators involved in the retinal clock, like that of the SCN, are thought to be melatonin and dopamine (Tosini and Menaker, 1996). As discussed earlier melatonin levels are high in the dark while dopamine levels are high in the light (for review see section 1.5.1.3).

The results of my studies indicate that both Gq/11α and Gtα protein concentrations are controlled by a circadian oscillator during a 24 hour light-dark cycle. It is of interest to speculate on what specific component of this circadian clock including neuromodulators may be involved in these regulatory processes. Having established that dopamine receptors are expresses in photoreceptor cells and that they are coupled to cAMP inhibition, cAMP levels in the photoreceptors should be high in the dark. Dark regulation of Gtα mRNA levels may therefore be mediated by increased cAMP. This may be facilitated by an inhibition of dopamine release by melatonin during the dark. In addition the low Gtα concentrations observed in animals kept under constant light conditions would therefore be due to an increase in dopamine and decrease in melatonin levels under these conditions.

It is more difficult to speculate why Gq/11α levels continue to increase in these animals and remain at a high level, partly because the dynamics of the Gq/11α mRNA levels during a 24 hour light-dark cycle are not known. One possible explanation is that light prevents the degradation of this protein. Since
melatonin receptors and Gq/11α are expressed in cells of the plexiform layers the lack of Gq/11α degradation could be the result of decreased melatonin levels. Melatonin levels are low in the light and its synthesis is inhibited by light. Again, because melatonin receptors couple to Gi and inhibition of adenylyl cyclase, this suggests a role for cAMP in the regulation of Gq/11α turnover and possibly transcription. The true interaction of these neuromodulators with the regulatory processes involved in the control of Gt and Gq/11α expression is not clear and warrants further investigations using dopamine and melatonin receptor antagonists.

4.4 The possible role of Gq/11α regulation in the vertebrate retina.

Until recently it was believed that G protein levels were static within any given system and that regulatory processes occurred primarily at the receptor level. However, increasing evidence now suggests that this may not be true and that G protein levels are subject to change under various conditions which can affect physiological responses within any given system. My studies indicate that there is a robust change in the concentration of Gq/11α during a 24 hour light-dark cycle and that these changes are highly regulated in a circadian manner. These highly regulated changes will probably result in variation of biological responses within the retina that may be important for its normal functioning. As to what these
responses may be I can only speculate at this stage based on my results and previous knowledge about Gq/11α proteins and the receptors that couple to them.

Gq/11α proteins are known to activate phospholipase C (for review see section 1.4.2). This leads to hydrolysis of PIP2 into two second messengers, IP3 and DAG. IP3 causes the release of calcium from intracellular storage sites (Rayer et al., 1990). DAG activates protein kinase C and results in phosphorylation of cellular components. This receptor mediated action of Gq/11α may serve as a clue to its possible function within the retina. Based on the localization of Gq/11α and G protein coupled receptors one can speculate as to which receptor couples to Gq/11α within the retina. The strongest candidates are the metabotropic glutamate receptors (mGluR). In fact preliminary studies using group I mGluR agonists in our laboratory indicates that the receptor involved may be the group I mGluRs. As mentioned before all subtypes of mGluRs with the exception of mGluR3 are found in the retina (see section 1.3). mGluR1 and mGluR5 which are known to couple to PLC activation are found in the inner plexiform and ganglion cell layers and are thought to be expressed by amacrine and ganglion cells within the retina (Hartveit, 1995). The functional role of the mGluRs are not clearly established but they are thought to facilitate the activation of the ionotropic glutamate receptors within the retina possibly through activation of protein kinase C (Tachibana and Okada, 1991). Ionotropic glutamate receptors are the primary mediators of glutamate action in the neuronal transduction pathways of the retina (for review see section
1.2). In the light glutamate is released by depolarized ON-bipolar cells onto amacrine and ganglion cells within the inner plexiform layer of the retina. Glutamate mediated depolarization of amacrine and ganglion cells result in the opening of ion channels and cation conductance through these channels. Since excessive calcium within a neuron has been shown to result in neurotoxicity (for review see Orrenius and Nicotera, 1994) it becomes especially important to regulate calcium levels within the neurons of the retina. This may possibly reflect why Gq/11α fluctuates during the light-dark cycle. Fluctuations in Gq/11α levels might be necessary to insure sub-toxic levels of calcium within the retina. If circadian regulation is blocked then Gq/11α levels will continue to increase and possibly lead to more efficacious activation of ionotropic glutamate receptors through mGluRs. Indeed, a role for mGluR 1 and 5 in neuronal survival and toxicity has been found in the retina (Price et. al., 1995).

4.5 Conclusion

In conclusion, my studies have shown that Gq/11α is expressed in the vertebrate retina and that it is mainly distributed in the inner and outer plexiform layers. My results also showed that both Gq/11α and Gtα protein concentrations are highly regulated in a circadian manner. The disruption of the circadian processes have been shown to cause retinal damage (Noell, 1980). Given the role of Gq/11α activation of protein kinase C as mediators of neurotoxicity my
identification of the regulation of Gq/11α may indicate a neuroprotective role for regulation of this protein. Much remains to be known about rhythmic retinal physiology. However, having identified some of the components of these circadian processes, there is a particularly exciting possibility that pharmacological approaches could be used to manipulate these rhythmic events in retinal diseases and that it may be possible to prevent or delay the progression of retinal degeneration by using drugs to manipulate or mimic the retinal circadian clock.
5. References


