Based on a Symposium on Pineal and Retinal Relationships held in Sarasota, Florida, May 3–5, 1985, sponsored by the National Institute of Child Health and Human Development and the National Eye Institute.

PINEAL AND RETINAL RELATIONSHIPS

Edited by

Paul J. O'Brien

Laboratory of Vision Research
National Eye Institute
National Institutes of Health
Bethesda, Maryland

David C. Klein

National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland

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Preface

It has long been known that the pineal and the retina share common structures, namely, photoreceptor elements, that are apparent in the mature pineal organ in lower vertebrates and embryologically in some higher vertebrates. More recently it has become clear that they also share certain physiological functions, such as circadian rhythms, and biochemical features, such as melatonin synthesis, rhodopsin kinase activity, and the highly antigenic S-antigen. These discoveries have suggested strongly that there could be many more processes shared by these organs which are otherwise unique in the vertebrate organism.

To explore these possibilities, the National Institute of Child Health and Human Development and the National Eye Institute sponsored a Symposium on Pineal and Retinal Relationships in Sarasota, Florida, May 3–5, 1985. This preceded the annual meeting of the Association for Research in Vision and Ophthalmology which was attended by investigators from North America, Europe, and Asia.

The purpose of the symposium was to bring together pineal and retinal investigators who have worked primarily in their separate fields. Our hope was that discussion of the unusual properties of the pineal organ and the retina would reveal additional areas of overlapping interests as well as opportunities for further research based on a sharing of insights and methodologies. This collection of papers presented at the symposium provides a view of the extensive interactions already taking place and a hint of the opportunities yet to be explored.

Paul J. O'Brien
David C. Klein

HISTORICAL PERSPECTIVES OF PHOTONEUROENDOCRINE SYSTEMS*

Andreas Oksche

Department of Anatomy and Cytobiology
Justus Liebig University of Giessen
Giessen, Federal Republic of Germany

I. INTRODUCTION

There is an increasing body of evidence that the retina of the lateral eyes and the pineal organ share common photoreceptive structures, biochemical processes and physiological functions including biorhythmic phenomena. The aim of this Symposium is to elucidate the features shared by these organs and to establish strategies for further research. This overview is intended to cast light on the development of the complex concept of photoneuroendocrine systems and to pay tribute to the great masters in the field.

According to our present knowledge, photoneuroendocrine systems encompass (1) a retino-hypothalamic projection to the suprachiasmatic nucleus; (2) primary photoreceptive pineal organs and pineal glands receiving a photic input from the retina; and (3) deep hypothalamic or encephalic photoreceptive elements not yet morphologically identified and only to a limited extent characterized physiologically.

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II. HISTORICAL DEVELOPMENTS

A. General outline - problems - definitions

The existence of an autonomic (vegetative) component of the optic system, capable of influencing a number of biological functions (e.g., color change, seasonal adjustment of reproductive mechanisms) was first defined by Ernst Scharrer (1937), based on his earlier experimental studies (Scharrer, 1928), the fundamental investigations of his scientific teacher Karl von Frisch (1911), and the elegant experiments of Benoit (1935). Later on, Hollwich (1952) defined this apparatus as the "energetic portion of the visual pathway" (cf. Hollwich, 1979). Finally, a general concept of photoneuroendocrine systems was introduced by Ernst Scharrer (1964).

Scharrer (1964) emphasized that photoneuroendocrine functions are controlled by a component of the optic system distinct from the apparatus for vision and optic reflexes. By involving neuroendocrine mechanisms this component participates in the control of autonomic functions that respond to periodic changes in the environment. Among the extrinsic and intrinsic inputs serving the control of endocrine mechanisms, photoperiodic (seasonal and daily) information appears to play a prominent role. For this

Figure 1. Letter of thanks (unpublished autograph) showing Karl von Frisch (1886-1982) at an age of 95.

Translation (A. O.) of the handwritten comment: "Dear Herr Oksche, Many thanks for your kind note of congratulations and for the set of reprints. I am indeed very pleased that my early observations on the photosensitivity of the pineal organ of minnows have now found increasing attention. At that time, Sigmund Exner* came to the Biological Laboratories (Biologische Versuchsanstalt **) in the Prater, and I showed him the experiments, only then was he really convinced.***"

* Sigmund Exner (1864-1926), Professor of Physiology and Head of the Department of Physiology at the prestigeous Medical Faculty of the University of Vienna. Distinguished neurophysiologist. Among his numerous publications are important contributions to visual physiology.

** The Biologische Versuchsanstalt belonged to the institutes of the Austrian Academy of Sciences.

*** Karl von Frisch published his classical treatise on mechanisms of color change (based on the material of his Ph.D.-thesis) in 1911, at an age of 25.

Es steigern sich die guten Wünsche
Je höher sich die Jahre ranken.
So hab' ich vielen lieben Worten
Diesmal noch herzlicher zu danken.

Sie fügen sich harmonisch ein
In meine späten Lebensstunden
Und halten mir den alten Kreis,
So hoff' ich, weiterhin verbunden.

München, 20. November 1981

K. v. Frisch

finden. Seinerzeit ist Sigmund Exner in Wien zu der biol. Versuchsanstalt im Prater gekommen, ich habe ihm die Versuche gezeigt, erst dann war er abgegangen.

Lieber Herr Oksche,
Vielen Dank für Ihren freundlichen Glückwunsch und die Sendung der Sonderdrucke. Es freut mich natürlich daß meine alten Beobachtungen über die Lichtempfindlichkeit des Pinealorgans bis heute gebührende Beachtung

K. v. F.

apparatus Scharrer postulated the existence of a retinohypothalamic pathway, at that time a hypothetical connection. Moreover, from his own experiments in teleosts he was familiar with light-sensitive pineal organs and deep diencephalic (encephalic) areas; this analysis was a natural extension of the concept of Karl von Frisch (1911).

Karl von Frisch can be regarded as the founder of the discipline of photoneuroendocrinology. Although the Nobel Prize (1973) was awarded to him in appreciation of his fundamental discoveries in the field of behavioral physiology ("language of the bees"), during his long lifetime he never lost interest in extraretinal photoreception (see von Frisch, 1970 and Fig. 1). In 1911 he published his comprehensive treatise of mechanisms of color change in teleosts. The European minnow, Phoxinus phoxinus (Phoxinus laevis), well known for its rapid color change, was one of his favorite experimental animals. Von Frisch observed that, like in intact individuals, bilaterally blinded minnows became pale in the dark and darkened almost immediately after re-exposure to light. In contrast with direct illumination of the trunk of blinded minnows, which had no effect on pigmentation, shading of the eyeless head led to a concentration of the melanin pigment, whereas illumination of the head resulted in an expansion of the pigment granules. Local anesthesia of the integument, transection of all portions of the trigeminal nerve and the stepwise removal of the entire skin of the head region were without effect on the light-dependent melanophore reaction. From these experiments it was evident that (1) the light-sensitive receptors are located within the skull, and (2) the parietal spot is the most effective extraretinal site in evoking response by melanophores. Von Frisch realized that a peculiar diencephalic structure, the pineal organ, lies directly beneath the parietal spot; for anatomical details he quoted the comprehensive treatise of Studnička (1905) on parietal organs, another milestone in the analysis of photoneuroendocrine systems. According to von Frisch, after pinealectomy the melanophore response was completely abolished for just one day; subsequently the light-dependent reactivity of the melanophores was restored. From experiments with surgical lesions he concluded that the "deep encephalic photoreceptor" must be sought in the diencephalon, in a layer close to the third ventricle. Furthermore, he suggested that the pineal sense organ might be regarded as a particularly dense accumulation of extraretinal photoreceptor cells. Finally, he drew attention to the fact that the lumen of the teleostean pineal organ, an evagination of the diencephalic (third) ventricle, is continuous with the rest of the ventricular system. He emphasized the necessity of

fine-structural investigations of this entire area.

The experimental approach with P. phoxinus was modified by Ernst Scharrer (1928) who examined light-dependent conditioned swimming and feeding reflexes. These reflexes appeared to furnish more reliable functional criteria than change in color. Scharrer was particularly attentive to the problem of the extra-retinal, extra-pineal sites of photoreception. During the course of his histological examinations he discovered secretory nerve cells in the preoptic nucleus of P. phoxinus. This observation became the inception of the concept of neurosecretion (Ernst and Berta Scharrer, 1937; cf. 1963), the fundament of our present knowledge of peptidergic neurons. However, it could not be proven that these neurosecretory cells are directly involved in extraocular photoreception. Several decades later, when presenting his general concept of neuroendocrine systems, Ernst Scharrer (1964) raised the question whether all neurons or only specialized elements are endowed with the capacity of recording photic stimuli. This crucial problem is still open to discussion. It is closely related to the existence of photopigments and other photolabile compounds; reversible change caused by photoactivation of enzymes may also represent the initial component in systems of biological photoreception (for literature, see Oksche and Hartwig, 1975, 1979). The microspectrophotometrically recorded photolabile compounds in diencephalic subependymal areas of Phoxinus phoxinus and tadpoles of Rana temporaria remain unidentified (Hartwig, 1975; cf. Oksche and Hartwig, 1979).

Evidently, the diencephalic primordium encompasses not only a "secretory field" (Wingstrand) but also a "photoreceptive field" (von Frisch). The latter might provide the matrix for the formation of retinal, pineal and "deep diencephalic" photoreceptor elements (cf. Oksche and Hartwig, 1975; Vigh-Teichmann and Vigh, 1983). After insertion of a methylene blue-stained colloidin lamella as a barrier at the level of the median eminence of the crested newt (Triturus cristatus), Sacerdote (1971) observed differentiation of ectopic retina-like structures in the hypothalamo-hypophyseal area. Unfortunately, to date, this exciting experiment has not been repeated by other workers. For a conclusive statement, ultrastructural and immunocytochemical methods must be applied in this type of study.

B. Deep encephalic photoreceptive elements

Already in his early experiments Benoit (1935) was able to show by use of bilaterally enucleated birds (domestic

mallards) that eyes are not necessary for the light-dependent gonadal response (see also Benoit, 1970). Since that time this phenomenon has been confirmed by a number of other investigators in different avian species (for references, see Yokoyama et al., 1978). To localize the encephalic sites of photoreception more precisely, chronically implanted light-conducting optic fibers have recently been used (cf. Yokoyama et al., 1978). Only implants within the mediobasal hypothalamus or close to this site were effective. However, the character of the photosensitive elements remains enigmatic; in avian species, thus far, the search with ultrastructural, microspectrophotometric and immunocytochemical methods has not provided positive results (cf. Oksche and Hartwig, 1979; Vigh-Teichmann and Vigh, 1983; personal communications, unpublished). The wide field of photobiology in avian endocrinology is beyond the scope of this historical review (for details, see Epple and Stetson, 1980). In the present context only the following points should be mentioned: (1) In photoperiodic responses of birds two aspects must be considered: (i) time and (ii) photosensitivity, the latter being a periodic function (Farner and Gwinner, 1980). (2) It is open to discussion whether the known retinohypothalamic tracts of birds have a photoneuroendocrine function, whereas we do know that there are photoneuroendocrine mechanisms that do not involve a retinohypothalamic tract (cf. Oksche and Hartwig, 1975).

C. Retinohypothalamic projections

Keeping this in mind, only a few general comments should be made on the retinohypothalamic projection. Scharrer (1964) emphasized the uncertainty concerning the precise origin, course and termination of this pathway. A number of earlier statements on the existence of such connections was based on artifacts (for references, see Oksche, 1970). However, in 1972 Hendrickson et al, as well as Moore and Lenn succeeded in demonstrating a retinohypothalamic projection to the suprachiasmatic nucleus of the mammalian hypothalamus; anterograde transport of radioactive amino acids and electron-microscopic visualization of degenerating nerve fibers were used in these studies (cf. Moore, 1973). Homologous projections were shown to exist also in the avian brain (cf. Oksche and Hartwig, 1979). In the light of this evidence the suprachiasmatic nucleus appeared to represent the exclusive port of entry for visual inputs to the hypothalamus. More recent research reports, however, mention accessory optic projections to circumscribed areas of the preoptic and mediobasal hypothalamus (cf. Mai and Teckhaus, 1982). Of particular

importance are the observations that the suprachiasmatic nucleus (1) contains populations of peptidergic neurons, especially vasopressin- (or vasotocin-) immunoreactive elements, and (2) can be regarded as an essential component of the biological clock (for references, see Aschoff et al., 1982) controlling also endocrine (e.g., adrenocortical) functions. Efferent fibers have been traced from the suprachiasmatic nucleus to neurohemal areas of the median eminence and to other nuclear areas of the hypothalamus and brainstem. Finally, the suprachiasmatic nucleus is an important link in the optic pathway serving the control of the sympathetic innervation of the mammalian pineal organ. In poikilothermic vertebrates various hypothalamic optic projections have been described. The role of these accessory bundles in neuroendocrine regulation (e.g., color change mechanisms via the intermediate lobe of the hypophysis) is, however, open to discussion.

The cellular origin of the retinohypothalamic tract is still an unsolved problem. It is, however, noteworthy that the retina may display nerve cells (mostly amacrine elements) containing biogenic amines or neuropeptides (Bruun et al., 1984; see references). To date, there is no evidence that these neurons might represent photoneuroendocrine elements; biogenic amines and neuropeptides may also function in a more conventional way as neurotransmitters and neuromodulators. At any rate, the retina is of diencephalic (hypothalamic) origin, and thus may be endowed with neuroendocrine properties.

D. Pineal complex

The pineal organ is a crucial component of photoneuroendocrine systems; in phylogenetic terms it can be considered as an extraretinal photoreceptor, biological clock and endocrine gland. Since structures and functions of the pineal organ constitute a central issue of this symposium, I shall restrict my comments to a few major aspects. These reflections are based on more than two decades of personal comparative work in this area (cf. Oksche, 1983, 1984). Outstanding comparative concepts of the pineal problem were presented by Studnička (1905) and Bargmann (1943). These eminent pioneers clearly recognized the photoneuroendocrine character of the pineal organ and came to highly predictive conclusions (for details, see Oksche, 1985):

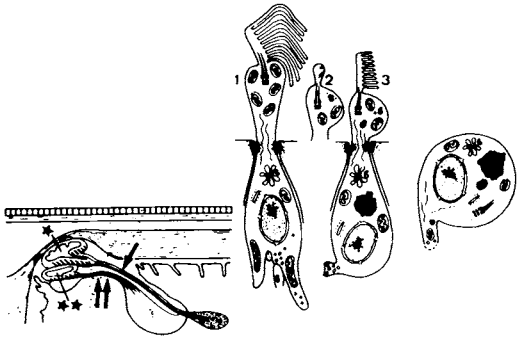
"These organs served originally, as one may assume with a certain degree of certainty, photoreceptive functions, however, they persisted in this form only in a relatively few groups of vertebrates. One of the parietal organs, the

pineal organ, has undergone a strange change in function; a sense organ was transformed into a gland of complex structure and enigmatic function. This functional alteration was accompanied by changes in the structural pattern of the organ; however, the onset of these changes is difficult to recognize, namely the structure of a functioning sensory pineal organ may also alter in a manner that it, at least partly, resembles a gland." (Studnička, 1905; translated by A.O.)

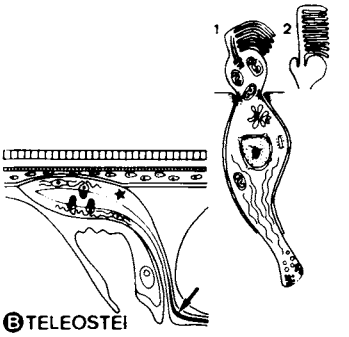
"The specific cellular elements of the pineal organ of the lower vertebrates display the character of sensory cells. Their morphological features allow the assumption that they are capable of secretory functions. One may tend to attribute to these elements, which both genetically and functionally resemble neurons, a synthetic capacity, last but not least with reference to the secretory (neurocrine) activity of nerve cells, particularly characteristic of the diencephalon. One may assume that in the surface-exposed pineal organs of lower forms the secretory activity of pineal receptors depends directly on illumination. The transformation of the pineal organ into a compact, highly vascularized body is accompanied also by a change in the structure of pinealocytes; after regression of their receptive segments the sensory cells are transformed into pinealocytes of the mammalian type. This results in a loss of the polar structure of the cells. It is an experimental task to investigate whether pinealocytes, after prevention of direct exposure to light, are still capable of receiving impulses via neural pathways coupled to the optic system thereby serving synthetic functions. Thus, the comparative approach leads to a working hypothesis that will most likely be fruitful in understanding the pineal organ." (Bargmann, 1943; translated by A.O.)

Eakin and Westfall (1959) and Steyn (1959) were the first to show that in ultrastructural terms the receptor cells in the parietal eye of lizards resemble retinal cones. Moreover,

Figure 2. Comparative representation of pineal complexes. Diagrammatic midsagittal views (after Studnička, 1905) in relation to the respective features of the pinealocytes of the receptor line. Single star: pineal organ (epiphysis cerebri); double star: parapineal organ (cyclostomes, lacertilians) or frontal organ (anurans); single arrow: pineal tract; double arrow: parapineal (parietal) tract (cyclostomes, lacertilians) or frontal-organ tract (anurans). Note diversity in ultrastructural details of the pinealocytes (1-3) indicating transformation of outer segment structures and increasing secretory capacity. After Oksche (1983). Courtesy Plenum Press,New York.



A CYCLOSTOMATA, PETROMYZONTIDAE



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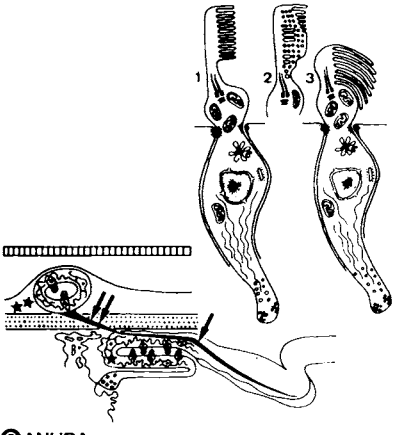

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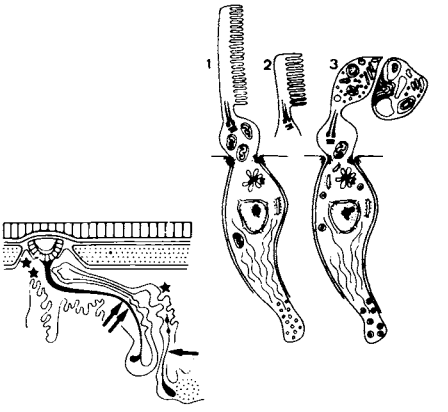

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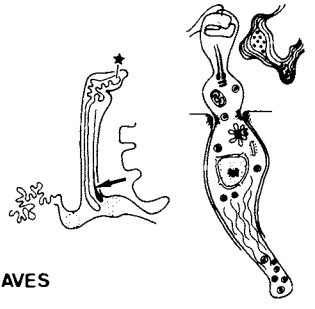

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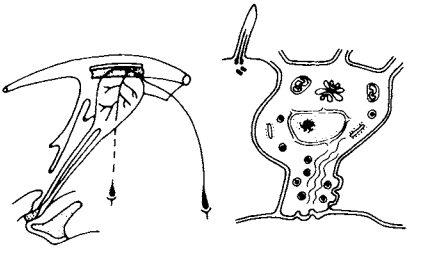

F MAMMALIA

Lerner et al. (1958, 1959) isolated and identified melatonin (5-methoxy-N-acetyltryptamine), the active principle in bovine pineal extracts capable of blanching the skin of frogs (see McCord and Allen, 1917). Finally, nearly a century after the discovery that the eye-like parietal organs of lower vertebrates are homologues of the pineal body of mammals (for references, see Studnička, 1905; Bargmann, 1943; cf. Stieda, 1865), Dodt and associates (1961) succeeded in demonstrating the direct electrophysiological response of these organs to light (cf. Dodt, 1973). My close interdisciplinary cooperation (since 1962) with Dodt and his colleagues (see Oksche, 1971; 1983) was paralleled by the systematic comparative studies (since 1966) of Collin (see Collin, 1971). Independently, we came to the conclusion that the secretory elements of the pineal gland (the pinealocytes) are derivatives of primary sensory (photoreceptive) cells (see Collin and Oksche, 1981 and Fig. 2). Furthermore, from our observations I suggested that in pineal photoreceptor cells of neuronal character the photic information can be translated into a neuroendocrine response, thus establishing photoneuroendocrine units (Oksche, 1970, 1971). This concept has continued to be an attractive working hypothesis (cf. Falcon, 1978) and it is still of actual interest (cf. Ekström, 1983; Falcon, 1984). Vigh-Teichmann and Vigh (1983) regard these cells as cerebrospinal fluid-contacting neurons. The phylogenetic process of the cytological transformation of pinealocytes (cf. Collin and Oksche, 1981) is closely correlated with the problem of the possible multiplicity of the pinealocytes of the receptor line (see Collin, Hartwig, Korf, van Veen, Vigh-Teichmann, this volume).

Molecular markers such as antibodies against opsin (Vigh-Teichmann), retinal S-antigen (Korf) and alpha-transducin (van Veen) are now used in comparative immunocytochemical work with retinal and pineal tissues. These investigations emphasize the existence of a number of common features of photoreceptor cells, both retinal and extraretinal. Furthermore, they raise the question of the physiological correlates of the individual structures and chemical compounds (photopigments, transmitters). Another important line of comparison is concerned with the intrinsic circuitries and neural projections in the retina and the different structural types of sensory pineal organs with particular consideration of their neurotransmitters (see Meissl, this volume). In this context the character of the avian pineal organ must be reconsidered in the light of new structural and functional

findings; there is now convincing evidence that it possesses a certain primary photoreceptive capacity (Deguchi, 1982; Takahashi, 1982; Vigh-Teichmann and Vigh, 1983; Semm and Demaine, 1983; cf. Oksche, 1985).

III. CONCLUSIONS

Photoneuroendocrinology cannot be separated from basic research on vision. The fundamental analyses of Eakin (1968, 1973) have clearly outlined the common ultrastructural features of retinal (vertebrate and invertebrate) and pineal receptors. This comprehensive ultrastructural work now needs an extension to the level of molecular mechanisms.

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MULTIPLE CELL TYPES IN THE PINEAL : FUNCTIONAL ASPECTS[1]

Jean-Pierre Collin, Paul Brisson, Jacky Falcón
and Pierre Voisin

Laboratory of Cell Biology and UA CNRS 290
Poitiers University
Poitiers, France

I. INTRODUCTION

Vertebrates use environmental factors as cues to synchronize circadian and circannual rhythms by which they adjust behavioural, physiological and biochemical functions. The circadian system, with its input sensors, oscillators and output machinery in turn aids in regulation of these important functions (47, 67, 69). The pineal organ is a component of this system. This diencephalic roof derivative is very responsive to light and is an important photosensor for this system. A second dorsal diencephalic derivative, the so called parapineal organ of lampreys and fish, or the frontal organ in anuran amphibians, or third eye in lizards will not be examined herein (12).

In non-mammalian vertebrates, the pineal organ is a light sensor. It may contain a more or less self-sustained circadian oscillator and produces nervous and hormonal signals. In contrast, the mammalian pineal gland seems to convey the photoperiodic information by means of hormonal outputs, under the control of an hypothalamic clock system, entrained by the photoperiod as perceived by the eyes.

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In order to illustrate the functional reorganization of the pineal organ (= PO)[2] during the course of evolution, we shall examine how photoreceptor cells and afferent innervation of the PO in lower vertebrates (Section II) are gradually replaced by other types of transducers (Section III). The development of efferent innervation during phylogeny will also be presented (Section IV). The interstitial cells (= supportive or glial cells) which are quantitatively well represented in the PO of all species will not be examined. In nonmammalian vertebrates, interstitial cells display analogies with retinal Müller cells and pigment epithelial cells (12, 75).

II. BINEURONAL CHAINS OF NON MAMMALIAN VERTEBRATES, FUNCTIONAL IMPLICATIONS

In contrast to the retina, where rods and cones are connected through bipolar cells to ganglion cells, the PO of lower vertebrates displays a bineuronal organization : the photoreceptor cells are synaptically connected to second-order neurons (= SN), projecting into the brain.

A. Cone-like photoreceptors (= CP and ICP in Fig. 1)

Similar to retinal rods and cones, the pineal photoreceptors are remarkably specialized, polarized and organized in a segmental fashion with outer and inner segments, cell soma and synaptic terminals (6-8, 12). One extremity (outer segment) is involved in phototransduction and the other one (synaptic terminal) in neurotransmission.

The outer segment displays a cilium - with 9 x 2 + 0 pattern of microtubules - containing a stack of disks or saccules. Disks consist of membrane limited spaces separated by thin layers of cytosol, remaining in open communication

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[2] **List of abbreviations used in the text :**

CNS : central nervous system ; CP : cone-like photoreceptors ; CRL : cells of the receptor line ; DCV : dense-cored vesicles ; EM : electron microscopy (or microscopical) ; ERP : early receptor potential ; HMP : highly modified "photoreceptor" cells ; ICP : indolergic cone-like photoreceptors ; MP : modified photoreceptor cell ; NAT : serotonin N-acetyltransferase ; PO : pineal organ ; SN : second-order neurons ; SR : synaptic ribbons.

with the extracellular space on the margins, i.e., the lumen of the PO in analogy to retinal cones. Around this common scheme, there are many variations in the shape and dimensions of the outer segment, as well as in the dimensions and number of disks (from few up to 300 disks) (12, 75). Electrophysiological, immunocytochemical and microspectrofluorometric data suggest that, in analogy with retinal photoreceptors, the outer segment membranes of pineal photoreceptors absorb light via photosensitive molecules (30, 41, 45, 70, 71). Several types of photopigments (45), possibly corresponding to several types of photoreceptors have been found. These appear to be built on the general pattern of visual pigments, a glycoprotein (= opsin) combined with a prosthetic group - a particular isomer of vitamin A. Like in retinal photoreceptors, the response to light seems to be amplified by means of interactions of photopigment molecules with amplifier proteins. Immunolabeling of the opsin (70, 71 ; cf. this volume), of the transducin α-subunit (Van Veen et al., personal communication) and of the S-antigen (identical to the "48K protein") (49, 56 ; cf. this volume) could be detected at least in outer segments. When the PO is stimulated by a flash of light, an almost instantaneous electrical response, the early receptor potential (ERP), is generated by photoreceptors, probably by charge displacement within the photosensitive molecules (45).

Also similar to retinal photoreceptors, the inner segment is often compartmentalized. An aggregation of mitochondria probably specialized for the production of energy-yielding substances (adenosine triphosphate) is present. The polymerized storage form of glucose is represented by glycogen particules which are scattered throughout the cytoplasm of photoreceptors ; occasionally clumps of glycogen (= paraboloid) can be found. The myoid region of the inner segment and cell soma contain the machinery (free polysomes, rough endoplasmic reticulum, Golgi area and vesicles) for production, processing and transit of macromolecules (e.g. opsin-like protein) and membranes. Two main flux of membranes may exist in photoreceptors : one involved in the renewal of disks (10, 29, 37 ; cf. this volume), the other involved in production of synaptic vesicles. The cell soma - with the nucleus - is found between the inner segment and the synaptic terminal (= pedicle).

Pedicles of photoreceptors terminate in areas of neuropiles, establishing synaptic contact with dendrites or soma of SN. In the synaptic terminals, the most frequent organelles are numerous electron lucent vesicles (synaptic vesicles : 35 to 60 nm in diameter), some dense-cored vesicles (80-180 nm, e.g. in lampreys), and synaptic ribbons (= SR) often perpendicular to the presynaptic membrane from which they are slightly separated by an arciform density (12, 75). In juxtaposition

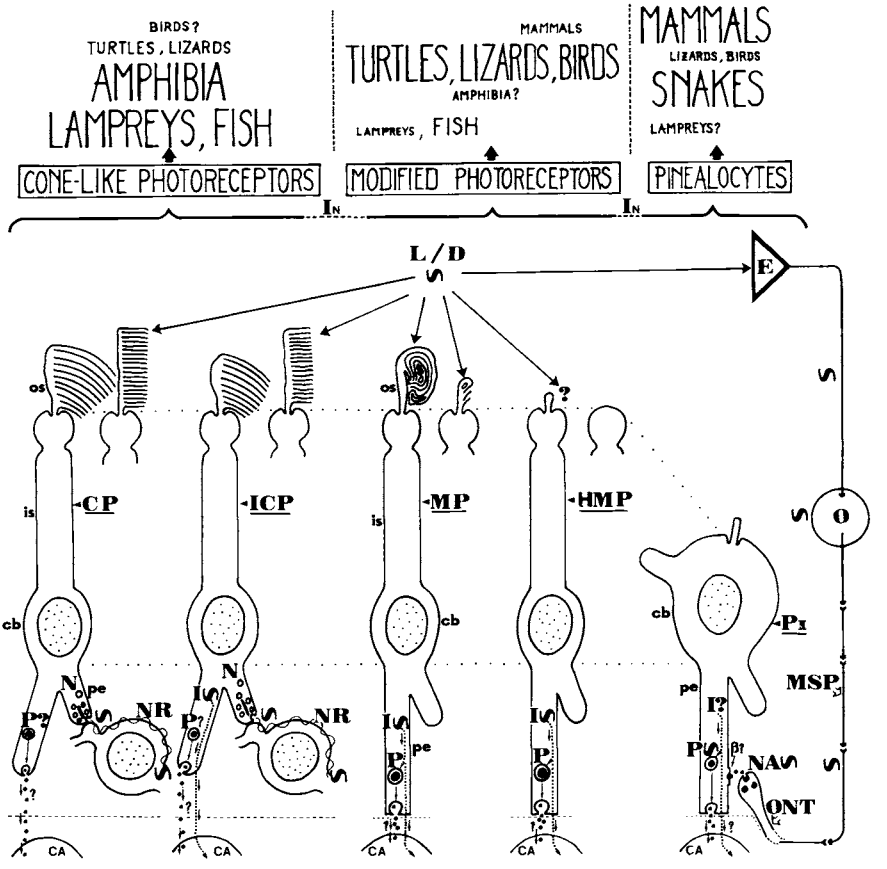

Fig. 1. Principal cell types of the receptor line (CRL) : CP, ICP; MP, HMP and Pi (see list of abbreviations), In : (intermediate cell types). Note on the top the distribution of the different types of CRL in the individual classes or orders of vertebrates.

INPUTS : Direct transduction of photoperiodic information (Light/Dark : L/D) may occur in CP, ICP and MP. Indirect transduction takes place in Pi. Among several receptors on Pi membranes only β_1-receptors (β) have been mentioned (E : eye ; NA : norepinephrine ; MSP : multisynaptic pathway ; O : oscillator ; ONT : orthosympathetic nerve terminal).

OUTPUTS : The physiological activity of the cells varies in a periodic manner (period of, or close to, 24 h :∿).

I : mainly ICP, MP, HMP of some species have been shown to be indolergic.

N : corresponds in CP and ICP to the excitatory neurotransmitter. The signals are passed to the brain via afferent neurons (NR), termed second-order neurons (SN) in the text.

P : proteic secretory material located in dense-cored vesicles ; its release from CRL remains to be established in most vertebrates.

(ca : capillary ; cb : cellular body ; is : inner segment ; os : outer segment ; pe : synaptic or asynaptic process) (11).

with synaptic ribbons, synaptic vesicles are often distributed in an ordered manner. At the putative active zones where SR face neurons, an increased density of the neuronal postsynaptic membrane and transverse filament-like structures, within the synaptic cleft, are found (6, 7). Axo-dendritic synapses (SR in the photoreceptor terminal facing a SN dendrite) are most common. In this type of synapse, one or several dendrites lie opposite one or several SR. Simple diad and triad complexes can be observed. Axo-somatic ribbon synapses may also be found. Synaptic ribbons occasionally described in the photoreceptor soma, and somato-dendritic ribbon synapses (SR of a photoreceptor soma facing a dendrite) have been mentioned. The synaptic transmission from pineal photoreceptors to SN is chemically mediated. Synaptic vesicles within pineal photoreceptors are believed to contain a neurotransmitter. In both retinal and pineal photoreceptors, synaptic ribbons are dynamic organelles exhibiting daily changes in number and size. This is believed to reflect circadian changes in storage and release of the neurotransmitter (44). In darkness, retinal and pineal photoreceptors are partially depolarized and release an excitatory neurotransmitter (16, 46). In contrast, light produces graded hyperpolarizations and suppresses the release of this neurotransmitter (16, 46). Therefore, signal transmission in the PO is mainly performed by the non-inverting synapses onto SN, a lateral interaction possibly being achieved by interneuron-like cells (46, and Section II.B.). Among several tested substances, aspartate and glutamate mimic the action of the natural neurotransmitter of most photoreceptors (16, 46). To date, it is believed that the excitatory neurotransmitter probably belongs to the acidic amino acids group. Besides sensory synapses, endowed with synaptic ribbons which transmit photoperiodic signals, other specific contacts exist : (i) symmetrically organized gap junctions between terminals of adjacent photoreceptors (53) that may be involved in an enhancement of electrical communication and signal encoding between photoreceptors ; (ii) conventional synapses, occasionally found between photoreceptor terminals and nerve fibers (75) and (iii) inhibitory or feedback synapses, suggesting chemical transmission from unidentified neurons to photoreceptors (53). The significance of the few dense-cored vesicles (DCV), originating from the Golgi complex and mainly found in the synaptic terminals, is

still unclarified (7, 11, 12).

There exists increasing data favoring the view that photoreceptors produce indolic signals. Data on indole metabolism have been reviewed in mammals (39). By using the Falck-Hillarp technique, serotonin was found to be stored in photoreceptors containing residual bodies (= ICP2 in the brook lamprey) and in photoreceptors of fish and frog (9, 10, 11, 16, 50). Serotonin-like and N-acetylserotonin-like immunoreactivities were located in ICP2 of the brook lamprey (26) and photoreceptors of the pike (24). A melatonin-like immunoreactivity was also found in the first category of photoreceptors (= ICP1) of the brook lamprey (26) and photoreceptors of the pike (23). Drugs acting on the synthesis or storage of serotonin altered the intensity of cytochemical reactions related to indoles (21). Monoamine oxidase activity was located in photoreceptors (21). After tritiated tryptophan, or tritiated 5-hydroxytryptophan or tritiated serotonin uptake in the pike PO, radioautography alone or used concurrently with thin layer chromatography has shown that photoreceptors are involved in indole metabolism (21). Daily variations have been reported for the immunoreactions described, concentrations of pineal indoles, serotonin-N-acetyltransferase activity (in the pike), hydroxyindole-O-methyltransferase activity (in lampreys and some fish species) and circulating melatonin (21, 23-25, 28). Serotonin may serve more as a precursor of physiologically active 5-methoxyindoles than as a neurotransmitter (46). This indoleamine is mainly found in the cytosol of the inner segment, perikaryon and synaptic processes of photoreceptors and, in addition, in residual bodies of ICP2 of the brook lamprey (16).

B. The second-order neurons (= SN = afferent innervation) and interneuron-like cells in non mammalian vertebrates

The intrinsic circuitry exhibits great variation within the phylum Vertebrata as well as within every vertebrate class. However, the general trend corresponds to a decrease in the number of intrinsic neurons during the course of vertebrate evolution. Although this number cannot be easely established, it seems to vary from a few units, to tens or up to approximately two thousand (6, 12, 21, 51, 52, 68, 75). In most species, neurons are not evenly distributed and do not appear to form a layered structure. Different types of pineal neurons were identified on the basis of their size and shape (unipolar, bipolar, multipolar). In the frog and the house sparrow, a distinction between multipolar (= amacrine-like neurons or interneurons) and pseudo-unipolar or unipolar neurons (= SN) contributing to the pineal tract, was proposed

(40, 76). Synaptic connections between neurons were found (53).

The responses of SN, after stimulation of photoreceptors by light, have been investigated (45). One system is capable of measuring solar radiation energy ; the other is concerned with the changing composition of day-light. Both types of responses are complementary to each other in giving information on the actual time of day.

(i) The achromatic (= luminance) response is the most common : with light stimuli of all wavelengths in the visible and ultraviolet spectrum, the responses of neurons are inhibitory.

(ii) The chromatic response : this mechanism reveals inhibitory responses upon illumination by stimuli of short wavelengths, whereas longer wavelengths produce excitation of neurons.

The pattern of connection between the different types of neurons is still poorly known in the PO. For example, the number of photoreceptors which connect dendrites and/or soma of one SN, or the number of SN connected to terminals of one photoreceptor, remain to be determined. In few species, the presence of synaptic contacts was suggested either between axons of extrapineal origin and photoreceptors or SN, or between extensions of local interneurons and SN or photoreceptors.

Trains of spikes generated in SN, travel through tens up to 2000 or more myelinated and unmyelinated axons of the pineal tract (analogous to the optic nerve) to the posterior and habenular commissures from which they project into the brain (12, 52, 68, 75). In teleosts, the tract reaches bilaterally into several major regions of the brain : pretectal area (which is part of the transition zone between di- and mesencephalon), dorsal and ventral thalamus, hypothalamus, habenular nuclei and the dorsal tegmentum (20). The afferent axons terminate to a large extent in areas that are innervated by or innervate the retina. A close association of retinal and pineal terminal fields was noted in pretectum, thalamus, hypothalamus and tegmentum. In the frog, the terminals of the pineal tract were localized in the pretectal area and the mesencephalic tegmentum (reticular formation : 54). In the house sparrow, pineal afferents apparently innervate the medial and lateral divisions of the habenular complex and the periventricular layer of the hypothalamus (40 ; see also 64, in the pigeon).

C. Distribution and function of bineuronal chains (Fig. 1).

Bineuronal chains were found in poikilotherms (except

ophidians). Possibly in relation with their relative scarcity in many species, their identification in birds remains extremely difficult ; in most mammals, they are apparently absent. Except in ophidians, it is now accepted that the PO of nonmammals is directly involved in the detection and integration of light stimuli (12, 45, 58 ; cf. this volume).

The above data allow the conclusion that photoreceptors translate the photoperiodic stimuli at least into two types of messages displaying daily variations : (i) signals transmitted to SN by means of an excitatory neurotransmitter (possibly an acidic amino acid), for projection to several major brain centers, and (ii) indolic signals (e.g. 5-methoxyindoles) that might act locally (autocrine and paracrine actions) and/or influence extrapineal targets via the bloodstream and the cerebrospinal fluid (11, 16, 18, 21, 27, 28). It is still uncertain whether all photoreceptors are involved in indole metabolism (= indolergic cells : 11). This is the reason why on Fig. 1, non indolergic cone-like photoreceptors (= CP) and indolergic cone-like photoreceptors (ICP) have been provisionally distinguished (11). The possible presence of an oscillator in bineuronal chains is discussed below (Section III).

The exact function of the afferent neural apparatus still remains obscure. This is due to the fact that it is not easy to distinguish between the neural and neurohormonal actions and that the photoreceptive properties of the PO are complemented by retinal and encephalic photoreceptors. The afferent projections to the mesencephalic tegmentum may serve in control of behavioural thermoregulation, phototactic reactions, without excluding other functions ; fibers reaching the hypothalamus may be involved in circadian and neuroendocrine functions (18, 20, 38, 52, 58, 59).

During ontogeny and phylogeny (12, 15), the number of photoreceptors and bineuronal chains decreases to be replaced, as mainly observed in amniotes, by other types of transducers (modified photoreceptors and pinealocytes) which are presented in the next section.

III. GRADUAL REPLACEMENT OF BINEURONAL CHAINS BY MODIFIED PHOTORECEPTORS AND PINEALOCYTES IN VERTEBRATES

A. Modified photoreceptors (MP) (Fig. 1)

Modified photoreceptors have been found in the PO of turtles, lizards, birds (12) and occasionally in fish (21) or in mammals (MP-like : 55). Homologies between photoreceptors and MP have been clearly established (6-8, 12, 51). From

electron microscopic (EM) studies, differences between photoreceptors and MP appeared mainly at the level of the receptor and transmission poles.

The outer segment corresponds to a cilium with a 9 x 2 + 0 pattern of microtubules, either bulbous or exhibiting cytoplasmic laminae extending into parallel stacks or irregular whorls, which may contain polymorphic inclusions. Intermediate types of outer segments contain a variable number of vesicles and vacuoles which vary in shape, dimension and electron density. Because these structures deviate from those of the regular outer segments of photoreceptors, MP outer segments were considered to be rudimentary (12). Early EM studies, did not allow the conclusion that outer segments of MP were directly photosensitive (6, 7, 12). However, (i) biochemical data (13, 75 ; cf. this volume), (ii) positive immunoreactions for opsin, α-subunit of transducin and S-antigen in the outer segments (41, 49, 70 ; Van Veen et al., personal communication) and (iii) evidence of electrical responses of MP to light in the medial PO of the pike (22), strongly suggest that, at least, one population of MP is photosensitive. Indirectly photosensitive cells, termed highly modified "photoreceptors" (HMP : Fig. 1) also might exist (11).

The fact that MP are independent of SN and possess an asynaptic process implies that they have lost the ability to generate a direct nervous response to light stimuli (6-8, 12). This interpretation also is supported by some electrophysiological data and by the evidence that the number of intrinsic neurons becomes considerably lower than the total number of transducers in sauropsids (6, 12, 13, 15, 62, 63). The asynaptic processes of MP terminate in the vicinity of the capillaries ; depending on reptilian and avian species, they contain more or less numerous DCV (see below). In addition to other data, these cytological features suggest that MP become adapted to neurohormonal transmission only (6, 7, 12, 51).

Similar to other secretory cells, the endomembranous system (rough endoplasmic reticulum, dictyosomes and vesicular material) plays an important role in the elaboration of secretory proteins (9, 10, 12). It has been hypothesized that the proteinaceous core of DCV, originating in dictyosomes, may contain a specific protein (= "pinealin") (10).

Data establishing that MP are also responsible for indole metabolism (= indolergic cells) are the following (9, 11, 13, 15, 16) :

(i) endogenous 5-hydroxytryptophan/serotonin (fluorophores), or endogenous serotonin and N-acetylserotonin-like indoles (immunoreactions) were located in MP of the pike, lizards and birds ; synthesis and storage of serotonin were modified by drug treatments (9, 11, 15, 16, 24).

(ii) monoamine oxidase activities were located in MP and other pineal cell types (21, 33).

(iii) irrespective of the season and the time of the 24 hr cycle, a selective uptake of tritiated 5-hydroxytryptophan or tritiated serotonin was observed in vitro and in vivo in MP of reptiles and birds. When radioautographic labeling was determined in conjunction with thin layer chromatography, in optimal conditions for biosynthesis and retention of 5-methoxyindoles, tritiated indoleamines appeared to be selectively metabolized in MP. In addition, the formation of tritiated 5-methoxyindoles from labeled precursors changed during the 24 hr cycle (2, 3, 9, 14, 34, 35, 72-74).

Therefore MP appear to be indolergic cells, at least in the species tested. In addition, the sauropsidian PO displays striking day/night changes in serotonin-N-acetyl transferase activity (= NAT ; involved in the circadian melatonin synthesis), and of pineal indoles ; the cyclicity of circulating melatonin also has been established (1, 16, 28, 57, 72 ; cf. this volume). Collectively, the present data allow the conclusion that MP translate the photoperiodic information at least into indolic signals displaying circadian variations. 5-Methoxyindoles (e.g. melatonin) act on extrapineal targets and possibly locally (9, 11, 27, 28, 58). In addition to the production of indoles, a proteinaceous secretion of still unknown significance is observed in MP (9, 11, 13).

Nevertheless, our above interpretation on the translation mechanisms in MP and in photoreceptors (or bineuronal chains) appears oversimplified. It is well known that the PO of two sauropsids (lizard and chicken) contains a circadian oscillator, not self-sustained in the chicken (1, 5, 47, 48, 67). Endogenous circadian rhythms of NAT activity (chicken) and melatonin release (lizard and chicken) are found in cultured PO. As shown at least in the chicken, the PO contains multiple redundant oscillators (66). Furthermore, from experiments performed on dissociated cultured pineal cells, it has been proposed that a photoreceptor, an oscillator and the output machinery for production of indoles may be found in a single cell (17).

Until now it is questioned which pineal cell type(s) might contain an oscillator (11, 16). Within sauropsids, several intrinsic populations of pineal transducers (photoreceptors of bineural chains, MP, HMP and pinealocytes = Pi) and interstitial cells (see introduction) have been found. Depending on species and on developmental stages of sauropsids, the percentage of each population of transducers might be different but MP and HMP are the most numerous. To date, HMP, Pi (see below) and interstitial cells are not supposed to be directly photosensitive. Although sauropsidian HMP and Pi are indolergic cells, it is still unknown how they may be

influenced by environmental changes. Unfortunately it is unknown whether the relatively scarce photoreceptors of sauropsids are indolergic in contrast with anamniotes. Therefore, it must be investigated whether photoreceptors (or bineuronal chains) and MP of nonmammalian vertebrates contain a more or less self-sustained oscillator, entrained by the light/dark cycle via the outer segment, and connected to the machinery for production of indolic signals. Pi and HMP are not supposed to contain an oscillator because the mammalian PO, consisting mainly of Pi and in some species of few HMP, does not express an inherent circadian rhythm of indoles and of enzymes involved in their metabolism.

B. Pinealocytes stricto sensu (Pi ; Fig. 1)

Pi, the chief cells of the PO of mammals and snakes, also have been found in peculiar portions of the PO of other amniotes. During the course of phylogeny or ontogeny, the four segments of a photoreceptor have been progressively transformed to give in Pi a cellular soma and cell processes (12, 15). When present, a cylindrical or bulbous cilium (9 x 2 + 0) and vesicle - crowned ribbons, homologs of synaptic ribbons, are the main cell components indicating a photoreceptor phylogeny. After cell differentiation (15), data favoring direct photosensitivity in the conventional sense, as well as the presence of second-order neurons synaptically connected to Pi are still lacking. Accumulated data show that Pi, MP, CP, ICP and intermediate cells (e.g. HMP) are homologous (7, 12, 51). In other words, Pi appear as extremely modified "photoreceptor" cells. In agreement with this previously established filiation, the S-antigen immunoreactivity appears as the first common "marker" for both pineal and retinal (cones and rods) transducers (49). In all these transducers, the S-antigen immunoreactivity was localized in outer segments and/or other cell compartments. Its functional significance in Pi is still unknown.

The process of protein secretion in Pi is very similar to that observed in MP (10). However, the number of DCV found in the processes oriented towards the capillaries is fewer than what is observed in the processes of some sauropsids. DCV fluctuate according to a circadian rhythm (19, 75).

Serotonin, monoamine oxidase activity and immunoreactivity for hydroxyindole-O-methyltransferase were found in Pi (9, 11, 43, 50, 77). When compared to ICP and MP our knowledge on indole metabolism at the cellular level is still fragmentary (36). However, considering the amount of data on Pi and on their forerunners in the nonmammalian vertebrates, many authors have accepted that Pi are indolergic cells.

In the rat, the photoperiodic information is perceived by the retina and then transfered via a retino-hypothalamic projection which entrains a circadian clock system (suprachiasmatic nuclei) (Fig. 1). The clock transmits the oscillatory information to the PO via a central pathway and orthosympathetic fibers (39 ; Section IV). The release of norepinephrine from these fibers is high at night and low during the day. The amine acts through α_1 and β_1 adrenoreceptors to increase cAMP which in turn induce changes in circadian levels of pineal NAT activity, pineal and circulating melatonin (39, 65 ; cf. this volume).

Therefore, an essential property of Pi is probably to convey the photoperiodic information, by translating chemical stimuli into 5-methoxyindolic signals, e.g. melatonin which acts on extrapineal targets and possibly locally (4, 60, 61).

IV. EXTRINSIC (= EFFERENT) INNERVATION

In the above sections, it has been shown that in response to photoperiodic information, the pineal transducers produce several types of signals, displaying daily variations and acting on the brain and possibly on other structures. However, hormones, neurohormones as well as agents of efferent fibers influence the pineal transducers. The following comments focus on the distribution and role of orthosympathetic and central innervation of the PO.

A. Orthosympathetic innervation

Catecholamine-containing axons invade the PO, mainly in amphibia and amniotes (6, 68, 75). This innervation quantitatively increases from lower to upper vertebrates. In a given class, considerable species dependent differences exist in the number of fibers and in their distribution throughout the pericapillary spaces, parenchyma and occasionally pineal lumens of poikilotherms. In the parenchyma, the fibers make appositional contacts with pineal cells ; visible synaptic differentiations are unusual (32). In homeotherms, axons originate from neuronal perikarya present in the superior cervical ganglia. In amniotes, norepinephrine is the neurotransmitter, but other agents (e.g. neuropeptides) might also be released from varicosities and endings. A serotonin uptake by sympathetic endings was also observed in several amniotes. The role of this innervation has been well investigated in the rat (Section III). In the PO of the chicken, it has been

suggested that the sympathetic innervation regulates the pineal's inherent rhythmicity (5).

B. Central innervation

The PO receives an efferent innervation originating from the central nervous system (CNS). In nonmammals, evidence on this type of innervation is still fragmentary : e.g. within the pineal tract of the frog (68) and within the PO of the house sparrow which receives fibers originating from neuronal perikarya located in the periventricular hypothalamic area (40). In some birds, fibers originating in the habenular complex (40, 64) might represent a pathway by which complementary photoperiodic information is conveyed from the retina to the PO, via the habenula (31). Because the PO might influence some hypothalamic centers, as well as the habenular complex, feed-back mechanisms have been suggested (40).

In agreement with some older work, recent cytological, electrophysiological and lesion experiments support the existence of projections of the CNS into the mammalian PO (42). Several types of fibers enter the PO via the habenular or the posterior commissure : free, appositional or synaptic endings have been located in several pineal portions. Peptide-like substances (vasopressin, oxytocin, neurophysin, vasoactive intestinal peptide, substance P, luteinizing hormone releasing factor, somatostatin) and serotonin have been identified in these fibers (11, 42). Perikarya were found in habenular, posterior commissural, paraventricular and central optic nuclei (42). To date, it is believed that pineal outputs are influenced by a direct control and feed-back mechanism (e.g. from the hypothalamus) originating from several CNS centers, themselves being influenced by endogenous and/or environmental inputs.

V. CONCLUDING REMARKS AND SUMMARY

In the pineal organ, bineuronal chains (= cone-like photoreceptors synaptically connected to second-order neurons) of nonmammalian vertebrates are gradually substituted, mainly in amniotes, by modified photoreceptors and pinealocytes. Based on ontogenetic and phylogenetic filiations between these pineal transducers and their intermediates, the concept of the "cells of the receptor line" (= CRL) (6, 7, 12, 15) has been strengthened by newly established common features of these cells ; i.e. molecules involved in phototransduction and amplification of photic stimuli, signaling molecules (5-methoxyindoles) and a proteinaceous secretion. Furthermore, daily

quantitative changes of CRL components (not reviewed here) and indole metabolism were found which could be altered by modifications of the photoperiod. Thus, increasing evidence favor the view that CRL are primarily involved in daily events entrained by light/dark cycle (16).

Although in the phylum Vertebrata, the existence of several phylogenetic lines of transducers cannot be excluded (11), it appears now that both structurally and functionally, the pineal transducers constitute a spectrum (Fig. 1). In relation with the gradual substitution of bineuronal chains, a plurality of transducers characterizes a given pineal organ. This means that either the entire spectrum or part of it may be found (7, 8, 12 ; and top of Fig. 1). When comparing species in a given vertebrate class, the percentage of transducers of each population seems to be different. This variability might aid in explaining the apparent inconstancy of function of the pineal organ in relation with adaptation (58).

The phototransduction mechanisms change during the course of vertebrate evolution : in contrast to their forerunners (cone-like and modified photoreceptor cells of non mammalian vertebrates) which are directly photosensitive, mammalian pinealocytes receive information from a clock system - present outside the pineal organ - entrained by the photoperiod. In response to photoperiodic information, two types of signals displaying daily fluctuations have been evidenced : (i) an excitatory neurotransmitter produced by cone-like photoreceptors and acting on second-order neurons, (ii) 5-methoxyindolic signals in most transducers (16). Indolic signals might have an autocrine, paracrine and neuroendocrine action (16). To date, cone-like photoreceptors appear, at least in some species, as multimessenger cells (16). It is questioned (i) whether a peptidic signal also is produced by CRL and (ii) whether bineuronal chains (or cone-like photoreceptors ?) and modified photoreceptors contain, in nonmammalian vertebrates, a more or less self-sustained circadian oscillator, entrained by the photoperiod and acting on the machinery for production of 5-methoxyindoles.

The second-order neurons (afferent innervation) decrease quantitatively during the course of evolution and ontogeny to disappear apparently in most mammals. They project into the brain to influence well-defined regions. Parallel to this phenomenon and to the structural/functional recycling of the transducers, the efferent (orthosympathetic and central) innervation appears to increase from lower to upper vertebrates. By conveying environmental and endogenous information, these important neural inputs control (or might control) the function of CRL.

Finally, an inherent property of pineal transducers is

their primary involvement either in translating (i.e. : cone-like and modified photoreceptors) or in conveying (i.e. : pinealocytes) the photoperiodic information by means of at least nervous and/or indolic signals. However, the production of signals seems to be influenced by (neuro)hormonal and neural inputs.

There exists increasing evidence that CRL are components of the vertebrate circadian system. The complexity of this system and of the interactions between its components is partly illustrated by that of pineal transducers and innervation.

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PHOTONEUROPHYSIOLOGY OF PINEALOCYTES

H. Meissl

Max-Planck-Institute for Physiological and
Clinical Research, W.G. Kerckhoff-Institute,
Bad Nauheim, FRG

I. INTRODUCTION

The first physiological account of an extraretinal photoreception mediated by the pineal organ was reported by von Frisch (1911). He observed that illumination of the pineal region of the minnow, *Phoxinus phoxinus*, previously blinded by removal of their lateral eyes, resulted in a darkening reaction of the skin. Following pinealectomy, the melanophore response was temporarily abolished. Von Frisch concluded that the pineal organ was one principal site of an extraocular photoreception in addition to deep-encephalic photoreceptors. Later on Scharrer (1928) confirmed these observations in the same species with behavioral experiments.

Up to the year 1960 the knowledge about a photosensory function of sub-mammalian species was based mainly on indirect evidence. Indubitable proof of a pineal light-sensitivity by electrophysiological recordings was accomplished by Heerd and Dodt (1961) when they succeeded to record wavelength-dependent light responses from the pineal nerve of the frog. A more extensive report was published by Dodt and Heerd in 1962 and in the same year Baumann and Miller and Wolbarsht recorded slow potentials from the frog's frontal organ and the reptilian parietal eye, the homologous structure of the frontal organ. These experiments provided direct physiological evidence of a pineal light sensitivity and gave rise to comparative studies in numerous species of lower vertebrates. Most of these were performed by extracellular recordings from pineal ganglion cells, pineal nerve fibers or have been obtained with mass slow potentials. The first intracellular recording from pineal cells was reported by Morita (1965) in the

epiphyseal stalk of the frog and by Hanyu et al. (1969) in the teleostean pineal. With intracellular dye injections Tabata et al. (1975) confirmed that most of the intracellular responses previously described were photoreceptor responses. In a short note Morita (1975) described intracellular recordings of lamprey photoreceptors, then Pu and Dowling (1981) gave a detailed description of the morphological and physiological characteristics of the pineal photoreceptor of larval lampreys. Intracellular recordings from other cell types are rare and have never been reported in detail.

II. ELECTROPHYSIOLOGY OF THE PINEAL SYSTEM

The position of the pineal system is of major importance for its photosensory function. Presupposition for a photoreception is that light reaches the visual pigment in the outer segments of the photoreceptor. Light that is scattered, absorbed or reflected by skin and skull is not disposable to the visual process. The skull of amphibians and fishes overlying the pineal region is largely cartilaginous. Furthermore, many species possess a specialized area on the head with a depigmented patch of skin overlying a bony tube which leads directly to the pineal area of the brain. These translucent pineal windows permit up to 1-50 percent of the incident light to enter the brain cavity (Morita, 1966). However, physiologically significant levels of light pass through even the ossified skulls of birds and mammals (Ganong et al., 1963; Hartwig and Van Veen, 1979). The comparatively thin skulls of lower vertebrates are therefore only an insignificant barrier against the penetration of light.

In lower vertebrates, which have developed an extracranial component of the pineal system the photoreceptors are only protected by the epidermis, which is often translucent.

A. Extracellular recordings from pineal nerve cells and fibers

Maintained spike activity of pineal ganglion cells or nerve fibers is a common characteristic of all vertebrate pineals studied so far (cf. Dodt, 1973; Dodt and Meissl, 1982). The maintained activity is a continuous discharge either in darkness or under constant conditions of illumination. The term "spontaneous activity" for this maintained discharges implies that the activity is autogenic. However, the random discharge pattern of most of the pineal cells distinguish them from neurons considered to possess autogenic activity which usually discharges regularly (cf. Rodieck, 1973). Furthermore, the maintained activity of pineal ganglion cells strongly de-

pends on a sustained synaptic input from other cells. Blocking the synaptic transmission in the pineal by treatment with Co^{2+} results in an isolation of pineal ganglion cells and a distinct inhibition of the maintained discharge (Meissl and George, 1984).

The maintained discharge rate of most pineal ganglion cells is inversely proportional to the luminance of the background. This relationship holds over a range of 6 to 8 log units in frogs and fishes (Morita and Dodt, 1965; Hamasaki and Esserman, 1976; Falcón and Meissl, 1981). The messages transmitted from pineal to brain are thus closely related to the ambient light level, which satisfies the requirements of a sensor acting as a dosimeter of solar radiation (Hamasaki and Esserman, 1976).

Light is the only effective stimulus which changes the activity of the pineal nerve or tract. Ineffective forms of stimulation include temperature, mechanical and chemical stimuli (Dodt and Heerd, 1962).

Two types of ganglion cell responses are recorded from pineal sense organs when the organ is exposed to light pulses. The first is best characterized as luminance off type and consists of a purely inhibitory response to light of all wavelengths (luminance or achromatic response type). The other response type is color-coded and exhibits inhibitory effects to short wavelengths and excitatory responses to longer wavelengths (chromatic response type). The distribution of chromatic and achromatic units varies among different species (Meissl and Dodt, 1981). Chromatic units are especially present in the extracranial part of the pineal complex, i.e., in the frontal organ of anurans and in the parietal eye of lacertilians. The intracranial pineal organ proper contains only in a few species a low number of cells showing this type of response (for details see Meissl and Dodt, 1981). Thus, the most common response type of the pineal organ proper is the luminance response.

Chromatic responses reveal an inhibitory reaction to light of short wavelengths and excitatory responses to medium and longer wavelengths (Fig. 1). In fishes and frogs the inhibitory component is most sensitive to ultraviolet light (λmax 355 nm) and in the parietal eye of lizards to blue light (λmax 450 nm). The excitatory component is most sensitive to mid-spectral light (λmax 520 nm). The chromatic response has two unique features. First, the responses to ultraviolet or green light are sustained, i.e., inhibition or excitation of the neural activity persists up to minutes after the end of the stimulus. Second, both components of chromatic responses interact and its neural activity depends on the stimulus, wavelengths and energy of previous illumination. For a detailed

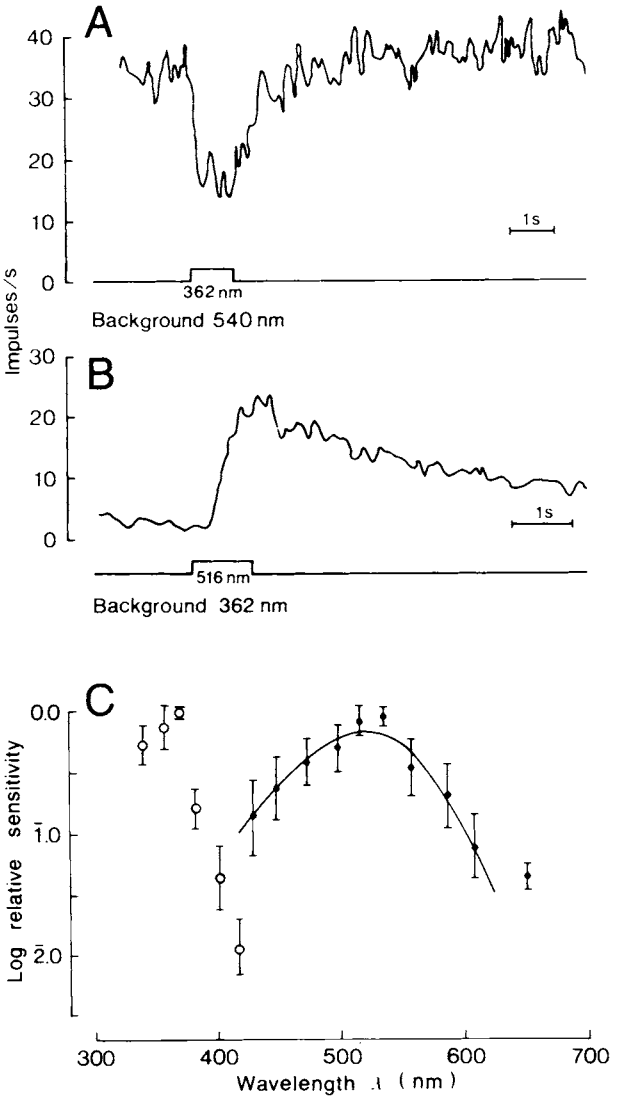

Fig. 1 (A, B) Peristimulus-time histograms of the chromatic response recorded from the pineal nerve of the frog. (A) Inhibition of the maintained discharge in response to ultraviolet radiation; (B) excitation in response to green light. Note different background illumination during test flashes. (C) Spectral sensitivity of the inhibitory (circles) and the excitatory (dots) component of the chromatic response during exposure to weak green and ultraviolet backgrounds.

discussion of chromatic response mechanisms see Dodt (1973) and Meissl and Dodt (1981).

The luminance response is the most common response type of pineal ganglion cells. Most cells of the pineal organ proper and also many pineal nerve fibers respond achromatically. Exposure of the pineal to brief flashes results in an inhibition of the maintained discharge which is followed by off-responses at cessation of the stimulus (Fig. 2). On-responses have never been observed in achromatic units of the pineal. The absolute threshold of the luminance response in a thoroughly dark adapted pineal of the frog is of the same order as the light threshold of the isolated retina of the dark adapted frog's eye (Dodt, 1973). The spectral sensitivity distribution of the luminance neurons varies slightly among different species with peak wavelengths between 500 and 540 nm. However, some species exhibit spectral sensitivity curves with maxima at longer wavelengths (frog: 560-580 nm; pike: 620 nm). Furthermore, two sensitivity curves are found in the pineal organ of frogs and some fishes exhibiting a Purkinje-shift during dark adaptation which suggests that rod- and cone-like photoreceptor mechanisms are present (for ref. see Meissl and Dodt, 1981).

B. Slow mass potentials

In addition to the impulse discharges of ganglion cells and nerve fibers illumination of the pineal complex evokes graded slow potential changes which are analogous to the electroretinogram of the lateral eyes. Both chromatic and achromatic responses are reflected in slow potential changes. If chromatic units are present the polarity of slow potentials changes with wavelength, whereas luminance neurons respond only with unidirectional graded potentials (cf. Dodt, 1973).

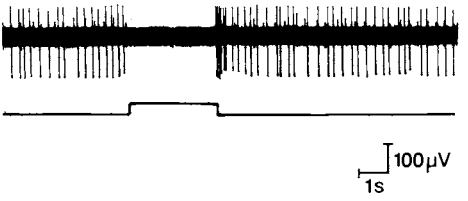

Fig. 2. Luminance response of a pineal ganglion cell obtained by extracellular recording. The light-induced inhibition of the maintained impulse discharge is followed by off-responses. Stimulus: 1 s duration, 590 μW/cm².

Slow mass potentials of the pineal closely resemble intracellularly recorded photoreceptor potentials. The shape of response varies with intensity, duration and wavelength of the stimulus as well as with the state of dark adaptation. Depending on the state of adaptation the amplitude increases up to a maximal voltage with increasing luminance of the stimulus. Analogue to photoreceptor potentials stimuli above saturation intensity produce a lengthening of the response. Under dark adapted conditions the amplitude-intensity relation of slow potentials fits a similar hyperbolic function earlier used to describe photoreceptor potentials (see section C).

Slow mass potentials can still be recorded after the synaptic transmission in the pineal organ is prevented by Co^{2+} and after aspartate. The nearly unchanged response characteristic after treatment with Co^{2+} supports the assumption that photoreceptors essentially contribute to the generation of these slow potentials (cf. Donley and Meissl, 1979; Meissl et al., 1986).

C. Intracellular recordings

Intracellular recordings from individual pineal cells meet difficulties because of their small size and their scattered distribution. Thus, the knowledge about the intracellular activity is fragmentary and confined only to a few species. However, the results hitherto obtained indicate that the response characteristics of pineal cells are similar to those of other photoreceptive structures (e.g. retina of the lateral eyes).

1. Photoreceptor cells

Numerous morphological and ultrastructural studies have established the presence of highly differentiated photoreceptor cells in the pineal organ of poikilotherms. In all these species the outer segments of receptors closely resemble retinal cones (cf. Collin and Oksche, 1981).

Intracellular recordings from pineal photoreceptors have been performed in species with well-developed photosensory pineal organ, i.e., in lampreys (Pu and Dowling, 1981; Meissl et al., 1982; Morita et al., 1985), in fishes (Hanyu et al., 1969; Tabata et al., 1975; Nakamura et al., 1985; Meissl et al., 1986) and frogs (Meissl, unpublished). Until now no successful recordings were presented from photoreceptors of the rudimentary type. Recordings from individual photoreceptors were distinguished from second-order neurons by their waveform and by intracellular application of dyes. Furthermore, photoreceptor responses outlast the treatment with Co^{2+} or aspartate which affect the synaptic transmission from recep-

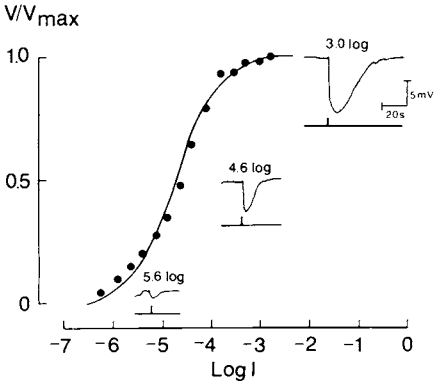

Fig. 3. Voltage-intensity plot of photic responses intracellularly recorded from dark-adapted photoreceptors of the pineal of the trout. Mean values of 16 individual receptors. Peak amplitudes of each response are normalized with respect to Vmax. The solid line represents the function $V/Vmax = I/I + \sigma$. Original recordings of three receptor responses are indicated besides plotted function. Log 0 light intensity corresponding to 2700 $\mu W/cm^2$ (from Meissl and Ekström, in preparation).

tors to second-order neurons (Meissl and Morita, 1982), which further indicates that the responses have their origin in the photoreceptor cell.

Pineal photoreceptors have resting potentials of 20 to 35 mV and respond by hyperpolarization to light stimuli. The responses to light are usually entirely monophasic without signs of an initial transient as shown in many retinal photoreceptors. Rapid initial transients have been observed only occasionally in pineal receptors in trouts (Meissl and Ekström, unpublished). The light-evoked intensity-graded hyperpolarization follows the function $V/Vmax = I^n/I^n+\sigma^n$, where V is the change in membrane voltage, Vmax the maximal amplitude, I the intensity of the light flash, and σ a constant equal to the intensity of Vmax/2 (Fig. 3). For brief flashes the value n=1 gives the best fit to the equation in pineal receptors in the lamprey (Pu and Dowling, 1982; Meissl et al., 1982) and in the minnow (Nakamura et al., 1985); a value of n=0.8 was found in the goldfish (Meissl et al., 1986).

Increasing flash intensity above saturation results in a lengthening of time required for membrane recovery. For flash intensities 2 log units above saturation the time increases up to 40 s.

Latencies of responses to brief flashes vary according to stimulus intensity (Fig. 4). For responses near threshold

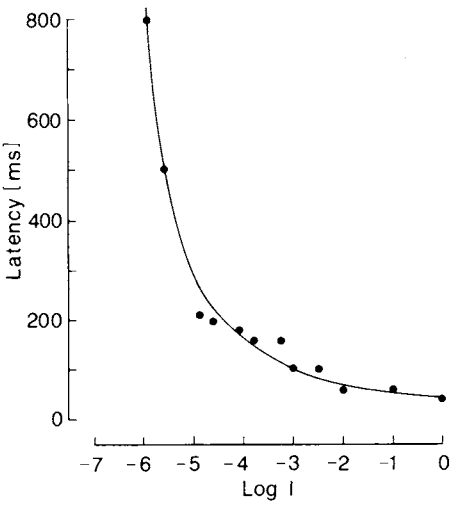

Fig. 4. Latency-intensity plot of a pineal photoreceptor response to brief light flashes. Stimulus duration : 200 ms; log 0 equal to 2700 μW/cm² (from Meissl and Ekström, in preparation).

latencies of up to 600 ms were recorded, and dropped to around 50 ms for saturating flashes. Shorter latencies in pineal photoreceptors were never described. In lamprey photoreceptors the minimum latency amounted to 200 ms (Pu and Dowling, 1981).

During the light-induced hyperpolarization the membrane resistance of pineal photoreceptors decreases, i.e., the conductance increases (Tabata et al., 1975; Nakamura et al., 1985; Morita et al., 1985). With increasing response amplitudes the change of resistance becomes thereby larger.

Action spectra of individual pineal photoreceptors were measured in the ayu (Hanyu et al., 1969), lamprey (Pu and Dowling, 1981; Morita et al., 1985), goldfish (Meissl et al., 1986), European minnow (Nakamura et al., 1985), and trout (Tabata et al., 1975; Meissl and Ekström, unpublished). Peak sensitivity is around 520-530 nm in goldfish, minnow, trout and ayu. In larval lampreys (*Petromyzon marinus*) the spectral sensitivity curve of pineal photoreceptors can be best fitted by a vitamin A_2 nomogram (λmax 545 nm), that of adult lampreys (*Lampetra japonica*) peaks at about 520 nm. Intracellular recordings indicate so far the presence of only one photopigment in all species studied. A second receptor population as predicted by immuno-electronmicroscopic studies (cf. Vigh-Teichmann et al., 1983) and extracellular ganglion cell recordings (cf. Meissl and Dodt, 1981) was never observed in intracellular recordings.

2. Ganglion cells

Pineal ganglion cells are defined in this section as neurons whose perikarya lie in the pineal tissue and whose axons form the pineal nerve or tract. The ganglion cells of the pineal organ apparently comprise an inhomogeneous population of neurons. While Wake et al. (1974) assumed pseudounipolar neurons as the exclusive source of pinealofugal axons in the pineal tract, it was later shown that different types of intrapineal neurons contribute to the pineal nerve and pineal tract of anurans (Eldred and Nolte, 1981) and teleosts (Vigh-Teichmann et al., 1982; Ekström and Korf, 1984). Despite the heterogeneity of the morphological appearance of pineal ganglion cells, extracellular recordings revealed usually only one response type best characterized as a luminance off-neuron, although occasionally chromatic responses have been observed in the intracranial pineal organ proper (cf. Meissl and Dodt, 1981).

In contrast to the accurate description of extracellular ganglion cell activity, no comprehensive analysis is available with intracellular techniques. Recordings of intracellular ganglion cell activity have been made in the frog pineal (Morita, 1965) and in the isolated pineal organ of lampreys (Morita, 1985). Morita (1965) recorded from spike generating cells in the frog's pineal organ an inhibition of spike activity and de- and hyperpolarizing slow potentials to photic stimuli. In the adult lamprey Morita et al. (1985) observed two types of responses from pineal ganglion cells. One type displayed maintained discharges in the dark and responded to light with a hyperpolarization and inhibition of spike activity. The other type responded only at cessation of light stimulation with transient off-discharges. During light stimulation these cells exhibited a hyperpolarization; when no light stimulus was presented the off-ganglion cells fired no action potentials.

In many pineal ganglion cells of the lamprey, the spike generating mechanism fails after the electrode penetrates the cell membrane. In these cells we observed action potentials only within the first seconds after penetration. Shortly thereafter the spike amplitude rapidly decreased leaving only the slow hyperpolarizing potential to flashes (Meissl, unpublished).

3. Interneurons

At present, the intrapineal neuronal circuitry is only partly clarified. Neurons that are homologous to retinal bipolar, horizontal or amacrine cells are assumed to be lacking in the pineal complex of anurans. However, it was concluded

from histological studies that there exists a limited number of interneurons in the pineal, which differ from the classical interneurons of the retina (Oksche, 1971; Paul et al., 1971).

A detailed study of acetylcholinesterase (AChE)-positive neurons in the frog's pineal favored a neuronal model of a basic bineuronal chain with photoreceptor cells, ganglion cells (pseudounipolar cells) and multipolar cells which were regarded as interneurons (Wake et al., 1974). In contradiction to this concept multipolar cells were also shown to contribute to the pineal tract (cf. Ekström and Korf, 1985). Until now, the synaptic connections of intrapineal neurons have not been elucidated in detail (Collin and Oksche, 1981).

The lack of a distinct laminar organization of cells and processes of most of the pineal organs prevented a definite functional clarification of the neuronal circuitry. The failure of intracellular recordings from pineal cells which have a different response characteristic from photoreceptors or ganglion cells is demonstrated by the extremely small number

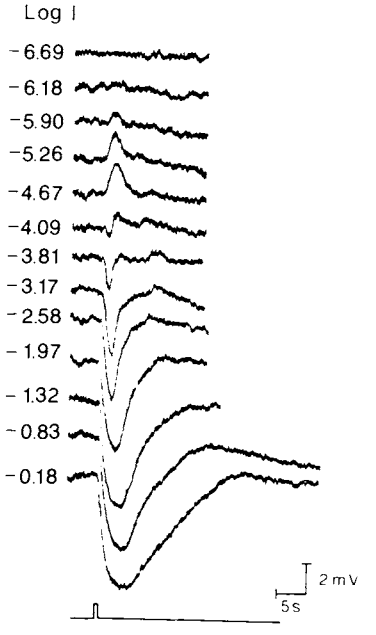

Fig. 5. Intracellular responses to light recorded from a pineal cell of *Phoxinus phoxinus* with presumed interneuron characteristic. Stimulus duration 500 ms, flash energy is indicated besides records, log 0 equal to 2200 μW/cm² (from Nakamura, unpublished).

of successful recordings. We observed in the goldfish only once light responses which apparently could not be attributed to photoreceptors or ganglion cells. A cell type exhibiting a similar response characteristic was occasionally penetrated in the pineal of the minnow, *Phoxinus phoxinus* (Nakamura et al., 1985). This cell type showed resting membrane potentials of 30 to 40 mV and in contrast to photoreceptors biphasic response to light stimulation (Fig. 5). Regardless of the stimulus wavelength the cells depolarized with dim light flashes and hyperpolarized with bright flashes. The amplitude of the hyperpolarizing potential increased over an intensity range of 5 log units (*P. phoxinus*), respectively 7 log units (goldfish), without saturation. Latencies and duration of responses corresponded to those obtained from photoreceptor cells. It is assumed that this rare cell type belongs to a small class of pineal interneurons.

4. Supportive cells

Ependymal supporting or interstitial cells bordering the pineal lumen usually lie interdispersed between photoreceptor cells. Details on the morphological appearance of supportive cells have been reviewed by Collin and Oksche (1981) and Vollrath (1981).

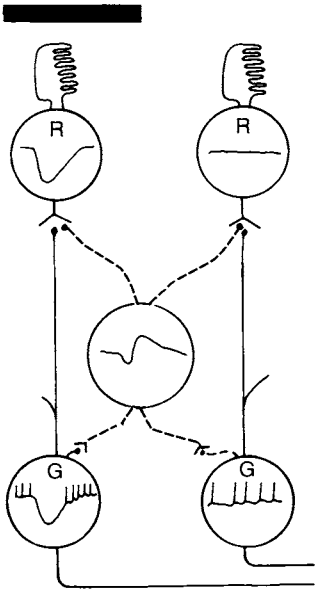

Fig. 6. Summary scheme of typical light responses from pineal cells. R photoreceptor, G ganglion cell, cell in the middle = interneuron, black bar = light flash.

Intracellular recordings from supportive cells have been mentioned by Pu and Dowling (1981) in the pineal of larval lampreys. The cells had a stable resting membrane potential of more than 60 mV and their activity was never modulated by light stimulation. Similar cells with stable resting potentials of more than 60 mV were frequently observed in the pineal organ of goldfish, the minnow, and trout (Meissl, unpublished). Recordings from such cells showed never a light-evoked potential change.

III. SUMMARY AND CONLUDING REMARKS

The pineal complex of poikilothermic animals is intrinsically photosensitive and capable of transmission of information concerning environmental lighting conditions to the brain. Photoreceptive pineal cells have many features in common with retinal cells, but they lack the complexity of neuronal information processing. The major neuronal pathway in the pineal organ is characterized by the information processed directly from photoreceptors to ganglion cells; only a small population of interneurons possibly forms an integrating network. Typical light responses from each of the neuronal types in the pineal are summarized in Fig. 6. Pineal photoreceptors respond to light with sustained hyperpolarizing potentials that lead to a hyperpolarization and lowered spike activity of ganglion cells. This relatively simple response scheme implies that the pineal is mainly a luminance detector and functions as an indicator of daylength and dosimeter of solar radiation.

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TURNOVER OF PINEAL PHOTORECEPTIVE MEMBRANES IN THE FROG, RANA ESCULENTA COMPLEX[1]

Hans-Georg Hartwig

Department of Anatomy II
University of Dusseldorf
Dusseldorf
Fed. Rep. of Germany

I. INTRODUCTION

Pineal receptor cells are highly polarized elements equipped with (1) receptor poles (modified sensory cilia bearing cone-like stacks of photoreceptive membranes), (2) cell bodies subdivided into perikarya and inner segment-like structures, and (3) neurotransmitter poles characterized by presynaptic vesicle-crowned rodlets (for further details, see Collin, this volume). Pineal sensory cells may show morphological characteristics (eg. dense-core vesicles) indicating a secretory activity (for details of their possible photoneuroendocrine function, see Oksche, this volume). Experimentally applied continuous light or darkness have been repeatedly shown to influence various compartments of sensory pinealocytes (e.g. receptor pole and/or neurotransmitter pole) as well as pineal sense organs in toto (e.g. size and weight) in fish, amphibians, and reptiles (for references, see Vollrath, 1981). In contrast to recent experimental observations demonstrating dynamic changes of retinal photoreceptor cells (for details and references, see LaVail; Pierce and Besharse; Dearry and Burnside, all this volume) apparently the possible plasticity of pineal sensory elements has not been studied

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under the influence of daily and seasonal changes of light. Although several results reported by different groups of researchers indicate a turnover of pineal photoreceptive membranes a study involving sufficient numbers of animals collected under standardized experimental conditions seems to be lacking. In the following report it will be shown that the outer segment membranes of the intracranial epiphysis cerebri in Rana esculenta exhibit a synchronized and complete daily turnover in summer, but not in other seasons of the year. The observations summarized in this report will be published in detail elswhere.

II. MATERIALS AND METHODS

Adult frogs of both sexes, Rana esculenta complex (Berger, 1983), with body weights between 35 and 55 g were purchased in April from a dealer in southern Germany. Groups of ten animals were kept in opaque plastic containers of 40 x 30 x 25 cm at a controlled temperature of 7-9° C in an artificially long day of 17L:7D. A vertically orientated fluorescent light tube served as a light source for every four plastic containers. Inside the containers a diffuse light with an intensity of approximately 90 Lux at a color temperature of 2800° K was recorded. At the end of June animals were collected at the beginning, middle and end of the day as well as at the beginning, middle and end of the night. Groups of six animals were sampled. Frontal organs, pieces of retinal tissue, and the dissected diencephalon were immersed for two hours in a modified Karnovsky fixative at room temperature (cf. Hartwig, 1973). Postfixation in cacodylate-buffered 1% OsO_4, dehydration, and penetration with epoxy resin followed standard procedures. Sampling for light and transmission electron microscopy took place in three subsequent years. In addition groups of six animals were investigated at the beginning of the day and night applying scanning electron microscopic techniques (for details and results, see Hartwig, 1984)

In April 1984 groups of 8-10 animals were subjected to one of the following surgical procedures: (1) transection of the frontal nerve running from extracranial frontal organ to intracranial epiphysis cerebri, (2) transection of both optic nerves, or (3) a combination of (1) and (2). Sham operated animals served as controls. Surgery was done under anesthesia with MS 222. Operated and sham operated animals were transferred to the experimental conditions mentioned above and sampled for transmission electron microscopy by the end of June 1984.

Furthermore, in late June 1984 animals adapted to the artificially long day mentioned above received 160 µCi of tritiated leucine (NEN: NET-460, L-(3,4,5-^{3}H(N)-Leucine; spec. act. 115 Ci/mmol) injected into the dorsal lymph sac. Injection took place near the end of the photophase (light period). Three animals were sampled at the beginning of the next photophase. The remaining three animals were injected with a chase of non-tritiated leucine at the end of the photophase of two subsequent days. Sampling took place at the beginning of the third photophase. In all these animals the fixation and embedding procedures followed the schedule mentioned above. Fixation and dehydration fluids did not contain measurable amounts of radioactivity (liquid scintillation counts). Light microscopic autoradiography followed standard protocols using Kodak NTB 2 emulsion exposed for periods of up to 6 months.

This report describes findings made in groups of animals living under outdoor conditions at the latitude of Kiel (Fed. Rep. Germany), as well as findings in animals transferred from laboratory adaptation to an artificially long day, to continuous darkness (for preliminary results, see Hartwig et al., 1985).

In a final experiment, animals were adapted to artificially long days (for technical details, see above) from (1) September to October, (2) November to December, or (3) February to March. Groups of 6 animals were investigated at light onset and light offset (for further details, see Hartwig et al., 1985).

III. RESULTS

Material was investigated by light and electron microscopy in a blind study. At the light microsopic level (semithin sections) in animals collected at the end of the scotophase (dark period) and beginning of the photophase (light period) the inner segments of pineal sensory cells were equipped with easily recognizable large and elongated outer segments (Fig. 1). Using high aperture optics, outer segments exhibited characteristic invaginations possibly indicating individual stacks of membrane lamellae. Only in approximately one tenth of the inner segments was there no apparent association with an outer segment. At the end of the photophase or beginning of the scotophase inner segments apparently no longer bore regularly lamellated outer segments comparable to those described in animals sampled at

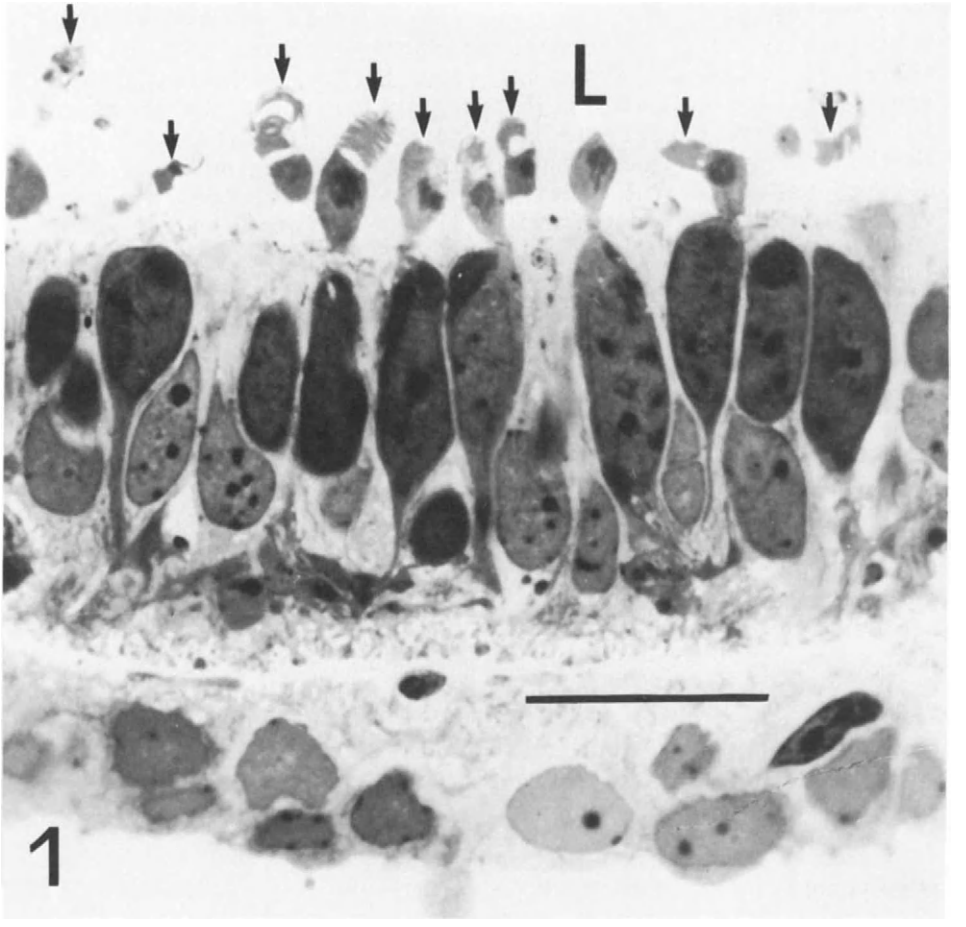

Fig. 1. Midsagittal semithin section showing ventral wall of the epiphysis cerebri and the roof of the diencephalon (bottom) in an animal sampled in the morning of an artificially long day. Inner segments of epiphyseal photoreceptor cells protruding into the pineal lumen (L) bear well developed outer segments (arrows). Scale marker: 10 µm.

the end of the scotophase and beginning of the photophase (Fig. 2). In association with approx. 15 % of the inner segments there occurred a weakly stained structure possibly

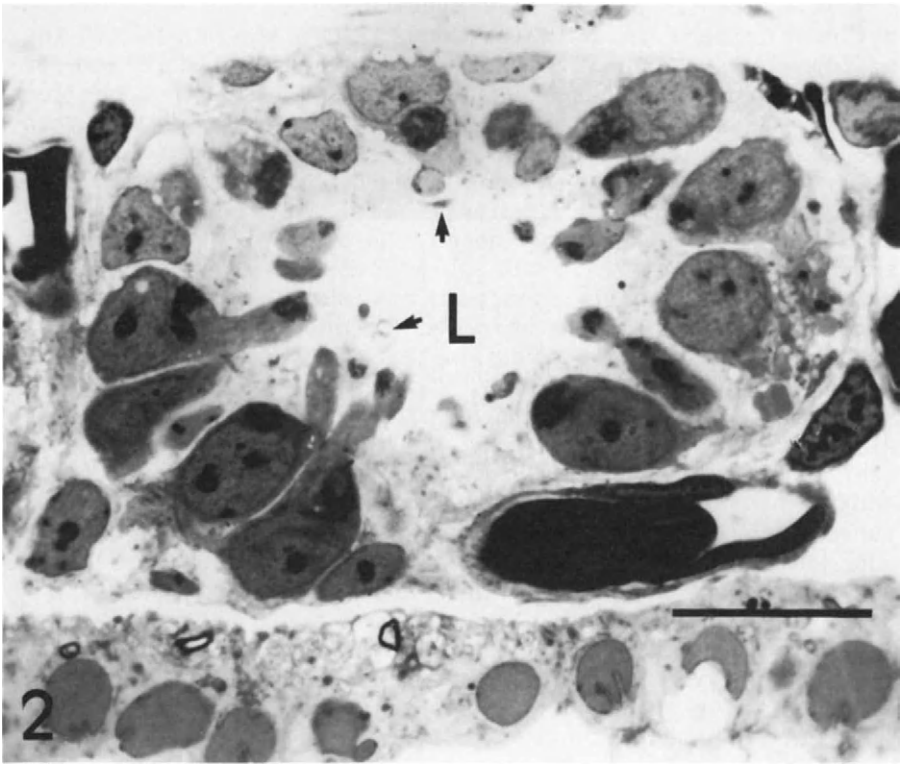

Fig. 2. Parasagittal semithin section showing epiphysis cerebri and the roof of the diencephalon (bottom) in an animal sampled in the late evening of an artificially long day. Inner segments of epiphyseal photoreceptor cells do not bear outer segments. Possible remnants of degraded outer segments are indiated by arrows. Scale marker: 10 µm.

representing an outer segment. The structures resembling outer segments that still remained (in drastically reduced numbers) in "evening" animals had ring-like profiles in semithin sections.

Scanning electron microscopic findings confirmed these light microscopic results (Hartwig, 1984). In the transmission electron microscope, outer segments predominantly

characterized by a cone-like structure could be observed in highest numbers in animals sampled at late hours of the scotophase. With light onset a gradual degradation of photoreceptive membranes was observed resembling light-dependent damage of retinal photoreceptive membranes. This process of "degradation" of outer segment membranes apparently was in progress during the photophase and, finally, resulted in a loss of regularly lamellated cone-like outer segments. It should be noted, however, that in animals transferred from 17L:7D to 20L:4D outer segment structures exhibited dramatically increased numbers of normal-appearing lamellar stacks at the expected time of the previously experienced scotophase hours, although light was still on in the new lighting schedule (M. Klockemann, unpublished). In a blind study it was possible to distinguish clearly in the electron microscope between the six groups of animals sampled around the clock (H.-J. Kock, unpublished). In addition to morphological changes in outer segment structures, daily changes in the appearance of the pineal lumina and neurotransmitter poles were noted. In animals sampled under outdoor conditions, changes of outer segment structures still permitted distinction between "morning" and "evening" groups in a blind study (M. Klockemann, unpublished), however, the differences in the number of intact or degraded outer segments were less pronounced.

Outer segment structures observed in frontal organs did not permit distinction between "morning" and "evening" animals. However, the neurotransmitter poles underwent similar dynamic changes in frontal and epiphyseal photoreceptor cells. These changes were related to size and number of synaptic ribbons as well as to size and packing density of synaptic vesicles, which will be dealt with in a separate study.

In animals with transected optic or frontal nerves, as well as in animals with transected optic and frontal nerves, it was not possible to distinguish outer segment structures sampled at the end of the scotophase from those collected at the end of the photophase. However, the neurotransmitter poles showed light-dependent changes of ultrastructure in operated animals not different from those observed in sham-lesioned controls. Sham-operated animals could not be distinguished from normal controls.

Following injection of tritiated leucine at the end of the photophase, there was no labeling of outer segment structures (above background) in animals sampled at the beginning of the next photophase whereas perikarya and inner segments as well as cell bodies of supportive cells exhibited a strong labeling with silver grains. However, in

animals sampled at the beginning of the third photophase following application of tritiated leucine, with subsequent chase injections of non-tritiated leucine for two days, outer segments were heavily labeled with silver grains.

IV. DISCUSSION

These light and electron microscopic observations clearly indicate that the outer segments of intracranial pineal photoreceptor cells in frogs, Rana esculenta complex, undergo synchronized changes of their size and shape in summer, but not during other seasons of the year. This conclusion is confirmed by results obtained in animals adapted in summer to an artificially short day (8L:16D). In a blind study "morning" and "evening" animals could not be distinguished with regard to the size and shape of epiphyseal photoreceptor cell outer segments under these experimental conditions (Hartwig, unpublished). Considering concepts of photoneuroendocrine cellular elements in pineal sense organs (Oksche or Collin, this volume) it is most interesting to note that intracranial pinealocytes undergo morphological changes so as to resemble "classical" photoreceptor cells in the morning and "regressed" photoreceptor cells in the evening. The latter are thought to exhibit a secretory activity. The reason why such synchronized morphological changes occur only in summertime is not yet understood. It remains enigmatic why frogs adapted in winter to an artificially long day, inducing a synchronized renewal of outer segment structures in summer, do not exhibit these phenomena. Frogs must be able to sense seasons of the year when kept in an artificial environment using an unknown entraining signal (Zeitgeber). In this context it should be noted that in general the circadian variation in pineal levels of melatonin and its precursor serotonin exhibit large amplitudes in summer but very small amplitudes in winter. Increase of indoleamine levels takes place regularly in evening and early night hours (for review, see Vollrath, 1981; for details in turtles, see Vivien-Roels et al., 1979). At that time of day epiphyseal photoreceptor cells in frogs exhibit morphological characteristics of "regressed" or "photoneuroendocrine" receptor cells.

Photoreceptor cells of the frontal organ apparently represent a specialized population of pineal photoreceptors: their outer segments show no synchronized changes in shape and size. In contrast to the intracranial epiphysis cerebri, the frontal organ, as an extracranial component of

the pineal complex, does not contain indoleamines in amounts discernible by histochemical techniques (for review and details, see Hartwig and Reinhold, 1981). Thus, epiphyseal photoreceptor cells are characterized by a daily gradual and synchronized degradation of outer segment structures in parallel with a daily increase of pineal indoleamine levels. The frontal organ apparently does not contain indoleamines and does not show a synchronized renewal of photoreceptor membranes. Future experiments will investigate the role of pineal indoleamines in the frog, Rana esculenta. Results described in the present contribution might indicate a local role for indoleamines in synchronizing turnover of pineal photoreceptor membranes (for the role of indoleamines in the control of retinal photoreceptor membrane renewal, see Pierce and Besharse, this volume). In this context it should be mentioned that the total amount of photoreceptor membranes daily renewed per photoreceptor cell is much higher in retinal rods and cones than in pinealocytes. Outer segments of pinealocytes are rather small when compared with retinal outer segments.

Following lesions of frontal and optic nerves it was not possible to distinguish between animals sampled at the beginning and at end of the photophase on the basis of outer segment morphology. This observation indicates that both frontal and optic nerves influence the rhythmic activity of the epiphysis cerebri. Since observation times were restricted to the beginning and end of the photophase it is difficult to draw further conclusions. Most probably this experimental interruption of retinal and extraretinal pathways conducting light-dependent information interfered with circadian clock mechanisms. This assumption is further supported by observations of Adler (1971), who showed that sectioning the frontal nerve prevents sun compass orientation in Rana pipiens. Another finding strongly indicates that circadian clock mechanisms are indeed involved in the control of synchronized renewal of epiphyseal photoreceptor outer segments: In frogs transferred from 17L:7D to 20L:4D for a period of 4 days, epiphyseal photoreceptor outer segments were remodeled from bulbous cilia to regularly lamellated structures at the middle of the previously entrained night, although the lights were on at this time of the new lighting schedule.

Results obtained in a pilot study (pulse chase labeling with tritiated leucine) indicate that synthesis of membrane material used for the night time formation of regularly lamellated outer segments must take place before the end of the previous light phase. The labeling of outer segments following a chase injection of non-tritiated leucine most

probably indicates a recycling of labeled substances. However, electron microscopic autoradiography and additional experiments are required for a detailed analysis of the mechanisms of membrane turnover in epiphyseal photoreceptor cells. In general, mechanisms of turnover of photoreceptive membranes must be rather different in retina and epiphysis cerebri; eg. in the retina the pigment epithelium plays a major role in this process (for review, see Bok, 1985) but the epiphysis cerebri contains no pigment epithelium. Light-dependent changes of ultrastructural details (frequency and size of lamellar bodies) observed in pineal supportive cells (= glial cells) strongly indicate that these elements play a major role in the turnover of photoreceptive membranes in the epiphysis cerebri. Autoradiographic techniques probably represent adequate methods for elucidating processes of turnover of pineal photoreceptor membranes.

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REGULATION OF TELEOST RETINOMOTOR MOVEMENTS BY CYCLIC AMP, CALCIUM, AND DOPAMINE[1]

Allen Dearry
Beth Burnside

Department of Physiology-Anatomy
University of California
Berkeley, California

I. INTRODUCTION

Retinal cell motility plays a critical and dynamic role in retinal function. Motility participates in such fundamental processes as development of cell shape, synaptogenesis, axon transport, synaptic modulation, photoreceptor outer segment disk turnover, and photoreceptor alignment (cf. Burnside and Dearry, 1986). Among the most striking examples of retinal motility are the movements of photoreceptors and retinal pigment epithelial (RPE) cells found in lower vertebrates (fish, frogs, and birds). In response to changes in light conditions and to circadian signals, photoreceptors elongate and contract and pigment granules migrate in and out of the long apical projections of the RPE cells (cf. Burnside and Nagle, 1983; Burnside and Dearry, 1986). Although these "retinomotor movements" have been extensively described over the last century, we have only recently begun to find clues to the mechanisms of their regulation by light and circadian signals. In this chapter, we describe our current understanding of the physiological regulation of fish retinomotor movements by intra- and extracellular messengers. We examine the effects of cAMP,

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Ca^{++}, and dopamine on retinomotor movements in green sunfish, Lepomis cyanellus, and consider their possible roles in mediating diurnal and circadian regulation of these movements. More detailed reviews of the literature concerning retinomotor movements and other aspects of retinal cell motility have recently appeared (Burnside and Nagle, 1983; Burnside and Dearry, 1986).

A. Retinomotor Movements as Models of Retinal Motility

Retinomotor movements provide valuable models for studying light and circadian regulation of retinal cell motility. They include both of the basic types of cell motility: cell shape change and intracellular transport. They exhibit large excursions so they are particularly amenable to quantitative analysis. In addition, they continue to occur in cultured retinas in vitro so they can be easily manipulated experimentally. Since cycles of retinomotor movements persist in constant darkness in many species, these movements also provide models for investigating the mechanisms underlying circadian regulation of retinal processes. Recently, we have been analyzing the neurochemical regulation of these movements in order to provide clues to light/dark or circadian changes in retinal transmitters and hormones. Thus, we believe that studying retinomotor movements in lower vertebrates can provide new insights into the light and circadian regulation of retinal physiology.

B. Characteristics of Retinomotor Movements

Retinomotor movements serve to reposition photoreceptor outer segments and RPE shielding pigment in order to optimize bright-light or dim-light vision (cf. Ali, 1975; Burnside and Nagle, 1983). In most fish, amphibians, and birds, the pupillary aperture is fixed. Instead of adjusting pupil diameter, these animals increase or decrease the amount of light incident upon the retina by morphological rearrangements of photoreceptors and RPE pigment. Photoreceptors elongate and contract and melanin pigment granules migrate in and out of the long apical projections of the RPE cells (Fig. 1). In the dark, rods contract and cones elongate, thereby placing the rods first-in-line to receive any incoming light, while RPE pigment granules aggregate into the cell bodies so that rod outer segments

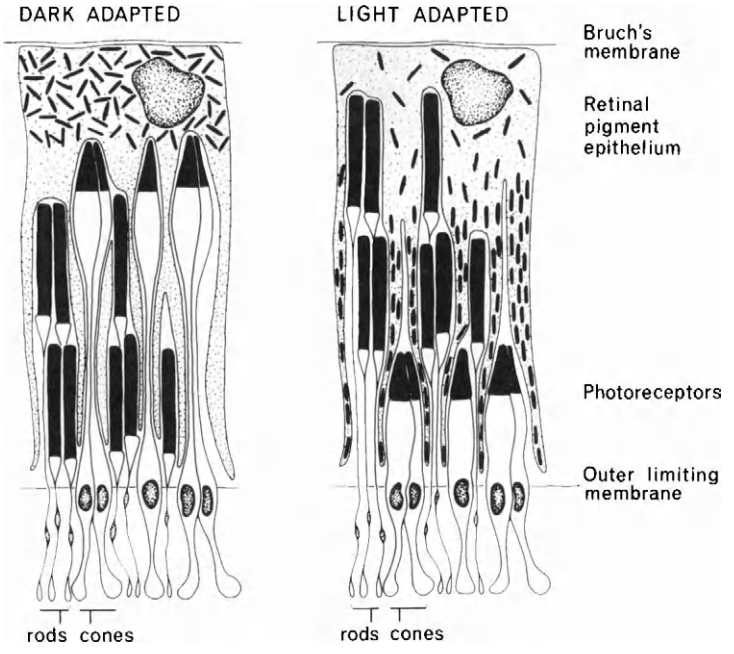


FIGURE 1. Schematic diagram of retinomotor movement in the green sunfish, Lepomis cyanellus. In the dark-adapted state at night, cone myoids elongate, rod myoids contract, and RPE pigment is aggregated into the scleral part of the RPE cells. In the light-adapted state at day, these movements are reversed: cones contract, rods elongate, and RPE pigment disperses into the cells' long apical processes. (Reprinted with permission from Burnside and Nagle, 1983, Prog. Retinal Res., Pergamon Press.)

are fully exposed (Fig. 1). In the light, each of these movements is reversed. Rods elongate and cones contract so that the contracted cones are now first-in-line to receive incoming light, and the elongated rods are shielded by the dispersed pigment granules in the long apical processes of the RPE cells (Fig. 1).

The in vivo rates of cone, rod, and RPE retinomotor movements are relatively slow: they vary among species and among cell types, but all those reported range between 0.2 and 4.5 μm/min (cf. Burnside and Nagle, 1983). In green sunfish, Lepomis cyanellus, cones elongate and contract at 1.1 - 1.5 μm/min (Burnside et. al., 1982a, b). In blue-

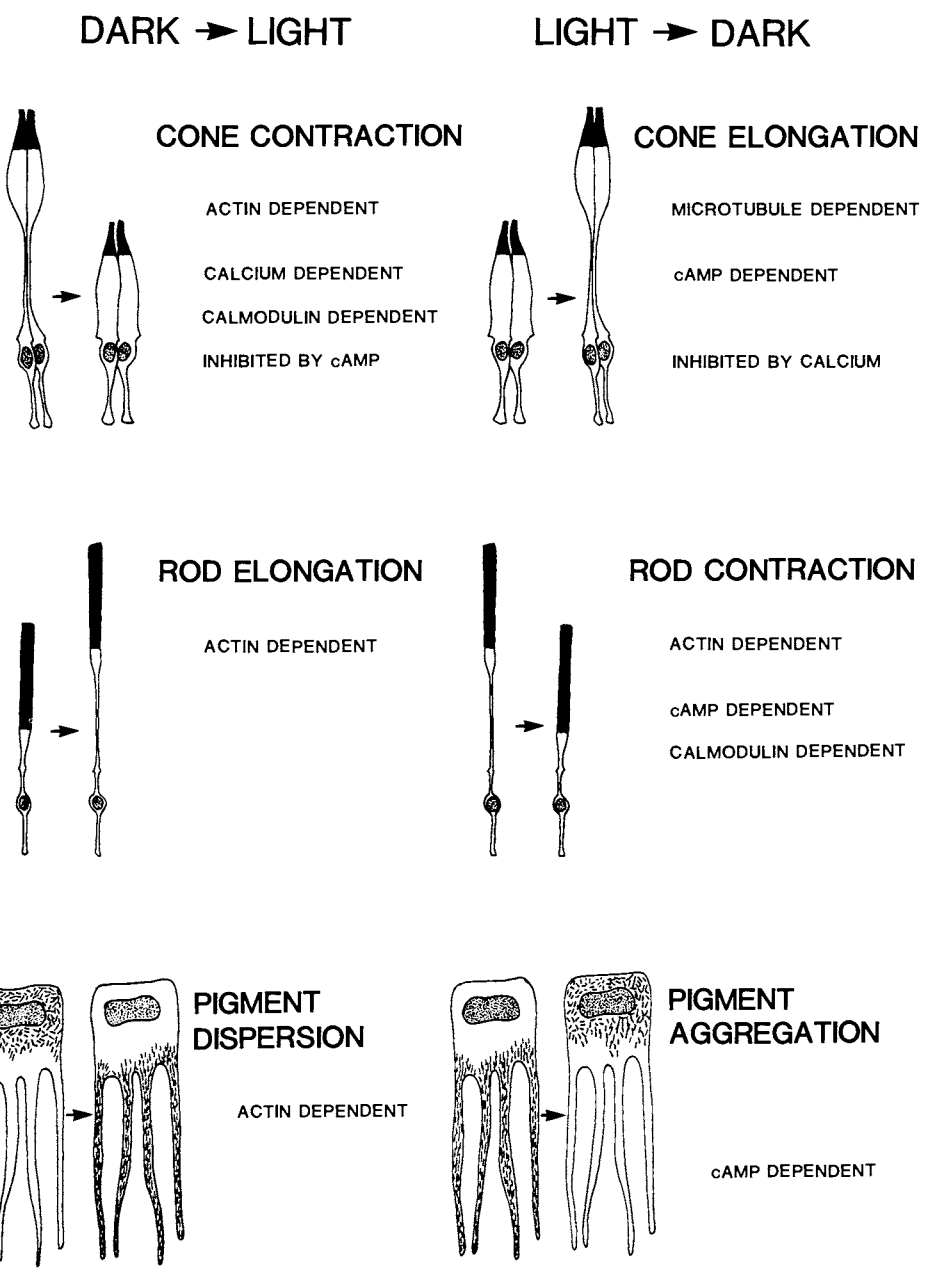
striped grunt, Haemulon sciurus, cones elongate at 2.0 μm/min but contract at 4.4 μm/min (Burnside et al., 1983). The pigment granules of this species aggregate or disperse at a rate of 3.5 μm/min. In the cichlid, Saratherodon mossambicus, rods elongate at 0.2 μm/min and contract at 1.3 μm/min (O'Connor, 1982). It is interesting to note that cone elongation is not initiated until 10-20 min after dark onset, while pigment aggregation and rod contraction begin immediately after dark onset. This staggered timing along with the relatively faster rate of pigment migration insures that RPE pigment is mostly aggregated before cone elongation begins. These relationships may be functionally advantageous in the wild since both cone and rod outer segments would be exposed during dusk.

The differential rates of retinomotor movements among cell types within one species suggest that these movements are mechanically independent. Recently we have shown directly that the machinery for movement is localized within each cell type. Retinomotor movements can be observed in enzymatically isolated RPE cells (Bruenner and Burnside, 1986) and in mechanically detached cone outer/inner segments (Dearry and Burnside, 1986a) and rod outer/inner segments (Nagle and Burnside, 1984; Dearry and Burnside, 1986a). Regulation of retinomotor movements, on the other hand, most likely entails interactions between photoreceptors and RPE as well as input from more proximal retinal cells (see below).

C. Cytoskeletal Elements Responsible for Retinomotor Movements

Initial investigations of retinomotor movements in our laboratory were aimed at elucidating which cytoskeletal elements were responsible for photoreceptor movements and

FIGURE 2 (opposite). A summary of the force-producing mechanisms and intracellular regulation of vertebrate retinomotor movements. Light onset initiates cone contraction, rod elongation, and pigment dispersion; all these movements are actin-dependent. Dark onset initiates cone elongation, rod contraction, and pigment aggregation; all these movements are associated with an increase in cytoplasmic cAMP level. (Reprinted with permission from Burnside and Dearry, 1986, Adv. Cell. Neurobiol., Academic Press.)


DARK → LIGHT
LIGHT → DARK
CONE CONTRACTION
ACTIN DEPENDENT
CALCIUM DEPENDENT
CALMODULIN DEPENDENT
INHIBITED BY cAMP
CONE ELONGATION
MICROTUBULE DEPENDENT
cAMP DEPENDENT
INHIBITED BY CALCIUM
ROD ELONGATION
ACTIN DEPENDENT
ROD CONTRACTION
ACTIN DEPENDENT
cAMP DEPENDENT
CALMODULIN DEPENDENT
PIGMENT DISPERSION
ACTIN DEPENDENT
PIGMENT AGGREGATION
cAMP DEPENDENT

pigment granule transport. Teleost cone and rod myoids and RPE apical projections contain longitudinally oriented actin filaments and microtubules (cf. Burnside and Nagle, 1983). Specific inhibitors and disrupters of these cytoskeletal components were employed to determine which parts of the motile machinery mediated contraction, elongation, and intracellular particle transport (see Fig. 2). Disruption of actin filaments with cytochalasin D prevented light-induced cone contraction, dark-induced rod contraction, and light-induced rod elongation (Burnside, 1976; O'Connor and Burnside, 1981, 1982; Burnside et al., 1983). The finding that rod and cone contraction is actin-dependent is consistent with reports for other nonmuscle cells in which essentially all contractile shape changes have been shown to be actin-dependent.

Disruption of microtubules with colchicine or nocadazol prevented dark-induced cone elongation but had no effect on rod elongation (Burnside, 1976; Warren and Burnside, 1978; O'Connor and Burnside, 1981; Burnside et al., 1983). It was somewhat surprising that rod elongation was dependent on actin filaments not microtubules. However, other examples of actin-dependent cell elongation have been described, e.g., acrosomal process formation in echinoderm sperm and the extension of microspikes by axonal growth cones (Yamada et al., 1971; Tilney et al., 1973).

Both actin filaments and microtubules appear to have roles in pigment granule transport in RPE cells. Cytochalasin D completely blocked light-induced pigment dispersion and also induced pigment aggregation in light-adapted fish (Burnside et al., 1983). However, cytochalasin D had no effect on dark-induced pigment aggregation. These results suggest that actin filaments are required for dispersion and maintenance of dispersion, but not for aggregation. Colchicine disrupted microtubules within RPE cell bodies, and both dispersion and aggregation of pigment were blocked in this region (Burnside et al., 1983). Because colchicine failed to disrupt microtubules in RPE apical projections, we were not able to assess the role of microtubules in transport within the projections.

In summary, inhibitor studies have shown that light onset initiates actin-dependent movement in all three cell types: cone contraction, rod elongation, and RPE pigment dispersion (Fig. 2). Rod contraction is also actin-dependent, whereas cone elongation is microtubule-dependent (Fig. 2). The machinery responsible for pigment aggregation has not been identified.

II. LIGHT AND CIRCADIAN REGULATION OF RETINOMOTOR MOVEMENTS

A. Regulation by Light

Light onset induces cone contraction, rod elongation, and RPE pigment dispersion. Brief flashes of 2.5 msec induced light-adaptive cone and RPE movements in trout indistinguishable from those induced by 40 min of continual light exposure (Muntz and Richard, 1982). This finding suggests that light onset activates a light-adaptive process which continues in the dark. This light-adaptive influence decays with time since goldfish cones and RPE began to return to their dark-adapted positions within 60 min of a 1-2 min light exposure (Wagner and Douglas, 1983).

The effect of light on retinomotor movements appears to be mediated locally within the retina. A small circular spot of light induced light-adaptive cone and RPE movements only in the light-exposed area of the retinas of dark-adapted fish; retinal areas outside the light spot remained dark-adapted (Easter and Macy, 1978). This result was also obtained following optic nerve section. Furthermore, light onset produces cone contraction in isolated dark-adapted retinas and dark onset produces cone elongation in isolated light-adapted retinas (Dearry and Burnside, 1984a,b). These observations indicate that light and dark can evoke retinomotor movements in the absence of efferent input to the retina.

The effect of light on cone and rod retinomotor movements may require input from retinal cells other than the photoreceptors themselves. Easter and Macy (1978) reported that a transition zone of approximately 50 μm surrounded the light-adapted retinal area when the eyes of dark-adapted fish where illuminated by a narrowly focused light beam. In this annular region, graded dark-adapted cone and RPE retinomotor positions were observed. They suggested that the presence of such a transition zone argued against a purely intracellular control system and in favor of intercellular regulation, e.g., by some form of cell-to-cell communication or by local diffusion of a released substance.

We have recently found that light can elicit appropriate retinomotor movements in isolated cone and rod fragments containing inner and outer segments (CIS-COS and RIS-ROS) (Dearry and Burnside, 1986a). Thus light can clearly act directly on photoreceptors to initiate retinomotor movements.

In contrast, the effect of light on RPE cells appears to be indirect. Light had no effect on pigment distribution in isolated frog RPE (Snyder and Zadunaisky, 1976) or in isolated fish RPE cells (Bruenner and Burnside, 1986). Two observations suggest that regulation of RPE retinomotor movements is mediated by rods: 1) in frogs the action spectrum of RPE pigment movement matched the absorption spectrum of rhodopsin (Liebman et al., 1969); and 2) in young fishes RPE retinomotor movement did not develop until after rods appeared even though RPE and cones were present earlier (Blaxter and Staines, 1970). It thus seems likely that some signal must be transmitted from retina to RPE to mediate light induction of pigment migration.

B. Regulation by Circadian Rhythm

Onset of light or dark is not the only stimulus capable of evoking retinomotor movement. In fish kept in constant darkness, cones continue to exhibit retinomotor movement, i.e., cones contract during subjective day and elongate during subjective night (cf. Burnside and Nagle, 1983). Such circadian cone movements in constant darkness were first observed by Welsh and Osborne (1937) in catfish and have since been observed in all teleost species studied, including the green sunfish used in our laboratory (Burnside and Ackland, 1984). Circadian responses in rods and RPE are present in some species but absent in others. Since cones undergo cycles of contraction and elongation in constant darkness, it is clear that not only light but also some circadian signal can regulate their movements.

In fish populations living under cyclic light conditions, this circadian influence may be the primary regulatory signal for cone contraction. In green sunfish sampled at intervals throughout the first 24 hours in constant darkness, cones contracted before expected dawn (Burnside and Ackland, 1984). In tetra kept in constant darkness, retinomotor movements also preceded the expected time of light onset (John and Haut, 1964; John and Kaminester, 1969). Predawn cone contraction has also been reported in fish in the wild (McFarland et al., 1979; Kavaliers and Ross, 1981). These results suggest that in real life cone contraction may occur mainly in response to a circadian rather than a light signal.

This circadian rhythm in retinomotor movement may be reflected in circadian changes in retinal physiology and in

visually guided behavior. We have recently found that green sunfish exhibit a circadian rhythm in retinal sensitivity as well as cone movement (Dearry and Barlow, 1986). Sensitivity was 3- to 10-fold higher during subjective night than during subjective day. John and Haut (1964) reported that black-banded tetra possess a circadian rhythm not only in cone, rod, and RPE retinomotor movements but also in schooling behavior. Light-induced school formation had a latency of 15 sec during subjective day and of 6 min during subjective night. Although a variety of mechanisms may contribute to circadian rhythms in sensitivity and behavior, the occurence of circadian rhythms in anatomy, physiology, and behavior suggests that they may be functionally related as has been postulated for Limulus (see Barlow et al., 1984).

III. INTRACELLULAR REGULATION OF RETINOMOTOR MOVEMENTS BY Ca^{++} AND cAMP

In order to investigate the mechanisms of intracellular regulation of retinomotor movements, we have used a variety of technical approaches. The most powerful technique for studying intracellular regulation of motility is the production of lysed cell motile models. This technique, exemplified by the glycerinated myofibril that played such an important role in the investigation of muscle cell motility, allows us to lyse the plasma membranes with detergent or glycerine, thus making the cells permeable to experimental media while nonetheless retaining functionally intact motile machinery. Detergent treatment inactivates the cell's control over its own internal millieu and makes it possible for us to analyze the effects of ions, nucleotides, and various drugs on the motile process. We have produced motile models of teleost retinal cones which have been lysed with the nonionic detergent Brij-58 so that they are permeablized but retain their ability to elongate and contract (Burnside et al., 1982b; Porrello et al., 1983; Gilson et al., 1986). By varying the components of the medium bathing the lysed cone models we can induce them either to elongate or to contract, and thus we can characterize the conditions which are required for producing elongation or contraction in vivo (see Table I). We have found that elevated cAMP is required for cone elongation and elevated Ca^{++} is required for cone contraction (see Fig. 3 and below).

TABLE I

LYSED CELL MOTILE MODELS

Characteristics:

membranes permeabilized
motile machinery functional
movement quantifiable

Method:

lyse for 3 min in 1% Brij-58, a non-ionic detergent
then incubate 15 min in media without detergent
then fix and measure cone lengths

Reactivation Media:

0.1 M PIPES buffer, pH 6.9
10mM EGTA
$<10^{-8}$ to 10^{-5}M free Ca^{++}
0-1mM ATP
0-1mM cAMP

Results with motile models are consistent with observed effects of various treatments which influence cAMP and Ca^{++} levels in intact cells. We have studied movements both in situ by intraocular injection and in vitro with isolated retinas. To avoid possible indirect effects mediated by other retinal cells, we have also examined motile processes in isolated RPE cells and in detached fragments of rods and cones which contain only inner and outer segments (RIS-ROS and CIS-COS). These photoreceptor fragments retain their ability to elongate and contract.

A. Cones

Lysed cell model studies have demonstrated that reactivated cone contraction is activated by Ca^{++} and inhibited by cAMP (Burnside et al., 1981; Porrello and Burnside, 1984).

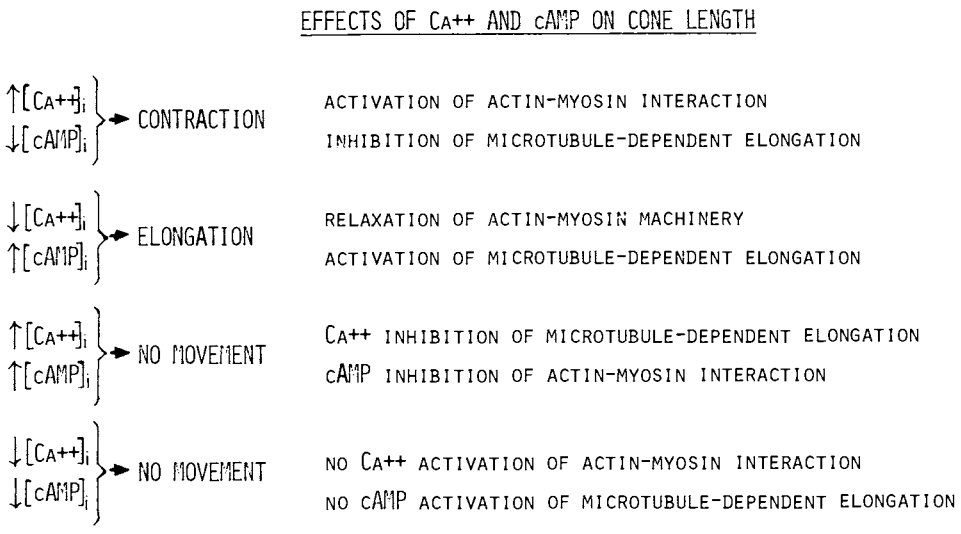


FIGURE 3. Ca^{++} and cAMP have antagonistic effects on cone myoid length. Studies with lysed-cell models of teleost cones have revealed that actin-dependent cone contraction requires both an increase in $[Ca^{++}]_i$ and a decrease in $[cAMP]_i$. In contrast, microtubule-dependent cone elongation requires both a decrease in $[Ca^{++}]_i$ and an increase in $[cAMP]_i$. Decreasing or increasing both Ca^{++} and cAMP levels has no effect on cone retinomotor movement.

The rate of reactivated cone contraction was Ca^{++}-dependent, with maximal rates occuring at $\geq 10^{-6}$M free Ca^{++}, and no contraction occuring at $<10^{-8}$M free Ca^{++} (Porrello and Burnside, 1984). The Ca^{++} dose-response curve for cone contraction resembled that for smooth muscle (Kerrick et al., 1980) with even slightly greater sensitivity to calcium in cones. The maximal rate of contraction in lysed cell models was identical to that observed during light-induced contraction in vivo (Burnside et al., 1981). These results suggest that a rise in cone myoid free Ca^{++} to at least 10^{-6}M is likely to accompany light onset in vivo.

Numerous other observations from the lysed cone model studies (not described in detail here) strongly suggest that Ca^{++} regulation of cone contraction is mediated by myosin phosphorylation as has been observed in smooth muscle and other nonmuscle cells (Adelstein et al., 1982; Porrello and Burnside, 1984). In this form of regulation, Ca^{++} binds

to calmodulin and the Ca^{++}/ calmodulin complex then activates myosin light chain kinase to phosphorylate myosin light chains. Only when mysoin light chains are phosphorylated can myosin interact with actin to activate myosin ATPase and produce contraction.

Reactivated contraction in lysed cone models was inhibited by cAMP at submicromolar concentrations (Porrello and Burnside, 1984). In smooth muscle and nonmuscle cells cAMP has been shown to inhibit contraction by catalyzing the phosphorylation of myosin light chain kinase, thereby inhibiting the interaction of myosin light chain kinase with Ca^{++}/calmodulin and preventing myosin phosphorylation (Adelstein et al., 1982). Several observations with lysed cone models suggest that cAMP plays a similar role in inhibiting reactivated cone contraction (Burnside and Ackland, 1986). These findings with lysed models do not of course rule out additional effects of cAMP in intact cones; for example, cAMP might also inhibit contraction indirectly by enhancing Ca^{++} sequestration and lowering intracellular free Ca^{++} levels. In intact cells light-induced cone contraction was blocked by culturing whole retinas with a phosphodiesterase inhibitor (IBMX), dibutyryl cAMP, or the adenylate cyclase activator forskolin (Dearry and Burnside, 1984a, 1985).

Several observations from our laboratory suggest that at least some of the calcium responsible for activating cone contraction in vivo is derived from internal stores. Intact cones of isolated retinas underwent normal light-induced contraction in Ca^{++}-free media containing 5mM EGTA for at least 10 minutes after light onset; thus it seems clear that light can induce the release of sufficient Ca^{++} from internal stores to initiate contraction (Porrello and Burnside, 1984). In longer incubations cones failed to contract completely when isolated retinas were cultured in the light in either Ca^{++}-free or Co^{++}-containing media (Dearry and Burnside, 1984a, 1986a). These results could indicate that an external source of Ca^{++} is required for completion of contraction over a long (30 min) time course or that Ca^{++}-dependent synaptic transmission in the inner retina is required for completion of cone movement. Since light can evoke partial cone contraction in the absence of extracellular Ca^{++}, it seems likely that light onset induces release of Ca^{++} from intracellular storage sites in cones.

Studies of the effects of extracellular Ca^{++} and Ca^{++} ionophore on intact cones further corroborate the suggestion that cone cytoplasmic Ca^{++} levels are low in the dark and rise at light onset. Cone contraction was induced in the

absence of light in dark-cultured retinas by elevating extracellular Ca^{++} or by application of the Ca^{++} ionophore A23187 (Dearry and Burnside, 1984a).

The intracellular conditions required for cone elongation are precisely opposite those required for cone contraction. In lysed cone models, reactivated elongation required elevated cAMP and low free Ca^{++} levels (Gilson et al., 1986). Cones elongated at a rate proportional to the cAMP concentration between 10^{-6} and 10^{-3}M. Ca^{++} inhibited cone elongation in a dose-dependent fashion between 10^{-7} and 10^{-5}M. These observations are consistent with our earlier findings that retinal cAMP levels were three-fold higher in dark-adapted than in light-adapted green sunfish retinas (Burnside et al., 1982b). Furthermore, in intact cones, elongation can be induced in the light by treatments that elevate cytoplasmic cAMP levels. Using both intraocular injections and cultured retinas, we found that cAMP analogs, when administered along with phosphodiesterase inhibitors, induced cone elongation in a dose-dependent fashion; cGMP and its analogs had no effect on cone movement in intact cells or in models (Burnside et al., 1982b; Burnside and Basinger, 1983; Burnside and Ackland, 1984). Forskolin, an activator of adenylate cyclase, and IBMX, an inhibitor of phosphodiesterase, elicited dose-dependent cone elongation in isolated light-adapted retinas (Dearry and Burnside, 1985). Forskolin also induced myoid elongation in isolated cone fragments containing only inner and outer segments (CIS-COS) (Dearry and Burnside, unpublished observations). Dark-induced cone elongation was inhibited in intact cultured retinas by elevated extracellular Ca^{++} concentrations (Dearry and Burnside, 1984a).

Taken together (Fig. 3) these observations suggest that in the cone myoid cytoplasm in vivo, light onset produces conditions of elevated free Ca^{++} and lowered cAMP levels, whereas dark onset produces low Ca^{++} and high cAMP levels. Since cycles of cone elongation and contraction continue in constant darkness (Burnside and Ackland, 1984), it follows that circadian signals trigger similar changes in cone myoid cAMP and Ca^{++} levels at subjective dawn and dusk.

B. Rods

Rods undergo opposite cell shape changes from cones at light and dark onset: in the light rods elongate and in the dark they contract (O'Connor and Burnside, 1981,1982). However, in rods as in cones, treatments which elevate cAMP

induced dark-adaptive retinomotor movements: rod contraction and cone elongation (O'Connor and Burnside, 1982). In preliminary studies with lysed cell models of rods, we have found that both cAMP and Ca^{++} are required for contraction (Lo and Burnside, unpublished observations). This is not surprising since all known actin-dependent contractile processes are activated by Ca^{++} (cf. Adelstein and Eisenberg, 1980). Thus in rods cAMP and Ca^{++} would appear to be elevated simultaneously at darkness onset, in contrast to cones where cAMP and Ca^{++} act antagonistically. Further studies are needed to characterize the roles of Ca^{++} and cyclic nucleotides in regulating rod retinomotor movements.

C. RPE

As with rods and cones, RPE cells undergo dark-adaptive retinomotor movements in response to treatments that elevate cAMP. Dibutyryl cAMP, the phosphodiesterase inhibitor IBMX, or the adenylate cyclase activator forskolin induced pigment aggregation in intact light-adapted retinas or in isolated RPE cells (Burnside and Basinger, 1983; Dearry and Burnside, 1984a, 1985; Bruenner and Burnside, 1986). Thus the signal to the RPE which notifies the cell that light is on or off appears to influence the cell's cAMP level.

IV. EXTRACELLULAR REGULATION OF RETINOMOTOR MOVEMENTS BY DOPAMINE

A. Inhibition of Adenylate Cyclase Activity

After finding that cAMP and Ca^{++} regulate cone retinomotor movement and that a circadian signal induces cone movement, we began to investigate whether any of the hormones or transmitters known to affect intracellular cAMP or Ca^{++} levels might influence retinomotor movements. We initially tested to see if dopaminergic or alpha-adrenergic agonists were capable of inhibiting forskolin-induced cone elongation and RPE pigment aggregation. Forskolin, an adenylate cyclase activator, induced concentration-dependent dark-adaptive cone and RPE movements (rods were not analyzed for technical reasons) in isolated light-adapted retinas (Dearry and Burnside, 1985). Forskolin-stimulated cAMP production can be inhibited by dopaminergic or alpha-

adrenergic agonists in cells with appropriate receptors (e.g., see Burns et al., 1982; Miyazaki et al., 1984). This inhibitory effect has generally been attributed to dopaminergic D-2 or alpha$_2$-adrenergic receptors (see reviews by Creese et al., 1982; Bylund and U'Prichard, 1983). We found that dopamine or apomorphine inhibited the extent of forskolin-induced movements by 57-70%; phenylephrine, an alpha$_1$-, and clonidine, an alpha$_2$-adrenergic agonist, were unable to inhibit forskolin-induced cone and RPE movements (Dearry and Burnside, 1985). Dopamine also inhibited dark-adaptive movements induced by IBMX, a phosphodiesterase inhibitor, but not those induced by the cAMP analog dibutyryl cAMP. Since dopamine inhibited forskolin- and IBMX-induced but not dbcAMP-induced retinomotor movements, it seems likely that dopamine inhibition of movements results from dopamine inhibition of adenylate cyclase activity. These results also suggest that dopamine has a direct effect on the cones and RPE cells themselves. If dopamine were inhibiting forskolin- and IBMX-induced movements through an indirect pathway involving second-order retinal neurons, then dopamine should have inhibited dbcAMP-induced movement through this same pathway. More conclusive evidence for a direct effect of dopamine on photoreceptors and RPE cells comes from recent work on isolated cells (see below).

B. Mediation of Cone Contraction by D-2 Receptors

Having found that dopamine could inhibit forskolin-induced dark-adaptive cone and RPE movements, we next asked whether or not dopamine could induce light-adaptive movements in the dark. When delivered via intraocular injection, dopamine produced maximal extents of cone contraction and pigment dispersion in dark-adapted fish (Dearry and Burnside, 1986a). In order to more fully investigate the effect of dopamine, we removed isolated retinas from dark-adapted fish and incubated them in constant darkness in a Ringer solution containing varying concentrations of dopaminergic agonists and antagonists. Dose-response studies revealed that apomorphine and dopamine induced concentration-dependent cone contraction with half-maximal effects at 10^{-9} and 10^{-8}M, respectively (Fig. 4). At 10^{-6}M, LY171555, a D-2 dopamine agonist, induced full cone contraction while SKF38393, a D-1 dopamine agonist, was ineffective. Clonidine, an alpha$_2$-adrenergic agonist, was also ineffective. Dopamine-induced cone contraction was

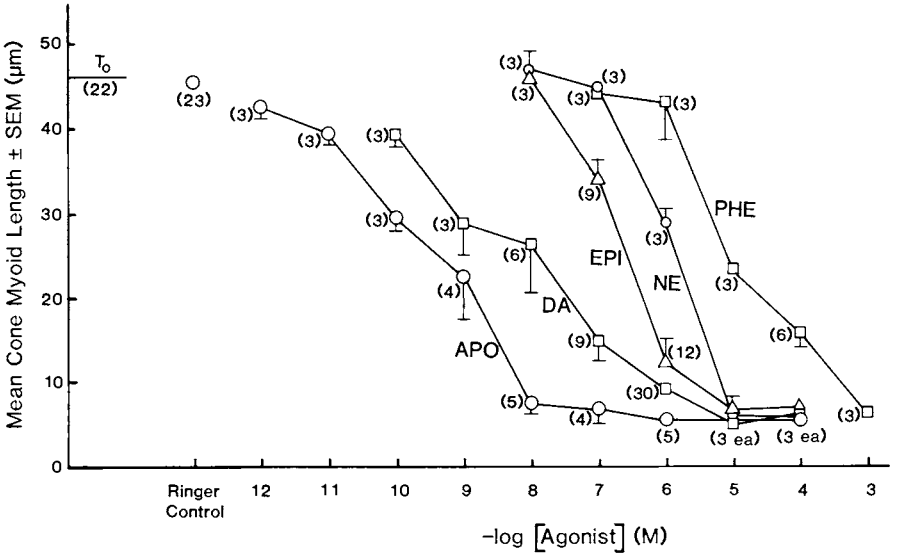


FIGURE 4. Catecholamines induce concentration-dependent light-adaptive cone contraction in isolated dark-adapted retinas cultured in constant darkness. Retinas were removed from dark-adapted fish under infrared illumination and fixed immediately after dissection (T_0) or after a 45 min culture in constant darkness in Ringer solution alone (control) or in Ringer solution containing varying concentrations of apomorphine (APO), dopamine (DA), epinephrine (EPI), norepinephrine (NE), or phenylephrine (PHE). Numbers in parentheses indicate number of retinas examined. (Reprinted with permission from Dearry and Burnside, 1986a, J. Neurochem., Raven Press.)

inhibited by antagonists with the following potency order: sulpiride > haloperidol >> domperidone = metoclopramide > fluphenazine > SCH23390. This hierarchy is characteristic of D-2 receptors. Sulpiride, a selective D-2 antagonist, also completely blocked cone contraction due to apomorphine, LY171555, and other catecholamines including epinephrine, norepinephrine, and phenylephrine. Thus both production and inhibition of agonist-induced cone contraction in dark-adapted retinas were selective for agents interacting with D-2 dopamine receptors.

This evidence for D-2 dopamine receptor-mediated cone contraction was initially surprising since previous reports indicated that dopamine activated adenylate cyclase activity

in carp and goldfish retinas, an effect mediated by D-1 rather than D-2 receptors (Redburn et al., 1980; Dowling and Watling, 1981). If dopamine acted similarly on green sunfish cones, it would be expected to produce cone elongation (which we have shown to be cAMP-dependent) not cone contraction. However, dopamine-stimulated adenylate cyclase has been found to be predominantly localized in inner retinal layers with little if any activity in the photoreceptor layer (Lolley et al., 1974; Thomas et al., 1978). In addition, sulpiride inhibited dopamine-induced cone contraction in our experiments, but in other reports sulpiride failed to inhibit dopamine-induced retinal cAMP accumulation (Watling and Dowling, 1981; Makman et al., 1982). Furthermore, we found that dopamine induced cone contraction with an EC_{50} of 10 nM, a value 1000-fold lower than the 10 μM EC_{50} reported for dopamine stimulation of adenylate cyclase in whole carp retinas (Dowling and Watling, 1981). Dopamine generally serves as a D-1 agonist with uM potency and as a D-2 agonist with nM potency (Kebabian et al., 1984). Our results thus strongly suggest that dopamine elicits cone contraction through an interaction with D-2 receptors.

In addition to finding that exogenous dopamine mimicked light by inducing cone contraction in the dark, we also found that dopamine is normally released from sunfish retinas in the dark but that concomitant uptake prevents its having an effect on cones. Blocking dopamine uptake with benztropine induced cone contraction in dark-adapted retinas cultured in constant darkness without addition of exogenous dopamine (Dearry and Burnside, 1986a; Fig. 5). This effect of benztropine was completely blocked by the D-2 antagonist sulpiride (Fig. 5). These observations indicate that cultured retinas can produce sufficient dopamine to induce cone contraction without addition of exogenous dopamine even in the dark.

We also examined the effects of the D-2 antagonist sulpiride on light-induced cone contraction and maintenance of the contracted cone position in retinas cultured in constant light. Sulpiride inhibited the extent of light-induced cone contraction in previously dark-adapted retinas by approximately 80% (Fig. 6). In addition, sulpiride also induced cone elongation in isolated light-adapted retinas cultured in constant light (Fig. 7). Since prior work has shown that light enhances retinal dopamine synthesis, turnover, and release (Kramer, 1971; Iuvone et al., 1978; Cohen et al., 1983), it seems probable that light-induced dopamine release contributes to light-induced cone contraction. In addition, our results suggest that dopamine must be

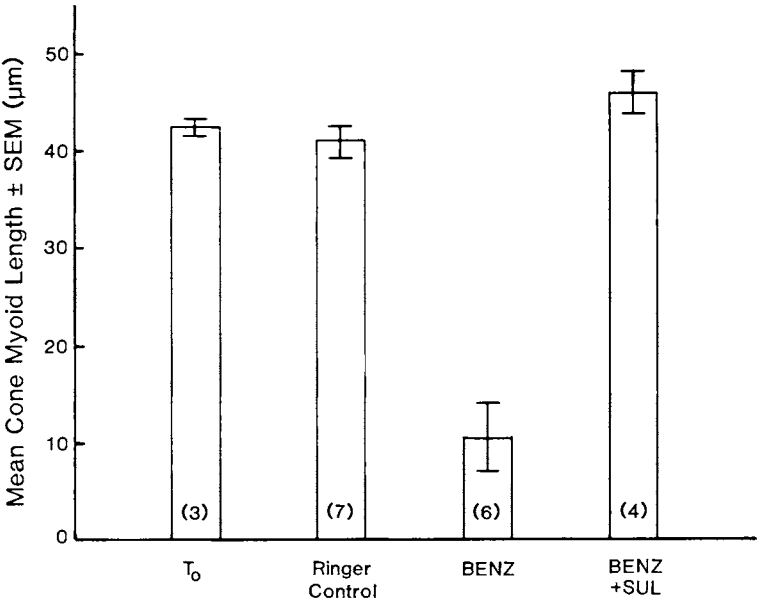


FIGURE 5. Blocking dopamine uptake with benztropine induces light-adaptive cone contraction in isolated dark-adapted retinas cultured in constant darkness. Retinas were removed from dark-adapted fish and fixed immediately after dissection (T_0) or after a 45 min culture in the dark in Ringer solution alone (control) or Ringer solution containing 10^{-5}M benztropine (BENZ) with or without 10^{-4}M sulpiride (SUL). Benztropine induced cone contraction without the addition of exogenous dopamine. The effect of benztropine was blocked by the D-2 dopamine antagonist sulpiride. (Reprinted with permission from Dearry and Burnside, 1986a, J. Neurochem., Raven Press.)

continually released in the light in order for cones to maintain their light-adapted positions.

To investigate whether the effect of dopamine on retinomotor movement was direct or mediated through other retinal cells, we prepared suspensions of isolated photoreceptor inner/outer segments from dark-adapted fish (Fig. 8). Rod and cone fragments were mechanically detached from isolated retinas and cultured in darkness with or without dopamine. We found that dopamine induced light-adaptive cone myoid contraction and rod myoid elongation (Dearry and Burnside, 1986a; Fig. 9). In addition, by sectioning plastic-embedded tissue, we observed that dopamine induced

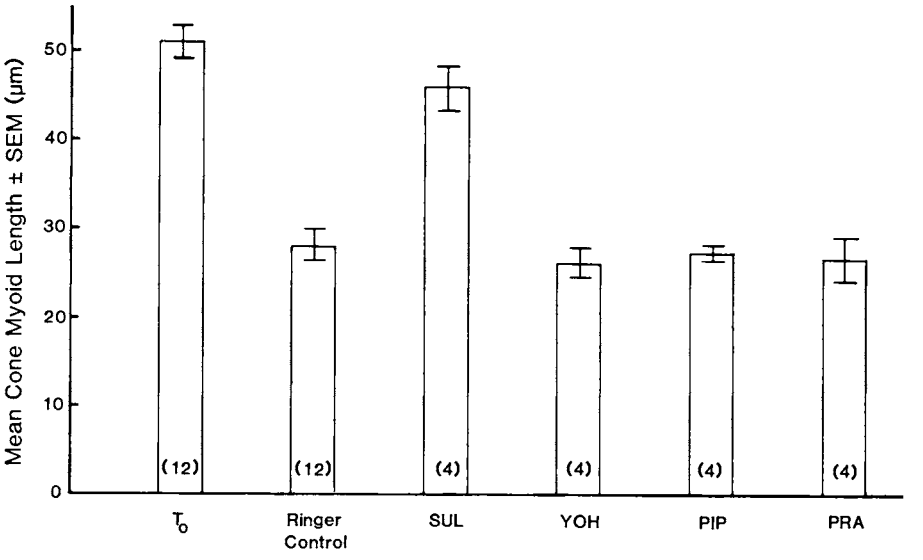


FIGURE 6. Sulpiride inhibits light-induced cone contraction. Retinas were removed from dark-adapted fish and fixed immediately after dissection (T_0) or after a 45 min culture in the light in Ringer solution alone (control) or in Ringer solution containing 10^{-4}M sulpiride (SUL); 10^{-4}M yohimbine (YOH); 10^{-4}M piperoxane (PIP); or 10^{-4}M prazosin (PRA). Sulpiride, a selective D-2 dopamine antagonist, inhibited the extent of light-induced cone contraction by 80%. Alpha-adrenergic antagonists were ineffective. (Reprinted with permission from Dearry and Burnside, 1986a, J. Neurochem., Raven Press.)

light-adaptive rod elongation in dark-cultured whole retinas and that the effect of dopamine was inhibited by sulpiride. Other work in this laboratory has demonstrated that dopamine and apomorphine induce light-adaptive pigment dispersion in enzymatically isolated RPE cells (Fig. 10) (Bruenner and Burnside, 1986). Thus our observations suggest that cones, rods, and RPE cells each possess D-2 receptors and that dopamine acts on the D-2 receptors to induce light-adaptive retinomotor movements in all three cell types.

C. Modulation of Dopamine Release

Several neural and hormonal agents have been reported to influence retinal dopaminergic activity. For example, GABA and melatonin have been found to inhibit dopamine

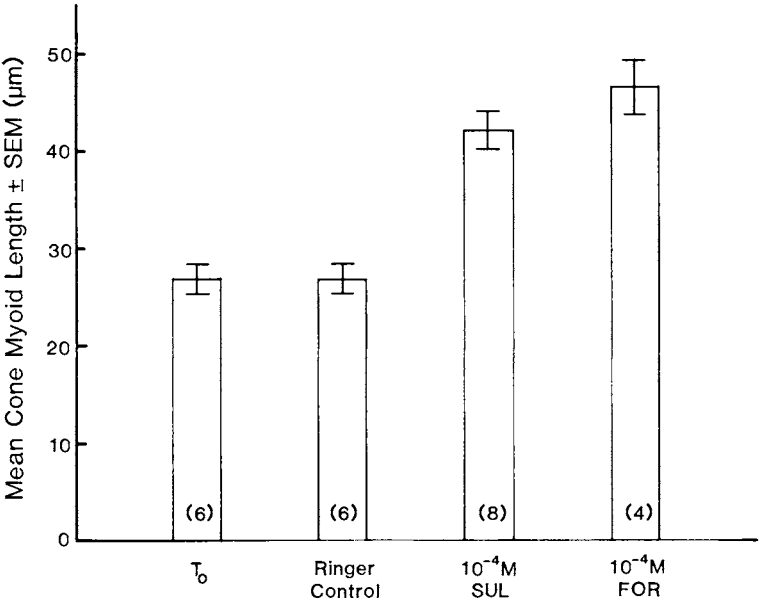


FIGURE 7. Sulpiride induces dark-adaptive cone elongation in isolated light-adapted retinas cultured in constant light. Retinas were removed from dark-adapted fish in order to obtain isolated retinas without RPE. All isolated retinas were cultured in the light for 45 min. T_0 retinas were then fixed, and the remaining retinas were cultured an additional 60 min in the light. Experimental solutions contained 10^{-4}M sulpiride (SUL) or 10^{-4}M forskolin (FOR). Both sulpiride, a D-2 dopamine antagonist, and forskolin, an adenylate cyclase activator, induced cone elongation. (Reprinted with permission from Dearry and Burnside, 1986a, J. Neurochem., Raven Press.)

release (Negishi et al., 1983; Dubocovich, 1983), whereas serotonin and quisqualate have been found to stimulate dopaminergic neurons (Kato et al., 1982; Kamp and Morgan, 1983). We therefore tested the effects of such agents on cone retinomotor movement in isolated green sunfish retinas.

Since GABA input reportedly inhibits dopaminergic neurons in dark-adapted vertebrate retinas (see review by Morgan, 1982), we first tested to see if GABA antagonists could induce cone contraction in dark-adapted retinas. If GABA antagonists block GABA-mediated inhibition of dopaminergic neurons, then the resultant increase in dopamine release from the disinhibited cells might be

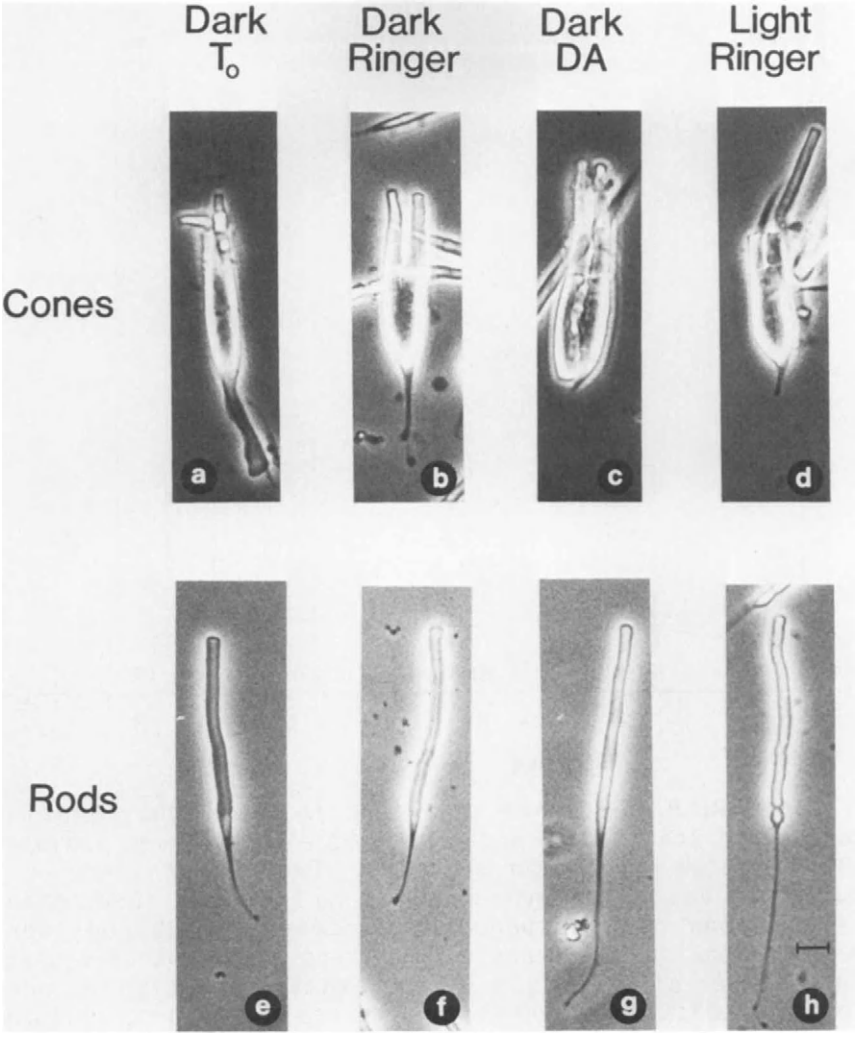


FIGURE 8. Dopamine and light induce light-adaptive retinomotor movements in isolated cones and rods. Retinas were removed from dark-adapted fish and photoreceptor inner-outer segments were isolated in constant darkness. Cells were fixed immediately after isolation (a and e); or after a 45 min culture in constant darkness in Ringer solution alone (b and f); darkness plus 10^{-6}M dopamine (c and g); or light (d and h). The bar in (h) corresponds to 10 μm. Both dopamine and light induced cone myoid contraction and rod myoid elongation. (Reprinted with permission from Dearry and Burnside, 1986a, J. Neurochem., Raven Press.)

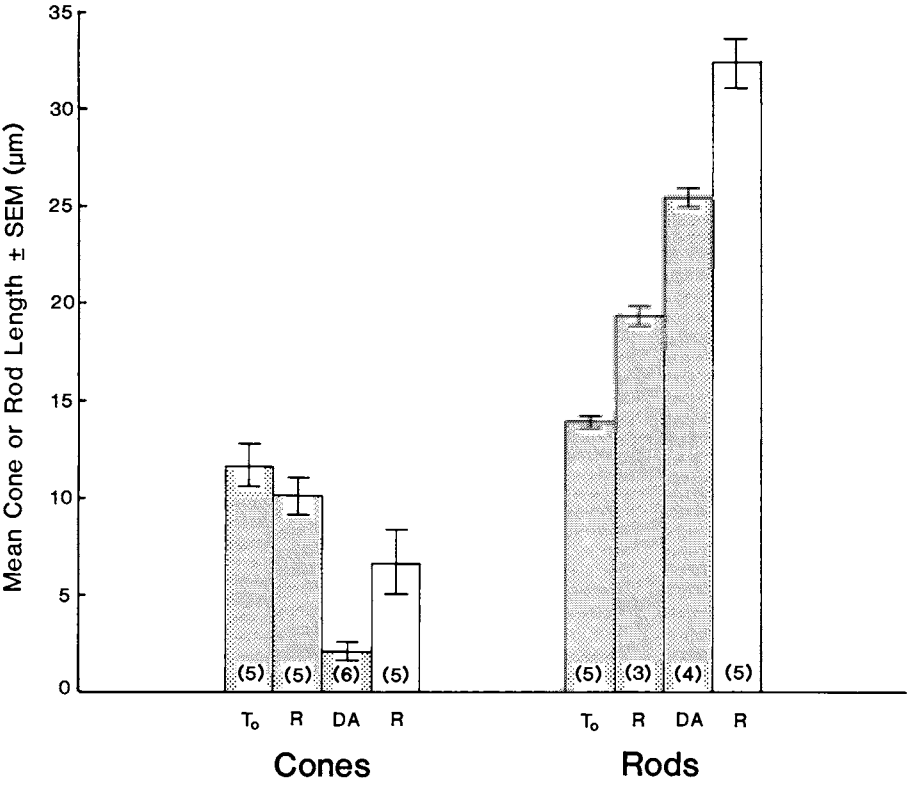


FIGURE 9. Dopamine and light induce light-adaptive cone myoid contraction and rod myoid elongation in isolated photoreceptor inner/outer segments. Experiments were conducted as described in the legend to Figure 8. One retina yielded one cell suspension; 20 cones and 20 rods were measured per cell suspension. Numbers in parentheses indicate number of cell suspensions examined. Stippled bars indicate cultures in constant darkness; clear bars, cultures in light. T_0 cells were fixed immediately after isolation; remaining cells were cultured in Ringer solution alone (R) in dark or light or in 10^{-6}M dopamine (DA) in darkness. (Reprinted with permission from Dearry and Burnside, 1986a, J. Neurochem., Raven Press.)

expected to induce cone contraction. We found that the GABA antagonists bicuculline and picrotoxin induced maximal cone contraction, and that their effects were blocked by the D-2 dopamine antagonist sulpiride (Dearry and Burnside, 1986b). This latter observation suggests that the influence of these

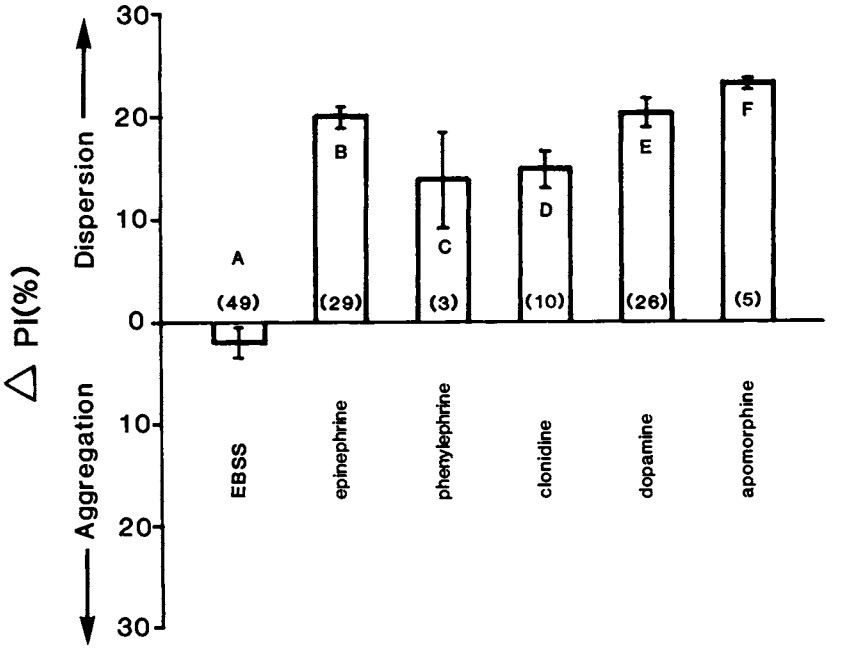


FIGURE 10. Catecholamines induce pigment dispersion in isolated RPE cells. RPE cells were enzymatically isolated from dark-adapted green sunfish and fixed immediately after isolation (T_0) or after a 45 min culture in constant darkness in Ringer solution alone (EBSS) or Ringer solution containing 10^{-6}M of the indicated catecholamine. Pigment index (PI) is the length of the cell occupied by pigment (%). The PI value for the T_0 cells was 74.7 $\pm$ 1.3% (n = 22) and is represented by the horizontal line. Numbers in parentheses indicate number of fish examined. (Reprinted with permission from Bruenner and Burnside, 1986, Invest. Ophthalmol. Vis. Sci., J. B. Lippincott.)

GABA antagonists on cone movement is mediated by dopamine acting on D-2 receptors.

Since GABA antagonists induced cone contraction in dark-adapted retinas, we next tested to see if muscimol, a GABA agonist, could inhibit light-induced cone contraction. We found that 10^{-4}M muscimol inhibited light-induced contraction by approximately 90% (Dearry and Burnside, 1986b). Sulpiride, a selective D-2 dopamine antagonist, also strongly inhibited light-induced cone contraction. These results are consistent with the idea that in sunfish retinas

GABA normally inhibits dopamine release. They are also consistent with previous suggestions that a decrease in GABA input to dopaminergic neurons may be an intermediate step in light-stimulated dopamine release (cf. Morgan, 1985).

Since serotonin reportedly increases dopamine release from carp retinas (Kato et al., 1982), we also tested the ability of serotonin to induce cone contraction in dark-adapted retinas. We found that 10^{-4}M serotonin induced about 50% of the full extent of *in vivo* light-adaptive movement (Dearry and Burnside, 1986b). The effect of serotonin was blocked by either mianserin, a serotonin antagonist, or by sulpiride, a D-2 dopamine antagonist. These results suggest that in green sunfish, as in carp retinas, serotonin enhances dopamine release through a receptor-mediated mechanism.

Several other putative modulators of retinal dopaminergic neurons were examined. The following had no effect on cone retinomotor position in dark-adapted retinas: 50 μM alpha-MSH, 100 uM carbachol, quisqualic acid, kainic acid, N-methyl-D-aspartate, and glycine (Dearry and Burnside, 1986b).

Our results thus suggest that GABA and serotonin influence the activity of dopaminergic neurons in green sunfish retinas. To further test whether GABA and serotonin effects were directly on cones or were possibly mediated through dopaminergic neurons, we used Co^{++} as a presynaptic blocking agent. If GABA antagonists and serotonin promote cone contraction in dark-adapted retinas by stimulating dopamine release, then their effects should be inhibited by Co^{++}. We found that 10^{-4}M bicuculline and 10^{-4}M serotonin failed to induce cone contraction in the presence of 5 mM Co^{++}; 10^{-6}M dopamine still produced contraction under these conditions (Dearry and Burnside, 1986b). These results suggest that GABA antagonists and serotonin act indirectly on cones by a mechanism requiring synaptic release in order to elicit cone contraction, whereas dopamine acts directly on cones to produce contraction without the need for synaptic transmission.

In summary, we have shown that dopamine, GABA antagonists, and serotonin induce light-adaptive cone contraction in isolated dark-adapted green sunfish retinas. The effect of dopamine is mediated through a direct interaction with D-2 receptors. The effects of GABA antagonists and serotonin appear to be mediated through a stimulation of dopamine release. Thus dopamine acts directly on cones as the final extracellular messenger, while GABA inhibits and serotonin stimulates dopamine release.

IV. CONCLUDING REMARKS

In this review we have presented a large number of observations describing the roles of cAMP, Ca^{++}, and dopamine in regulating retinomotor movement in the green sunfish Lepomis cyanellus. These observations provide a consistent pattern of regulation which is summarized briefly below for cones, rods, and RPE.

Cones: Our understanding of the regulation of cone length is illustrated in Fig. 11. In the dark (night) cytoplasmic cAMP levels are high and cones elongate by a microtubule-dependent mechanism. Light onset is associated with a fall in cAMP level and a (perhaps transient) rise in free Ca^{++} concentration. These light-induced changes in cAMP and Ca^{++} activate the cone's actin machinery and inhibit its microtubule machinery. Light can induce contraction of the cone myoid directly in isolated inner-outer segment fragments (CIS-COS) by some unidentified mechanism associated with Ca^{++} release from internal stores. Since CIS/COS lack a nucleus or synapse, the entire transduction cascade from light reception to actomyosin contraction must be mediated by structures and molecules present in the inner and outer segments. Dopamine induces cone contraction by D-2 receptor-mediated inhibition of adenylate cyclase and a resultant fall in cytoplasmic cAMP level. The effectiveness of dopamine on CIS-COS indicates that D-2 receptors and the regulated adenylate cyclase are found on the cone inner and/or outer segments (this, of course, does not rule out their presence on the rest of the cone).

Rods: In the dark, cytoplasmic cAMP levels are high and rods contract by an actin-dependent mechanism (presumably actin-myosin interaction). In the light, cytoplasmic cAMP levels are low and rods elongate by an actin-dependent mechanism (presumably actin filament assembly and bundling). Ca^{++} effects have not been fully characterized but preliminary results suggst that calcium rises at darkness onset to initiate actin-dependent contraction. Since light can induce rod myoid elongation in isolated inner-outer segment fragments (RIS-ROS), the necessary machinery for motility must be present in inner and outer segments. Dopamine induces rod elongation and its effect is mediated by D-2 receptors. Thus in rods as in cones, the D-2 receptors and adenylate cyclase must be located on the inner and/or outer segments.

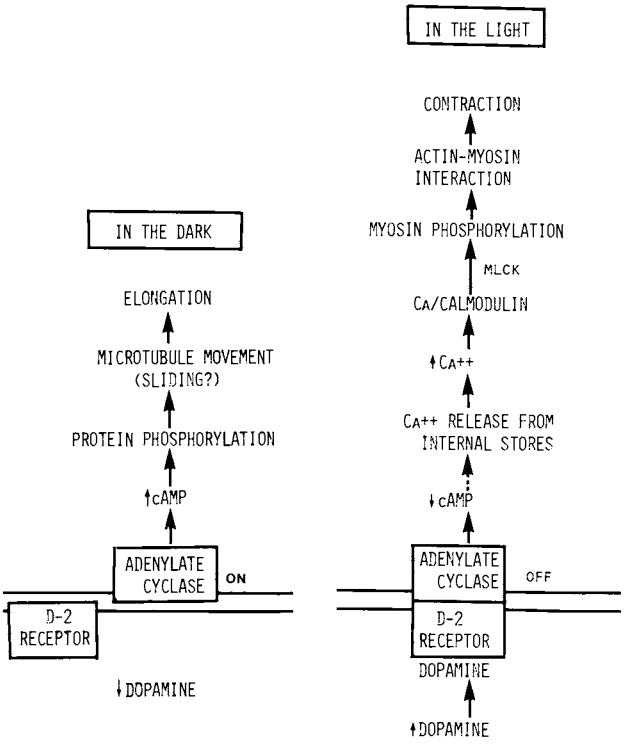


FIGURE 11. Scheme of extra- and intracellular regulation of cone retinomotor movement. In the dark or during subjective night, retinal dopamine release is low and adenylate cyclase activity is relatively high. Intracellular cAMP levels are thus relatively high, and cones elongate by a microtubule-dependent mechanism. In the light or during subjective day, retinal dopamine release is enhanced. Dopamine binds to D-2 receptors or cones, thereby inhibiting adenylate cyclase activity and decreasing intracellular cAMP content. Cytoplasmic Ca^{++} levels are elevated, and cones contract by actin-myosin interaction.

RPE: In the dark, cytoplasmic cAMP levels are high and pigment granules aggregate to the basal surface of the RPE cells by an unknown mechanism at least partially dependent on microtubules. In the light cytoplasmic cAMP levels are low and pigment granules disperse into the long apical projections by an actin-dependent mechanism. The role of Ca^{++} in RPE pigment migration has not yet been characterized. Light does not induce pigment dispersion in RPE

cells in the absence of the retina. Thus the light signal must be transferred from the retina to the RPE in order to induce pigment dispersion. Dopamine induces maximal pigment dispersion in isolated RPE cells. Since dopamine has an effect opposite to cAMP, its effect may be mediated by D-2 receptor inhibition of adenylate cyclase and a resultant fall in cAMP level. The effectiveness of dopamine on isolated RPE cells indicates that dopamine receptors are present on the RPE cell surface.

These observations raise several obvious questions:

1) What is the source of dopamine?

Experiments with the dopamine uptake blocker benztropine demonstrate that sunfish retinas make and release dopamine. In other fish retinas interplexiform cells have been identified as the primary candidates for dopaminergic neurons (Dowling and Ehinger, 1978). These cells have their perikarya in the inner plexiform layer and send processes into the outer plexiform layer where they have been shown to synapse with horizontal and bipolar cells. In rat retinas, tyrosine hydroxylase-containing interplexiform cells have recently been found to have processes extending beyond the outer limiting membrane into the photoreceptor layer (Negishi et al., 1985). Our results suggest that dopamine released from interplexiform cells diffuses past the outer limiting membrane to reach both photoreceptors and RPE cells. Preliminary studies in our laboratory indicate that the bathing medium of light-cultured retinas induces light-adaptive RPE pigment dispersion when added to isolated RPE in the dark; this effect is blocked by the D-2 dopamine antagonist sulpiride. Thus dopamine is a likely candidate for a signal from retina to RPE that communicates light onset.

2) What role does dopamine normally play in light and circadian regulation of retinomotor movements?

Although light can initiate cone contraction, dopamine may be required to obtain the full extent of light-induced contraction and to maintain contraction in constant light. Light induces partial contraction in isolated CIS/COS in the absence of dopamine. However, dark-adaptive movements are induced by the D-2 dopamine antagonist sulpiride in light-adpated retinas cultured in constant light. Thus, light onset may produce transient changes in Ca^{++} and cAMP levels

in the myoid (thereby initiating contraction), but dopamine may be required to inhibit adenylate cyclase and keep cAMP levels low enough to maintain the contracted state.

Green sunfish have a circadian rhythm in cone retinomotor movement and in retinal sensitivity: Cones contract and retinal sensitivity is low during subjective day. Rats have a circadian rhythm in retinal dopamine content (Wirz-Justice et al., 1984): Dopamine levels are elevated during subjective day. Thus, dopamine may serve as a light-adaptive signal for the circadian modulation of retinomotor movement and retinal sensitivity. Since dopamine acts as a "lights-on" messenger from green sunfish retina to RPE, it is also possible that dopamine has a similar role in stimulating the circadian burst of shedding observed during subjective day in rats kept in constant darkness (LaVail, 1980). Dopamine may also have a role in generating the RPE light peak potential in cats (Dawis and Niemeyer, 1984). In addition, dopamine has been reported to decrease the receptive field of horizontal cells (Piccolino et al., 1984) and the surround responses of bipolar and ganglion cells (Hedden and Dowling, 1978; Thier and Alder, 1984). Thus, dopamine may have a role in regulating the general adaptational state of retina and RPE. Dopamine may act as a general light-adaptive retinal transmitter of both circadian and light signals.

3) How do light and a circadian clock modulate dopamine release?

Our findings suggest that dopamine release in green sunfish retinas is inhibited by GABA and enhanced by serotonin. In fish retinas, both of these transmitters are localized in amacrine cells (Lam et al., 1979; Osborne et al., 1982) and hence may influence the activity of dopaminergic interplexiform cells. Light or a circadian "day" signal may decrease GABA and/or increase serotonin release, which in turn increases dopamine release. In addition, another indoleamine, melatonin, may have a role in regulating dopamine release. Melatonin has been found to suppress electrically evoked dopamine release in rabbit retinas (Dubocovich, 1983). Further, the activity of N-acetyltransferase, an enzyme controlling melatonin synthesis, has a circadian rhythm in Xenopus retina; its activity is high during subjective night (Besharse and Iuvone, 1983). Thus, an increase in melatonin release during subjective night may inhibit dopamine release. However, several workers have reported that retinal melatonin levels in

trout, ground squirrels, and rats do not have a diurnal rhythm (Gern et al., 1978; Reiter et al., 1981, 1983), and we have provided evidence that melatonin does not inhibit dopamine release in either dark- or light-adapted green sunfish retinas (Dearry and Burnside, 1986b). These discrepancies may be species differences, but clearly more work is needed to resolve such issues.

Finally, recent evidence suggests that teleost fish retinas receive efferent input from the nervus terminalis and that these efferent fibers synapse with amacrine cells (Munz et al., 1982; Springer, 1983; Stell et al., 1984). Although light and dark can induce retinomotor movements in isolated retinas, efferent input may well have a role in circadian regulation. Efferent signals may result in an inhibition of dopamine release during subjective night or a stimulation of dopamine release during subjective day. In Limulus photoreceptors, efferent input is high during the night and promotes dark-adaptive anatomical and physiological changes (Barlow et al., 1984). We have noted that isolated retinas removed from dark-adapted green sunfish undergo light-adaptive retinomotor movements independently of light when cultured in constant darkness in a normal teleost Ringer solution (Dearry and Burnside, 1984a). We have suggested that this movement may be due to dopamine release since it is inhibited by either low $[Ca^{++}]_o$ or by sulpiride (Dearry and Burnside, 1986a). These results suggest that green sunfish retinas, like Limulus photoreceptors, may receive efferent input at night. This input may inhibit dopamine release and thereby promote dark-adaptive retinomotor movements.

In conclusion, we have shown that the intracellular second messengers cAMP and Ca^{++} regulate cone, rod, and RPE retinomotor movements through direct effects on each cell's motile machinery. Dark-adaptive movements in each cell type are associated with an increase in cAMP content. Dopamine acts as an extracellular messenger to induce light-adaptive retinomotor movement in each cell type. Light or a circadian "day" signal increases retinal dopamine release. Dopamine binds to D-2 receptors on cones, rods, and RPE cells, thereby inhibiting adenylate cyclase activity and decreasing intracellular cAMP concentration. It seems likely that dopamine acts as a "light" messenger to modulate a variety of light-induced or subjective day retinal processes.

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CIRCADIAN PHOTORECEPTOR OUTER SEGMENT DISC SHEDDING IN THE RAT[1]

Matthew M. LaVail
Bonnie A. P. Lord
Douglas Yasumura

Departments of Anatomy and Ophthalmology
University of California San Francisco
San Francisco, California

I. HISTORICAL PERSPECTIVE AND INTRODUCTION

In the mid-to late-1960's, Richard Young discovered the remarkable renewal mechanism whereby rod outer segment (ROS) disc membranes are continuously synthesized at the base of the outer segment, thereby displacing previously synthesized discs away from the photoreceptor cell body (Young, 1967; Young and Droz, 1968). Soon thereafter, Young and Bok (1969) defined a new role of the retinal pigment epithelium (RPE), i.e. to phagocytize and degrade membranes that are shed (or removed by the RPE) in packets from the apical tip of the ROS. In the late 1960's and early 1970's it was discovered that in the RCS rat with inherited retinal dystrophy, the RPE failed to carry out its phagocytic function, a failure that resulted in an accumulation of shed ROS membranes (Herron *et al.*, 1969; Bok and Hall, 1971; LaVail *et al.*, 1972). This underscored the importance of the balance between synthesis and disposal of ROS membranes and hinted that perturbations of ROS synthesis or disposal might underlie some forms of nherited and environmentally induced retinal degenerations. With further research on various vertebrates, the decade following the discovery of ROS renewal saw a substantial gain in our knowledge of ROS synthesis and turnover (see reviews in Young, 1976; Bok and Young, 1979).

Despite the progress, however, several major questions remained: 1) How are ROS synthesis and disposal coupled to maintain a uniform ROS length? 2) What

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controls the ROS disc shedding mechanism? 3) What is the precise role of the RPE in ROS disc shedding? Do the RPE cells actively remove packets of discs or simply phagocytize discs that are already shed by the photoreceptor? 4) What are the kinetics of disc shedding and disposal of ROS membranes? The problem in resolving these issues was the assumption that disc shedding was a continuous, random event. There was no way to determine the sequence or timing of events, and no way to synchronize or experimentally perturb the system. For this reason, the observation that ROS disc shedding is rhythmic (LaVail, 1976a, b) met with considerable enthusiasm.

The impetus to explore the rhythmic nature of ROS disc shedding came from some preliminary electron microscope observations on the RPE of experimental rat chimeras (Mullen and LaVail, 1976). The finding of some presumptive phagosomes of ROS membranes in RPE cells derived from mutant RCS rats led us to suspect that the adjacent, wild-type RPE cells were partially alleviating the phagocytosis defect in the mutant cells. We asked how the number of phagosomes in these RPE cells compared with the number in normal RPE cells. When we examined normal rat retinas, we were surprised to find a paucity of phagosomes in the RPE, especially since it was known that the RPE had to degrade a large volume of ROS membrane (Young, 1971), and Dean Bok had shared unpublished calculations with us which showed that each RPE cell in the rat had to degrade 25,000-30,000 ROS discs each day (Bok and Young, 1979). It seemed possible that we and others had been taking tissues at inappropriate times of the day, so we removed eyes at different points of the light-dark cycle, fully expecting that if any differences existed, more disc shedding would occur at night since no previous studies had been done at that time. It was then that we found the now familiar burst of ROS disc shedding soon after the onset of light (LaVail, 1976a, b).

In this chapter, we will review what has been learned about ROS disc shedding in the rat by considering the directions research efforts have taken since 1976. We will primarily restrict our comments to studies on the rat to reduce overlap with other chapters in this volume on rhythmic disc shedding in amphibians. A thorough review of these studies is presented by Besharse (1982).

II. DIRECTIONS OF DISC SHEDDING RESEARCH

A. Characteristics of the Burst of Disc Shedding

From the original experiments (LaVail, 1976a, b) it became apparent that ROS undergo a burst of disc shedding beginning about 30 minutes to about 2.5 hours after the onset of lighting (Figs. 1 and 3). Thereafter, the number of large phagosomes present in the RPE drops to a low basal level throughout the rest of the day and night (Figs. 2 and 3). From the phagosome size during the burst of shedding, it became apparent that some ROS shed their daily complement of discs (usually about 10% of the ROS length; Young, 1967; Bok and Hall, 1971; LaVail *et al.*, 1972) in a single packet, but most ROS must shed more than one packet of discs each day (LaVail, 1976b). From the slope of the post-burst curve shown in

Fig. 3, it was also apparent that the lifetime of large phagosomes during the peak disc shedding period is short, perhaps only about 2 hours. It should be noted that these and all other disc shedding studies on the rat have used albino animals, and the burst of disc shedding usually results in phagosome counts 2.5-5 times the basal level. When pigmented rats are examined, they display a burst of disc shedding that is sometimes less than 2 times the basal level, and the basal level later in the day and during the night is somewhat higher than in albino animals (LaVail, 1981).

The burst of disc shedding in the rat appears to be independent of the intensity of light, since Tamai and Chader (1979) found virtually the same number of phagosomes during the burst of shedding when animals were maintained in either low (<0.5 ft-c) or moderate (45-50 ft-c) illuminance levels. In addition, these workers demonstrated that in the developing rat, the burst of disc shedding first appears at postnatal day 15, at about the time of eye opening and before the ROS are fully mature.

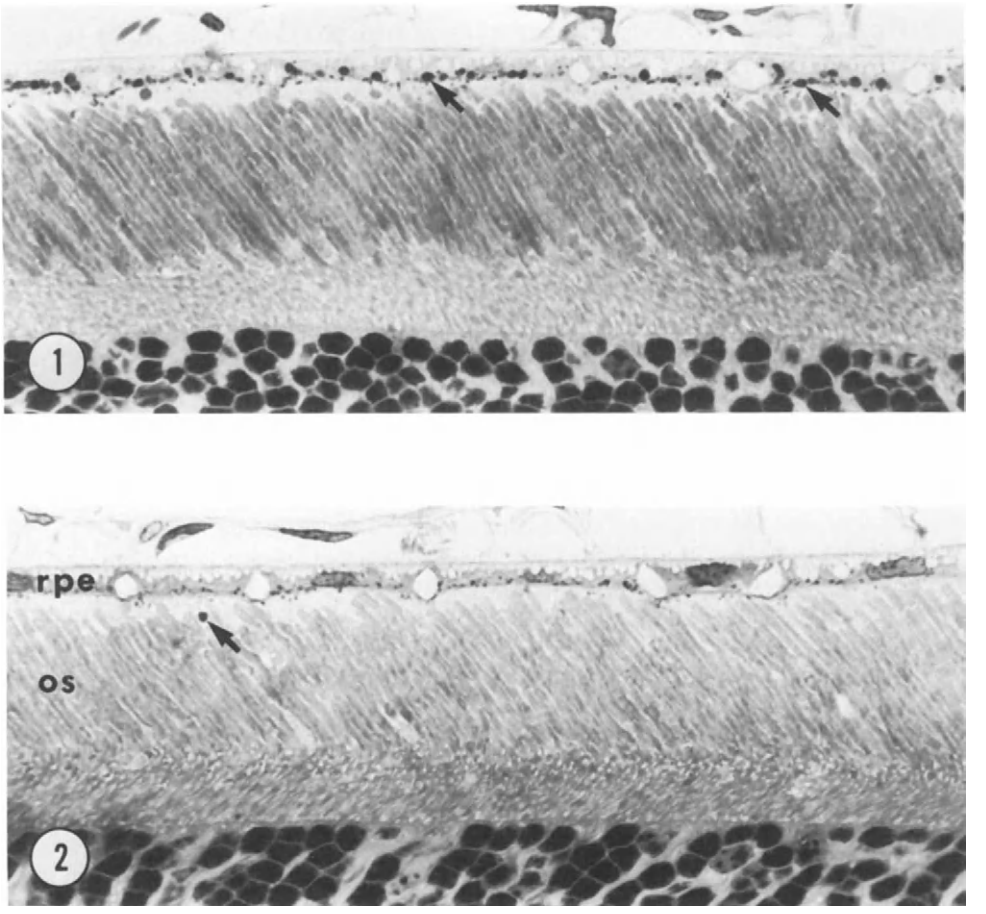
The rhythmic nature of ROS disc shedding has subsequently been examined in a variety of species of all vertebrate classes. In virtually all cases, ROS show a burst of disc shedding soon after the onset of light (reviewed by Besharse, 1982; Herman and Steinberg, 1982). Thus, this phenomenon appears to be a universal characteristic of rod photoreceptor cells. In some instances, however, small bursts also occur at different times of the day or night.

In some species, cone photoreceptors also show a major burst of disc shedding, but soon after light *offset*, 12 hours out of phase with rods (Western fence lizard: Young, 1977; goldfish: O'Day and Young, 1978; chick: Young, 1978). Additional studies on other species with duplex retinas, particularly mammals, suggest the timing of cone shedding may be variable from species to species. However, the basic conclusion is that cones do at least exhibit rhythmic disc shedding behavior (Anderson *et al.*, 1980; Tabor *et al.*, 1980; Fisher *et al.*, 1982; Besharse, 1982).

B. Disc Shedding as a Circadian Event

1. Discovery of a circadian rhythm in ROS disc shedding

After it was recognized that a rhythmic burst of disc shedding occurs in the morning following light onset, an obvious question was whether this phenomenon was controlled by a direct effect of light on the photoreceptors or by a systemic or humoral factor. For several months we attempted in several ways to occlude one eye of rats before the onset of light, including monocular eye suture alone or in combination with black felt-tip markers or black eye patch. In no case was the burst of shedding in the occluded eye reduced in magnitude from that in the open eye. This was true even if the animal was anesthetized and the half of the head with the occluded eye was buried under the cage bedding. Just when we were about to conclude that a humoral factor must be regulating the disc shedding in both eyes via a light-mediated mechanism, we carried out a "final control" of not turning on the lights in the morning, fully expecting to see no burst of disc shedding in either eye. To our surprise, both eyes still showed a large burst of



Figures 1 and 2. Light micrographs of retinas from normal albino rats that were sacrificed at different times of the day. Soon (1.5 hours) after the onset of light in the morning (Fig. 1), many large, intensely stained phagosomes are present in the RPE cell somas (arrows) and among the RPE cell processes and apical tips of the outer segments. Later (9 hours after light onset) in the day (Fig. 2), very few large phagosomes are present (arrows). os, outer segments; rpe, retinal pigment epithelium. (x850). (Fig. 2 from LaVail, 1976a; Copyright Academic Press, Inc.)

disc shedding at the usual time even in the absence of light onset (LaVail, 1976b). This induced us and others to become familiar with the features of circadian rhythmicity and to explore the possibility that ROS disc shedding was displaying a circadian rhythm.

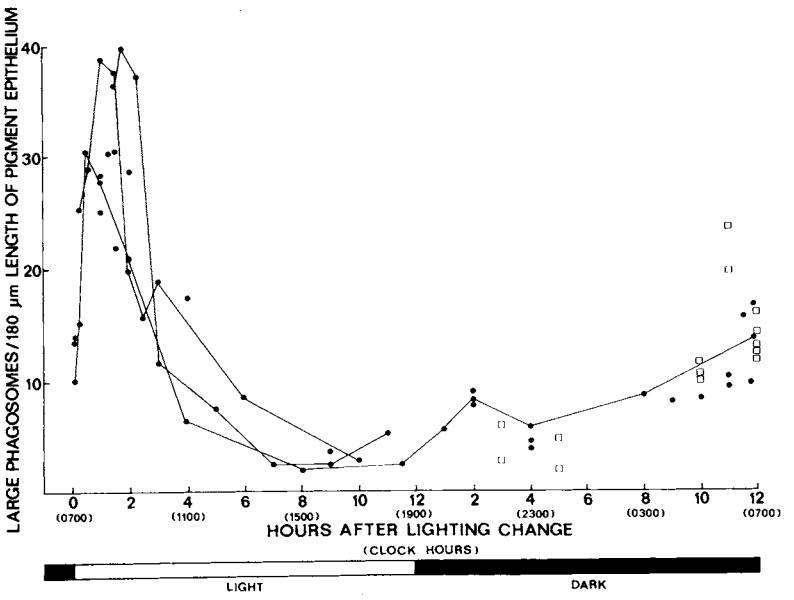


Figure 3. Counts of large phagosomes at different times of the lighting cycle. Each point represents the mean number of phagosomes found in ten 180-μm lengths of RPE in the eye of a single animal. The 3 curves represent separate experiments. The open squares represent rats that were taken from the dark and placed in the light 1 hour before sacrifice. (From LaVail, 1976b; Copyright American Association for the Advancement of Science).

2. Circadian characteristics of the disc shedding rhythm

One feature of circadian rhythms is that they are a self-sustaining oscillation that continue in the absence of lighting cues. We initially showed (LaVail, 1976b) that ROS disc shedding continued at the presumptive time of light onset for 3 days in continuous darkness (Fig. 4), and later we (LaVail, 1980) and Goldman *et al.* (1980) found that the rhythm persisted in darkness for at least 12 or 14 days, respectively.

Another characteristic of circadian rhythms is that they free-run in darkness, i.e., their period changes slightly from the 24-hour cycle that they maintain in a cyclic light environment. In our animals kept in continuous darkness for 12 days, the disc shedding rhythm displayed a free-running pattern, since it became slightly longer than 24 hours, with an estimated period of 24 hours 8 minutes to 24 hours 19 minutes (LaVail, 1980).

Many circadian rhythms are abolished by maintaining animals in constant illumination. Goldman *et al.* (1980) found that when albino rats are placed in

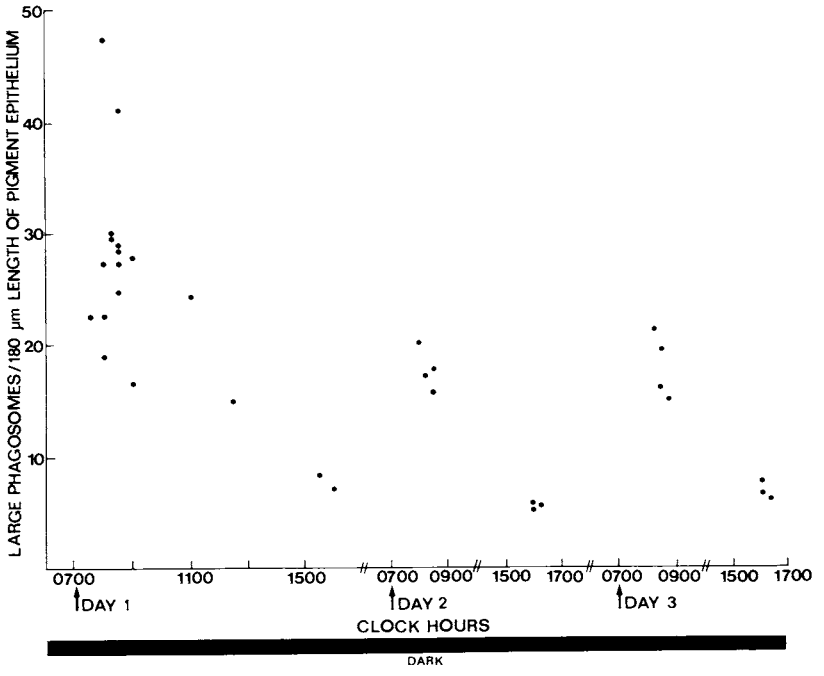


Figure 4. Counts of large phagosomes, as in Fig. 3, but from rats maintained in continuous darkness for 3 days. The arrows indicate when the lights would have come on if the rats had been kept in 12:12 cyclic lighting, as in Fig. 3. (From LaVail, 1976b; Copyright American Association for the Advancement of Science).

constant light for more than 24 hours, the shedding peak is abolished. Likewise, when rats are reared in constant light, a burst of disc shedding is not seen (Tamai and Yagi, 1980).

An additional feature of circadian rhythms is that they re-entrain to a shift in the time or duration of the so-called zeitgeber (time-giver), light being the most dominant zeitgeber for mammalian rhythms. The ROS disc shedding rhythm also shows the property of re-entrainment to light. When we shipped rats to our laboratory from the Zivic-Miller Laboratories in Allison Park, PA, we effected a 3-hour phase delay in the light cycle. Remarkably, at least 4 weeks, and perhaps longer, was required for complete re-entrainment to the phase shift (LaVail, 1980). This has obvious ramifications for vision scientists who use experimental animals soon after shipment across time zones. Moreover, the long re-entrainment stands in contrast to most circadian rhythms, where 8-10 days is considered a relatively long time (Bünning, 1973). Goldman *et al.* (1980) also found that a prolonged re-entrainment period (>3 weeks) was required for the ROS disc shedding rhythm after a 5-hour phase delay. However, with a 5-hour phase *advance*, these authors noted a faster re-entrainment time, and following a

10-hour phase advance, only 5 days was necessary for complete re-entrainment (Goldman *et al.*, 1980). Thus, re-entrainment of the disc shedding rhythm occurs more slowly to phase delays than to phase advances, and more rapidly to large advances than smaller ones.

The studies described in this section clearly demonstrate that the ROS disc shedding rhythm in the rat meets all the major criteria of a circadian rhythm. It 1) is a self-sustaining oscillation, 2) free-runs in darkness, 3) is abolished by constant light and 4) can re-entrain to a phase-shift of the zeitgeber.

3. Light-triggering vs. circadian regulation

We wondered how strongly the disc shedding rhythm was regulated by circadian factors versus how strongly the rhythm was associated with light onset. We were particularly concerned with this issue, because very soon after our discovery of rhythmic disc shedding and the first hints of its circadian nature, Scott Basinger found in frogs (*Rana pipiens*) that the ROS disc shedding could be triggered by light onset at different times of the day rather than being regulated by circadian factors (Basinger *et al.*, 1976). A related question was how much dark time, if any, was required for another burst of shedding to occur.

We explored these issues by taking rats from the dark at different times of the night and exposing them to light for 1 hour, a period of light exposure that produced maximal disc shedding in the morning of the regular 12:12 light cycle. Remarkably, little or no burst of shedding occurred until light onset in the morning was approached (Fig.3). Our conclusion from these experiments was that very close to a full 12 hours of dark adaptation may be required before light can induce the burst of disc shedding (LaVail, 1976b). This proved to be an incorrect interpretation, because when Goldman *et al.* (1980) did the experiment by sacrificing all animals at the peak shedding time after variable periods of dark adaptation followed by 1.25 hours of light, they found that only 2 hours of darkness was required to elicit a burst of disc shedding.

Following these studies, Goldman *et al.* (1980) tested whether the burst of disc shedding could be light-triggered in the rat by subjecting previously entrained animals to various periods of constant light, followed by 2 hours of darkness then 1.5 hours of light. They found evidence for light-triggered shedding, but could not induce a complete shedding burst prior to the time of the circadian peak in the morning. This and the other studies described above show that ROS disc shedding in the rat is much more strongly regulated by circadian factors than by light-triggering.

C. Search for Circadian Control Mechanism(s) of Disc Shedding

1. Involvement of the pineal gland

When we began to suspect circadian factors in the regulation of ROS disc shedding, Alan Laties suggested we try to disrupt the rhythm using reserpine systemically, since this drug abolishes pineal-mediated circadian rhythms by

depleting the neurotransmitter from afferent nerve terminals to the pineal gland that arise from the superior cervical ganglion. When we did this experiment, reserpine did, in fact, block the burst of disc shedding the morning following its injection (LaVail, 1976b). This meant that the disc shedding mechanism could at least be influenced, if not controlled, by humoral factors. Unfortunately, the reserpine experiment also erroneously suggested involvement of the pineal gland, which led our laboratory and those of Paul O'Brien and Gerald Chader to many months of experiments that gave only negative findings. When the pineal gland was surgically removed or was deafferented by bilateral superior cervical ganglionectomy, the burst of ROS disc shedding was unaffected (Tamai *et al.*, 1978; LaVail and Ward, 1978). In addition, when other possible sources of hormonal influence such as the pituitary, thyroid and parathyroid glands were removed, the disc shedding burst was also unaffected (LaVail and Ward, 1978).

2. Local vs. central control

To approach the question of intraocular (local) versus humoral (central) control of the disc shedding rhythm, Teirstein *et al.* (1980) carried out a series of experiments using monocular occlusion and surgical procedures combined with different light regimens. When monocularly occluded rats were exposed to constant light, the burst of disc shedding was suppressed in the open eye that was exposed to light, whereas the burst continued in the occluded eye. In addition, when monocularly occluded rats were subjected to a 5-hour phase advance in the light cycle, the open eye re-entrained to the phase shift, whereas the occluded eye retained the original rhythm. Since each eye was shown to exhibit its own separate circadian rhythm, these studies virtually ruled out a humoral factor in the direct regulation of disc shedding and suggested the presence of a circadian oscillator within each eye or orbit.

Teirstein *et al.* (1980) also found that sectioning the optic nerve did not alter either the normal rhythmic burst of disc shedding or the results of the monocular occlusion with constant light suppression of the open eye. This argues further for local control within the orbit, and perhaps within the retina. However, Teirstein *et al.* (1980) found that optic nerve section did prevent re-entrainment of the disc shedding rhythm to a phase shift in the lighting cycle. This suggests a possible role for central control for some aspects of the circadian disc shedding rhythm, perhaps through efferent nerve fibers in the optic nerve (Tierstein *et al.*, 1980).

Some further evidence in support of different sets of control mechanisms for different aspects of the disc shedding circadian rhythm is available. By using different light intensities, Goldman (1982) found that the inhibition of disc shedding by constant light required substantially higher levels of illumination than did phase shifting.

It should be noted that the precise cellular localization and neural or chemical mediator(s) for both the local and central disc shedding control mechanisms remain to be identified. For local control, evidence for an intraretinal source is accumulating in amphibians (Besharse and Dunis, 1983; see other chapters in this volume). In the rat, Remé *et al.* (1984) have found that although the monoamine oxidase inhibitor, clorgyline, reduces the magnitude of the ROS disc shedding burst, it does not phase delay the rhythm as it does some other behavioral and

neurochemical circadian rhythms. This substance presumably acts on the suprachiasmatic nucleus of the hypothalamus, the putative central pacemaker for most mammalian circadian rhythms. Remé *et al.* (1984) argue that these data support the notion of local intraocular control of retinal rhythms. Some evidence for intraretinal regulation of another rhythmic event exists in the rat, i.e., phosphatidylinositol synthesis (Dudley *et al.*, 1984), and it is possible that the local control mechanism for this rhythm is related to that of ROS disc shedding.

D. Disc Shedding in the RCS Rat

Following the initial reports of rhythmic ROS disc shedding in the rat, Goldman and O'Brien (1978) reasoned that just as retinal researchers previously had not taken eye tissues at the opportune time to see substantial disc shedding in normal rats, perhaps the apparent absence of phagocytosis in RCS rats with inherited retinal dystrophy (Herron *et al.*, 1969; Bok and Hall, 1971; LaVail *et al.*, 1972) could be explained on the same basis. When they examined the RPE of RCS rats taken at different times of the day, Goldman and O'Brien (1978) found, indeed, that a small burst of disc shedding and phagocytosis occurred with a peak at 1 hour after light onset, but the number of phagosomes at all times was only about 5% of that found in normal animals. Tamai and O'Brien (1979), also found that the maximal number of phagosomes in RCS rats was reached at P15, whereas the adult level in normal rats was not reached until P35. These studies provide an example of how the rhythmic nature of disc shedding can be used as a tool to probe photoreceptor-RPE interactions. In this case, such studies altered our concept of the RPE defect in an important experimental animal model of human disease. The irony here is that a question posed by this mutant led to the discovery of the circadian nature of ROS disc shedding in the first place (see Introduction).

E. Other Rhythmic Events in Photoreceptors

When it became evident that ROS disc shedding was rhythmic event, a number of investigators began to search for other rhythmic events in vertebrate photoreceptor cells. Several of these studies in the rat are mentioned here briefly to give a flavor of the rhythmic events found in photoreceptor cells.

1. Autophagy in photoreceptor cells

The incidence of autophagic vacuoles in rod inner segments was examined in rats at different times of the light cycle by Remé and Sulser (1977), who found that autophagic activity followed a diurnal variation with a peak about 3 hours after the disc shedding peak. In a later study, Remé *et al.* (1983) showed that the autophagic degradation in rod inner segments of rats follows a circadian rhythm, but unlike the burst of disc shedding the autophagy peak 1) is not abolished by

constant light, 2) is eliminated by constant darkness and 3) can be induced by light in either the light or dark phase of the light cycle. Thus, the autophagy rhythm is substantially more dependent on light stimulation than is the disc shedding rhythm (Remé *et al.*, 1983). Although the disc shedding and autophagy rhythms are temporally linked, a direct relationship of the two processes has yet to be established.

2. Photoreceptor metabolism

Besharse *et al.* (1977) demonstrated in *Xenopus laevis* an accelerated membrane synthesis at the base of the ROS after light onset in the morning, as well as a burst of disc shedding at the tip of the ROS. This led to a number of subsequent studies on possible rhythmic features of photoreceptor metabolism, primarily in amphibians. In one report on the rat, Chan *et al.* (1984) used autoradiographic methods to study leucine uptake *in vitro* at different times of the day. These authors purported to show two peaks of uptake in photoreceptor cells, one in the morning and one at night, but the data seem less than compelling.

Dudley *et al.* (1984) have sought biochemical correlates of circadian ROS disc shedding in the rat, particularly in opsin and phospholipids, the major components of ROS. They found no diurnal variation in the synthesis of opsin, glycoproteins or proteins in general. However, they did find a diurnal variation in rates of phospholipid synthesis. Phosphatidylcholine, phosphatidyl-ethanolamine and phosphatidylinositol all showed high synthetic values in the light and low values in the dark. Moreover, when rats were maintained in continuous darkness for 3 days, all 3 phospholipids still showed high synthetic values in the presumptive light phase and low values in the presumptive dark phase, thereby suggesting that phospholipid synthesis in photoreceptors follows a circadian rhythm.

3. Synaptic ribbons

Soon after our initial findings of circadian disc shedding, we became aware of the findings of Wagner (1975) that in the fish retina, the synaptic ribbons of cone pedicles undergo a circadian fluctuation in number. Since Vollrath (1973) had previously demonstrated light-induced circadian changes in the number of synaptic ribbons in pinealocytes of Guinea pigs, we wondered whether or not this phenomenon occurred in rod photoreceptors of the rat, cells that were displaying a circadian rhythm in disc shedding at the opposite end of the cell. We prepared a large number of electron microscope photomicrographs of the outer plexiform layer of rats that had been sacrificed at different times of the day and night. When we quantified the synaptic ribbons, we found no significant difference in number per unit length of retina at different times of the light cycle. During the course of this work, however, Spadaro *et al.* (1978) published a report based on about twice the area of tissue that we had examined, and they found a major diurnal fluctuation in the incidence of synaptic ribbons. We are puzzled at the unexplained difference

in our findings and theirs. It should be noted that Spadaro *et al.* (1977) also have reported diurnal structural changes in mitochondria of photoreceptor synaptic terminals in the albino rat.

4. Interphotoreceptor matrix

The interphotoreceptor matrix (IPM) that surrounds the photoreceptor cells is composed of a mixture of glycoproteins and proteoglycans that can be demonstrated histochemically. The IPM has shown to be abnormal in concentration or distribution in several forms of inherited retinal degeneration (LaVail *et al.*, 1981, 1985). Since it now appears that the photoreceptors synthesize at least some components of the IPM (Gonzalez-Fernandez *et al.*, 1984; Hollyfield *et al.*, 1985), we examined rat retinas histochemically that had been taken at different times of the day or night. We could find no consistent differences in concentration at different times of the day. However, our histochemical methods are far too crude to detect small concentration changes, and our results clearly are inconclusive. We present them here only to illustrate how we have shifted our thinking in the past decade. In the mid-1970's, we were surprised to find a rhythmic event in photoreceptors and the adjacent RPE. In the mid-1980's, we are now surprised when we *fail* to find rhymicity in dynamic events in the retina!

III. CONCLUSION

As noted above, when the rhythmic nature of ROS disc shedding was discovered it was hoped that the phenomenon could be used as a tool to resolve a number of questions about the control mechanisms of disc shedding and phagocytosis. In this concluding section, we will consider to what extent studies on the rat have provided new information in this area.

Hall (1978) used the burst of disc shedding to great advantage when he isolated ROS from rats either just before or after the onset of light and fed the ROS to cultured RPE cells. The ROS obtained soon after the onset of light were phagocytized far more avidly than those obtained before the onset of light. This strongly suggested that some chemical change in the ROS occurred that "primed" or "activated" them and allowed them to be recognized and phagocytized by the RPE cells. The fact that this change is under circadian control was underscored by Hall's (1980) additional finding that ROS taken from rats kept in continuous darkness were also avidly phagocytized when they were isolated soon after the presumptive onset of light. The finding by Goldman *et al.* (1980) of a requisite 2-hour "dark factor" before a burst of disc shedding can occur is also consistent with a biochemical change occurring at or near the time of light onset.

The nature of the presumptive biochemical change remains a mystery. O'Brien (1976) found incorporation of fucose and galactose into rhodopsin *in vitro* and speculated that these sugars might be added to the ROS plasma membrane to serve as a marker. This is consistent with the generally accepted view that the oligosaccharide side chains of surface glycoproteins are important in

cell-cell recognition. Bernstein (1979) has described a change in malachite green staining of ROS of rats as early as 10-30 minutes after the onset of light, suggestive of a change in ROS phospholipids at the time of disc shedding. Clearly, the elucidation of the specific biochemical change that mediates outer segment disc shedding is an outstanding problem in photoreceptor biology.

Despite these and other studies described above, we have yet to resolve any of the major questions posed at the outset in studies on the rat, except for learning about the kinetics of disc shedding and phagocytosis. The reason for this may lie in the limitations imposed by the rat as an experimental model. The rat has very small photoreceptor outer segments, so rods and cones are difficult to distinguish from one another as in the mouse (Carter-Dawson and LaVail, 1979). Autoradiographic studies of ROS renewal in rodents are difficult to carry out, because a far less discreet band of labeling is formed than in amphibians (Besharse and Hollyfield, 1979). Since rodents are warm-blooded animals and the retina has high metabolic demands, tissue culture studies are far more restricted in scope than are those using cold-blooded animals. Perhaps most significant is the fact that ROS disc shedding in the rat is driven by a circadian rhythm and as such, an investigator cannot easily or precisely control the onset of the event. This stands in contrast to amphibians, particularly *Rana pipiens*, where light triggers the event (Basinger *et al.*, 1976). Thus, in many respects the use of amphibians (see other chapters in this volume) offers a greater chance of resolving questions of disc shedding control mechanisms than does the rat. However, the major strengths of the rat, i.e., its circadian rhymicity and potentially useful mutant models, should not be overlooked. Some common features of the control of disc shedding and other circadian rhythms in the body will probably be found. At that point, insights from the more established field of pineal-mediated circadian rhythms can be applied to the problems of circadian ROS disc shedding in the rat.

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INDOLE BIOCHEMISTRY IN PINEAL AND RETINAL MECHANISMS

W. B. Quay

Department of Physiology-Anatomy
University of California, Berkeley, and
Bio-Research Laboratory
Napa, California

I. INTRODUCTION

Chemical mediators having an indolic structure occur in, and have actions upon, both pineal and retinal tissues. Increasing study of indolic mediators is broadening our ideas of cellular and molecular mechanisms not only in pineal and eye but also in most regions and organs of the body. This near ubiquity of influence is dominated by neural and glandular localizations of synthesis and release of indolic mediators. Nevertheless, fundamental mechanisms are likely to be shared at cellular and molecular levels.

Present appreciation of, and research concerning, pineal and retinal indolic mediators have developed within little more than twenty-five years. The intellectual course of this scientific development often has been indirect, and more like the pulling at loose threads than the organized attempting to perceive or interpret the fabric as a whole. Thus the fundamental relationships and recurring patterns in indole mediator mechanisms are only just beginning to be revealed.

II. HISTORICAL OVERVIEW

A. Pineal Indole Mediators

Isolation and structural identification of melatonin (5-methoxy-*N*-acetyltryptamine) by Lerner and coworkers (1, 2) started the investigation of pineal indolic constituents. Their work was based upon progressive purification of the factor in beef pineal tissue that had the greatest potency in stimulating pigment movement toward the nuclei within amphibian skin melanocytes in their bioassay system. Axelrod and Weissbach then elucidated the biosynthetic pathway to melatonin (3). This was the beginning of still continuing important studies on mechanisms regulating pineal melatonin synthesis. Most of the accompanying papers about pineal indoles are within this scientific heritage.

The work of Lerner, Axelrod and coworkers, noted above, provided for the first time evidence of a biologically active and at first thought uniquely pineal compound. Concurrently Giarman and colleagues (4) showed that human and monkey pineals have exceptionally great concentrations of serotonin (5-hydroxytryptamine, 5-HT). This is both an intermediate in pineal synthesis of melatonin, and a more widely occurring indolic chemical mediator in its own right. The above three sets of findings indicated to me at that time that of all known pineal compounds, indoleamines provided the most promising entrée to the revelation of the pineal's functional relations and biochemical mechanisms (5, 6). This led to the discovery of high amplitude 24-hour rhythms in pineal 5-HT and its derivatives, 5-hydroxy-indole-3-acetic acid (5-HIAA) and melatonin, and the phase shifts in these rhythms with modified timing of illumination (7, 8). Axelrod's group at the NIH rapidly extended these findings by intensive study, first of mechanisms governing the activity of hydroxyindole-*O*-methyltransferase (HIOMT), the last enzymatic step in pineal synthesis of melatonin (9-11), and also by study of mechanisms affecting pineal synthesis and metabolism of 5-HT (12-14). At about this time too, Owman, using the Falck-Hillarp histochemical method, made important discoveries concerning cellular localizations of 5-HT and other biogenic amines within mammalian pineals (15-17). In a number of respects Owman's observations and interpretations anticipated what was later appraised and confirmed by autoradiographic and quantitative biochemical methods.

The beginning of a third phase in research on pineal indole biochemistry can be assigned to the discovery that the rate-limiting, and to the highest amplitude rhythmic, step in

pineal synthesis of melatonin resided in serotonin-*N*-acetyltransferase (NAT) rather than in HIOMT activity (18, 19). This enzyme activity in pineal, and more recently shown in retina, has been the basis of experimental designs testing the compounds and mechanisms in biosynthesis of melatonin under a wide array of environmental and pharmacological conditions. This line of investigation is represented by several of the papers in this volume, and these can be consulted for references.

Melatonin is not the only pineal indole mediator. It is certainly the most studied one, however. It is possibly the most widely occurring pineal indole mediator among vertebrate animals as a pineal hormone. Serotonin (5-HT) occurs in at least the most studied pineal glands at far greater concentrations than melatonin (20). It is not only an intermediate in pineal synthesis of melatonin and related products, but it functions as well as a paracrine chemical mediator within pineal tissue. Here it is in part released from pinealocytes to be taken up in part by adrenergic nerve terminals, and to also affect the perfusability of the pineal's intercellular canaliculi (20, 21). Pineal 5-HT appears also to represent a storage phase in the daily cycle of pineal melatonin synthesis and release, since there is very little storage of melatonin itself, and pineal 5-HT contents build up during the daily light phase in most of the studied species. 5-HT in the general circulation originates from gastrointestinal mucosa, and is neither taken up, nor augmented, by the pineal.

B. Retinal Indole Mediators

John Welsh, a comparative physiologist at Harvard University, demonstrated early with bioassays that the retina and pigment epithelium+choroid of many vertebrates contain 5-HT (22). Retinal 5-HT occurs in inner plexiform and nuclear layers, and here immunohistochemical methods have revealed its finer localizations within some amacrine neurons and processes within the inner plexiform layer (23). Extensive and important early work on this was accomplished by Ehinger and Florén (24-26), but there has been controversy until recently concerning neurotransmitter status for retinal 5-HT. Binding characteristics of [^{3}H]5-HT to retinal membranes suggest that the [^{3}H]-ligand is binding to the physiological receptors for 5-HT. These and related findings have been reviewed recently by Osborne (23).

The evolutionary origin of the pineal gland from median eye-like structures led me to suspect that the melatonin-synthesizing enzyme, HIOMT, should be demonstrable also in the

lateral eyes. This was found to be true; HIOMT activity was found in the eyes as well as the pineal of diverse vertebrates (27) and its developmental changes were examined in an amphibian (28). Retinal HIOMT activity was found by other investigators too, and in mammals as well as lower vertebrates (29, 30). Both melatonin and NAT are now well known in the retina (34, 35). Retinal content of melatonin, and its 24-hour rhythm, persist after surgical removal of the pineal gland (pinealectomy), showing that retinal melatonin is not pineal dependent (36, 37). This does not exclude the possibility, however, that pineal or exogenous melatonin can affect the retina or other parts of the eye. The cat's iris+ choroid has a high uptake capacity for administered [^{3}H]melatonin (38), and administered melatonin can have gross effects on weights of parts of the eye in immature hamsters (39).

III. NEUROCHEMICAL CROSSCURRENTS: SELECTIVE SURVEY

Research on pineal and retinal indole mediator biochemistry has followed rather narrow conceptual paths. Pineal investigators have been predominantly concerned with melatonin, its control and evidence for its hormonal mode of chemical mediation. Investigators of visual and photic mechanisms of the eye also appear to have considered relatively few indole mediators and to have not yet been able to integrate the actions of any of these into concepts of normal physiological control mechanisms common to more than one or few species. This situation is complicated for both fields by the comparative diversity among investigated subject species in aspects of indolic compound levels, localizations, fluctuations and suggested functional relationships.

I wish here to briefly and selectively note some of the topics and approaches that may aid in broadening our perspectives and facilitate more expeditious progress in pineal and retinal biochemistry and physiology.

A. Non-enzymatic Reactions of Indole Mediators

Knowledge of non-enzymatic reactions of indole mediators can be important in several kinds of common circumstances: avoidance of artifactual formation of particular indole derivatives in vitro and the mistaking these for natural products or metabolites; planned derivativization in order to obtain quantitatively a more stable and improved compound for

TABLE I. Examples of Non-enzymatic Reactions of Indoles

Reaction(s)	Products	References
A. With certain ionizable salts (mercuric acetate and others)	Addition and/or coordination compounds	(40)
B. Pictet-Spengler reaction with an aldehyde: e.g. formaldehyde	Diverse Tricyclic derivatives, such as tetrahydro-β-carbolines	(41, 42)
C. Conjugation to protein, using formaldehyde	Multiple bonding sites: methylene bridges, etc.	(43-45)
D. Complex with flavins	Diverse	(46)
E. Complex with pyridine nucleotides and related compounds	Diverse	(47)
F. Flavin-sensitized photo-oxidation	Oxidized indole derivatives	(48)
G. Oxidation and/or peroxidation with polymerization	Melanin pigments	(49)

detection or measurement; planned derivativization for the purpose of removing or isolating particular indolic compounds or groups of compounds, either to remove them as potentially interfering agents or to provide a step in their isolation and purification; and interpretation and manipulation of fates and interactions in either natural (cytosolic or other) or artificial (in vitro) systems. Examples of some of the kinds of non-enzymatic reactions that can involve indole mediators, either accidentally or by plan, are listed in Table I. These are important to keep in mind.

B. Additional Pineal and Retinal Indolic Derivatives

Results from the use of newer analytical methods having greater specificity and sensitivity are likely to contradict or modify some of the recent beliefs about natural, and experimentally modified, pineal and retinal indoles and their derivatives. Some examples of this are seen with the application of gas chromatography+mass spectrometry to pineal and CNS tissues (50, 51). However, with increasing numbers of technical choices in detectors and other possible steps and components, evaluation of comparative merits and relative practicality of reported procedures can be difficult (52-55).

In contrast with some results from earlier analytical methods, 5-methoxytryptophol's concentrations in the rat pineal are now reported to be as great as those of melatonin, and to similarly change with time of day (50). Another example is provided by the tetrahydro-β-carbolines (THBC, or tryptolines). There is now confirmation of McIsaac's early (56) claim that under physiological conditions the pineal contains and forms such compounds as methoxy-THBCs. Endogenous human pineal levels of 6-methoxy-THBC have been reported to range from 0.2 to 2.4 μg/pineal (8.8 to 42.0 μg/g)(57). 6-Methoxy-THBC also has been reported in the retinas of pigs, rabbits and humans, and at concentrations comparable to those of melatonin (58, 59). Many compounds of this family are potent psychopharmacologic agents and affect enzymes and binding as well as receptor sites influencing the actions and metabolism of 5-HT and related indolic mediators. This has been most extensively studied in the CNS (60-67), and should now be extended in studies of pineal and retinal mechanisms.

C. Dietary and Precursor Effects

It has been known for many years that brain 5-HT concentrations can be lowered when animals are on either a tryptophan-deficient diet or one with excessive L-phenylalanine, and can be raised with greatly increased intake of L-tryptophan (68-71). The phenomenon occurs also in the mammalian pineal (72), but its possible ramifications there have not been studied. In the retina such effects appear to be even less studied, although elevated plasma tryptophan is reported in patients with cataracts (25.9±1.9) or aphakia (26.2±3.1 μg tryptophan/ml)(73). Recent indications suggest that although competing amino acids can impair the transport of tryptophan into the brain, they do not notably impair its transport at the 5-HT neuronal membrane in vivo (74). It is both increasingly important and increasingly feasible technically to

define regional differences in these effects and their mechanisms at neuroanatomical, cellular and molecular levels.

D. Alternative Metabolic Pathways and Fates

This subtopic is closely related to B above; one complements the other. Especially in pineal indole biochemistry, recent interpretations and reviews recapitulate the early noted (75) and well known major biosynthetic and metabolic pathways for 5-HT, melatonin and their relatives. But consideration of possible alternative paths and fates are omitted. Some alternative metabolites from melatonin are listed in Table II. Some of these are biologically active and merit particular study in pineal and retinal tissues.

TABLE II. Alternative Melatonin Metabolites

Metabolite	Suggested derivation	Subjects	References
A. *N*-Acetylserotonin	Demethylation	Rats & men	(76, 77)
B. 5-Methoxytryptamine	Deacetylation by aryl acylamidase	Rats	(78)
C. *N*-Acetyl-5-methoxykynurenamine	Indole 2,3-dioxygenase (pyrrolase), & foramidase actions	Brain tissue	(79, 80)
D. Methoxymelanin	Oxidation &/or peroxidation in part (see Table I, G)	Microsomal systems	(81)

E. Structure-Activity Relationships

The relationships between either chemical or biological activity on the one hand and differences in molecular structure on the other are important for a number of considerations, both theoretical and practical. In these considerations, distinctions between chemical and biological activities relate chiefly and superficially to the kind of assay system and end point studied rather than implying an intrinsic difference in the molecular origins of the observed activities. Nevertheless, whether the activities are studied in vivo or in vitro, biological activities, the diverse biological effects, of members of a group of compounds are usually more complex and indirect in their chemical origins and mediation.

Chemical structure-activity relationships should be considered comprehensively and in detail in the evaluation and validation of an assay system or technique. Historically, however, this kind of laborious pursuit often trails by some years the initial presentation, whether it has to do with direct chemical assay (82) or the use of complex biological systems (83-86). There is also the frequent difficulty of obtaining test samples of some compounds to complete a series of chemical analogues or congenors to be tested, without the necessity of custom synthesis. Even when availability is assured, the identities and purities of the compounds may need to be confirmed or established in one's own laboratory. Inspite of such circumstances, the study of structure-activity relationships remains fundamental to progress in our understanding of the measurement, and of the modes of action and interaction, of indole chemical mediators.

The currently most egregious example of the need for detailed study of structure-activity relationships among indole derivatives resides in psycho- and neuropharmacology. However, this topic area increasingly involves retinal and ocular therapeutics as well (87-90). More and more claims are made that this or that compound blocks, competes, or otherwise affects, uptake, binding, receptor or other particular mechanisms pertaining to a specific indole mediator, most frequently 5-HT. But the chemical specificity of the action or phenomenon is given token attention, if any. This problem enfolds considerations of the possible roles and uses of THBCs, and of melatonin itself, in health and disease.

IV. CONCLUSIONS

We are fortunate to be at the threshold of an expansive and promising field of regulatory and medical biology. This is indole chemical mediation in biological control mechanisms particularly as they are now being pursued within pineal and eye. Other light- and radiation-sensitive tissues and cells in diverse kinds of organisms will increasingly be brought into consideration in the study of these mechanisms (91).

Along with opportunities for important advances, we have the potential for needless handicapping. Avoidance of the latter will depend largely upon a multidisciplinary approach, one that takes advantage of errors as well as advances in other disciplines or specialties. This should aid in fostering foresight in conceptualizations and experimental designs.

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MELATONIN IN VERTEBRATES: ARGUMENTS FOR LOCAL FUNCTIONS AND HORMONAL EFFECTS[1]

William A. Gern
Jeanne M. Nervina

Department of Zoology and Physiology
University of Wyoming
Laramie, Wyoming 82071

I. INTRODUCTION

The observation that melatonin and the biochemical apparatus necessary to synthesize this compound has an ancestral association with vertebrate photoreceptive structures or their derivatives is intriguing. Our interest was sharpened through the implication this finding has on the evolution of melatonin function. Recently a hypothesis concerning the evolution of melatonin function has been advanced (19, 22) that is based principally on two aspects of melatonin biology, i) that vertebrate photoreceptive structures with evolutionary histories dating to the formation of the subphylum, synthesize melatonin, and ii) that melatonin has actions within such structures which could be termed functions (we follow the definitions proposed by Gould and Vrba, (23).

II. Sites of Melatonin Synthesis in Vertebrates: An Evolutionary Survey

Within vertebrates, melatonin has been localized in pineal (31, c.f. 35, 48, 22) and extrapineal locations (c.f. 39, 22) including retina, Harderian gland and gut. Enzymes necessary to convert tryptophan to melatonin (hydroxyindole-O-methyltransferase, HIOMT and serotonin N-acetyltransferase, SNAT) have been localized in the pineal (35, 28) retina (34,

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8, 3, 20, 25, 27,) and Harderian gland (47, 3). Melatonin has been localized in gut using fluorometry (38) and immunohistofluorescence (4, 26). Presently published data demonstrating melatonin in gut are from laboratory rats (we have observed melatonin in the gut of Kangaroo rats, Dipodomy ordi using immunohistofluorescence techniques; (Karn and Gern unpublished), and pigeons (45). To date only HIOMT has been identified in the gut (36) and no studies demonstrating conversion of radiolabeled substrates to melatonin by gut or Harderian gland have been advanced. While HIOMT has been demonstrated in the gut, it is not known if melatonin synthesis occurs at this site. From these studies a roster of locations for melatonin synthesis is beginning to emerge, but paleontological information indicates that some have been synthesizing melatonin longer than others.

It has been argued (19, 22) that the eyes and pineal-complex organs have the longest history of melatonin synthesis (and perhaps secretion) in the Vertebrata. For example, a pineal foramen is consistently present in the dorsal head skeleton of ancient ostracoderm fossils (41, 16). Lateral eyes are an equally ancient and consistent vertebrate feature. At present it is not possible to determine which of the two locations - lateral or medial photoreceptive structure - is the most ancient site of melatonin synthesis.

Harderian glands possess HIOMT (46) and SNAT (3) activity indicating the capability of melatonin synthesis from serotonin. No studies have been advanced demonstrating that melatonin synthesis can occur when Harderian gland tissue is incubated in the presence of radiolabeled substrates like serotonin or tryptophan. Because of the recent evolution of Harderian glands as vertebrate structures (Harderian glands evolved during the Devonian period as an adaptation to prevent desiccation of the eye; 48) relative to the pineal organs and to lateral eyes, it is argued (19, 22) that any action Harderian gland melatonin has within these structures is more recent than melatonin actions in pineal organs and lateral eyes. Also because pineal organs, and perhaps eyes (40) have been secreting melatonin into blood for millions of years prior to a Harderian gland contribution it is difficult to determine any new function for circulating melatonin from this source.

Gut melatonin is difficult to fit into these arguments because of the small amount of available information. Gut melatonin has been identified in rodent species, but it is not known whether melatonin is synthesized or accumulated by this structure. We have tried, unsuccessfully, to identify melatonin in the gut of teleost fishes. While it is difficult to interpret such data, our current thinking is that it

is not present in the species examined. But caution is warranted considering that there are 22,000 species of teleosts. There are no published data concerning gut melatonin in birds, reptiles or amphibians making phylogenetic arguments extremely tenuous. Gern and Karn (22) used the presence of serotonin-containing cells in the gut of vertebrates as an indicator for the possible presence of melatonin. Here it was found that such cells are common in tetrapod gut (amphibians, reptiles, birds and mammals). But they are rare or perhaps absent in teleosts, with very little information on their presence in other fish groups. From these data it is argued - with great caution - that gut melatonin is a tetrapod feature and therefore is phylogenetically more recent than the appearance of melatonin in the pineal-complex organs and retinas of vertebrates. Obviously considerable work needs to be done to determine the phylogenetic distribution of melatonin in the gut of vertebrates. Figure 1 summarizes the present understanding of melatonin distribution in the tissues of vertebrates.

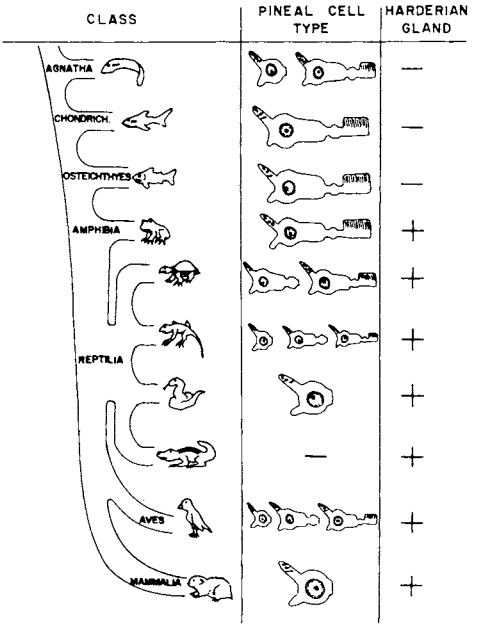


Figure 1. A summary of the pineal cell type and disposition of the Harderian gland is displayed. With few exceptions, most vertebrate species possess a pineal organ which synthesizes melatonin. Also, very few species of vertebrates lack eyes, and it is becoming clearer that vertebrate eyes,

in general, synthesize melatonin. Harderian glands are limited to tetrapod groups (+). The extent of melatonin synthesis by Harderian glands in these taxa of vertebrates has not been examined extensively. Both pineal organs and retinas have very long phylogenetic histories of melatonin synthesis, much longer than Harderian glands for example. Finally, gut melatonin (not depicted) has been observed only in pigeons and rodents (see text).

III. An Hypothesis

We have argued that melatonin is a secretory product of photoreceptor cells present within the epiphyseal complex and retina (19, 22). Investigations using melatonin immunohistofluorescence have demonstrated the most intense accumulation of melatonin in the retina occurs in the outer nuclear layer (5, 46). In addition Weichmann et al., (50) using antibodies to HIOMT have observed immunoprecipitation reactions in the outer nuclear layer of rat and bovine retinas. Because melatonin and the critical enzyme necessary for melatonin synthesis are found in the nuclear region of photoreceptor cells it now appears that this cell type is the location of melatonin synthesis.

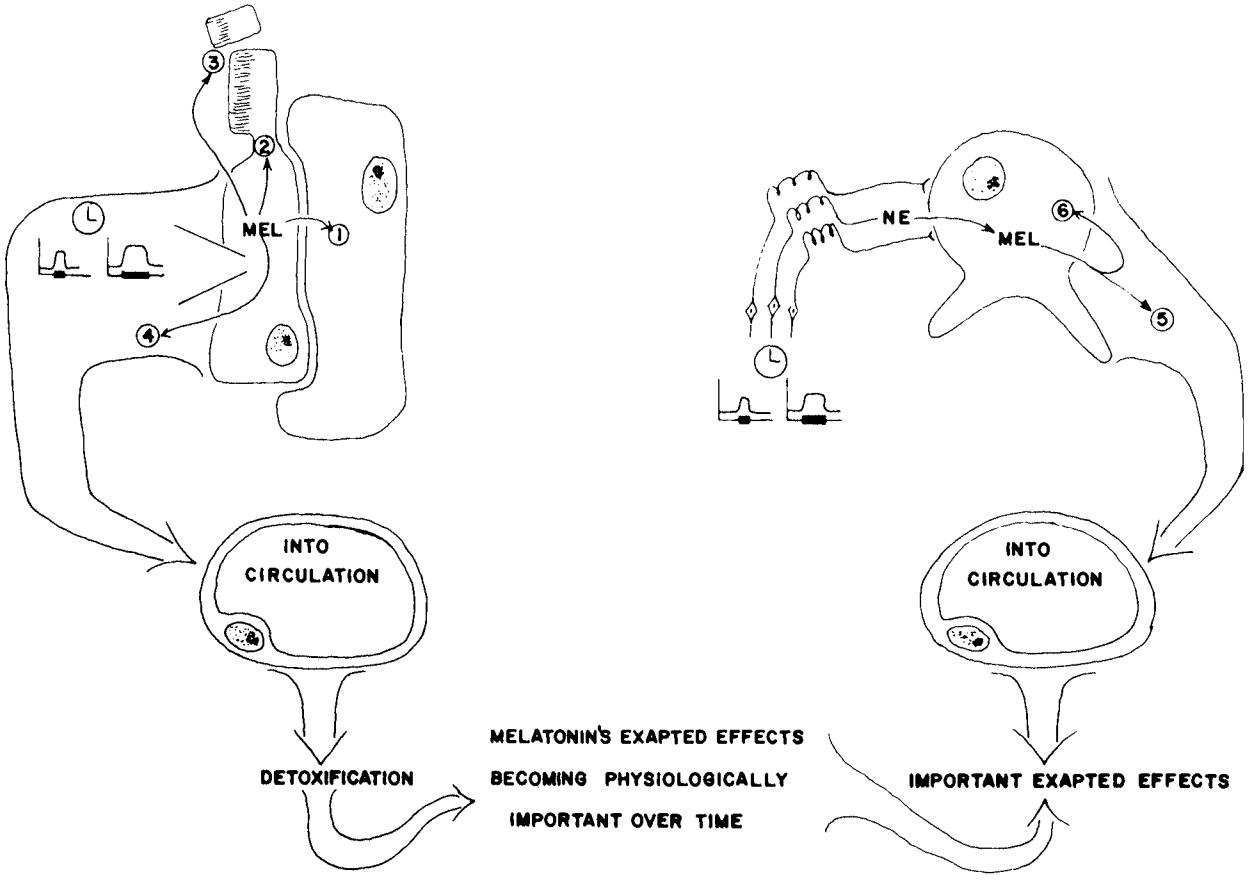
Pinealocytes are the location of melatonin synthesis in the pineal gland of mammals (c.f. 35, 28, 48). Through the extensive work of Collin and coworkers, (c.f. 11, 12) it appears that the mammalian pinealocyte is homologous to pineal photoreceptor cells (including photoreceptor and secretory rudimentary photoreceptor cells) present in species ancestral to the Mammalia. These data imply that the sensory line of cells are the location of melatonin synthesis in the pineal organs of nonmammalian vertebrates. Other evidence supports this contention. First, Vivien-Roels et al. (46) observed melatonin immunohistofluorescence in the pineal organs of teleosts and reptiles. In these studies, fluorescing material was dispersed in a spotty distribution around the lumen of the organ, in a pattern similar to the distribution of sensory line cells. More recently, Falcon et al., (17, 18) reported the ultrastructural distribution of melatonin-N-acetylserotonin and serotonin in the pineal organ of pike (Esox lucius: Teleostei) to be in the sensory line cells. Together these data indicate that the photoreceptor cells or their derivatives are the source of melatonin production in the pineal organs of nonmammals.

From these data it is concluded, that melatonin synthesis is another interesting property shared by medial and lateral photoreceptive structures. (Several other shared properties are discussed in this volume.) And, that photoreceptor cells

present within these structures are the sites of melatonin synthesis. Melatonin actions on photoreceptor cells, or adjacent cells within medial and lateral photoreceptive structures are under careful scrutiny. We ask if these roles for melatonin are important for another reason? Specifically, are melatonin actions in photoreceptor cells or adjacent cells ancestral, predating other actions observed for the molecule?

We hypothesize (19, 22) such actions are ancestral and that those roles for melatonin described as hormonal evolved at a later date through exaptation (23). It is assumed that most of the basic biology of melatonin synthesis has been conserved over time. Accordingly, melatonin synthesis would have occurred at night - the case in extant species examined to date. And, that melatonin synthesis was an entrained circadian biochemical system using light/dark cycles as a Zietgeber. With these assumptions we have hypothesized (Fig. 2) that melatonin functioned to facilitate the process of photoreception by acting on either the photoreceptor cells themselves, or on adjacent cells. Some of the melatonin synthesized at night appeared in the circulation. Initially circulating melatonin had no hormonal action. Melatonin leaving a photoreceptive structure may have been secreted specifically for detoxification by the liver. Or it may have "leaked" from the structure. Because of melatonin's structure and non-polarity, lipid bilayers do not present a substantial barrier to the molecule. Thus, melatonin is difficult to hold within secretion vesicles (perhaps this is why melatonin vesicles have not been described) and would argue for leakage from melatonin-synthesizing cells. In this case the end result would have been melatonin entering the circulation having no function and subsequently being detoxified by the liver. The nightly appearance of melatonin, originating from photoreceptive structures, in plasma would have been a very regular event - as regular as it is in extant animals. Also, circulating melatonin would have been an accurate marker of either the onset of the scotophase, its length, or both, just as it is today. Animals with cellular mechanisms capable of discriminating the nightly pulse of circulating melatonin - that is, use the timing information inherent in the pulse - could more precisely place physiological events with seasonal change. This would serve to increase fitness and therefore would be selectively advantageous. The contention of this hypothesis is that the paracrine events associated with the photoreceptive structures were ancestral and that subsequent hormonal actions, while being important because they served to increase fitness, developed at a later time.

It is unfortunate that most of the hypothesis is untestable because of parsimony in the information extractable


MEL
NE
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INTO CIRCULATION
DETOXIFICATION
MELATONIN'S EXAPTED EFFECTS
BECOMING PHYSIOLOGICALLY
IMPORTANT OVER TIME
IMPORTANT EXAPTED EFFECTS

Figure 2. The left side demonstrates paracrine effects melatonin may have had in ancestral medial or lateral photoreceptive structures. It is assumed that melatonin synthesis was regulated by the scotophase, as in extant lower amniotes and anamniotes causing nightly pulses of varying length. Synthesized melatonin has several known actions in lateral photoreceptors: 1) actions on adjacent cells, e.g., pigment condensation, or inhibition of neurotransmitter release, 2) actions on the contractile processes in the myoid region, 3) actions on outer segment disc shedding, or 4) secretion into blood, either specifically for detoxification or incidental secretion resulting in detoxification. In extant vertebrates, possessing pineal photoreceptor cells, hormonal actions for melatonin have been observed. Such actions are hypothesized to be the result of exaptation. Because the nightly pulse of melatonin would be extremely regular (as regular as night and day) animals capable of using the information contained within such a pulse, to regulate physiological events like reproduction, would be at selective advantage and thus display increased fitness. In the synapsid line leading to mammals pineal photoreceptor cells underwent morphological restructuring which included loss of the outer segments. Innervation from the brain (superchiasmatic nucleus (SCN) in particular) became more important (31) with the SCN generating a rhythm of nervous outflow to the pineal. Because the SCN was (is) entrained by light perceived by the eyes, photoperiod information to the pineal regulating melatonin synthesis is maintained. Hence, nightly pulses of melatonin synthesis occurred. Concurrently, the pineal organ lost most efferent nervous connections to the brain. Thus, photoperiod information perceived by pineal photoreceptor cells relayed to the brain centers in nonmammalian vertebrates is lost in mammals (and perhaps birds convergently). However, melatonin synthesis is unaltered, old paracrine functions associated with photoreception by the gland are lost, some actions are maintained (like #6, protein secretion see 24) and the hormonal effects are amplified (#5).

from fossil vertebrates, however some aspects can be examined with the scientific method. One way is to examine primitive vertebrates for melatonin actions that can be termed hormonal. For example, melatonin is quite potent in causing the condensation of melanin in the dermal melanophores of larval vertebrates including the ammocoete larvae of cyclostomes (15). This melatonin is of presumed pineal origin, and therefore satisfies the definition of a hormone. But what is the function of this condensation event? What is the selective benefit of becoming lighter at night? It is difficult to determine what advantage this would offer. Could it be that this effect of circulating melatonin is without advantage, that is nonaptive (23), without function just as the circulating melatonin may have been in the most ancient vertebrates? Another would be to examine lateral and medial photoreceptive structures for melatonin function. Melatonin has several reported actions in the eye. It causes cone movement (35), condensation of pigment in the PE (9), inhibits calcium-dependent dopamine release (14) and stimulates rod outer segment shedding (1). Few papers exist, describing melatonin actions in the pineal gland. One is the contribution of Halder-Misra and Pevet (35), who describe an effect of melatonin on the protein secretion from mammalian pineal glands (Fig. 2). Do other actions exist?

We have been addressing the question of melatonin action in photoreceptive structures by examining the trout retina for melatonin binding. In an initial investigation melatonin binding in 15,000 xg supernatants of trout ocular tissues was examined (21). Here a binding protein was described which displayed high affinity binding (Kd 1.25 nM) and that was destroyed by trypsinization, boiling or treatment with proteases. The binding displayed specificity as determined by the ability of unlabeled competitors to displace ^{3}H-melatonin binding. In these tests, unlabeled melatonin was most potent in displacing unlabeled melatonin binding, although 5-MTOH displayed considerable cross reactivity.

The ability of trout ocular membranes to bind melatonin is the subject of current investigations. In these studies, membranes from the neural retina and pigment epithelium were prepared using a procedure described by Cardinali et al. (8). Ocular tissue was homogenized in cold 0.32 M sucrose. Melanin was separated from other membrane components by centrifugation (33,000 xg; 4^{o}C) through a sucrose gradient of 0.32 M, 1.0 M and saturated sucrose (top to bottom of tube). Membranes were collected from the top two layers while the melanin-containing bottom layer was discarded. The membranes were washed twice in buffer (Tris 10mM, $MgCl_2$ 6mM, ascorbic acid 91.%) then diluted to a concentration of 1:120 with buffer. Incubations of membranes were carried out in conical centrifugation (1.5 ml

Eppendorf) tubes in which ^{3}H-melatonin (20-50 nMoles) in methanol had been added then reduced to dryness with a stream of nitrogen gas. Other tubes containing both ^{3}H-melatonin and a 100-fold excess of unlabeled melatonin were prepared in a similar way. Diluted membranes (250 ul) were added to these tubes and were allowed to incubate in the cold for 4-6 hours. Following incubation 1.0 ml of cold assay buffer was added to each tube and bound ^{3}H-melatonin was separated from unbound by centrifugation (12,000 xg, 10 min, 4^{o}C). The supernatant was removed by aspiration and the tube walls washed twice with assay buffer. Distilled water (1.0 ml) was added to each tube and following a brief incubation the pellet was resuspended, placed into a scintillation vial containing fluor and the amount of radiation present in each vial determined by liquid scintillation spectrophotometry. All values were subsequently calculated to fmoles bound.

From these investigations we found that a 100-fold excess of unlabeled melatonin reduced the amount of ^{3}H-melatonin bound by approximately 50-60%. As indicated (Fig. 3) unlabeled melatonin and 5-MTOH were equipotent in inhibiting ^{3}H-melatonin binding while N-acetylserotonin reduced ^{3}H-melatonin somewhat. None of the compounds significantly reduced the amount of ^{3}H-melatonin bound. However, an analog of melatonin, propionyl-5methoxytryptamine, also displayed significant cross reactivity. Saturation studies demonstrate the presence of two binding components, one which saturates at approximately 100 nMoles ^{3}H-melatonin and another that does not saturate but increases linearly with increased concentrations of ^{3}H-melatonin. Scatchard analysis yield curvilinear plots with one component displaying an average Kd of 2.0×10^{-10}. The binding component in the membrane preparation is heat sensitive, destroyed by trypsin and 3M urea and requires the presence of divalent cation (Mg^{2+} or Ca^{2+}).

Similar ocular binding of melatonin has been reported. Cohen et al., (10) observed melatonin binding in cytosol preparations of rat and hamster uterine, ovarian, liver and ocular tissue. These investigators observed binding of ^{3}H-melatonin saturated at a concentration of approximately 1 μM. Vacas and Cardinali (44) reported a melatonin saturated at approximately 750 to 1,000 nM and displayed a Kd of 7×10^{-7}M. The binding was temperature sensitive, decreased by trypsin and was enhanced by the addition of divalent cation. In pineal membranes N-acetylserotonin, serotonin and 2-methyl indole were major competitors.

Lang et al., (30) measured ^{3}H-melatonin in both cytosol and membrane fractions from various rat tissues including those from the eyes. These binding interactions had a Kd of 8.0×10^{-9} for the membrane fractions and 6.0×10^{-8} for the cytosol component. Membrane binding of 6-OH-melatonin was

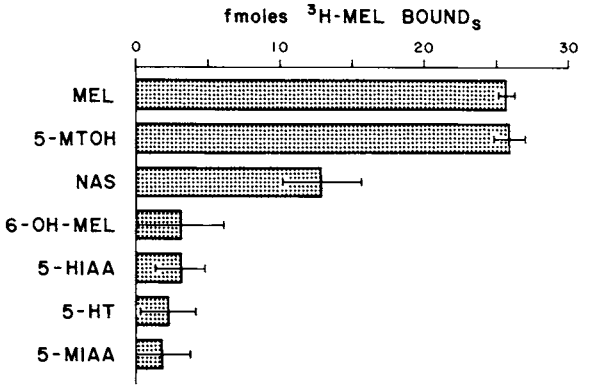

Figure 3. The effect of various indoleamines competing with ^{3}H-melatonin for binding sites on trout ocular membranes is depicted. All indoleamines tested were held at 100-fold excess over the amount of ^{3}H-melatonin in the incubation medium. The amount of ^{3}H-melatonin bound specifically (Bound$_S$) was determined by subtracting the amount of ^{3}H-melatonin bound without competition from the amount bound when competitor was present. The difference was considered to be specific binding. Unlabeled melatonin (MEL), 5-methoxytryptophol (5-MTOH) and N-acetylserotonin (NAS) displayed cross reactivity with MEL and 5-MTOH being equipotent. 6-hydroxymelatonin (6-OH-mel), 5-hydroxyindoleacetic acid (5-HIAA), serotonin (5-HT) and 5-methoxyindoleacetic acid (5-MIAA) did not significantly depress the amount of ^{3}H-melatonin bound compared to those incubations where no unlabeled indoleamine competitor was present.

more dependent on the presence of divalent cation than that in the cytosol. These investigations demonstrated that binding of ^{3}H-melatonin by ocular tissue membranes was greater than 200 fmoles/mg protein and that the amount of binding was reduced by trypsin.

The results we observed for ^{3}H-melatonin binding in trout ocular membranes are similar to those reported by others.

Besides melatonin, 5-MTOH was a major competitor of ^{3}H-melatonin binding. Similar cross reactivity results were observed in bovine medial basal hypothalamus (7). In all investigations melatonin binding was dependent on the presence of divalent cations. The thermal stability of the melatonin binding proteins are similar and binding remains stable for long periods when incubations are performed at low temperature. Also, binding of melatonin by ocular tissues has been reported by three groups, Cohen et al. (10), Gern et al. (21) and Lang et al (29, 30). An important question remains, that is, does the ^{3}H-melatonin binding assessed in the ocular tissue represent an interaction with a melatonin receptor? Examining the results from ^{3}H-melatonin binding in trout ocular membranes, certain aspects of the binding interaction indicate that a receptor is involved. Namely, a protein is involved in the binding event and this protein is trypsin, heat and urea sensitive. Also, nonspecific lipases do not decrease binding. The affinity of ligand for protein is in the range normally expected for receptors. Finally the dependency on the presence of divalent cations is similar to results observed for gamma-aminobutyric acid and glycine binding in brain and hypothalamic melatonin binding (7). And the amount of ^{3}H-melatonin bound in an eye is similar to the amount of melatonin that is present in a trout eye. However, there are some aspects of this interaction that give us pause. Specifically, the amount of ^{3}H-melatonin present in the incubation medium necessary to achieve saturation of melatonin binding is high, approximately 100 pMoles. At saturating concentrations, approximately 0.4 pMoles is bound. Cross reactivity studies indicate that both melatonin and 5-MTOH are equipotent in inhibiting ^{3}H-melatonin binding. Because both are hydrophobic molecules they may interact with outer segment membranes nonspecifically.

Arguing against the nonspecific interaction are measurable events due to the application of melatonin or 5-MTOH. For example, melatonin mimics light-induced rod outer segment shedding in Xenopus eye cup preparations (1). And in this preparation 5-MTOH is equipotent with melatonin in inducing the event. Melatonin causes tonic contractions of rat gut thus halting the peristaltic contractions of this organ (Harlow, personal communication). 5-MTOH is a synthetic product of the retina (33). We are continuing to examine melatonin binding in the retina and remain open as to whether this interaction is one between ligand and receptor.

IV. Summary

In summary, it is apparent that melatonin synthesis by vertebrate medial and lateral photoreceptors is phylogenetically ancient. At present it is difficult to determine which of these two sites is older in terms of melatonin production. It has been hypothesized that primordial melatonin action could be best described as parahormonal (or perhaps neuromodulatory), being synthesized in a specific location - photoreceptor cells - and acting on these cells or adjacent cells. We speculate that the circadian system controlling melatonin synthesis in the pineal organs of extant, nonmammalian vertebrates (2, 13, 42) also was present in the earliest vertebrates. (Focus is on the pineal organ because it is the chief source of circulating melatonin in vertebrates.) Thus, melatonin synthesis would have been a very regular event, and organisms capable of monitoring the nightly circulating melatonin pulse would be able to sequence physiological events into closer temporal position with seasonal climatic change. Such temporal sequencing would serve to increase fitness and fix this new melatonin effect (23) into the genome.

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MULTIPLE AROMATIC AMINE N-ACETYLTRANSFERASES IN THE PINEAL GLAND

M. A. A. Namboodiri

Department of Biology
Georgetown University
Washington, D. C.

P. Voisin

Zoologie Biologie Cellulaire Faculte des Sciences
Poitiers, France

D. C. Klein

Laboratory of Developmental Neurobiology
National Institute of Child Health and Human Development
Bethesda, Maryland

Acetylation of aromatic amines has been an area of active investigation during the last 30 years. Initial studies were focused on drug acetylation because it was observed that drugs such as isoniazid (INH) and dapsone (DDS) are excreted in urine in the N-acetylated form (1). These studies led to the discovery of acetyl CoA as the endogenous acetyl donor for acetylation reactions, and also resulted in a detailed description of the N-acetylating system in the liver, including its purification and characterization and the finding of genetic polymorphism (2-4). Acetylation of aromatic amines in the liver was initially considered to be primarily a detoxification mechanism. However, recent experimental evidence indicates that it is also an essential step in the metabolic activation process which converts aromatic amines to potent carcinogens (5).

Acetylation of aromatic amines has also been studied in some detail in blood, brain, retina and the pineal gland. The best understood is the acetylation of serotonin in the pineal gland because of the role N-acetylation plays in the synthesis and regulation of melatonin, a putative hormone (Fig. 1).

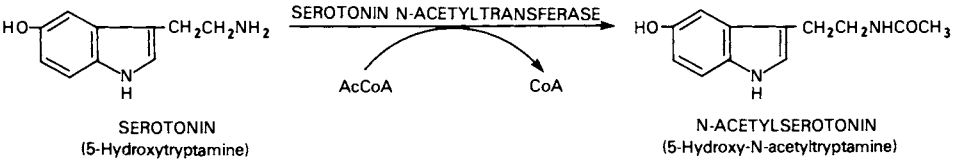

Fig. 1. N-Acetylation of serotonin.

I. SEROTONIN ⟶ MELATONIN CONVERSION

Interest in the N-acetylation of serotonin in the pineal gland started when Aaron Lerner discovered that melatonin, the skin lightening compound found in the pineal gland, is an N-acetylated derivative of 5-methoxytryptamine (6). Subsequently, Weissbach and co-workers demonstrated that serotonin was converted to melatonin in the pineal gland (7-8). The pathway involves the N-acetylation of serotonin by serotonin N-acetyltransferase[1] (serotonin NAT) to form N-acetylserotonin, which is O-methylated by hydroxyindole-O-methyltransferase (HIOMT) to form melatonin. HIOMT received most attention at that time because it was discovered that HIOMT activity and melatonin content of the pineal gland are regulated by a photoneural mechanism (9-10). This influenced early investigators to suspect that HIOMT was responsible for the regulation of melatonin synthesis, and the concentration of melatonin in the pineal gland and in the circulation.

Investigations of the serotonin N-acetylation in the pineal gland received a major stimulus when Axelrod and his colleagues discovered that the synthesis of melatonin from tryptophan was increased by adrenergic drugs (11). This observation prompted Klein and co-workers to study HIOMT under this condition to understand the mechanism involved in the

[1] The term, arylalkylamine N-acetyltransferase, has also been used in the text to denote the same enzyme.

regulation of melatonin biosynthesis and the role of HIOMT. It was soon discovered that HIOMT showed only a marginal increase when melatonin synthesis from tryptophan was increased several fold by adrenergic drugs (12). This was interpreted by Klein and co-workers that perhaps another mechanism was responsible for the increase in melatonin synthesis. They analyzed the intermediates in the conversion of tryptophan to melatonin and discovered that the increase in melatonin synthesis caused by treatment with adrenergic drugs was associated with a large increase in the melatonin precursor, N-acetylserotonin (12). This led to interest in serotonin NAT and the discovery that adrenergic agonists increase the activity of serotonin NAT 10- to 100-fold by a direct action on pineal cells (13-15). Subsequently, they found that a large increase in the activity of serotonin NAT occurred at night, and this appeared to be responsible for the concomitant decrease in serotonin as well as the increase in melatonin in the pineal gland (16-17, Fig. 2). These observations brought pineal serotonin NAT to the forefront of pineal research and led to extensive investigations of the neural regulation of this enzyme and the role it plays in the circadian regulation of indole metabolism in the pineal gland.

II. NEURAL REGULATION OF SEROTONIN NAT IN THE PINEAL GLAND

It is now well established that pineal NAT in the rat exhibits a circadian rhythm with 50 to 70-fold increase in activity at night (18-19). This increase is caused by the stimulation of the pineal gland by a circadian clock in the suprachiasmatic nucleus (SCN); a neural circuit involving both central and peripheral structures, including superior cervical ganglia (SCG), links the SCN to the pineal gland (20-22). The neurotransmitter which stimulates pineal cells physiologically is norepinephrine (23-24). It is released from sympathetic nerve terminals whose cell bodies are located in the SCG (23).

Norepinephrine acts through a mechanism involving both α- and β-adrenergic receptors to increase cAMP, which in turn increases serotonin NAT activity 50 to 70-fold (25-27). cAMP acts to increase serotonin NAT activity at least in two ways. First, it increases the synthesis of new proteins by a mechanism involving both transcriptional and translational processes (28-30). Second, it stabilizes serotonin NAT, probably by a cAMP dependent phosphorylation mechanism (31). The stabilizing effect of cAMP is apparent when the cAMP concentration in cultured pineal cells is decreased. This leads to a rapid

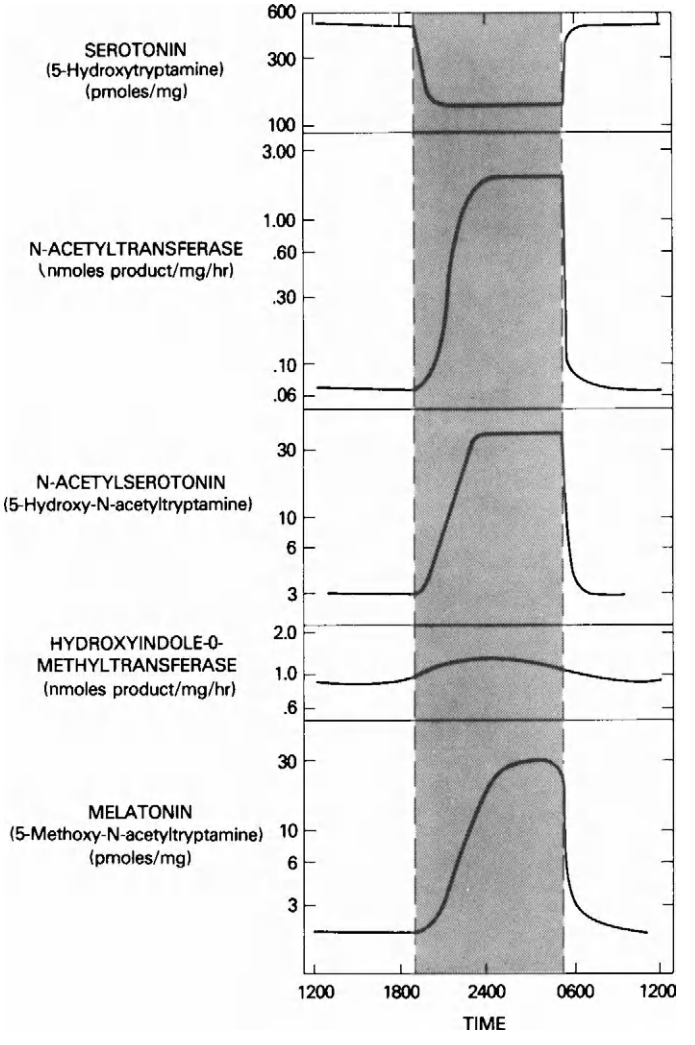

Fig. 2. Rhythms in indole metabolism in the rat pineal gland. The shaded portion indicates the dark period of the lighting cycle. The data have been abstracted from reports in the literature. 5-HT, 5-hydroxytryptamine, serotonin; NAct, N-acetyltransferase; NAc 5-HT, N-acetyl 5-hydroxytryptamine; HIOMT, hydroxyindole-O-methyltransferase; NAc 5-MT, N-acetyl 5-methyoxytryptamine, melatonin.

decrease in NAT activity (31). This decrease also occurs in intact rats when they are exposed to light at night; light appears to act by a gating mechanism at the level of the SCN to inhibit the neural stimulation of the pineal gland (32). These large and rapid changes in serotonin NAT activity generate the rhythm in melatonin concentration in the pineal gland and in the circulation (33).

Regulation of pineal serotonin NAT has been studied in several species other than the rat, including chicken, sheep and hamster. These studies show that all exhibit a circadian rhythm in serotonin NAT activity. However, notable differences in the mechanism of its regulation exist among these species. Thus, in chicken the serotonin NAT rhythm is not controlled entirely by the SCN, but to a large degree by a biological clock located within the pineal gland (34). Also, the mechanism of the noctural increase in serotonin NAT does not always seem to involve norepinephrine (34). There appear to be two notable differences in sheep. They are the rapid nocturnal increase in serotonin NAT activity (maximum reached in about 30 min.) and the small magnitude (3-5-fold) of the rhythm (35). In addition, pineal serotonin NAT in sheep appears to be only partly responsible for the regulation of melatonin synthesis (36). The hamster pineal serotonin NAT rhythm has characteristics of that in both the rat and sheep; the apparent amplitude of the rhythm is small, as in sheep, while the nocturnal response exhibits a lag as in the rat (37).

The molecular mechanisms involved in the regulation of the serotonin NAT rhythm are only beginning to be understood. As noted above, the increase in activity at night appears to involve transcriptional and translational processes. However, it is not clear whether new molecules of serotonin NAT are produced or some regulatory proteins are synthesized under this condition. The rapid decrease in the enzyme activity, which occurs on exposure to light, still remains a mystery. While a protein thiol:disulfide exchange mechanism has been proposed to be involved in this process, direct experimental support for this hypothesis is lacking (38-39). Progress toward understanding the molecular mechanisms of serotonin NAT regulation has been rather slow because of the lack of availability of the enzyme in the purified form. Purification of serotonin NAT has been difficult because of the unstable nature of the enzyme and the scarcity of adequate amounts of tissue containing the active enzyme (40-41). Recently, however, we have succeeded in purifying the enzyme from rat and sheep pineal glands using disulfide exchange, size exclusion and affinity chromatography (42-43). Accordingly, more information on the molecular mechanisms of serotonin NAT regulation is expected to become available in the near future.

III. REGULATION OF SEROTONIN NAT IN THE EYE

The retina is another tissue in which serotonin NAT exhibits a true circadian rhythm. This rhythm has been detected in a variety of species, although it has been studied in some detail only in chicken and frog (44-45). In both chicken and frog the rhythm is of small magnitude (5-10-fold), persists in constant darkness, and is suppressed by light (45-47). It has been possible to demonstrate the above characteristics of the rhythm in cultured frog eye cups, indicating that the rhythm is controlled by a biological clock located inside the eye (48). cAMP appears to be involved in the increase in the enzyme activity at night, although the mechanism generating the cAMP is not known (45). In addition, catecholamines appear to exert an inhibitory influence on the avian retinal serotonin NAT rhythm, opposite to their stimulatory effects in the rat pineal gland (46,49). The rhythm in retinal serotonin NAT seems to regulate a similar rhythm in melatonin, which is believed to be involved in the control of rhythmic retinal functions, including disk shedding (49).

IV. ARYLAMINE AND ARYLALKYLAMINE NATS IN THE PINEAL GLAND

In the early stages of the investigations of biosynthesis of melatonin in the pineal gland, it was assumed that pineal serotonin NAT was the same as the relatively nonspecific aromatic amine NAT in the liver. The discovery of the adrenergic regulation of pineal serotonin NAT, however, began to change this view. It seemed possible that adrenergic regulation of pineal NAT could involve a different molecular species of NAT. In pursuit of this, Klein and Weber found in unpublished studies that the increase in pineal NAT caused by isoproterenol, an adrenergic agonist, was detectable only if serotonin or tryptamine was used as the amine substrate, not when INH, a known substrate for the liver enzyme, was used.

This possibility was further pursued and it was discovered that both the rat and sheep pineal gland contain two distinct types of NATs which can act on aromatic amines (50). One type acetylates arylamines such as aniline and p-phenetidine preferentially, whereas the other acts on arylalkylamines such as serotonin, tryptamine and phenylethylamine with high specificity. Both enzymes are located in pineal cells. The Km of acetyl CoA for arylamine NAT is unusually high (2 to 4 mm), about 20-fold higher than that of arylalkylamine NAT; this suggests that perhaps the endogenous acetyl donor for this enzyme

may not be acetyl CoA. As described below, these two enzymes can be distinguished further by their (1) regulation, (2) stability, and (3) molecular size.

A. Regulation

Regulation of pineal NATs has been studied under three different conditions: 1) isoproterenol treatment, 2) exposure to darkness at night and 3) cycloheximide treatment (50). Treatment of rats with isoproterenol during the day increased arylalkylamine NAT about 100-fold, without affecting arylamine NAT to any significant extent (Table 1). This indicates that the nocturnal increase in NAT activity in the rat pineal gland is specific for arylakylamine NAT. Another instance in which the differential regulation of the two enzymes is evident is in their rapid response to darkness at the onset of night in the sheep pineal gland. The arylalkylamine NAT increased about 5-fold 30 min. after the exposure to darkness at night; arylamine NAT remained unchanged under this condition (Table 2). Further, treatment of sheep with cycloheximide 30 min. before exposure to darkness caused a 90% reduction in arylalkylamine NAT measured 30 min. after exposure to darkness, but did not have any effect on arylamine NAT (Table 3). These observations show that pineal arylamine and arylalkylamine NATs are differentially regulated.

TABLE I. Effect of isoproterenol treatment on arylamine and arylalkylamine NAT activities in rat pineal gland

Substrate	NAT activity (nmol/min./mg protein)	
	control day time	isoproterenol
Tryptamine	0.011 ± 0.007	1.5 ± 0.25
Phenylethylamine	0.019 ± 0.005	1.5 ± 0.15
p-Phenetidine	0.20 ± 0.036	0.13 ± 0.006
Aniline	0.14 ± 0.007	0.08 ± 0.018

Rats received isoproterenol (10 mg/kg) or saline and were killed during the daytime. Enzyme activity was measured with 0.5 mM AcCoA and 10 mM amine substrate. Results are expressed as the mean ± S.E. of triplicate determinations (from Ref. 50).

TABLE II. Ovine pineal arylalkylamine and arylamine NAT activities 30 min. before and after lights off at night

Substrate	NAT activity (nmol/min./mg protein)	
	Day	Night
Tryptamine	0.18 ± 0.04	0.71 ± 0.07
Phenylethylamine	0.13 ± 0.05	0.66 ± 0.07
p-Phenetidine	0.38 ± 0.11	0.42 ± 0.10
Aniline	0.12 ± 0.04	0.12 ± 0.05

Groups of five sheep were killed 30 minutes before lights off and 30 minutes after lights off (1800 hours). NAT activities were measured with 0.5 mM AcCoA and 10 mM amine substrate. Results are expressed as the mean ± S.E. of triplicate determinations (from Ref. 36).

TABLE III. Effect of cycloheximide treatment on arylamine and arylalkylamine NAT activities in the ovine pineal gland at night

Substrate	NAT activity (nmol/min./mg protein)	
	Control Night	Cycloheximide
Tryptamine	0.71 ± 0.07	0.06 ± 0.02
Phenylethylamine	0.66 ± 0.07	0.075 ± 0.08
p-Phenetidine	0.42 ± 0.11	0.35 ± 0.11
Aniline	0.12 + 0.05	0.10 ± 0.03

Sheep received cycloheximide (4 mg/kg) or saline 30 minutes before lights off (1800 hours) and were killed in the dark 30 minutes after lights off. NAT activities were measured with 0.5 mM AcCoA and 10 mM amine substrate. Results are expressed as the mean ± S.E. of triplicate determinations (from Ref. 50).

B. Stability

During the early stages of these investigations we found that arylamine NAT activity in the rat pineal homogenate was almost completely lost on storage overnight in the cold. This prompted us to compare the inactivation of arylamine and arylalkylamine NAT in the broken cell preparations in the cold. We found that the activity of arylamine NAT from both rat and sheep pineal glands decreased more quickly at 4°C than arylalkylamine NAT. Complete loss of arylamine NAT activity occurred after 24 hours in the rat and over 95% of the activity disappeared after 48 hours in sheep (Fig. 3). The corresponding inactivation of arylalkylamine NAT was 50% in the rat and 25% in sheep (Fig. 3).

Arylalkylamine NAT is more unstable at 37°C in broken cell preparations; about 90% of the activity is irreversibly lost in about 30 min. (40). It is not known if arylamine NAT also undergoes a similar inactivation at 37°C. Acetyl CoA and polyanions protect arylalkylamine NAT from this inactivation whereas disulfides such as cystamine and insulin, accelerate it (38-41). The rate of inactivation is reduced considerably in partially purified preparations of the enzyme suggesting

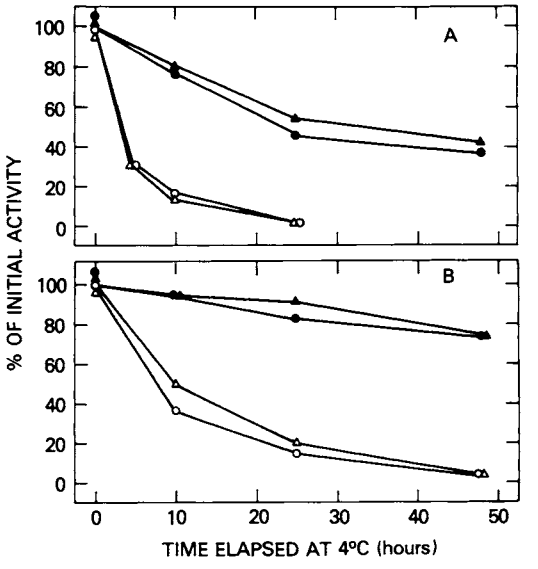

Fig. 3. Time course of inactivation (4°C) of pineal arylamine and arylalkylamine NATs. Samples of pineal supernatant preparations (10,000 xg) were stored at 4°C for the indicated time and assayed with 0.5 mM AcCoA and 10 mM amine substrate (from Ref. 50).

that some soluble factors, perhaps disulfides, present in the pineal supernatant are involved in the inactivation. However, our attempts to prevent the inactivation by treating the preparation with thiols has not been successful. Thus, the mechanism of this inactivation remains unclear.

C. Molecular size

Proof that the pineal gland contains separate arylamine and arylalkylamine NATs has come from the demonstration that these enzymes can be resolved using size exclusion HPLC. Chromatography of the 100,000 xg supernatant from isoproterenol treated rat pineal glands reveals that arylalkylamine NAT activity is present in two peaks (Fig. 4A). One contained molecules with an apparent molecular weight of about 100,000 and accounted for 75% of the recovered activity. The second peak contained molecules with an apparent molecular weight in the 10,000 to 20,000 range. Arylamine NAT activity was detected in a single peak of intermediate molecular size (Mr = 35,000). Recovery of activity was about 75% in both cases. As observed in the rat, size exclusion HPLC of the night sheep pineal gland supernatant also showed clear separation between arylamine and arylalkylamine NAT activities (Fig. 4B). However, the elution pattern of ovine arylalkylamine NAT activity was slightly different from that of the rat pineal preparation; the higher molecular weight peak contained 90% of the recovered activity; a distinct lower molecular weight peak was not apparent. As in the case of the rat pineal preparation, the arylamine NAT was detected in a single molecular form with an apparent molecular weight of about 35,000.

V. MULTIPLE FORMS OF ARYLALKYLAMINE NAT

An interesting characteristic of arylalkylamine NAT is that it can exist in different molecular forms depending on the ionic environment (42, 43, 50, 51). Three distinct forms of arylalkyamine NAT are detected during size exclusion chromatography using Sephacryl S-200 (low pressure) as well as using TSK 3000 columns (high pressure). In the presence of ammonium acetate (0.1 M, pH 6.5), DTT (10mM) and BSA (0.1 mg/ml), the enzyme is separated into two molecular forms as described above. However, in presence of sodium citrate (0.1 M, pH 6.5) containing DTT (10 mM) and BSA (0.1 mg/ml), it is detected in a single molecular form of intermediate molecular size (Mr = 30,000).

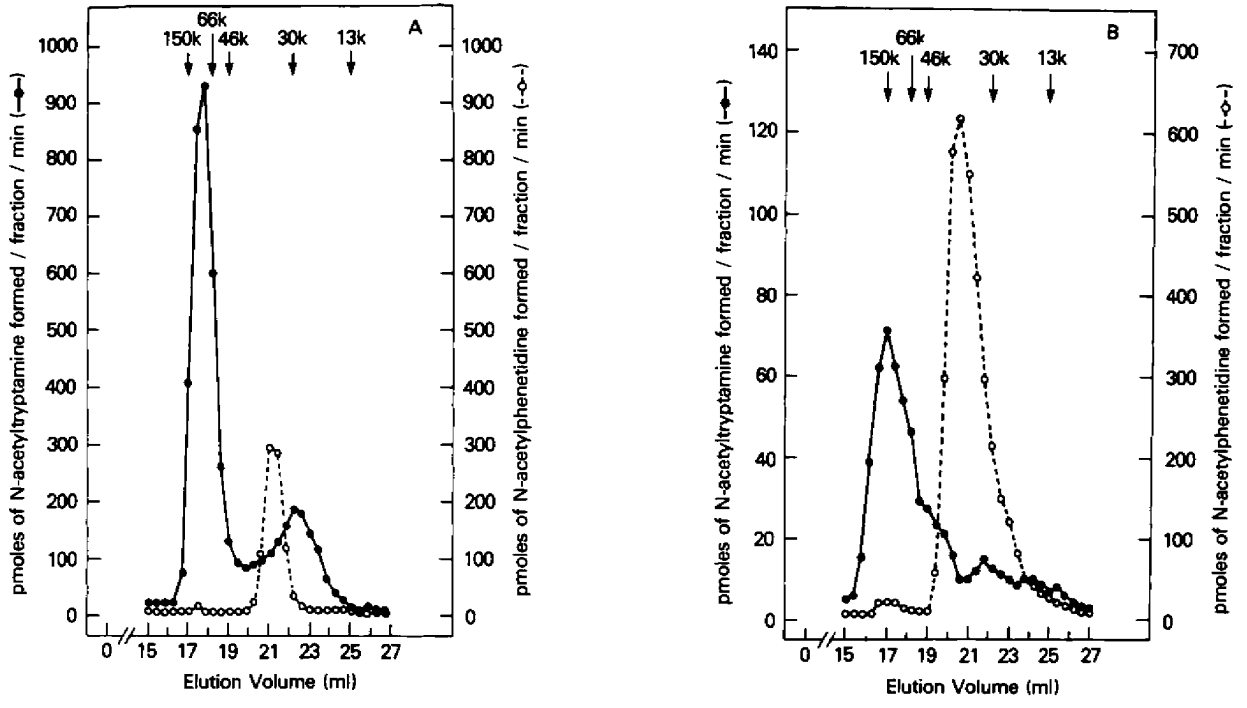

Fig. 4. Size exclusion HPLC profiles of arylamine and arylalkylamine NATs from rat and sheep pineal glands. The supernatants (100,000 xg) from rat (A) and sheep (B) pineal homogenates were chromatographed. The fractions were assayed with 0.1 mM AcCoA and either 10 mM tryptamine or 3 mM p-phenetidine. Upper arrows indicate molecular weight markers: γ-globulins, 150,000; bovine serum albumin, 66,000; ovalbumin, 46,000; carbonic anhydrase: 30,000; cytochrome c, 13,000 (from Ref. 50).

Another interesting observation is that the ratio of the two forms of the enzyme obtained on chromatography in the presence of ammonium acetate is changed after purification of the enzyme (42,50). The small molecular weight form becomes the predominant species after purification of the enzyme using chromatography based on disulfide exchange principles (42,51). In an earlier report Morrissey et al. have reported that rat pineal NAT in the 100,000 xg supernatant can be separated into two forms (Mr = 39,000 and Mr = 10,000) on Sephadex G100 chromatography in the presence of potassium phosphate (50 mM, pH 6.5) containing 4 mm mercaptoethylamine (52). Our results indicate that the multiple forms of the enzyme depend on the ionic environment whereas the results of Morrissey et al, seem to indicate that interaction between sulfhydryl groups is involved in this process.

The important variable in the ionic environment is not ionic strength. An increase in the concentration of ammonium acetate from 50 mM to 400 mM does not convert the Mr = 100,000 and Mr = 10,000 forms of the enzyme to the Mr = 30,000 form, whereas treatment with sodium citrate does (Fig. 5). Independent of these effects on molecular size are effects on activity. Increasing the ionic strength of buffers by adding sodium chloride, sodium citrate, sodium phosphate, ammonium acetate, potassium chloride or ATP causes as much as a 5-fold increase in enzyme activity (41). Thus, we believe there are two independent effects of ions on NAT. All salts appear to activate the enzyme in a dose-dependent manner, perhaps through a mechanism altering the physical characteristics of the active site environment. In contrast, it appears that certain salts which stabilize the enzyme, including sodium citrate, can convert the enzyme from the Mr = 100,000 and Mr = 10,000 forms to the Mr = 30,000 form; other salts including ammonium acetate can convert the Mr = 30,000 form to the Mr = 100,000 and Mr = 10,000 forms. One obvious difference between these salts is that one is monoionic and the other is polyionic. Perhaps this is the critical difference which determines the molecular form of arylalkylamine NAT. It is also possible that the apparent sensitivity of NAT to differences in salt composition may influence the intracellular form, activity and stability of this enzyme.

VI. MULTIPLE AROMATIC AMINE NATS IN OTHER TISSUES

There is now convincing evidence indicating that multiple NATs having narrow specificity exist in different tissues. These have been studied extensively in liver in relation to

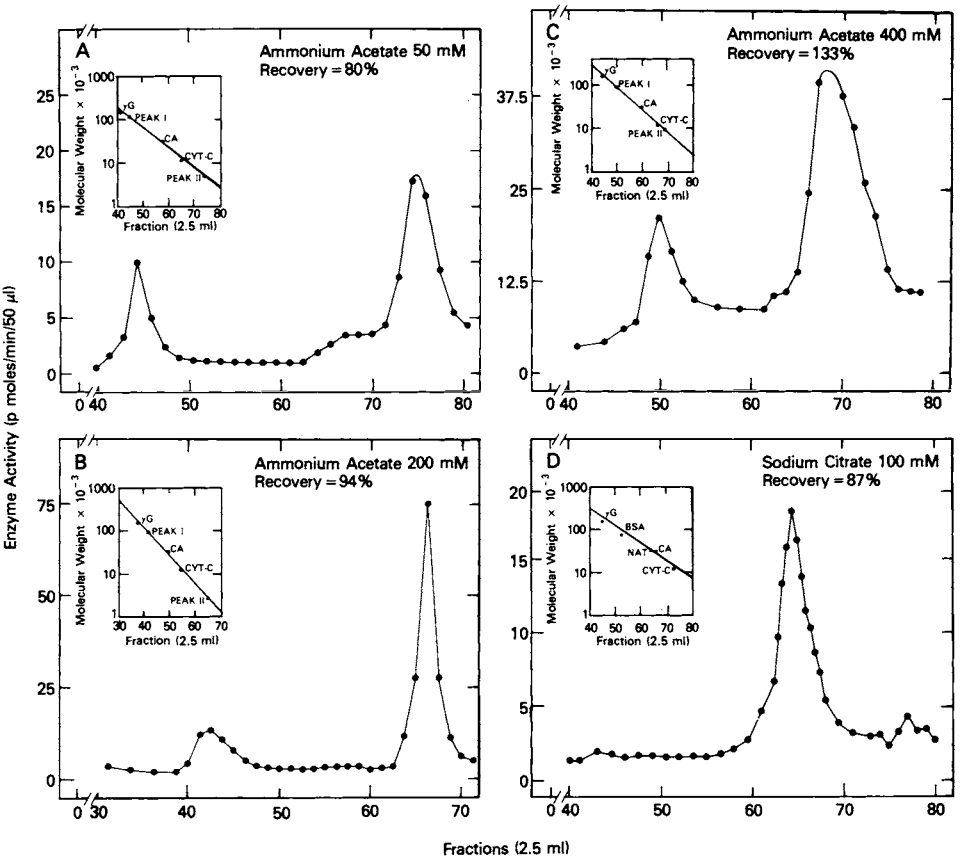

Fig. 5. Size exclusion chromatography of NATs using sephacryl S-200 under different ionic conditions. Four S-200 columns were equilibrated with four different buffers namely, ammonium acetate buffer, pH 6.5, 50 mM (1.5 x 100cm), 200 mM (1.5 x 95cm) and 400 mM (1.5 x 110cm), and sodium citrate buffer, pH 6.5, 100 mM (1.5 x 110cm), all containing DTT (10 mM) and BSA (0.1 mg/ml). A 2.5 ml enzyme sample containing BSA (1 mg/ml) was applied to each column, the fractions were collected (flow rate 5 ml/h), and assayed for enzyme activity. The columns were calibrated using three proteins, namely γ-globulin (Mr = 150,000, ^{14}C-methylated), carbonic anhydrase (Mr = 30,000, ^{14}C-methylated), and cytochrome C (Mr = 12,400). Samples containing the three proteins (2.5 ml, 5 mg/ml) were applied to the columns and the fractions were collected. Cytochrome C was detected by absorption (400 nM) and the other two by radioactivity (from Ref. 42).

genetic polymorphism in drug acetylation. In rabbit liver, the most extensively studied system to date, two types of NAT activity can be distinguished. One type acetylates drugs such as DDS and INH, whereas the other acts on p-aminobenzoic acid (PABA) with high specificity; only the former exhibits genetic polymorphism with the rapid and slow acetylator phenotypes (5). Recent experimental evidence indicates that both these activities are located on the same protein (53). A similar pattern exists in mouse liver (54). However, in hamster liver the pattern appears to be exactly opposite; the PABA NAT activity exhibits polymorphism while the INH NAT activity remains unchanged in the various individuals (55). Recently, it has been possible to separate these two activities by ion exchange chromatography, showing that they represent distinct molecular forms (56). Preliminary studies in the rat indicate that its NAT pattern in the liver may be similar to that in the hamster (5).

Blood and brain NAT have also been studied in some detail. The blood enzyme is unusual because it acetylates PABA with very high specificity (57). The blood enzyme exhibits genetic polymorphism in mouse and hamster but fails to do so in rabbit, although the general properties of the enzyme are comparable in all the three species (5). The brain enzyme has been studied in some detail only in the rat. Substrate specificity and inhibition studies suggest that it is similar to the liver enzyme rather than to the pineal enzyme (58-59). It is not clear if it exhibits genetic polymorphism.

Genetic regulation of aromatic amine NATs has been studied primarily in mice and hamster using inbred strains. The results of genetic analysis of liver and blood NAT activities are consistent with simple autosomal Mendelian inheritance of two co-dominant alleles at a single locus (60-61). These studies have also demonstrated the existence of minor modifying genes that segregate independently of the major NAT gene (60). Based on these results, it is tempting to speculate that the multiple NATs in different tissues are expressed from a single gene. It is possible that post transcriptional and/or post translational modifications play important roles in the formation of the multiple NATs. Thus, post translational modification, perhaps controlled by the modifying genes, may be involved in the slow and rapid acetylator phenotypes in the rabbit and mouse liver. The qualitative differences between the NAT activities of slow and rapid acetylator phenotypic rabbits combined with the lack of quantitative changes between them in the amount of the enzyme protein, determined using immunological methods, support this view (62-63). Post transcriptional modifications, perhaps involving differential splicing, may explain the different molecular forms of NAT in the pineal gland and hamster liver. Finally, it is also

possible that the multiple separable NATs in the pineal gland and hamster liver are altogether different gene products. These possibilities can be tested when appropriate monoclonal antibodies and cDNA probes become available.

VII. CONCLUSION

The pineal gland contains at least two types of NATs which can act on aromatic amines. One type acetylates arylalkylamines, such as serotonin, tryptamine and phenylethylamine, while the other acts on arylamines, such as aniline and phenitidine. The presence of these two enzyme activities in the pineal gland can be considered an evolutionary adaptation to generate the circadian rhythm in melatonin, without affecting the "house keeping" metabolism of arylamines. Therefore, one would predict that a similar enzyme system is present in other tissues exhibiting melatonin rhythm, such as the retina. Understanding the molecular mechanisms involved in the tissue specific expression of NATs in general and pineal NATs in particular is the next important step in this line of investigation.

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LOCALIZATION OF MELATONIN SYNTHESIS AND BINDING IN THE VERTEBRATE RETINA[1]

Allan F. Wiechmann[2]
Department of Anatomy
UCLA School of Medicine
Los Angeles, California

Dean Bok
Departments of Anatomy and Ophthalmology
UCLA School of Medicine
Los Angeles, California

Joseph Horwitz
Department of Ophthalmology
UCLA School of Medicine
Los Angeles, California

I. INTRODUCTION

Prior to 1965, most investigators assumed that the melatonin synthesizing enzyme, hydroxyindole-O-methyltransferase (HIOMT), was unique to the mammalian pineal gland. This assumption was altered when Quay (1965) reported the discovery of HIOMT in the retinas and pineals of several nonmammalian vertebrates (fish, amphibians, reptiles and birds). Cardinali and Rosner (1971 a,b) later reported that

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[2]Present address: Department of Ophthalmology, Baylor College of Medicine, Houston, Texas.

HIOMT activity was present in the mammalian retina. HIOMT could be detected in the retina as early as the 17th day of gestation, whereas no HIOMT activity was found in the rat pineal until the 12th postnatal day. Ocular HIOMT activity increased to the 30th postnatal day and then leveled off. These authors also demonstrated that radioactive serotonin was converted to melatonin in rat retinas.

Baker et al. (1965) showed that most of the HIOMT activity in the developing Xenopus laevis larvae was in the lateral eyes. HIOMT activity in the mouse eye peaked during the first week postpartum, fell during the following week, then gradually rose to adult levels during the following weeks (Smith and Baker, 1974). HIOMT activity was detected in the retinas of 15 day chick embryos (Wainwright, 1979), and rose steadily to adult levels. The most rapid increase in HIOMT activity was observed to occur immediately prior to hatching.

Cardinali and Wurtman (1972) compared the biochemical properties of HIOMT activities in pineal gland, retina, and harderian gland of the rat. Based on enzyme kinetics, they suggested that the pineal and retinal HIOMTs were closely related enzymes, whereas the harderian gland HIOMT may have a different substrate specificity. Nagle et al. (1972) reported that the highest ocular HIOMT activity was during the light period, which was in opposition to the pineal HIOMT activity. Constant light suppressed HIOMT activity after several days, and animals exposed to constant darkness still presented cyclic changes in retinal and pineal HIOMT activity. A timing shift occurred in retinal HIOMT activity during constant darkness, in which the maximal retinal HIOMT activity coincided with that of the pineal gland.

Cardinali et al. (1972) demonstrated that exposure of rats to continuous green light for 17 days resulted in almost total loss of pineal HIOMT, and in stimulation of retinal HIOMT. Since long term exposure of rats to similar intensities of green light has been shown to promote photoreceptor degeneration (Noell et al., 1966), the authors suggested that HIOMT is not present in retinal photoreceptors. No histological examination was performed on these tissues, however.

Retinal HIOMT activity was subsequently studied and characterized in duck (Cardinali and Rosner, 1972), frog (Eichler and Moore, 1975), trout (Gern et al.,1978a, 1984) and hamster (Pevet et al., 1980). No day-night rhythm of retinal HIOMT acivity was detected in frog (Rana pipiens), trout, or hamster.

An unusual study was recently performed by Cremer-Bartels et al. (1983). They subjected birds to variations of low magnetic field strength, and observed an effect on retinal and pineal HIOMT activity. They also observed an influence of

magnetic field variation on human night vision acuity. The authors concluded that magnetic field sensitivity is present in avian and human retinas, and suggested that inhibition of methoxylation of indolamines (HIOMT activity) is involved in the magnetic field sensitivity in humans.

Melatonin and N-acetylserotonin immunofluorescence was first observed by Bubenik et al. (1974) in the retina, pineal gland and cerebellum of the rat. Since the melatonin antibody cross reacted completely with N-acetylserotonin, no distinction could be made between melatonin immunoreactivity and N-acetylserotonin immunoreactivity. This N-acetylindole immunofluorescence was localized to the outer nuclear layer of the retina, which is the layer containing the photoreceptor nuclei. Later, using a more specific antibody to melatonin, Bubenik et al. (1976) observed melatonin immunofluorescence in the outer nuclear layer of the retina, optic nerve, optic chiasma, suprachiasmatic nucleus, and the optic tract of the rat. Using a quantitative fluorescence method, Bubenik et al. (1978) reported that the amount of melatonin immunoreactivity in the outer nuclear layer of the retina was higher during the dark phase than during the light phase. Vivien-Roels et al., (1981) observed melatonin immunoreactivity in the outer nuclear layer of fish, tortoise and hamster. Some immunofluorescence was occasionally observed in the inner nuclear layer.

Using a specific melatonin radioimmunoassay, Pang et al. (1976, 1977) observed detectable levels of melatonin in the pineal, retina and brain of rats and chickens. Pang et al. (1980b) later demonstrated a diurnal rhythm of melatonin in the rat retina, with peak levels during the dark period. The diurnal rhythm of N-acetylserotonin in the rat retina was very similar to the melatonin rhythm, except that the overall levels were slightly higher (Pang et al., 1981). Pang et al. (1983) also observed that the pineal, serum, retina and brain of quails, pigeons and chickens showed diurnal rhythms of melatonin, with peak levels at night. Diurnal rhythms of N-acetylserotonin were observed only in the pineal gland and retina. Reiter et al. (1981) observed a very dramatic nocternal rise in ground squirrel pineal melatonin levels measured by radioimmunoassay, but did not observe a statistically significant rhythm in melatonin content in the retina. Recently, melatonin has been detected in the human retina by radioimmunoassay (Osol and Schwartz, 1984) and gas chromatography mass spectrometry (Leino, 1984), and in bovine retina by high pressure liquid chromatography (Hall et al., 1985)

Gern and Ralph (1979) used organ cultures of whole trout retinas to show that the retina was capable of synthesizing melatonin from radioactive serotonin. The radioactive product

was identified as melatonin by its binding to a specific melatonin antibody. The authors suggested that retinally synthesized melatonin was released into the circulation, since plasma melatonin levels were lower in bilaterally enucleated animals, than in intact animals. Experimental (sham-operated) controls, however, showed no statistically significant difference. Several studies have shown that pinealectomy does not completely abolish the occurrence of melatonin in the serum of rats (Ozaki and Lynch, 1976), turtles (Owens et al., 1978) and trout (Gern et al., 1978b). Radioimmunoassay of plasma melatonin levels in alligators revealed that these animals have circulating melatonin even though they lack a pineal organ (Roth et al., 1980). These studies lend some support to the hypothesis that the retina secretes melatonin into the circulation.

Yu et al. (1982) examined the effect of light and darkness on the release of N-acetylserotonin and melatonin by cultured guinea pig retinas. After 12 hours of light or darkness, N-acetylserotonin and melatonin was extracted from the medium and measured by radioimmunoassay. The release of N-acetylserotonin and melatonin by retinas incubated in the dark were higher than those in the light. The authors suggested that this study supported the hypothesis that the retina may be an important extrapineal source of melatonin in the circulation.

In 1979, Binkley et al. reported the presence of N-acetyltransferase (NAT) in the retinas of chick, rat, sparrow and hamster. They used a radioenzymatic assay to show a diurnal rhythm of retinal NAT, with highest levels during the dark period. Constant light suppressed NAT activity, whereas NAT levels remained high in constant darkness. These authors observed that the neural retina and pigment epithelium of the chick retina contained approximately equal amounts of NAT activity. Hamm and Menaker (1980) demonstrated a circadian rhythm of NAT activity in the chick retina, with peak levels during the dark period. Using a melatonin radioimmunoassay, they showed that retinal melatonin levels had a daily fluctuation similar to NAT. Bright light given during the dark period, when NAT activity is highest, caused NAT activity to decrease to daytime levels within one hour. The rhythm of NAT activity and melatonin levels persisted in constant darkness, suggesting that there is a circadian modulation of melatonin synthesis that is not dependent upon cyclic light. The pigment epithelium contained much less NAT activity than did the neural retina, and did not show a day-night change. After pinealectomy, NAT activity persisted in both a light-dark cycle and in constant darkness. This suggested that melatonin is rhythmically synthesized in the chick retina by NAT, rather than taken up from the circulation.

Recently, Iuvone and Besharse (1983) reported a very similar circadian rhythm of NAT activity in Xenopus laevis. The rhythm of NAT activity fluctuated on a diurnal rhythm, with peak levels at night. The NAT rhythm was suppressed with constant light, but persisted for up to three days in constant darkness, suggesting that retinal NAT activity occurs as a circadian rhythm that is entrained by light and dark. NAT activity was observed only in the neural retina; none was in the pigment epithelium-choroid. The dark-induced rise in NAT activity was blocked by protein synthesis inhibitors in vitro. Cyclic AMP analogs administered during the light period caused a rise in NAT activity. These results suggested that the increase in NAT activity during the dark period is dependent on synthesis of new protein, and that the induction of NAT in the retina is regulated by a cyclic AMP-dependent mechanism. Besharse and Iuvone (1983) also demonstrated that an endogenous circadian rhythm of NAT activity was sustained in vitro, and could be entrained to a new lighting schedule. The authors suggested that the Xenopus retina should be regarded as the locus of a circadian clock.

Since light-induced photoreceptor damage is more severe in sexually mature rats than in immature rats (O'Steen et al., 1974), some investigators thought that pituitary or gonadal hormones may influence the photoreceptor resistance to the damaging effects of light. O'Steen and Kraeer (1977) showed that light-induced photoreceptor destruction was reduced by hypophysectomy. Injection of pituitary homogenates or prolactin reversed this protection from photic damage. The authors concluded that hypophyseal hormones appear to have a regulatory influence on the severity of light-induced retinal photoreceptor damage in the rat. Pinealectomized rats showed less light-induced photoreceptor damage than did sham-operated rats (Rudeen and O'Steen, 1979). This suggested that a pineal hormone, possibly melatonin, may affect the susceptibility of the retina to photic damage. In 1980, Bubenik and Purtill examined the effects of melatonin on retinal damage induced by continuous illumination. Melatonin-treated rats showed more intense retinal damage than the control animals. Rats treated with bromocriptine, a stimulator of dopamine receptors, showed less light-induced retinal damage than controls. Bromocriptine was also reported to reduce degeneraton in the outer nuclear layer of rats with inherited retinal degeneration (Royal College of Surgeons rat). Since melatonin has been observed to to induce pigment granule aggregation in the retinal pigment epithelium (Pang and Yew, 1979), it has been postulated that the action of exogenously applied melatonin might increase the damaging effects of light by reducing the amount of protective pigment surrounding the photoreceptors.

Nagle et al. (1973) demonstrated that pinealectomy increased retinal HIOMT activity in rats, and abolished the diurnal fluctuations in retinal HIOMT. The effect of pinealectomy on retinal melatonin levels in the rat was studied by Yu et al. (1981). A diurnal rhythm of melatonin in the retina persisted one week after pinealectomy. An increase in retinal melatonin levels was observed one month after pinealectomy, indicating a compensatory rise in melatonin synthesis in the retina after removal of the pineal gland. The authors suggested that melatonin biosynthesis in the retina may be modulated through a negative feedback system. Reiter et al. (1983) showed that peak retinal melatonin levels were increased in the rat following pinealectomy. Gonadectomy abolished the peak retinal melatonin levels. These observations supported the findings of Yu et al. (1981), and also suggested that gonadotrophins or gonadal hormones may influence retinal melatonin synthesis.

In 1976, LaVail demonstrated that a burst of rod outer segment shedding occurs in the retina after the onset of light, and is blocked by reserpine, a drug that interferes with the pineal circadian rhythm by depletion of norepinephrine in afferent nerve terminals. LaVail suggested that humoral factors may therefore influence disc shedding, but required more definitive experiments. The effect of pinealectomy on photoreceptor outer segment disc shedding was subsequently studied in several laboratories (LaVail and Ward, 1978; Currie et al., 1978, Tamai et al., 1978; Goldman, 1982). All laboratories reported that pinealectomy was without effect on disc shedding. These findings, however, do not preclude the possibility that endogenously synthesized retinal melatonin may be involved in disc shedding.

Cohen et al. (1978) provided evidence for a cytoplasmic melatonin receptor in the hamster eye. Melatonin binding was saturable and specific, and appeared to be comprised of high and low affinity binding sites. The binding kinetics, however, were performed on ovarian tissue, in which binding was also detected. The authors did not state if they analyzed tissue fractions other than the cytosol for melatonin binding. Bubenik et al. (1978) reported that, after application of exogenous melatonin, higher levels of melatonin immunoreactivity were observed in the outer nuclear layer of the rat retina, suggesting that some kind of uptake mechanism for melatonin is present in the retina.

Gern et al. (1981) demonstrated saturable, specific binding of melatonin in the membrane-cytosol fraction of trout retinal pigment epithelium-choroid. Some melatonin binding by the trout neural retina was mentioned, but no data was shown for this observation. The authors suggested that, although a melatonin receptor may truly exist in the neural retina, the

observed binding may be artifact due to contamination by the pigment epithelium. Ehinger and Floren (1978) did not observe active uptake for melatonin in the rabbit retina. The incubation times used by Ehinger and Floren, however, were of a much shorter duration than those used by Cohen et al. (1978) and Gern et al. (1981).

The results of the studies discussed so far suggest that melatonin is synthesized in the retina, and perhaps has a physiological role within the retina. An increasing number of studies have suggested that melatonin may indeed be involved in retinal physiology.

In 1965, Kraus-Ruppert and Lembeck reported that the pigment granules of the retinal pigment epithelium (RPE) in isolated eyes of dark-adapted Rana pipiens disperse upon exposure to light. The pigment granules in isolated light-adapted eyes show some contraction when brought into darkness. These results are similar to the in vivo response of pigment granule migration to light and dark, as discussed by Walls (1942). Melatonin administration in vitro in the light, or injection of melatonin into light-adapted frogs mimics dark-induced pigment granule aggregation (Kraus-Ruppert and Lembeck, 1965).

Cheze and Ali (1976) observed no effect of pinealectomy or optic nerve transection on RPE melanosome migration in fish. They did observe, however, that intraocular injection of melatonin caused aggregation of melanosomes within the RPE. Injection of serotonin was without effect. In a similar study using guinea pigs, Pang and Yew (1979) demonstrated that ocular perfusion, as well as intraocular and intracarotid injections of melatonin induced melanosome aggregation within the RPE and choroid. Pang et al. (1978) had previously demonstrated that the melanosomes of the guinea pig RPE-choroid responded to changes in illumination.

To determine if an endogenously synthesized retinal compound such as melatonin could influence RPE melanosome migration, Flight (1979) performed a very simple but interesting experiment on the retina of Xenopus laevis. The neural retina was separated from the RPE, and was placed in suitable medium in darkness. A piece of tail fin of a Xenopus larvae, of which the melanosomes were dispersed (light-adapted), was placed at the photoreceptor surface of the frog neural retina. A rapid and complete aggregation of melanosomes occurred in the Xenopus tail fin. Melatonin is known to have a potent melanosome-condensing effect on the tail fin of Xenopus larvae (Bagnara and Hadley, 1970). This experiment indicated that a pigment-aggregating substance was secreted from the neural retina, which reached the melanophores through the medium.

Homogenates of chick retina were observed to lighten Rana pipiens tadpoles (Mull and Ralph, 1972). After characterization with column chromatography and thin-layer chromatography, the active compound was tentatively identified as melatonin. A recent study by Krasovich and Benson (1983) reported that intraocular injection of melatonin induces melanosome aggregation in RPE of the hamster. From these studies, it appears that endogenously sythesized retinal melatonin may have a site of action on the melanosomes of the RPE-choroid. During the dark period, when melatonin levels are high in the retina, melatonin may bind to sites on the RPE-choroid, to induce aggregation of pigment granules away from the photoreceptor outer segments to allow light to sensitize more photoreceptors. During the light period, the reduced levels of melatonin in the retina may allow the RPE melanosomes to disperse into the apical microvilli to protect the photoreceptor outer segments from light damage.

It has been suggested that, because of the cyclic nature of melatonin synthesis and photoreceptor outer segment disc shedding, ocular melatonin might possibly be involved in the shedding process. White and Fisher (1980) observed that melatonin implants increased the frequency of outer segment disc shedding during the shedding peak in rats. Using an *in vitro* eye cup preparation of Xenopus laevis, Besharse and Dunis (1982) reported that outer segment disc shedding occurred in a non-permissive (low bicarbonate) medium in the presence of colchicine (a microtubule synthesis inhibitor). The authors suggested that colchicine activates a cytoskeletal process which is normally involved in the control of disc shedding. It is interesting to note here that several studies have suggested that melatonin may have an effect on microtubule formation (Banerjee *et al.*, 1972; Banerjee and Margulis, 1973; Malawista, 1973; Freire and Cardinali, 1975; Cardinali and Freire, 1975), which prompted Besharse (1982) to suggest that melatonin may affect microtubules in a manner similar to colchicine.

Besharse and Dunis (1983) demonstrated that melatonin, 6-chloromelatonin, and 5-methoxytryptophol all activate photoreceptor outer segment disc shedding in isolated light-adapted Xenopus eye cups in both permissive and non-permissive medium. Ogino *et al.* (1983) reported that melatonin inhibited phagocytosis of polystyrene latex beads in cultured chick RPE, at a concentration of 10^{-16} M. Cyclic AMP inhibited phagocytosis at 10^{-11} M. The authors suggested that melatonin is an intercellular signal for stopping phagocytosis.

Dubocovich (1983) demonstrated that melatonin inhibits the calcium-dependent release of dopamine from the isolated rabbit retina. This suggested that melatonin could play a role in

modulating the activity of dopamine-containing neurons in the retina. Recently, Dubocovich (1984) showed that the melatonin-induced inhibition of dopamine release from the chicken retina is antagonized by N-acetyltryptamine. Since N-acetyltryptamine competitively antagonizes the effect of melatonin (Heward and Hadley, 1975), this study suggests that N-acetyltryptamine and melatonin bind to the same receptor in the retina; melatonin exerting a physiological response, and N-acetyltryptamine blocking that response.

A considerable amount of data has accumulated during the past decade which suggest that melatonin is involved in some aspects of retinal physiology. The results of these studies are difficult to interpret without a knowledge of the cellular location of retinal melatonin synthesis, and its site of action within the retina. Even though the outer nuclear layer demonstrates melatonin immunoreactivity, it is not clear whether the presence of melatonin at that site is a result of melatonin synthesis or uptake. Since melatonin exerts physiological effects within the retina, a receptor for melatonin must be present to mediate these responses. Unambiguous demonstration of the sites of melatonin synthesis and uptake in the retina is necessary for an understanding of the regulation and functional role of melatonin in the vertebrate retina.

II. MATERIALS AND METHODS

A. Preparation of HIOMT Antibody

The methods used to prepare antibodies to HIOMT were a modification of those used by Kuwano and Takahashi (1978) and have been described in detail previously (Wiechmann et al., 1985). Bovine pineal glands (Pel Freez; Rogers,AR) were homogenized in five volumes of 0.2 M sucrose, 0.02 M potassium phosphate pH 7.3, with or without proteolytic inhibitors. The homogenate was centrifuged at 105,000g for one hour at 4°C, then the pellet was discarded. The supernatant (pineal extract) was used to immunize rabbits. Bovine brains and retinas were obtained from a local slaughterhouse, and extracts prepared in the same manner as described for the pineal.

Bovine pineal extract (containing 5 mg protein) was emulsified with an equal volume of Freund's complete adjuvant (Difco; Detroit,MI), and injected subcutaneously into New Zealand white rabbits every two weeks. Rabbits were bled from

the ear vein, the blood was clotted and then centrifuged at 35,000g for 30 minutes at 4°C, and the antiserum decanted and frozen.

CNBr-activated Sepharose 4B (Pharmacia; Piscataway,NJ) was swollen with 1mM HCl, then suspended in two volumes (40 ml) of 0.02 M sodium bicarbonate pH 9.5. Pineal extract (30 ml) dialyzed against 0.1M sodium bicarbonate, 0.5 M NaCl pH 8.5, was added to the gel suspension. After coupling for 24 hours at 4°C, the gel was filtered and resuspended in 0.38 M glycine, 100 mM Tris pH 8.3 for 4 hours at 4°C to bind unreacted groups. The gel was washed and resuspended in 0.15 M potassium phosphate, 0.5 M NaCl pH 7.3 and packed into a 1.5x20cm column.

Brain-Sepharose was prepared in the same manner as described above. Dialyzed brain extract (200 ml) was coupled to 70 ml swollen gel suspended in 130 ml 0.2 M sodium bicarbonate pH 9.5. The washed gel was packed into a 2.5x30cm column.

Antiserum was dialyzed against 0.1 M Tris HCl pH 7.4, and applied to a QAE-A50 Sephadex (Pharmacia) column (1.5x11cm), which had been equilibrated with the same buffer. The eluted immunoglobulin fraction was concentrated and dialyzed against 0.02 M potassium phosphate, 0.15M NaCl pH 7.2 (PBS) on a MicroProDiCon apparatus (Bio-Molecular Dynamics; Beaverton, OR), and was then applied to the pineal-Sepharose column and washed with 0.15 M potassium phosphate, 0.5 M NaCl pH 7.3 until all the unbound protein had washed through the column. The bound anti-pineal antibodies were eluted with 0.2 M glycine-HCl, 0.5 M NaCl pH 2.4 buffer, and were concentrated and dialyzed against PBS on a MicroProDiCon apparatus. The anti-pineal antibodies were then applied to the brain-Sepharose column. The first immunoglobulin peak was collected and concentrated as described above.

B. Biochemical Analysis of HIOMT Antibody

The proteins of bovine pineal and retina extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), and thereafter electrophoretically transferred to a nitrocellulose sheet (Schleicher and Schuell; Keene,NH) (Towbin et al., 1979). The nitrocellulose sheet was incubated with the purified antibody (first peak from the brain-Sepharose column), and subsequently labeled with goat-anti rabbit IgG conjugated to horseradish peroxidase (Bio-Rad). After reaction with the HRP color development reagent (Bio-Rad), the antigen-antibody complexes became visible. Nonimmune rabbit IgG was used as a control to eliminate the possibility of non-specific interactions.

To demonstrate that the purified antibody was directed against HIOMT, immunotitration was performed with the pure antibody. Constant amounts of the bovine pineal extract (20 µl) were incubated with increasing amounts of purified antibody (0-35 µl , 0.7 mg/ml). Appropriate amounts of normal rabbit IgG (Cappel; Malvern,PA) were added to the tubes to result in a constant amount of gamma globulin in each tube. This mixture was incubated for 45 minutes at 37°C, then for seven hours at 4°C. Goat anti-rabbit gamma globulin (GARGG, 27.0 mgs, E-Y Laboratories; San Mateo,CA) was added to each tube, and incubated overnight at 4°C to precipitate the antigen-antibody complex. The samples were centrifuged at 4000g at 4°C for 30 minutes to pellet the antigen-antibody complex, and the supernatants were measured for HIOMT activity by radioenzymatic assay.

Purified HIOMT antibodies were coupled to CNBr-activated Sepharose 4B according to the method described by Kuwano et al.(1978). Pineal and retinal extracts were applied to the antibody-Sepharose immunoadsorbant column, washed with PBS, and the bound proteins were eluted with 0.2 M glycine HCl, 0.5 M NaCl pH 2.4. SDS-PAGE was performed on the proteins as described previously. HIOMT activity was measured by a modification of the method of Axelrod et al.(1965). Samples were incubated with 50 ug N-acetylserotonin (Sigma) and 2 nmoles ^{3}H-S-adenosylmethionine (73 mCi/mmole, Amersham; Arlington Heights,IL) in a final volume of 0.3 ml. After 30 minutes of incubation at 37°C, the reaction was terminated by the addition of 1 ml of 0.2 M borate buffer pH 10, followed by 8 ml of chloroform. The aqueous phase was removed by aspiration, and the organic phase washed with 1 ml of buffer. The aqueous phase was removed and the chloroform was evaporated to dryness, and redissolved in 1 ml of ethanol. The radioactivity measured by liquid scintillation spectrometry.

Peptide mapping was performed on the purified pineal 39,000 mol.wt. subunit and the retinal 25,000 mol.wt. subunit by the limited proteolysis method of Cleveland et al.(1977). Briefly, 100 ng of the purified subunits were incubated with 2 ug of Staphylococcus aureus V8 protease (Miles; Elkhart,IN) in 20 mM Tris-HCl pH 7.9 for one hour at room temperature. The reaction was stopped by addition of SDS and beta-mercapto-ethanol to final concentrations of 2% and 10%, respectively, and boiling of the samples for 5 minutes. The samples were loaded onto a 12.5% polyacrylamide gel and the peptide fragments were separated by SDS-PAGE, and silver stained.

C. HIOMT Immunocytochemistry

Tissues were immersion fixed overnight at 4°C in Perfix (Fisher; Pittsburgh,PA), then embedded in paraffin (Paraplast, Ted Pella; Tustin,CA). De-paraffinized sections (10 μm) were treated with blocking solutions, and incubated in either the HIOMT antibody (74 ug/ml) or nonimmune rabbit gamma globulin (74 μg/ml), for two hours at room temperature. After the primary incubation, the sections were rinsed in TBS, and incubated in GARGG (68 μg /ml) for 0.5 hours at room temperature. The sections were rinsed again in TBS, then incubated in peroxidase-anti-peroxidase complex (40 μg/ml, Sternberger-Meyer; Jarrettsville, MD) for 0.5 hours at room temperature. After rinsing in TBS, the sections were incubated in 0.06% diaminobenzidine (Sigma) and 0.03% H_2O_2 in 0.05 M Tris HCl pH 7.4, for 1-5 minutes. The sections were rinsed, and counter stained with Mayers-hematoxylin.

D. ^{3}H-Melatonin Autoradiography

^{3}H-melatonin (2-aminoethyl-2-^{3}H-N-acetyl-5-methoxy-tryptamine; 26.4 Ci/mmole) was purchased from New England Nuclear (Boston,MA), and its radiopurity was checked by thin layer chromatography prior to use. Adult Rana pipiens were sacrificed by decapitation, and the eyes enucleated. The anterior segment and sclera were removed, and the retinas were incubated in 3 ml modified Ringer's solution containing 400 nM ^{3}H-melatonin (10 μCi/ml) with or without 400 μM unlabeled melatonin (Sigma; St.Louis,MO) oxygenated with 95% O_2 and 5% CO_2 at 5°C for one hour. After incubation, the tissue was placed in fresh, nonradioactive incubation medium, and the RPE-choroid was separated from the neural retina. The tissues were frozen in liquid nitrogen-cooled Freon 22 slush (Matheson; Cucamonga,CA), freeze-dried at -50°C, fixed with osmium vapors, and embedded in Spurr resin (Polysciences; Warrington,PA). Tissue sections (0.5 um) were prepared for autoradiography and quantified as decribed previously (Wiechmann et al., 1986).

E. Saturation Binding Assays

Melatonin binding assays were performed by a modification of methods used by Cardinali et al. (1979) and Cohen et al. (1978). Adult Rana pipiens were sacrificed, and neural retina and RPE-choroid isolated as decribed above. The tissues were sonicated at 50 watts for 30 seconds in 10 volumes cold 50 mM

Tris-HCl, 6 mM $CaCl_2$, 0.1% ascorbic acid pH 7.4, then centrifuged at 3000g for 5 minutes at 4°C. The RPE-choroid supernatant was decanted, and the pellet resuspended in assay buffer to yield a melanosome-enriched fraction. The RPE-choroid supernatant was also assayed for melatonin binding. The neural retina membrane-enriched supernatant (nuclei-free) was decanted for use in the binding assay and the neural retina pellet was resuspended in buffer. In some experiments, the neural retina supernatant was centrifuged again at 27,000g for 15 minutes at 4°C, and the pellet resuspended in assay buffer to yield a crude membrane fraction. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (Sigma) as the standard.

Aliquots (4.0 ml) of tissue pellet suspension were incubated in triplicate for 5 hours at 0°C in darkness with 10-1000 nM ^{3}H-melatonin with or without 100 μM unlabeled melatonin. The 3000g tissue pellet incubations were terminated by centrifugation at 3000g for 10 minutes at 4°C. The supernatant was aspirated, and the radioactivity of the pellet was measured by liquid scintillation spectrometry.

Tissue supernanant suspensions were incubated as described above for the tissue pellet suspensions. As a control, a parallel series of tubes containing no tissue suspensions were assayed. At the end of the tissue supernatant incubations, 0.8 mls of dextran-coated charcoal (5% activated charcoal, 2.5% dextran) was added to each tube and allowed to stand at 0°C for 10 minutes to adsorb unbound ligand. The samples were centrifuged at 3000g for 30 minutes at 4°C, then 0.8 ml supernatant was measured for radioactivity with 10 ml Aqueous Counting Scintillant. Specific melatonin binding was calculated as the difference between the total ^{3}H-melatonin bound and the ^{3}H-melatonin bound in the presence of 100 μM unlabeled melatonin (non-specific binding). Specific binding data were subjected to Scatchard analysis (Scatchard, 1949), and the line slope and intercepts determined by linear regression analysis using the method of least squares.

F. Thin Layer Chromatography (TLC) of Bound ^{3}H-Melatonin

To determine whether the bound radioactivity was authentic ^{3}H-melatonin after the binding assay incubation, the bound radioactivity was chromatographed on a Kodak silica gel TLC plate in a chloroform-methanol (9:1,vol:vol) solvent system.

G. Competitive Binding Assays

Displacement studies similar to the methods of Gern et al. (1981) and Cohen et al. (1978) were performed to determine the specificity of the binding sites for melatonin. Tissues were prepared as described above for the saturation binding assays. Aliquots (0.4 mls) of tissue suspension were incubated in triplicate for 5 hours at 0°C in darkness with 200 nM ^{3}H-melatonin with or without 100 uM unlabeled melatonin analog. The 6-choromelatonin was a gift of Dr. Michael Flaugh (Lilly Research Labs; Indianapolis,IN) (Flaugh et al., 1979). All other indole analogs were obtained from Sigma Chemical Co. Bound ^{3}H-melatonin was separated from unbound melatonin as described above, and the radioactivity was measured by liquid scintillation spectrometry.

III. RESULTS

A. Analysis of Purified Antibody

Protein electroblot-immunolabeling was performed to identify the protein to which the antibody was made. When bovine pineal and retina extracts were electroblotted, 39,000 and 25,000 mol.wt. protein bands of the pineal, and a 25,000 mol.wt. band of the retina (figure 1) were recognized by the antibody. The subunit molecular weight of bovine pineal HIOMT is generally considered to be 39,000 (Kuwano and Takahashi, 1978; Jackson and Lovenberg, 1971: Nakane et al., 1983) although a lower molecular weight subunit of about 25,000 has also been reported (Karahasanoglu and Ozand, 1972).

To determine if the HIOMT antibody was monospecific, and cross-reacting with two similar polypeptides (the 25,000 and 39,000 mol.wt. bands), we isolated the antibodies to the 39,000 mol.wt. band according to the method described by Talian et al.(1983). Antibody eluted from the nitrocellulose strip containing the 39,000 mol.wt. band was used to label electroblotted bovine pineal and retina extracts. The same labeling pattern as seen on figure 1 was observed, indicating that both proteins are recognized by a single population of the antibodies suggesting that they may be structurally related.

To determine whether the isolated antibody was directed against HIOMT, immunoprecipitin titration was performed with bovine pineal extract. Increasing amounts of pure antibody

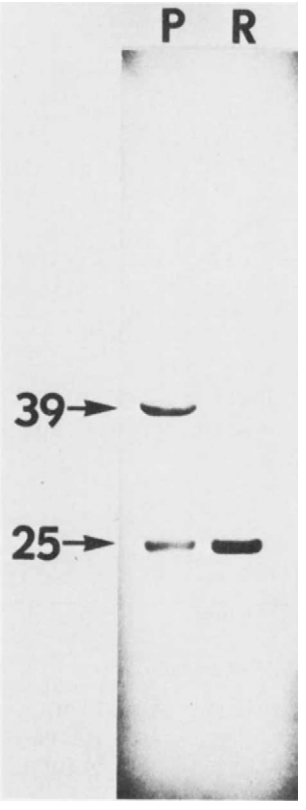


Fig. 1. Transblot immunolabeling of bovine pineal (P) and retina (R) extracts with the HIOMT antibody demonstrates the purity of the isolated antibody. The antibody labels two peptide bands of the pineal extract (39,000 and 25,000 mol.wt.), and only one of the retina extract (25,000 mol.wt.). From figure 3, Wiechmann et al. (1985) with permission of Lippincott.

reacted with the HIOMT in the extract, resulting in decreasing HIOMT activity in the supernatant (figure 2). This experiment demonstrated that the isolated antibody was indeed directed against HIOMT.

B. Analysis of Purified HIOMT

The purified pineal HIOMT obtained from the antibody immunoadsorbant column, when analyzed by SDS-PAGE, was shown to be composed of subunit bands of 39,000 and 25,000 mol.wt., whereas the retinal enzyme had a subunit molecular weight of

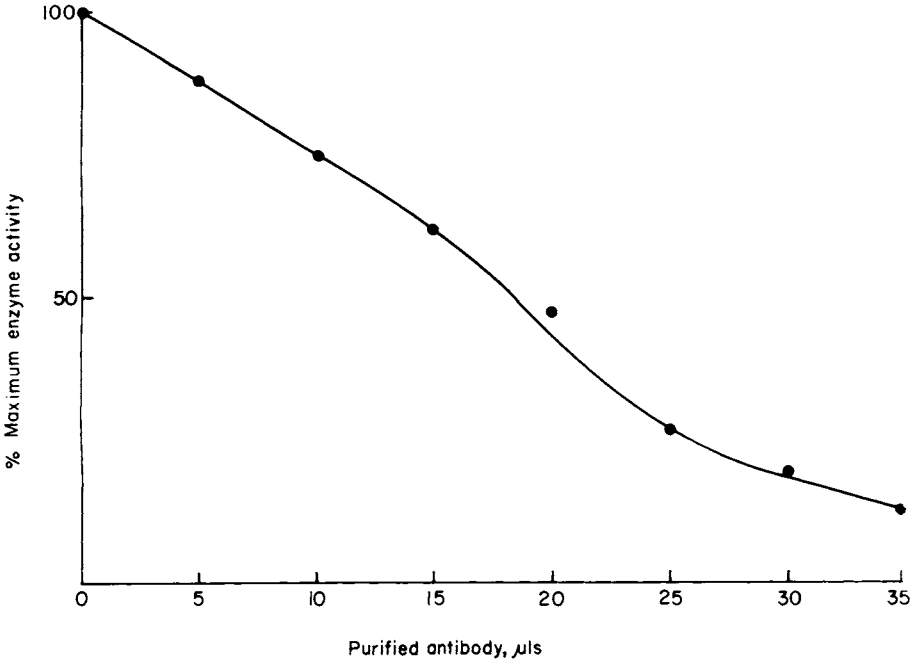


Fig. 2. Immunoprecipitin titration. HIOMT activity in reaction mixture supernatant is decreased by increasing amounts of the pure antibody. From figure 4, Wiechmann et al. (1985) with permission of Lippincott.

25,000 which was identical to the transblot labeling pattern we had observed. The purified pineal HIOMT from the immunoadsorbant column was measured for activity by radioenzymatic assay. Increasing amounts of the pure enzyme resulted in increasing HIOMT activity (figure 3).

To determine if sequence homology existed between the 39,000 mol.wt. subunit and the 25,000 mol.wt. subunits, one-dimensional peptide mapping was performed on purified pineal and retinal HIOMT. Staphylococcus aureus V8 protease specifically cleaves peptide bonds on the carboxy-terminal side of glutamic and aspartic acid. Similar peptide profiles are an indication of sequence homology between two proteins. In the peptide map (figure 4), the resulting peptide profile shows that there are identical peptide fragments in the two HIOMT enzymes (small arrows). The upper band (large arrow) in lane #5 is obviously undigested enzyme. The sequence homology indicated in the experiment suggests that the 25,000 mol.wt. enzyme peptide is similar or identical to a portion of the 39,000 mol.wt. enzyme peptide.

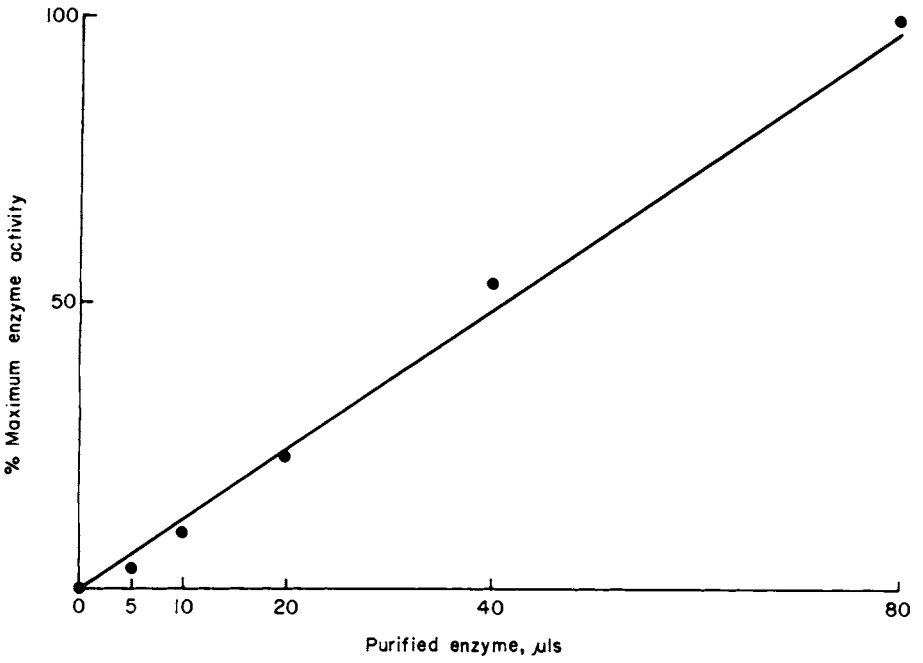


Fig. 3. Radioenzymatic assay of purified bovine pineal HIOMT. Increasing amounts of enzyme (5 µg/ml) results in increasing HIOMT activity. From figure 6, Wiechmann et al. (1985) with permission of Lippincott.

C. Immunocytochemistry

Tissues treated with nonimmune antibody showed no specific peroxidase labeling. Pinealocytes in bovine and human pineal glands treated with the HIOMT antibody were heavily labeled with the brown reaction product of the antibody-PAP complex (figure 5). The interstitial cells and blood vessels were not labeled. There appeared to be many gradations of antibody labeling of the bovine pinealocytes. HIOMT immunoreactivity was not observed in rat pineal glands obtained during the light period, but was observed in rat pineal glands obtained during the dark period (data not shown). The labeling in the rat was diffuse, and not as intense as seen in the bovine or human pineal.

Rat retina treated with the HIOMT antibody showed labeling of photoreceptor inner segments, the outer nuclear layer, and the outer plexiform layer, indicating that the photoreceptors contain the HIOMT enzyme (figure 6b). There also appeared to be some labeling of cells in the inner nuclear layer. There was no labeling of the photoreceptor outer segments. Within

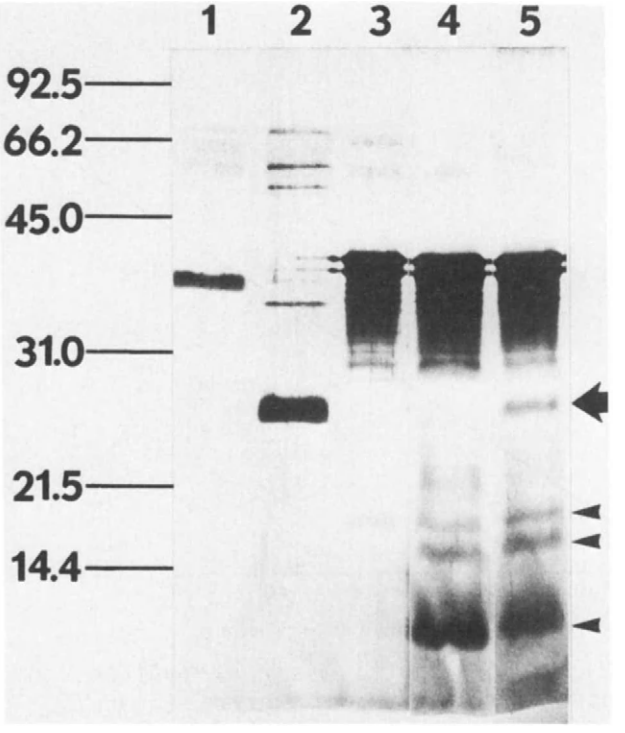


Fig. 4. Peptide map of purified bovine pineal and retinal HIOMT. The 39,000 mol.wt. HIOMT peptide is in lane #1, while the 25,000 mol.wt. HIOMT peptide is in lane #2. Protease (2 ugs) is in lane #3. Lane #4 contains the incubation mixture of the 39,000 mol.wt. pineal HIOMT and protease, and lane #5 contains the incubation mixture of the 25,000 mol.wt. retinal HIOMT and protease. From figure 7, Wiechmann et al. (1985) with permission of Lippincott.

the limits of sensitivity and cross reactivity of bovine and human HIOMT, specific HIOMT labeling in the human retina appeared to be limited to the photoreceptors (figure 6d).

D. Localization of Melatonin Binding

Retinas of adult Rana pipiens were incubated in media containing ^{3}H-melatonin with or without a 1000-fold excess of unlabeled melatonin. Bound radioactivity occurring in the tissue incubated with excess unlabeled melatonin was considered to represent non-specific binding of the ligand to the tissue. After processing the tissue for autoradiography,

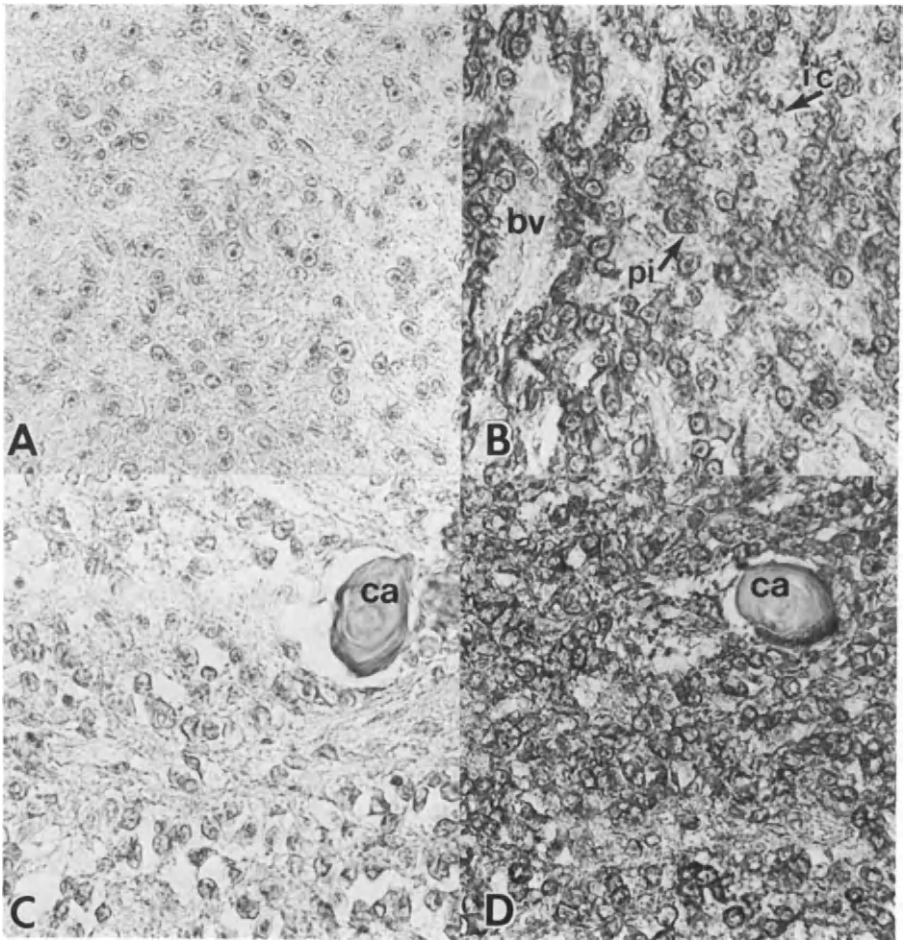


Fig. 5. Immunocytochemistry of bovine and human pineal glands. A. Bovine pineal gland incubated with nonimmune antibody. B. Bovine pineal gland incubated with the HIOMT antibody. HIOMT immunoreactivity appears in some pinealocytes (pi), but is lacking in others. Blood vessels (bv) and interstitial cells (ic) are not labeled. C. Human pineal gland (25 year-old female) incubated with nonimmune antibody. D. Human pineal gland incubated with the HIOMT antibody. HIOMT labeling appears in the pinealocytes, but not in blood vessels, interstitial cells, or corpora arenacea (ca) (x477). From figure 8, Wiechmann et al. (1985) with permission of Lippincott.

radioactivity was found associated with the melanosomes of the RPE-choroid (figure 7), and was concentrated in the outer plexiform layer of the neural retina (figure 8). The

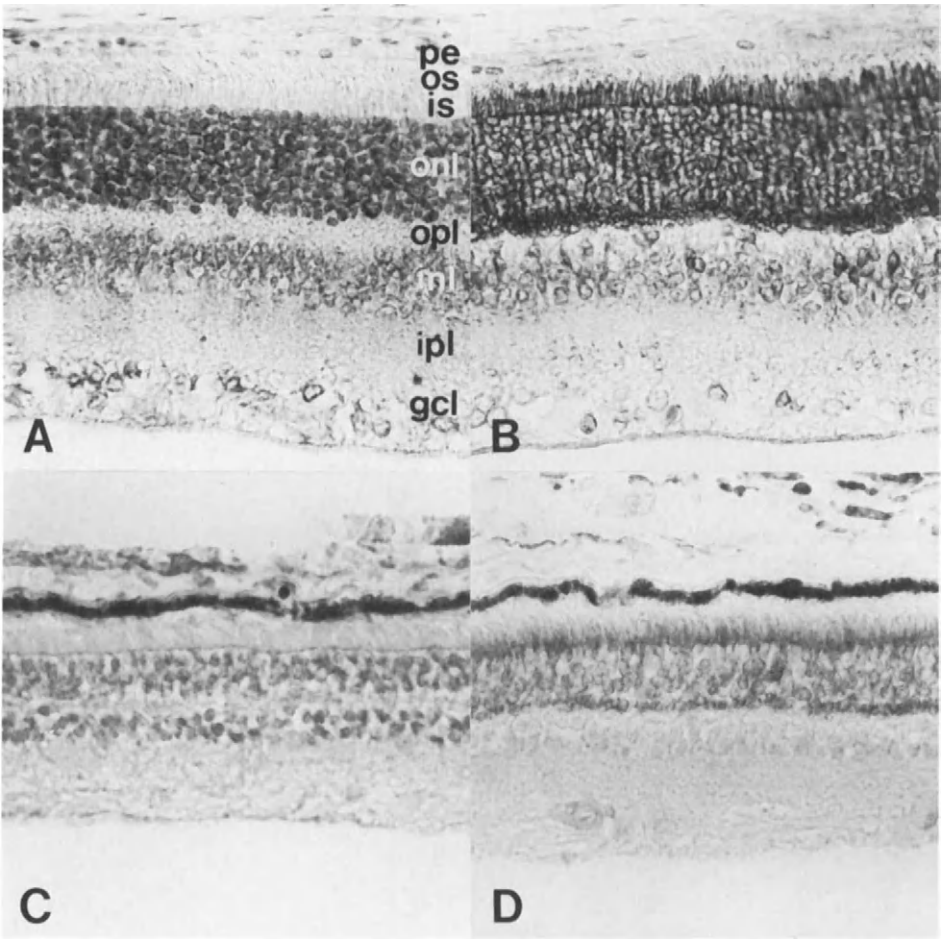


Fig. 6. Immunocytochemistry of rat and human retinas. A. Rat retina incubated with nonimmune antibody. B. Rat retina incubated with the HIOMT antibody. The photoreceptor cell bodies demonstrate HIOMT immunoreactivity. Some labeling also occurs in the inner nuclear layer. C. Human retina incubated with nonimmune antibody. D. Human retina incubated with HIOMT antibody. HIOMT labeling is observed only in the photoreceptors. Pigment epithelium (pe), photoreceptor outer segments (os), photoreceptor inner segments (is), outer nuclear layer (onl), outer plexiform layer (opl), inner nuclear layer (inl), inner plexiform layer (ipl), ganglion cell layer (gcl) (x477). From figures 10 and 11, Wiechmann et al. (1985) with permission of Lippincott.

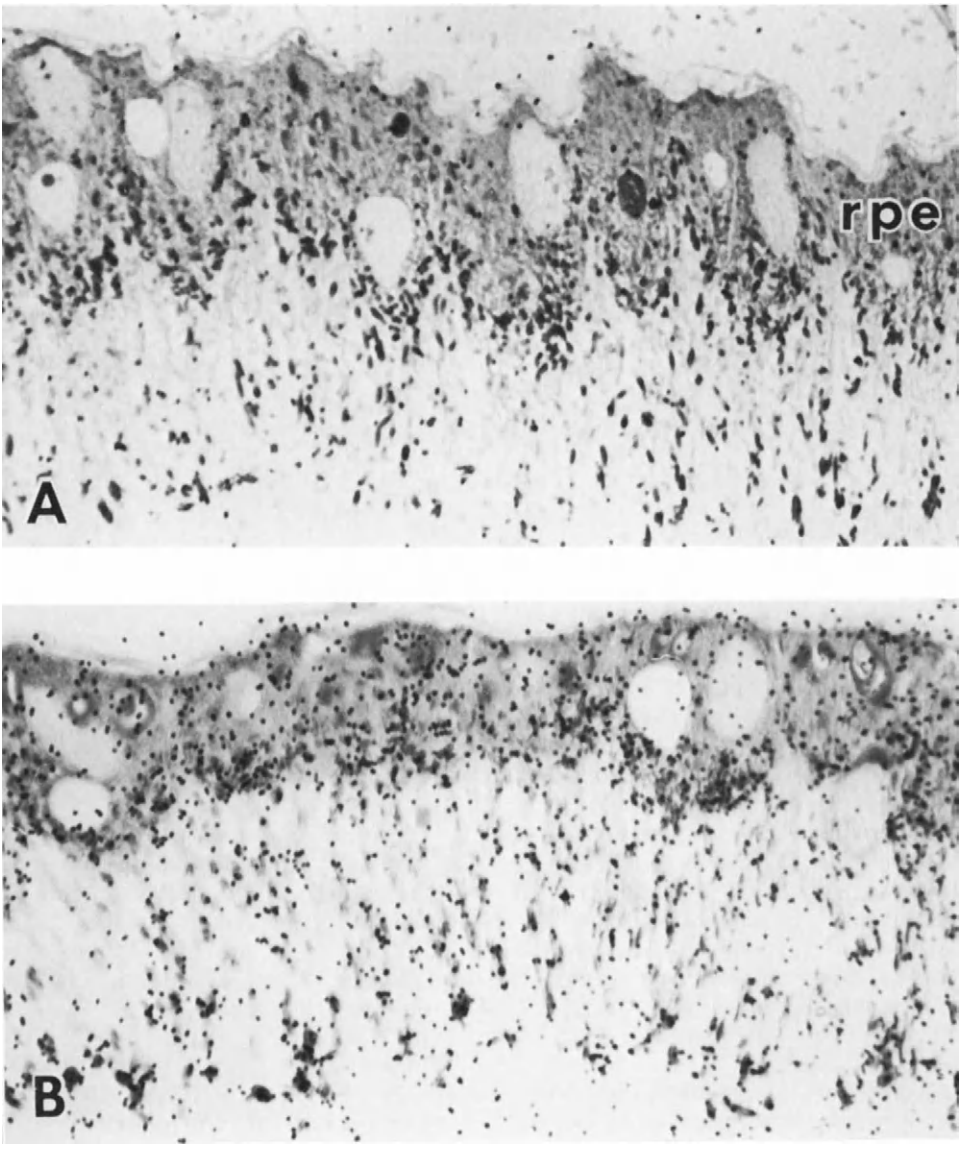


Fig. 7. Light microscope autoradiograms of RPE-choroid of Rana pipiens incubated for 1 hour with ^{3}H-melatonin in the light. A. Control tissue incubated with ^{3}H-melatonin in the presence of 1000-fold excess unlabeled melatonin. B. Tissue incubated with ^{3}H-melatonin. Radioactivity is associated with melanosomes of the RPE. The autoradiograms were exposed for 5 months. Retinal pigment epithelium (rpe) (x1330). From figure 4, Wiechmann et al. (1986) with permission of Lippincott.

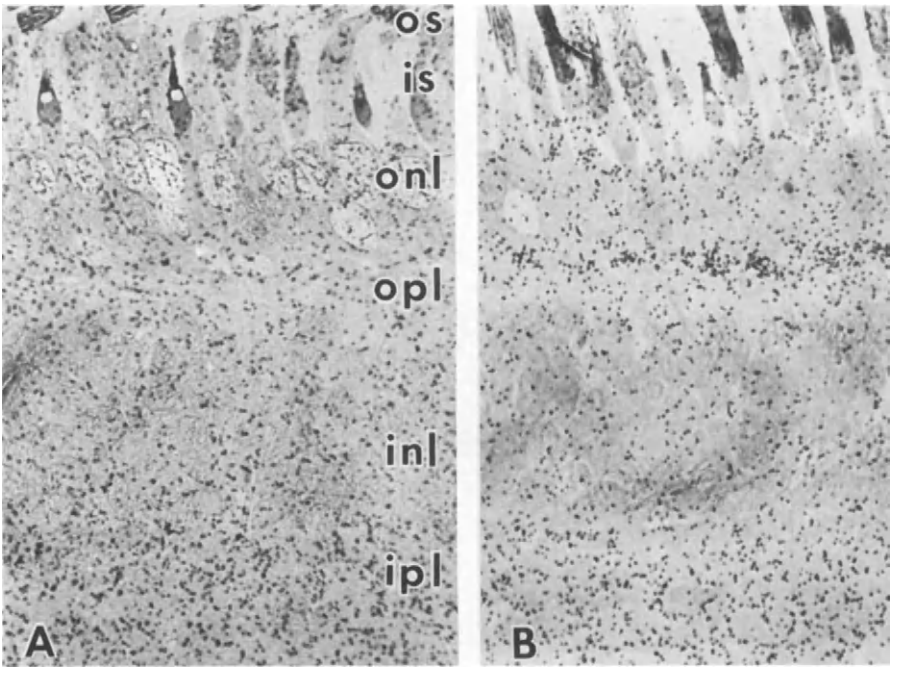


Fig. 8. Light microscope autoradiograms of neural retina of Rana pipiens incubated for 1 hour with ^{3}H-melatonin in the light. A. Control tissue incubated with ^{3}H-melatonin in the presence of 1000-fold excess unlabeled melatonin. B. Tissue incubated with ^{3}H-melatonin. Specific radioactivity is associated with the outer plexiform layer of the retina. The autoradiograms were exposed for 13 months to allow optimal visualization of radioactive labeling. Photoreceptor outer sements (os), photoreceptor inner segments (is), outer nuclear layer (onl), outer plexiform layer (opl), inner nuclear layer (inl), inner plexiform layer (ipl) (x1127). From figure 5, Wiechmann et al. (1986) with permission of Lippincott.

association of melanosomes with radioactive labeling, and the intense radioactive labeling of the melanosomes of the choroid (data not shown), indicated that melatonin bound to the melanosomes of the pigment epithelium and choroid.

Radioactive labeling was concentrated in the outer plexiform layer of the neural retina (figure 8b). Quantitative measurements of silver grain concentration in the various retinal layers are shown in figure 9. The outer plexiform layer was the only retinal layer in which the total binding was significantly higher ($P<0.0005$) than the non-specific binding.

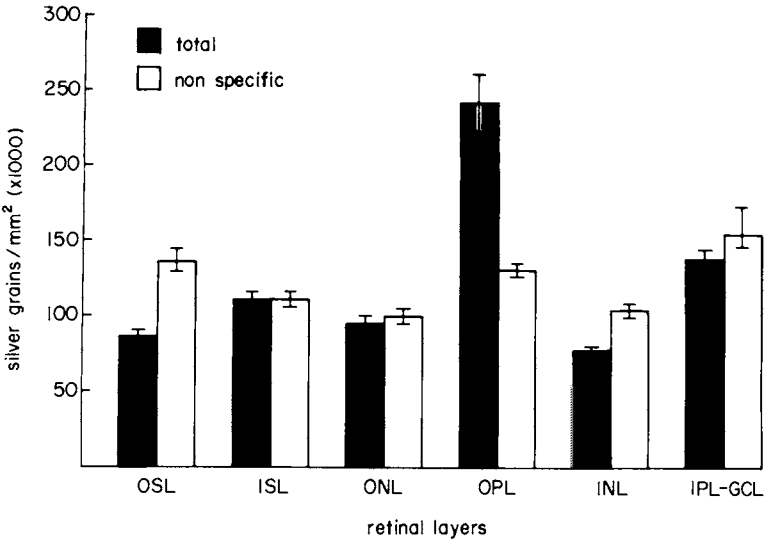


Fig. 9. Grain count distributions in retinas incubated for 1 hour in ^{3}H-melatonin with (open bars) or without (black bars) a 1000-fold excess of unlabeled melatonin. Each bar is the mean of values from nine samples + standard error of the mean. The autoradiograms from which these measurments were made were exposed for 5 months. Outer segment layer (OSL), inner segment layer (ISL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer and ganglion cell layer (IPL-GCL). The only region showing specific binding of ^{3}H-melatonin is the outer plexiform layer. From figure 6, Wiechmann et al. (1986) with permission of Lippincott.

E. Kinetics of ^{3}H-Melatonin Binding

Saturation of melatonin binding in the neural retina and RPE-choroid was determined by incubating tissue homogenates with increasing concentrations of ^{3}H-melatonin. Melatonin binding was almost completely saturated at 600 nM in the RPE-choroid (figure 10a), and at 1000 nM in the neural retina (figure 11a). The protein concentrations of the RPE-choroid melanosome-enriched fraction and neural retina membrane-enriched fraction were 1 mg/ml and 2.0 mg/ml, respectively. Scatchard analysis of the specific binding curves revealed linear plots (figures 10b and 11b), suggesting the presence of a single class of binding sites in both the neural retina and RPE-choroid. Both the RPE-choroid and neural retina binding sites had an apparent dissociation constant of 6×10^{-7}M. No

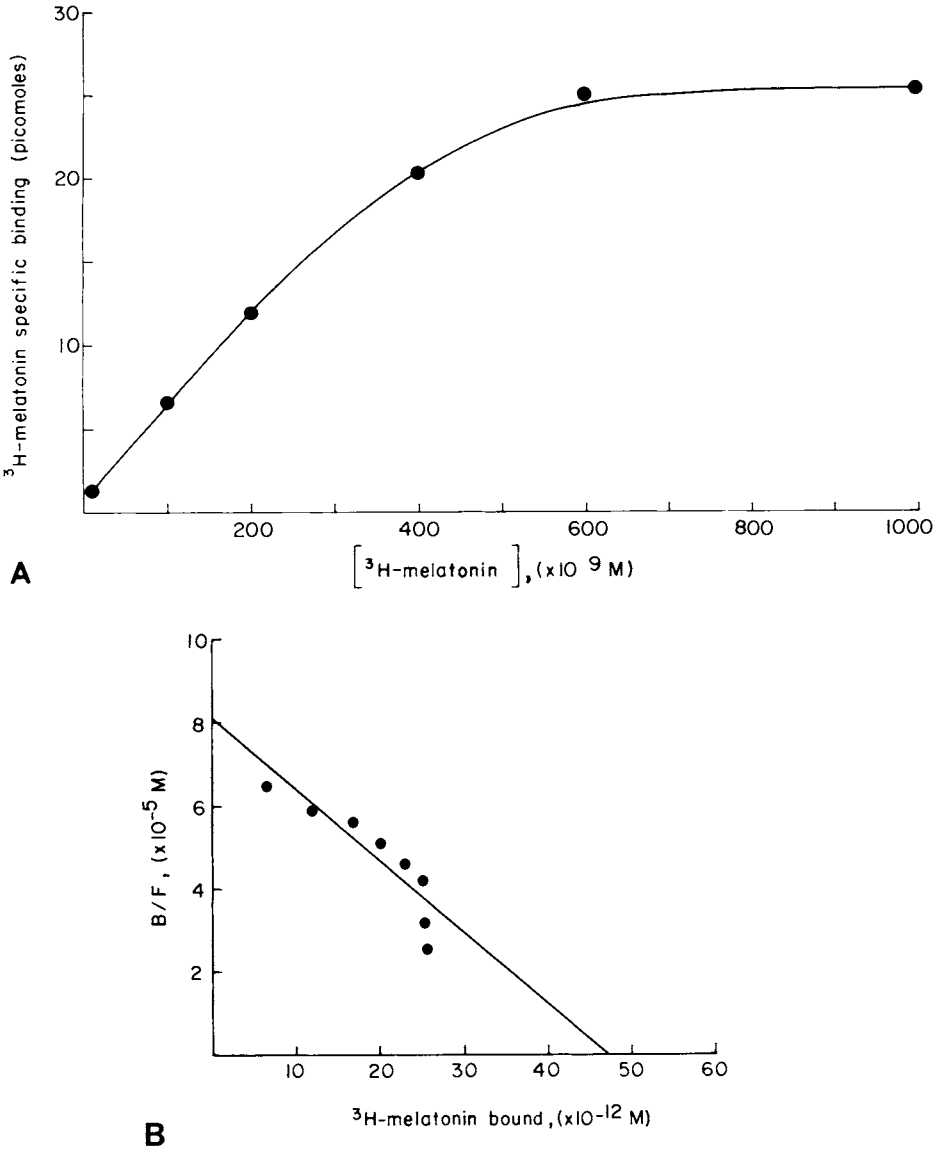


Fig. 10. ^{3}H-melatonin binding as a function of increasing ^{3}H-melatonin concentration in the melanosome-enriched fraction of frog RPE-choroid. A. Saturation analysis of ^{3}H-melatonin specific binding. Each point is the mean of triplicate samples. B. Scatchard analysis of the ^{3}H-melatonin binding. From figure 7, Wiechmann et al. (1986) with permission of Lippincott.

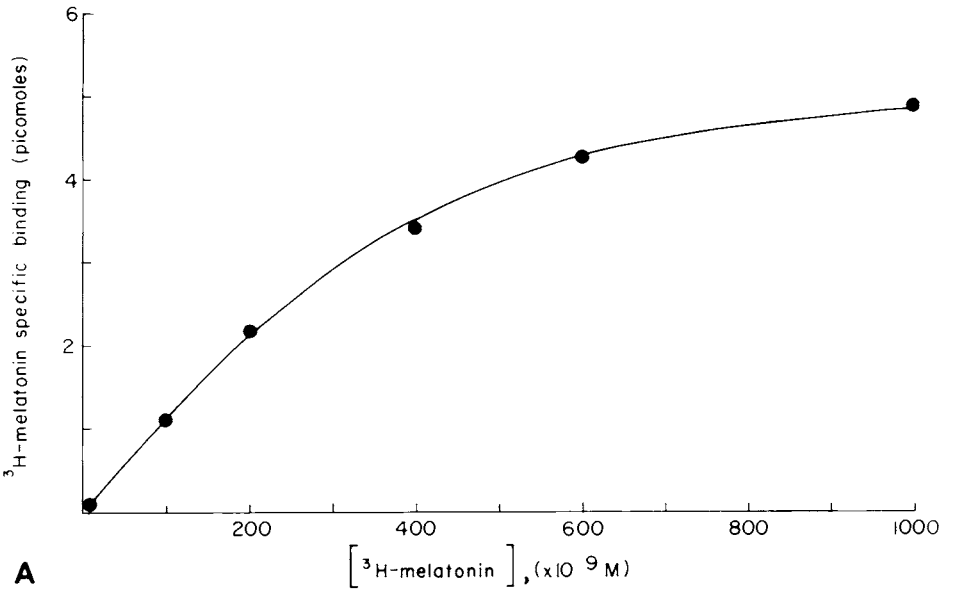
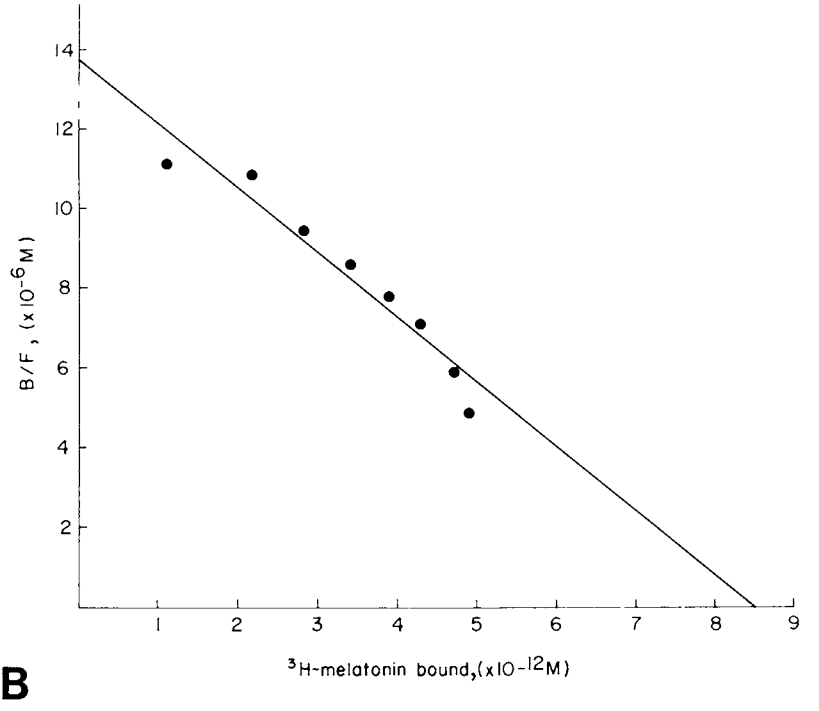


Fig. 11. ^{3}H-melatonin binding as a function of increasing ^{3}H-melatonin concentration in the membrane-enriched fraction of frog neural retina. A. Saturation analysis of ^{3}H-melatonin specific binding. Each point is the mean of triplicate samples. B. Scatchard analysis of the ^{3}H-melatonin binding. From figure 8, Wiechmann et al. (1986) with permission of Lippincott.

specific melatonin binding was observed in the RPE-choroid melanosome-free homogenate, or in the neural retina nuclei-enriched fraction. Specific melatonin binding was observed in the neural retina crude membrane fraction (27,000g pellet), but not in the cytosol fraction. This suggests that melatonin binding occurs on membrane-associated components of the neural retina. ^{3}H-melatonin bound to RPE-choroid and neural retina tissue fractions was examined for purity by TLC. More than 98% of the extracted radioactivity cochromatographed with authentic melatonin.

Displacement studies were performed to determine the specificity of the melatonin binding sites in the retina. RPE-choroid melanosome-enriched fractions and neural retina membrane-enriched fractions were incubated with constant amounts of ^{3}H-melatonin in the presence of various indole analogs. In the neural retina, both 6-chloromelatonin and 5-methoxytryptophol demonstrated complete displacement of



B

Fig. 11b.

melatonin binding. Colchicine did not displace melatonin binding, suggesting that the melatonin binding we have observed is not due to binding to microtubules. The order of degree of displacement by indole analogs were quite different for the RPE-choroid and neural retina. There appeared to be significant displacement of melatonin binding by several indole analogs.

IV. DISCUSSION

Using the immunoadsorption method of Kuwano and Takahashi (1978), we have isolated specific antibodies to HIOMT. Previous immunodiffusion analysis of the pure antibody showed only one precipitin line against the pineal extract (Wiechmann et al., 1985). Electroblot analysis of the HIOMT antibody, and SDS-PAGE of the purified HIOMT suggest that there may be

two separate HIOMT subunits. Although both the 25,000 and 39,000 subunit molecular weights have been reported in bovine pineal gland (Kuwano and Takahashi, 1978; Jackson and Lovenberg, 1971; Nakane et al., 1983, Karahasanoglu and Ozand, 1972), only the 39,000 mol.wt. subunit has been reported in chicken pineal gland (Nakane et al., 1983). Based on the immunological identity and sequence homology indicated by the immunodiffusion analysis, electroblot immunolabeling, and peptide mapping, the 25,000 mol.wt. polypeptide appears to be similar or identical to a portion of the 39,000 mol.wt. band. The 25,000 mol.wt. band may be a true subunit of HIOMT, or a degradative product of the 39,000 mol.wt. subunit.

The pinealocytes have long been presumed to be the site of melatonin synthesis in the mammalian pineal gland. Our immunocytochemistry data demonstrate that the enzyme, HIOMT, and therefore melatonin synthesis, is located in the pinealocytes of the bovine pineal gland. This is in agreement with the results of Kuwano et al. (1983). Due to the varied degree of labeling of the pinealocytes for HIOMT, we suggest that not all pinealocytes are actively involved in the synthesis of melatonin.

We have also observed that HIOMT immunoreactivity in the rat pineal gland is higher during the dark period than during the light period (Wiechmann et al., 1985). Other laboratories have reported that HIOMT activity in the rat pineal is higher during the dark period than during the light period (Axelrod et al., 1965; Nagle et al., 1972). Their observation that puromycin blocks the dark-induced rise in HIOMT activity, suggests that the increase in HIOMT activity during the dark period may be mediated by protein synthesis (Axelrod et al., 1965). Our data support the hypothesis that the higher HIOMT activity in the rat pineal gland observed during the dark period is due to higher levels of HIOMT in the tissue rather than some chemical modification in activity of the enzyme.

The observation that HIOMT is present in the retinal photoreceptors supports the hypothesis that the photoreceptors are the site of retinal melatonin synthesis. Since photoreceptors and pinealocytes have many similarities, and pinealocytes are capable of melatonin synthesis, many investigators have predicted that the photoreceptors would be the most likely site of retinal melatonin synthesis. The presence of HIOMT labeling in the entire photoreceptor, with the exception of the outer segment, suggests that HIOMT is located in the cytosol of the cell. Reports from other laboratories have shown that HIOMT activity is confined mainly to the cytoplasmic fraction of the retina and pineal (Cardinali and Wurtman, 1972). The observation that HIOMT is present in both the pinealocytes and the retinal

photoreceptors supports the hypothesis that the mammalian pinealocytes belong to a sensory cell line that has evolved from the sensory pineal photoreceptors of lower vertebrates.

The autoradiographic and biochemical results suggest that melatonin binding occurs in the melanosomes of the RPE-choroid and in the outer plexiform layer of the neural retina. Other investigators have demonstrated saturable melatonin binding in the cytoplasmic fraction of the hamster eye (Cohen et al., 1978), and in the membrane-cytosol fraction of trout RPE-choroid (Gern et al., 1981).

Although melatonin binding to melanosomes has not been demonstrated previously, the action of melatonin on melanosomes of RPE cells has been demonstrated. Intraocular administration of melatonin induces melanosome aggregation in the RPE of frogs (Kraus-Ruppert and Lembeck, 1965), trout (Cheze and Ali, 1976), and guinea pigs (Pang and Yew, 1979). Since retinal melatonin levels and degree of RPE melanosome aggregation are highest during the dark period (Pang et al., 1978; Walls, 1942), endogenous retinal melatonin may be involved in regulation of pigment granule migration in the RPE. In addition to the specific melatonin binding component of melanin granules, a low affinity, high capacity binding component is also apparent. Many investigators have reported that ring compounds, such as chloroquine, prochloroperazine, and kanamycin accumulate in melanin granules of the RPE and choroid (Potts, 1962; Dencker, 1973). This drug affinity for melanin granules indicates that these granules may have sites for nonspecific binding or storage of physiological substances such as melatonin.

Melatonin binding has been demonstrated in several neural tissues. Cardinali et al. (1979) have shown saturable binding of melatonin to membranes of bovine hypothalamus, and Niles et al. (1979) have shown melatonin binding to the cytosol of several brain regions of the rat. Binding of melatonin also occurs to membranes of bovine pineal gland (Vacas and Cardinali, 1980). Melatonin inhibits neurotransmitter uptake in synaptosome-rich homogenates of rat hypothalamus (Cardinali et al., 1975), and dopamine release from rat hypothalamic slices (Zisapel and Laudon, 1982). Based on these observations, it was suggested that melatonin modulates neurotransmission in the brain. Melatonin has been shown to inhibit the calcium-dependent release of dopamine from isolated rabbit retina (Dubocovich, 1983), suggesting that it may modulate neurotransmission in the retina, as well as in the brain.

The dissociation constant obtained on this study suggests that melatonin binds with low affinity in both the RPE-choroid and neural retina. This observation is similar to those of other studies on melatonin binding in the eye (Cohen et al.,

1978), pineal (Vacas and Cardinali, 1980), and midbrain (Niles et al., 1979). The competition experiments indicate that several indole analogs displace melatonin binding in the RPE-choroid and neural retina. This is in agreement with the results of Gern et al. (1981) and Cohen et al. (1978) which show that several indole analogs cross reacted with a melatonin receptor. Reasons for the differences between the neural retina and RPE-choroid in degree of displacement by the various indole analogs are not presently understood.

The lack of displacement of colchicine with the melatonin receptor of the retina suggests that the observed melatonin binding is not due to binding to microtubules. Also, since colchicine does not appear to bind to the melatonin receptor, the action of colchicine on disc shedding reported by Besharse and Dunis (1982) may be mediated through a mechanism different from the methoxyindole-induced disc shedding (Besharse and Dunis, 1983). It is interesting to note that both 6-chloromelatonin and 5-methoxytryptophol demonstrate complete cross reactivity with the melatonin receptor of the neural retina, since Besharse and Dunis (1983) have reported that melatonin, 6-chloromelatonin and 5-methoxytryptophol all activate photoreceptor outer segment disc shedding in Xenopus eyecups.

Reppert and Sagar (1983) suggested that the most likely function of retinally synthesized melatonin was in diurnal phenomena within the eye, since the contribution of retinal melatonin to the blood is quite small. We have shown that photoreceptors are the site of retinal melatonin synthesis. Melatonin (and some indole analogs) have been shown to inluence outer segment disc shedding and phagocytosis (Besharse and Dunis, 1983; Ogino et al., 1983), photomechanical movements (Krause-Ruppert and Lembeck, 1965; Cheze and Ali, 1976; Quay and Mcleod, 1968; Pierce et al., 1984), and retinal neurotransmitter release (Dubocovich, 1983). The present study has demonstrated the presence of saturable, low capacity hormone binding in the retina. We suggest that in the frog, the sites of action of endogenous retinal melatonin are the melanosomes of the RPE-choroid, and elements of the outer plexiform layer of the neural retina.

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MELATONIN AND N-ACETYLTRANSFERASE RHYTHMS IN PINEAL AND RETINA[1]

Sue Binkley

Biology Department, Temple University
Philadelphia, Pa 19122

I. INTRODUCTION

Pineal function has invited retinal comparisons because (i) environmental lighting modifies pineal biochemistry, (ii) nonbiochemical aspects of pineal function involve environmental lighting schedules, (iii) some species have a 'third eye', (iv) there are anatomical similarities in development (1), and (v) anatomical likenesses (1). Direct comparisons between retinas and pineals of several species have been made in three types of studies: (i) in vivo studies where animals have been exposed to lighting regimens and injected with compounds followed by measurement of pineal and retinal N-acetyltransferase activity (NAT) and/or melatonin; (ii) in vitro studies where pineals and retinas have been exposed to lighting regimens followed by measurement of NAT and/or melatonin; and (iii) studies of homogenates of pineals or retinas subjected to chemical and/or temperature treatments. Where the data can be directly compared, there are remarkable similarities in pineal and retina. The techniques used in these studies have been described previously (2-11).

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II. THE RHYTHM, COMPARATIVE BIOCHEMISTRY

In 1979 (10) we reported that retinas were like pineal glands in having a well-defined daily cycle in NAT in animals kept in 24h light-dark cycles -- thus proving that retinas of several species are capable of cyclic melatonin synthesis (Figs.1-4). Two avian species (chickens and quail) have NAT and melatonin cycles in their retinas that quantitatively rival those simultaneously measured in their pineal glands (3,8,10,11). Other species so far examined (lizards, rats, hamsters, sparrows) have so far been observed to have small rhythms in their retinas in contrast to well defined pineal NAT and melatonin cycles (3,12,13). In the species that have retinal cycles in NAT and melatonin comparable to pineal, several observations made previously for the pineal hold true for the retina: melatonin and NAT values are positively correlated with peaks in the dark-time.

III. REGULATION BY LIGHT AND DARK

Properties of regulation by light and dark of pineal NAT cycles established for young chickens include (i) persistence of a rhythm (freerun) in constant dark, DD, (ii) attenuation of amplitude in constant light (LL), (iii) refractoriness to stimulation by dark (L/D) encompassing the time of subjective dawn, (iv) a decline in NAT to near day values in less than 30 minutes (rapid plummet) in response to light in the subjective dark-time (D/L), (v) entrainment by light-dark cycles, (vi) modification of waveform by photoperiod, and (vii) phase shifting by light and dark pulses. Some of these properties have been measured for retinas:

The rhythms freerun in DD (constant dark). Chicks (3 weeks old) placed in DD exhibit continued cycles of NAT in both pineal and retina for at least 3 days (10). The excursion, or amplitude, of the NAT cycle is less in the eye than in the pineal (Fig. 5).

LL (constant light) attenuates the rhythms (Fig. 6). The amplitude or the excursion of retinal NAT is attenuated by LL (10). Moreover, a discernible rhythm was not present in retina though there was a very low amplitude cycle in the pineal.

There is a refractory period. NAT in pineal glands cannot be stimulated by dark at all times. In the early light-time, pineal NAT does not rise in response to 4h dark (14,15). Retinal NAT exhibits the refractory period (10) (Fig. 7).

Light causes a rapid plummet. NAT falls rapidly in

pineals and retinas exposed to light in the middle of their dark-time (10).

Extension of light into the normal dark-time suppresses NAT of both pineals and retinas (10).

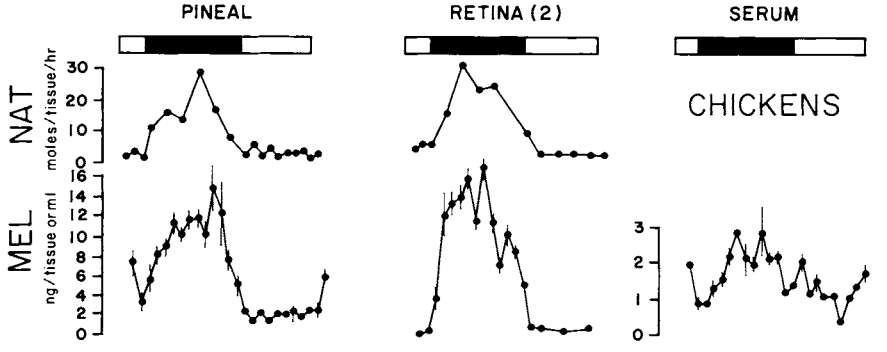

Fig. 1. NAT and melatonin in chick pineal, retina, and serum (16).

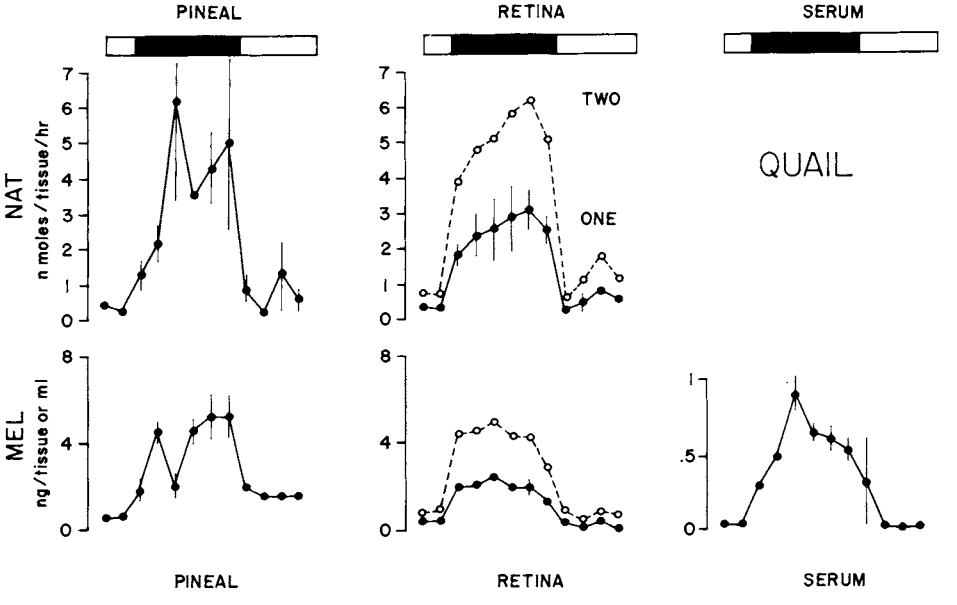

Fig. 2. NAT and melatonin in quail pineal, retina, and serum (unpublished data of Binkley, Underwood, Mosher, Siopes).

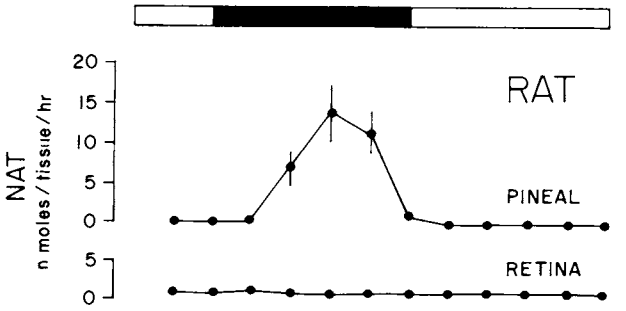

Fig. 3. NAT in pineal and retina of laboratory rats (13).

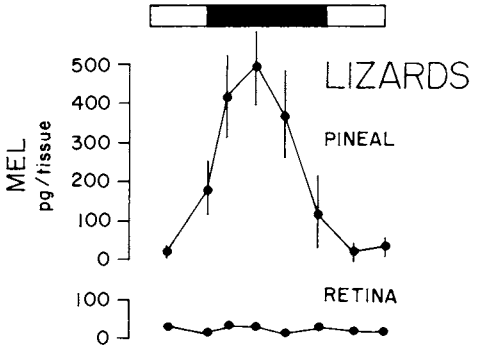

Fig. 4. Melatonin in pineal and retina of Anolis carolinensis (Redrawn after Underwood, 12).

IV. PROPERTIES OF NAT IN RETINA AND PINEAL

The chemical properties of NAT have been studied in homogenates of pineals and retinas following pre-assay incubations and during assays themselves by modifying the physical or chemical milieu -- light, temperature, phosphate concentration, substrate concentration, potential inhibitors, potential activators, etc.

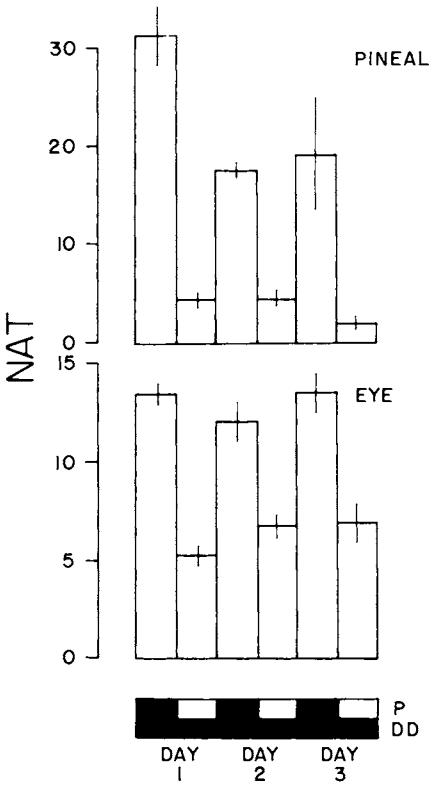

Fig. 5. NAT was rhythmic in the eyes and pineals of chicks kept in DD for 3 days following LD12:12 (9).

Light had no effect on homogenate of pineal glands (8).

Temperature does affect NAT (6,8). NAT in homogenates of dark-time chick pineal and retina is 'protected' (activity maintained) by cold, 0C. Activity is lost rapidly when the homogenate is placed at 37C (Fig.8). The response to temperature is similar in the two tissues. The performance of retinal and pineal NAT were compared in homogenates of chick tissues under assay conditions (Fig. 9) and it appeared that pineal NAT reached maximal levels faster than retina (possibly an artifact of NAT concentration).

Enzyme concentration affected NAT. Pineal NAT is higher in diluted homogenates than in more concentrated solutions. This observation is also true for retina NAT (Fig. 10). The dilution effect supports the possibility that lower NAT is due to the presence of an inhibitor or protease.

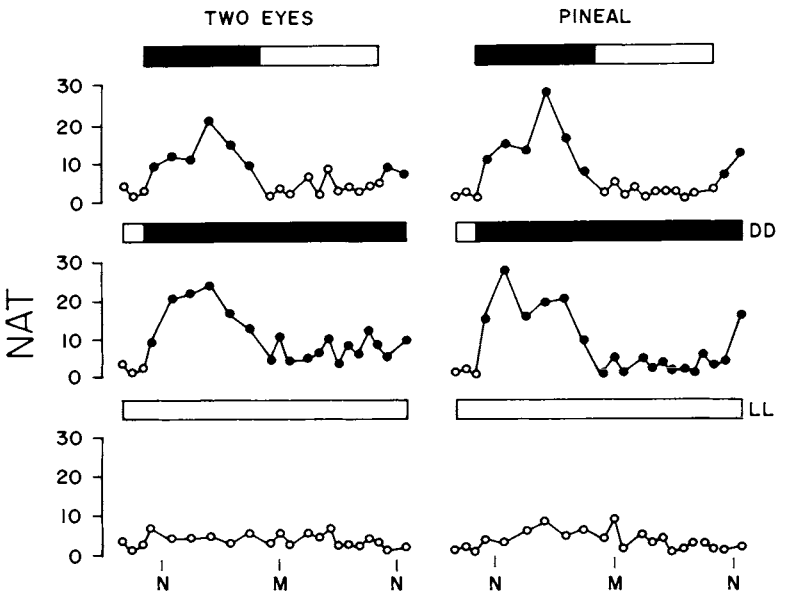

Fig. 6. NAT (nmoles/tissue/hr) in chick pineal and retina in LD12:12, the first 24h of constant dark (DD), and the first 24h of constant light (LL) (10).

AcCoA and its analogues protect NAT. Rat and chick pineal NAT were protected in homogenates against the thermal loss of activity which occurs at 37C by one of the substrates of NAT, acetyl coenzyme A (AcCoA)(6,8). Some compounds which contain structural sequences similar to AcCoA also protect NAT in homogenates of chick retina and pineal (Table 1). Serotonin inhibited chick retinal NAT and cystamine inhibited pineal NAT.

High phosphate protects chick pineal NAT. Hamm and Menaker (17-19) reported that chick pineal and retinal NAT were higher in 0.3M phosphate buffer than in 0.05M phosphate buffer. Because the effect was not present in rat pineals or other tissues, they suggested that the phosphate effect might be associated with some aspect of chick pineal-retinal physiology that is not present in rats -- light sensitivity and/or the ability to generate cycles.

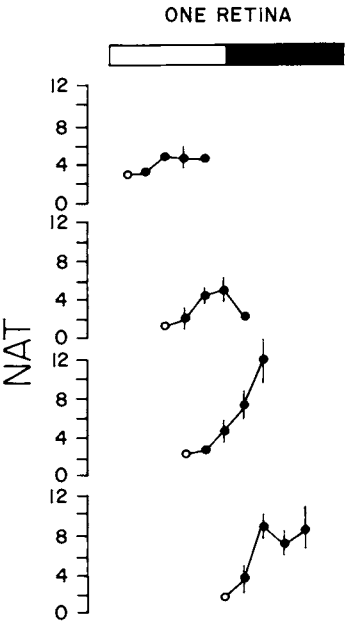

Fig. 7. NAT(nmoles/tissue/hr) time sequences in retinas of chicks placed in dark at successive times relative to their prior LD12:12 -- in the early light NAT did not initiate (refractory) whereas when the time of expected dark approached, NAT rose in response to dark (sensitive)(10). The phenomenon also occurs in pineal (16,15,20).

V. INTERACTION OF PINEAL AND RETINA

Potential signal exchange between pineal and retina have been studied in chicks using (i) patches, and (ii) inactivating agents, isoproterenol and cycloheximide (10,11).

The eyes and pineal region were covered with various combinations of patches (11). Extension of light into the dark-time suppresses NAT (e.g. from 14 to 3 nmoles/eye/h); this effect was used to test for light sensitivities in the patched birds. The eyes respond to light independently of each other and independently of the pineal gland. The eyes, however, do influence the pineal (as found in a previous study where blinding did not prevent but did alter the rapid plummet response to light, 15).

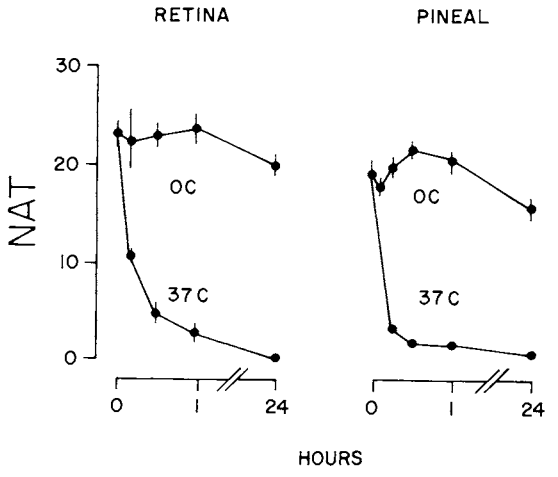

Fig. 8. NAT(nmoles/tissue/hr) in homogenates is protected by cold.

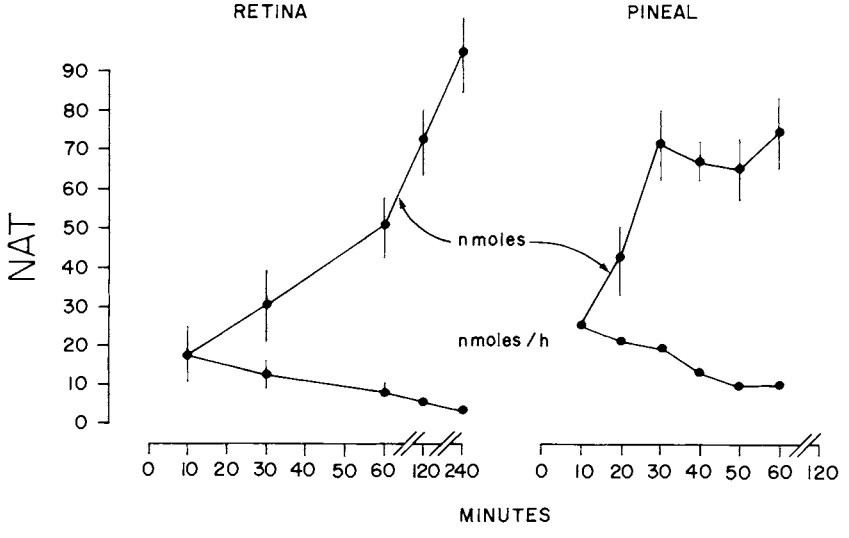

Fig. 9. The time course of NAT (nmoles/tissue/hr) under assay conditions.

Subcutaneous isoproterenol injections lower pineal but not retinal NAT; intraocular isoproterenol lowers ocular but not pineal NAT(10). Thus, NAT of both retina and pineal is inhibited by adrenergic agents such as isoproterenol. There appears to be a blood-ocular barrier for isoproterenol.

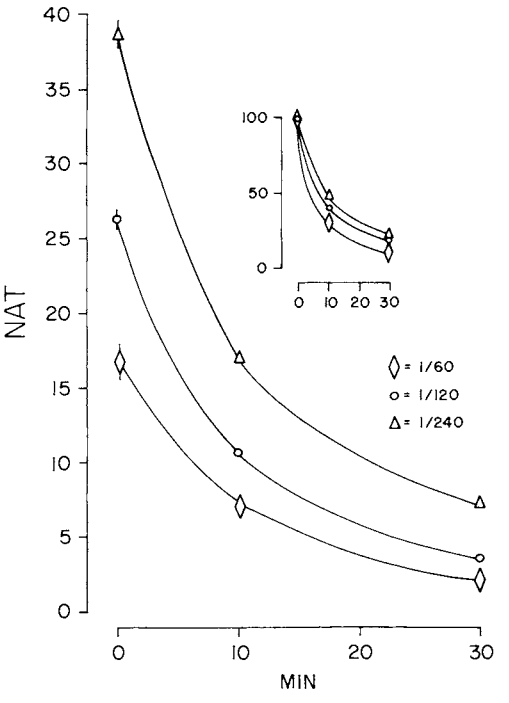

Fig. 10. Dilution (1 retina/x microliters homogenate) results in higher NAT in retinas; inset normalized.

Intraocular cycloheximide, a protein synthesis inhibitor, lowers both pineal and retinal NAT, but subcutaneous cycloheximide only reduced pineal NAT (10). Thus, cycloheximide inhibits both retinal and pineal NAT. Cyclohexmide can apparently get out of the eye. Because protein synthesis inhibitors reduce NAT, high NAT probably requires protein synthesis. However, cycloheximide is poisonous and any effects of the compound could be secondary.

VI. PINEAL AND RETINA ARE SIMILAR

In the chick alone, a dozen parameters are similar between pineal and retina including (i) photoreceptor-like cells, (ii) presence of melatonin, serotonin, HIOMT, and NAT, (iii) high NAT and melatonin in the dark-time, (iv) persistence of NAT and melatonin rhythms in DD, (v)

TABLE I: NAT of chick pineal (10) and retina are stabilized by some compounds and inhibited by others. Retinas were homogenized (1 retina/60 microliters) in 6.8 phosphate buffer and incubated for 20 min with one of the compounds listed (8 mM). NAT values are given for the retina to show the type of data that were obtained. The % columns show the per cent protection against loss of activity at 37C, or, in two cases, inhibition (*) of NAT. Cysteamine and penicillamine are sulfhydryl compounds related structurally to acetyl coenzyme A (AcCoA); dithiothreitol (DTT) is a sulfhydryl agent; cystamine is a disulfide.

Compound	Retina NAT	Retina %	Pineal %
None	3.6 +/- 0.4		
AcCoA	11.4 +/- 1.4	70	97-131
Cysteamine	8.2 +/- 0.5	41	21-25
Serotonin	1.4 +/- 0.1	60*	
Cystamine	4.7 +/- 0.2	10	55*
Penicillamine	4.5 +/- 0.2	8	31
DTT	3.7 +/- 0.2	1	21-45
0C	14.9 +/- 1.7		

attenuation of NAT rhythms by light, (vi) refractoriness of NAT response to dark encompassing dawn, (vii) rapid plummet response of NAT to light at night, (viii) suppression of NAT by extension of light into the dark-time, (ix) NAT inhibition by cycloheximide, (x) NAT inhibition by isoproterenol, (xi) thermal NAT inactivation, and (xii) NAT protection by acetyl CoA and cysteamine. It seems reasonable, then, to seek functional explanations for the similarity between pineal and retina.

VII. PINEAL AND RETINA FUNCTION

Pineal roles are well established in (i) reproduction, (ii) circadian rhythms, and (iii) skin pigmentation. In considering the functions of ocular melatonin these possibilities exist since some ocular melatonin does enter the circulation. An alternative (or additional) possibility is (iv) that ocular melatonin has functions in the eye (8).

The pineal functions in reproduction. The pineal is a source of an antigonadal hormone. Melatonin is probably this hormone. Darkness causes regression of gonads in some

species; the regression is prevented by pinealectomy. Blinding, like darkness, results in gonad regression.

Circadian behavior is regulated by the pineal. Circadian rhythms (e.g. in perch-hopping) are abolished by pinealectomy in some species (e.g. sparrows) producing arrhythmic animals. Blinding produces arrhythmia in chicken locomotor activity (21). Melatonin injections synchronize cycles of some species so it may be the signal by which the pineal conveys rhythm information to the rest of the body. In other species (e.g. pigeons, quail), pinealectomy does not abolish rhythms, but a combination of blinding and pinealectomy produces arrhythmic locomotor activity (22,23). Thus the pineal and eyes may be acting in the same way with respect to circadian rhythm control.

Skin color may be a consequence of melatonin. Melatonin was named for the fact that it lightens skin of some lower vertebrates by concentrating the pigment in cells called melanophores (24). The hypothesis is that the pineal secretion of melatonin in the dark-time is responsible for dark-time blanching. Some of the lower vertebrates have been blinded and shown to retain the dark-time blanching reaction. Anolis carolinensis did not have a melatonin rhythm in retina (12) so it seems that the retina may not secrete melatonin in Anolis.

The retina may have an "endocrine" role. Retinal melatonin accounts for 20-50% of the amount of melatonin that gets into the bloodstream in quail and chickens (3,25) which have substantive ocular melatonin cycles. Extraocular melatonin appears to be important at least in the control of behavioral circadian rhythms of quail, chicks, and pigeons.

Intraocular roles of melatonin seem likely. Rhythms and effects of melatonin have been found in several parameters: (i) disk shedding, (ii) photoreceptor cell length, (iii) pigment migration, and (iv) ocular pressure. Notably, some of the intraocular functions are reminiscent of melatonin effects on skin pigment cells (24,27).

Clocks may have NAT as a component (2,16,26). Pineal glands of chickens displayed spontaneous NAT cycles when they were placed in culture (2,16). Moreover, the performance of pineals in vitro was dependent upon the time the glands were removed and placed in culture (which the author has called timekeeping, 26). Xenopus retinas have the ability to generate NAT cycles in vitro (27). However, retina NAT may be responsive to inhibition by regulating agents -- adrenergic agents such as isoproterenol and serotonin -- these could come from cells within the retina or have non-ocular origins. Retinas, then, like pineals, may be 'circadian biological clocks' in some species.

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RHYTHMS OF MELATONIN BIOSYNTHESIS IN RETINA: INVOLVEMENT OF CALCIUM, CYCLIC AMP AND DOPAMINE IN THE REGULATION OF SEROTONIN N-ACETYLTRANSFERASE[1]

P. Michael Iuvone

Department of Pharmacology
Emory University School of Medicine
Atlanta, Georgia 30322

I. INTRODUCTION

Convincing evidence supports the hypothesis that melatonin is synthesized in the retinas of vertebrates, as well as in their pineal glands. Melatonin-like immunoreactivity has been observed in the retinas of mammals, amphibians, and birds (Bubenik et al., 1974, 1976; Vivien-Roels et al., 1981; Osol and Schwartz, 1984; Pang et al., 1985; Dubocovich et al., 1985; Underwood and Siopes, 1985; Wiechman et al., 1986). The presence of authentic melatonin in retinas of cows, chickens, and humans has been established using high pressure liquid chromatography with electrochemical detection and gas chromatography-mass spectroscopy (Reppert and Sagar, 1983; Leino, 1984; Hall et al., 1985). The levels of melatonin-like immunoreactivity in retina do not decrease following pinealectomy (Hamm and Menaker, 1980; Yu et al., 1981; Reiter et al., 1983). Thus, retinal melatonin is not synthesized in the pineal gland, the major source of circulating melatonin. Retina contains the enzymes for the synthesis of melatonin from serotonin, serotonin N-

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acetyltransferase (EC 2.3.1.87) and hydroxyindole O-methyltransferase (HIOMT; EC 2.1.1.4) (Baker et al., 1965; Cardinali and Rosner, 1971b; Wainwright, 1979; Binkley et al., 1979; Miller et al., 1980; Iuvone and Besharse, 1983). Furthermore, conversion of [^{3}H]serotonin to [^{3}H]melatonin in retina has been demonstrated (Cardinali and Rosner, 1971a; Gern and Ralph, 1979). Both melatonin-like immunoreactivity and HIOMT-like immunoreactivity have been localized in the photoreceptor layer of the retina, consistent with the hypothesis that melatonin is synthesized in retinal photoreceptors (Bubenik et al., 1974, 1976; Vivien-Roels et al., 1981; Wiechman et al., 1985).

In the pineal gland, melatonin biosynthesis occurs as a diurnal rhythm. N-Acetylation of serotonin appears to be a key regulatory step in pineal melatonin biosynthesis (Klein and Weller, 1970; Binkley et al., 1973). Diurnal rhythms of melatonin-like immunoreactivity and of N-acetyltransferase (NAT) activity have also been observed in retinas of rats, chickens, quails, and frogs (e.g., Binkley et al., 1979; Hamm and Menaker, 1980; Miller et al., 1980; Pang et al., 1980, 1985; Iuvone and Besharse, 1983; Reiter et al., 1983; Underwood and Siopes, 1985; Wiechman et al., 1985). The rhythms in retinas of these animals are characterized by peak levels of melatonin and NAT activity at night. The rhythms in chickens and African clawed frogs (Xenopus laevis) have been described as circadian (Hamm and Menaker, 1980; Iuvone and Besharse, 1983); that is, the daily rhythm persists in constant darkness. In other species the rhythm may be dependent on exposure to light and dark (Wiechman et al., 1986).

This paper reviews the evidence for the regulation of NAT activity in the retina of Xenopus laevis by a circadian pacemaker that is localized in the eye. Evidence for specific roles for dopamine, calcium, and cyclic AMP in the regulation of retinal NAT activity is also discussed.

II. CIRCADIAN RHYTHM OF RETINAL NAT ACTIVITY

Melatonin is synthesized from the biogenic amine serotonin (5-hydroxytryptamine) by the sequential actions of NAT and HIOMT. NAT, an acetyl coenzyme A-requiring enzyme, converts serotonin to N-acetylserotonin. HIOMT, which uses S-adenosylmethionine as cofactor, methylates

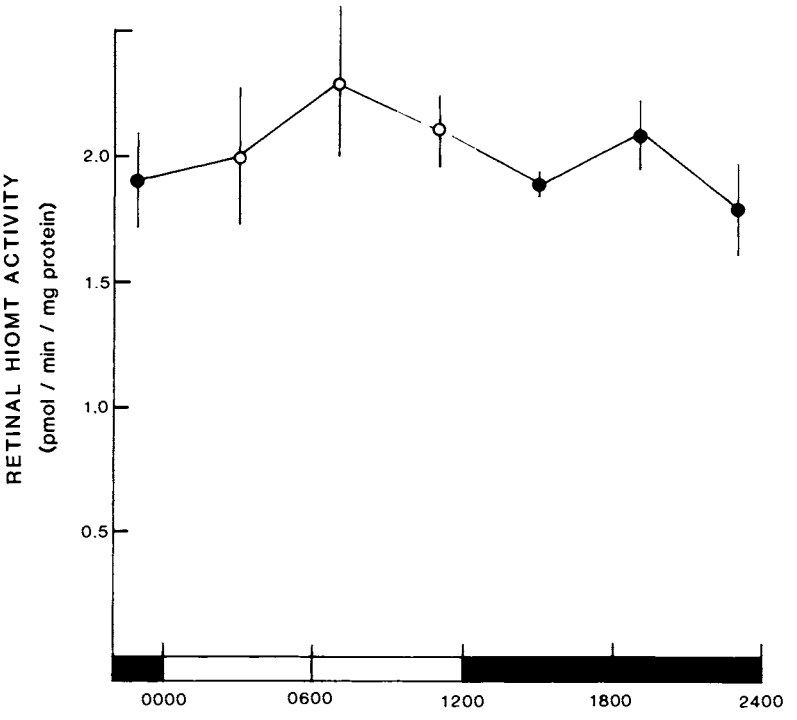


Figure 1. HIOMT activity in Xenopus retina. Frogs were housed on a 12 hr light:12 hr dark cycle, and were killed at the times indicated. Open circles represent activity in retinas prepared in light, and filled circles represent that of retinas prepared under dim red light (Kodak Wratten No. 2). HIOMT activity was determined in the supernatant fraction of retinal homogenates by measuring the transfer of a [^{14}C]methyl group from [^{14}C]-S-adenosylmethionine to N-acetylserotonin (Axelrod and Weissbach, 1961), essentially as described by Yang and Neff (1976). The concentrations of S-adenosylmethionine and of N-acetylserotonin were 70 μM and 0.8 mM, respectively. N = 5/time point.

the hydroxyindole moiety of N-acetylserotonin to form melatonin (5-methoxy-N-acetyltryptamine).

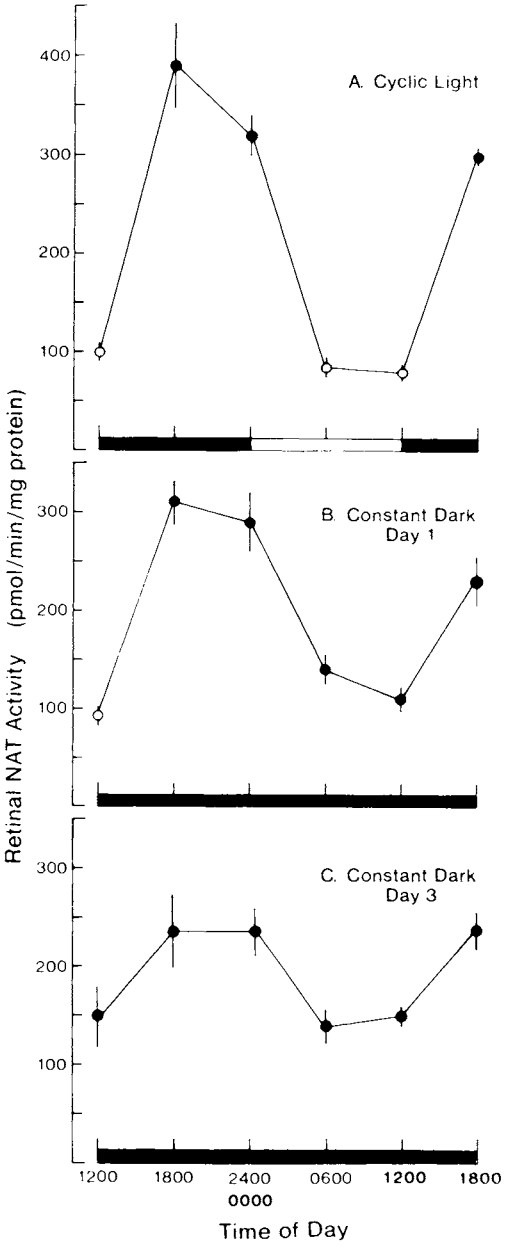
Retinal NAT activity of frogs (X. laevis) maintained on a 12 hr light:12 hr dark cycle, occurred as a diurnal rhythm (Iuvone and Besharse, 1983). NAT activity was lowest at the middle of the light period, began to rise shortly before light offset, and peaked after approximately 9 hr of darkness. NAT activity during the 9th hr of

darkness was 6-7 fold higher than that during the 9th hr of the light period. Retinal NAT activity continued to cycle for at least 3 days in constant darkness (Iuvone and Besharse, 1983), indicating that the rhythm was circadian. Constant light exposure for 1 day dramatically suppressed the amplitude of the rhythm, while constant light for 3 days abolished it.

In contrast to the high amplitude rhythm of retinal NAT activity, no significant differences in HIOMT activity of Xenopus retina, measured in retinal homogenates, were observed over the 12 hr light: 12 hr dark cycle (Fig. 1). Diurnal changes in cofactor availability could result in a rhythm of HIOMT activity in situ, but no evidence presently exists for such changes. The available data suggest that production of N-acetylserotonin by NAT is the key regulatory step in the synthesis of melatonin in Xenopus retina.

To determine if the circadian clock regulating the rhythm of retinal NAT activity is intrinsic to the eye, the activity of NAT was examined in cultured eye cups to eliminate possible contributions of neural or endocrine inputs to the retina (Besharse and Iuvone, 1983). A rhythm of retinal NAT activity, similar to that observed in intact animals, was seen in eye cups cultured under conditions of 12 hr light: 12 hr dark, and in eye cups cultured in constant darkness for 1 or 3 days (Fig. 2).

Figure 2. Retinal NAT activity in eye cups maintained in culture. Eye cups were prepared 2 hr prior to the time of normal light offset, and were cultured under conditions of 12 hr light:12 hr dark or in constant darkness. Open circles represent retinas dissected in light, and filled circles represent those dissected under dim red light. The first time point (1200 h) is the time of normal light offset. In A and B, this corresponds to a period 2 hr after preparation of the cultures whereas in C it corresponds to 50 hr after culture preparation. NAT activity was determined in retinal homogenates by measuring the transfer of a [^{14}C]acetyl group from [1-^{14}C]acetyl coenzyme A to tryptamine (Deguchi and Axelrod, 1972), as described in Iuvone and Besharse (1986a). N = 5/time point. Data were analyzed by analysis of variance and the rhythms in each case were significant (for A and B, $p < 0.001$; for C, $p < 0.01$). Reproduced from Besharse and Iuvone (1983) by permission from Nature, Vol. 305, No. 5930, pp. 133-135, Copyright (c) 1983 Macmillan Journals Limited.


A. Cyclic Light
B. Constant Dark
Day 1
C. Constant Dark
Day 3
Retinal NAT Activity (pmol/min/mg protein)
400
300
200
100
300
200
100
300
200
100
1200
1800
2400
0000
0600
1200
1800
Time of Day

In intact animals, the rhythm of NAT activity could be re-entrained to a new light-dark cycle (Iuvone and Besharse, 1983). To determine if the phase of the *in vitro* rhythm could be modified, a phase reversal experiment was conducted. Eye cups were cultured for 48 hr on a light-dark cycle that was reversed relative to the cycle to which the animals had been previously exposed. Samples taken at 6 hr intervals over a subsequent 30 hr period in constant darkness revealed that the rhythm of retinal NAT activity had completely reversed during the 48 hr re-entrainment period *in vitro* (Besharse and Iuvone, 1983). Thus, the eye cup preparation contains both the circadian clock and the entrainment pathway for the regulation of the rhythm of retinal NAT activity.

III. EFFECT OF CYCLIC AMP ON RETINAL NAT ACTIVITY

In the pineal gland, NAT activity is stimulated by a cyclic AMP-dependent mechanism (Klein and Berg, 1970; Deguchi and Axelrod, 1973; Deguchi, 1973,1979). Cyclic AMP also appears to be involved in the regulation of NAT activity in retina. Light suppresses the nocturnal increase of NAT activity. However, when dibutyryl cyclic AMP and 8-bromocyclic AMP were added to cultures of light-exposed eye cups, retinal NAT activity increased (Iuvone and Besharse 1983,1986a; Fig. 3). Dibutyryl cyclic GMP, adenosine, and 5'AMP had no effect on NAT activity under identical conditions, suggesting a specificity for the cyclic adenine nucleotide. The increase of NAT activity that occurs in dark-adapted retinas was not additive to that elicited by dibutyryl cyclic AMP (Fig. 3), suggesting that darkness and dibutyryl cyclic AMP increase NAT activity by similar mechanisms. The phosphodiesterase inhibitor 3-isobutylmethylxanthine (IBMX) increased cyclic AMP levels and stimulated NAT activity of light-exposed retinas. Forskolin, an activator of adenylate cyclase, also increased both cyclic AMP levels and NAT activity of light-exposed retinas (Fig. 4). The effects of forskolin on both cyclic AMP and NAT were synergistically potentiated by IBMX, supporting the hypothesis that forskolin increases NAT activity by stimulating cyclic AMP synthesis. Collectively, these data suggest that NAT activity in the retina is regulated by a cyclic AMP-dependent mechanism, as it is in the pineal gland.

The mechanisms responsible for the cyclic AMP-mediated

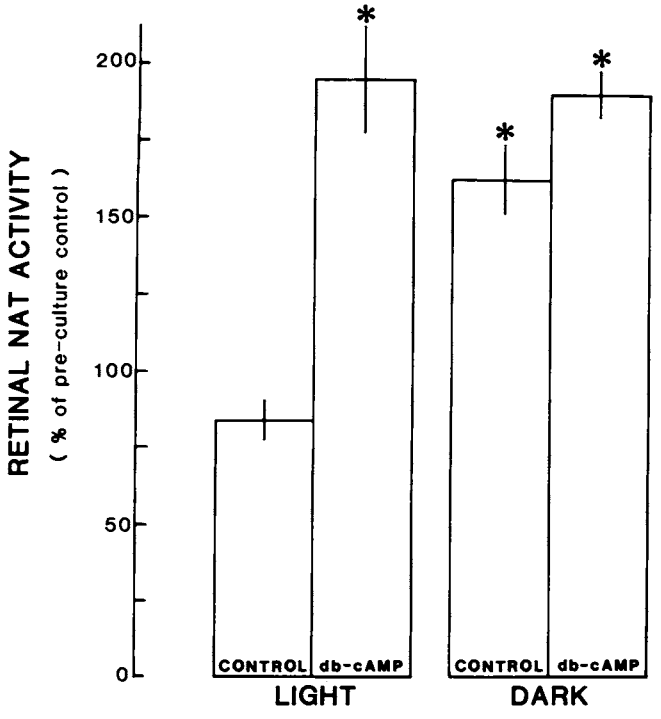


Figure 3. Effect of dibutyryl cyclic AMP on NAT activity of retinas cultured in light or dark. Eye cups were prepared just prior to the time of normal light offset, and were subsequently cultured for 4 hr in light or dark, with (db-cAMP) or without (control) 2.0 mM dibutyryl cyclic AMP. Data are expressed as percent of preculture control, which was the activity in eye cups prepared at the time of light offset but not subsequently cultured: 75 ± 8 pmol/min·mg protein. N = 5/group. *$p < 0.01$ vs pre-culture control. Reproduced from Iuvone and Besharse (1983), Brain Research 273, 111-119, Elsevier, Amsterdam.

increase of NAT activity are not fully understood. The stimulation of NAT activity by dibutyryl cyclic AMP, IBMX, or forskolin was not inhibited in calcium-free medium (Iuvone, 1986; Iuvone and Besharse, 1986a), which blocks most types of synaptic transmission. Thus, cyclic AMP probably elicits its effect directly within the NAT-containing cells, rather than on another cell type that communicates with the NAT cells via Ca^{2+}-dependent

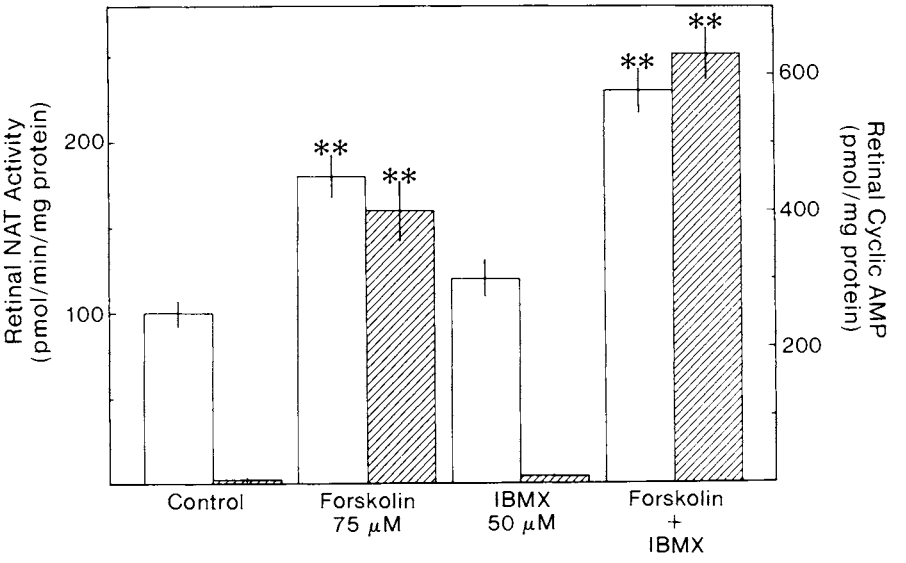


Figure 4. Forskolin stimulates retinal cyclic AMP and NAT activity in eye cups cultured in light. Eye cups were incubated in light in the presence of forskolin and IBMX for 10 min for cyclic AMP measurements (▨) and for 6 h for NAT activity measurements (□). In each group, N = 6-10. **p < 0.01. Reproduced from Iuvone and Besharse (1986a), J. Neurochem. 46, 33-39, Raven Press, New York.

synaptic transmission. Attempts to activate NAT in a cell-free system by cyclic AMP-dependent phosphorylation were unsuccessful (Iuvone and Besharse, 1986a), suggesting that phosphorylation of the enzyme by cyclic AMP-dependent protein kinase is probably not involved. Furthermore, the protein synthesis inhibitors cycloheximide and puromycin inhibited the dibutyryl cyclic AMP-evoked increase of NAT activity in light-exposed retinas (Iuvone and Besharse, 1986a), as well as the nocturnal increase of enzyme activity in dark-adapted retinas (Iuvone and Besharse, 1983). Thus, retinal NAT activity may be regulated by an effect of cyclic AMP-dependent protein kinase on the synthesis of the enzyme or of an effector molecule. A more definitive assessment of the molecular basis for the regulation of NAT activity awaits the development of specific antibodies for studies of enzyme induction.

IV. INVOLVEMENT OF CALCIUM IN THE REGULATION OF RETINAL NAT ACTIVITY

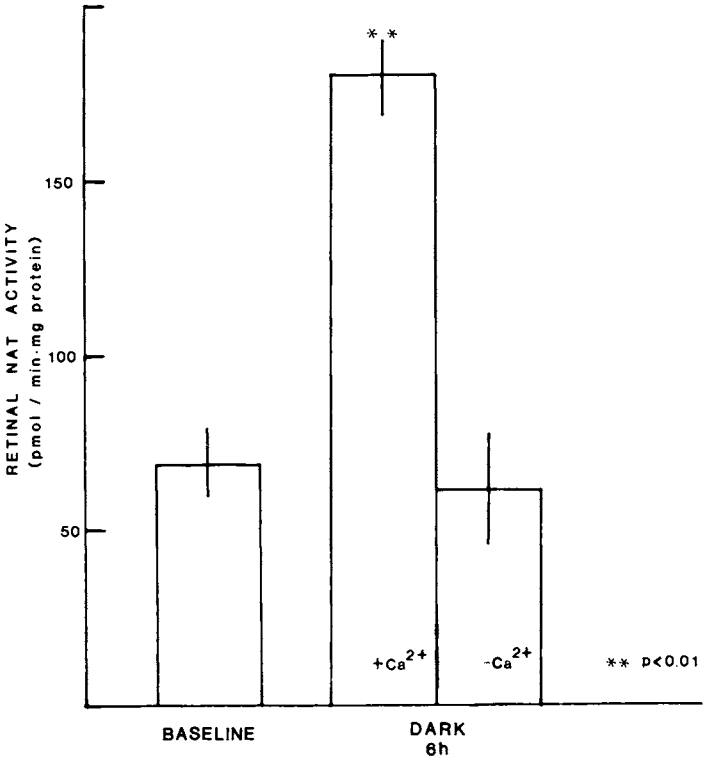


Figure 5. Calcium is required for the increase of retinal NAT activity in the dark. Eye cups were prepared in normal incubation medium containing 1.8 mM $CaCl_2$. The samples were preincubated in the light for 30 min in normal medium ($+Ca^{2+}$) or in medium without $CaCl_2$ ($-Ca^{2+}$). Following the preincubation, the samples were transferred to fresh '$+Ca^{2+}$' medium or '$-Ca^{2+}$' medium and were incubated in the dark for 6 hr. At the end of the incubation period, retinas were dissected, frozen, and assayed for NAT activity. Baseline samples were prepared as described above, but were dissected and frozen at the beginning of the incubation period. In each group, N = 5. $^{**}p < 0.01$. Reproduced from Iuvone and Besharse (1986b), J. Neurochem. 46, 82-88, Raven Press, New York.

Cyclic AMP is generally recognized as a second messenger for a variety of neurotransmitters and hormones. In the rat pineal gland, the stimulation of cyclic AMP synthesis and NAT activity is mediated by the action of norepinephrine, released from sympathetic nerve terminals,

on a β-adrenergic receptor in pinealocyte plasma membranes (Deguchi, 1973). The involvement of cyclic AMP in the regulation of NAT activity in retina suggested the possible involvement of Ca^{2+}-dependent release of a neurotransmitter or neuromodulator that increases cyclic AMP synthesis in the NAT-containing cells. Furthermore, photoreceptors, presumably the retinal cell type that contains NAT, possess voltage-sensitive Ca^{2+} channels that are open in darkness and closed as a consequence of light exposure (Fain et al., 1980; Corey et al., 1984). For these reasons, the involvement of Ca^{2+} in the regulation of NAT activity was examined.

Omitting $CaCl_2$ from the culture medium completely blocked the nocturnal increase of NAT activity in retinas incubated in the dark (Fig. 5). In EGTA-buffered medium, 10^{-3}-10^{-4} M free Ca^{2+} was required for the maximal increase of NAT activity in the dark (Iuvone and Besharse, 1986b). Other divalent cations, including Mn^{2+}, Ba^{2+}, and Sr^{2+}, did not substitute for Ca^{2+}. Mg^{2+} and Co^{2+}, divalent cations that block Ca^{2+} channels and inhibit synaptic transmission (del Castillo and Engbaek, 1954; Weakly, 1973; Dowling and Ripps, 1973; Cervetto and Picollino, 1974), completely inhibited the dark-dependent increase of retinal NAT activity in the presence of Ca^{2+} (Iuvone and Besharse, 1986b). The organic calcium channel blockers nifedipine and D600 also inhibited the dark-dependent increase of NAT activity. These results suggest that Ca^{2+} influx through voltage-sensitive calcium channels is involved in the dark-dependent increase of NAT activity.

Many of the intracellular effects of calcium are inhibited by trifluoperazine, which inhibits protein kinase C and calmodulin-requiring enzymes (Weiss and Levin, 1978; Schatzman et al., 1981). Trifluoperazine also inhibited the dark-dependent increase of NAT activity (Iuvone and Besharse, 1986b).

Although the dark-dependent increase of NAT activity is a calcium-dependent process, the stimulation of NAT activity by dibutyryl cyclic AMP, IBMX, and forskolin is not (Iuvone and Besharse, 1986a). These findings suggest that retinal NAT activity may be subject to regulation by redundant mechanisms, one Ca^{2+}-dependent and the other cyclic AMP-dependent, and that Ca^{2+}, rather than cyclic AMP, is responsible for the dark-dependent increase of NAT activity. Alternatively, Ca^{2+} influx and cyclic AMP accumulation may be components of a cascade of events leading to increased NAT activity, with Ca^{2+} influx preceding the increase of cyclic AMP. One possible cascade involves Ca^{2+} influx into nerve terminals triggering the release of

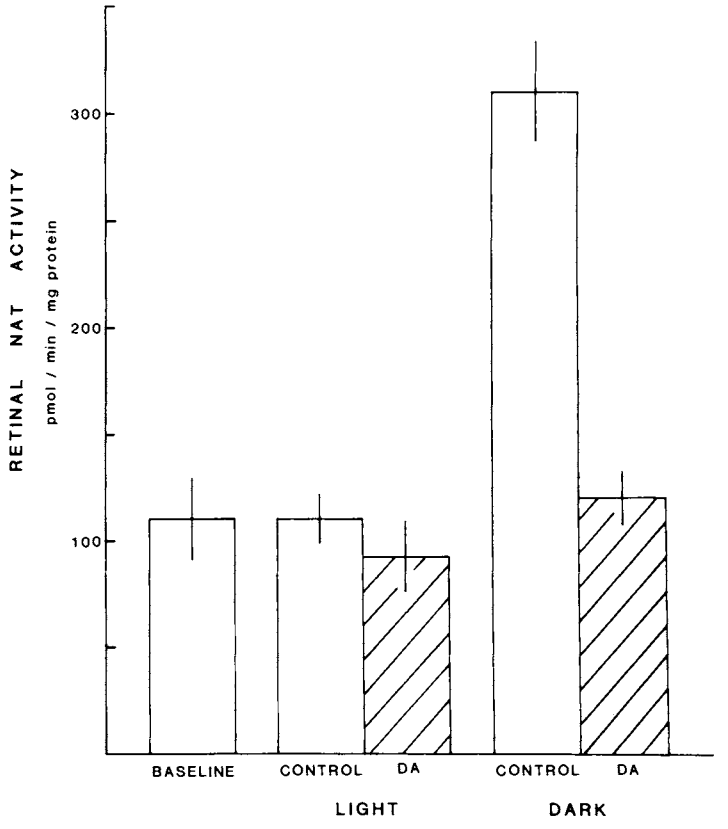


Figure 6. Effect of dopamine on retinal NAT activity in light and dark. Eye cups were prepared at the time of normal light offset and were incubated in light or dark for 6 hr. Dopamine was added to the incubation medium at a concentration of 10 μM. Following incubation for 6 h, retinas were dissected, frozen, and assayed for NAT activity. N = 5/group. Reproduced from Iuvone and Besharse (1986c), Brain Research 369, 168-176, Elsevier Biomedical Press, Amsterdam.

a neurotransmitter that increases adenylate cyclase activity in the NAT-containing cells. It is noteworthy that norepinephrine released from sympathetic nerve terminals increases cyclic AMP and, consequently, NAT activity in rat pineal (see Axelrod, 1974), and that blockade of voltage-sensitive calcium channels inhibits norepinephrine and epinephrine release from sympathetic nerve terminals and adrenal medullary cells (Kilpatrick et al., 1983; Callanan and Keenan, 1984). An alternate cascade might involve a depolarization of the NAT-containing cells,

presumably photoreceptors, in the dark. Depolarization would open voltage-sensitive Ca^{2+} channels; Ca^{2+} influx might subsequently result in activation of a Ca^{2+}/calmodulin-dependent adenylate cyclase. Consistent with this hypothesis, current through voltage-sensitive Ca^{2+} channels in photoreceptors is increased in darkness and is blocked by Co^{2+}, Mg^{2+}, and D600 (Fain et al., 1980) at concentrations similar to those that inhibit the dark-dependent increase of NAT activity.

V. THE ROLE OF DOPAMINE IN THE REGULATION OF RETINAL NAT ACTIVITY

The involvement of norepinephrine in the regulation of pineal NAT activity led us to investigate the possible role of norepinephrine and related catecholamine neurotransmitters in the regulation of NAT activity in the retina. Although norepinephrine increases NAT activity in the rat pineal gland (Klein and Berg, 1970), it decreases NAT activity in the pineal gland of chickens (Deguchi, 1979; Riebman and Binkley, 1979). Therefore, norepinephrine was tested for both stimulatory and inhibitory effects on NAT activity in Xenopus retinas. Norepinephrine (50 μM) had no effect on NAT activity of light-exposed retinas, but suppressed the dark-dependent increase of NAT activity (Besharse et al., 1984; Iuvone and Besharse, 1986c).

Dopamine is the predominant catecholamine in the retinas of several vertebrates (Haggendal and Malmfors, 1965; DaPrada, 1977; Iuvone et al., 1978a; Hadjiconstantinou et al., 1983), including Xenopus laevis (Iuvone, in preparation). Dopamine is localized in retinal amacrine and interplexiform cells (reviewed in Iuvone, 1984). Light stimulates the synthesis, release, and metabolism of retinal dopamine (Kramer, 1971; DaPrada, 1977; Iuvone et al., 1978a,b, unpublished observations; Cohen et al., 1983). Similar to norepinephrine, dopamine (10 μM) had no effect on retinal NAT activity in the light, but completely inhibited the increase of enzyme activity in the dark (Fig. 6). Thus, dopamine mimicked the inhibitory effect of light on NAT activity.

Half-maximal inhibition of the nocturnal increase of NAT activity occurred at 300 nM dopamine (Iuvone and Besharse, 1986c). The inhibitory effect of dopamine was not due to direct inhibition of the enzyme, but occurred

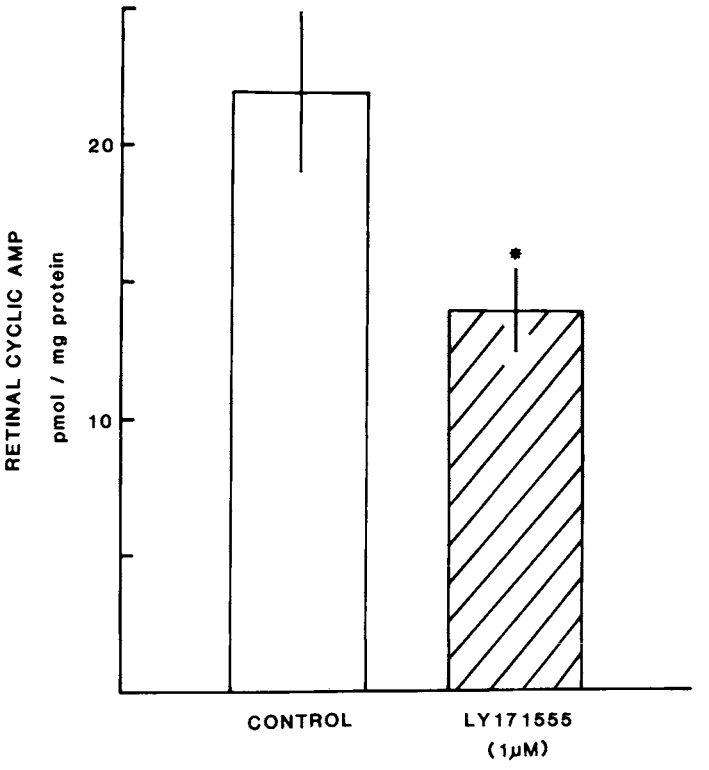


Figure 7. Effect of LY 171555 on retinal cyclic AMP accumulation. Eye cups were prepared at the time of light offset and were incubated in the dark for 30-40 min in medium, with or without LY 171555 (1 μM). Eye cups were dissected under infra-red light. N = 9-10/group. *$p < 0.05$. Reproduced from Iuvone (1986a), Life Sci. 38, 331-342, Pergamon, New York.

through a receptor-mediated mechanism. Because both norepinephrine and dopamine inhibited the dark-dependent increase of enzyme activity, selective receptor agonists and antagonists were used to characterize the receptor. These studies indicated the involvement of specific dopamine receptors, rather than α- or β-adrenergic receptors (Iuvone and Besharse, 1986c). Studies with benztropine, a dopamine uptake inhibitor, indicated that endogenous retinal dopamine can elicit the inhibition.

Further experiments were conducted to characterize the dopamine receptor subtype and to probe the mechanism of dopamine's action on NAT activity. Kebabian and Calne

(1979) have classified DA receptors into two subtypes based on pharmacologial and biochemical criteria: D_1 receptors, which are coupled to adenylate cyclase in a stimulatory fashion, and D_2 receptors, which are either not coupled to adenylate cyclase or mediate an inhibition of the cyclic AMP-synthesizing enzyme. The majority of dopamine receptors in retina appear to be D_1 receptors (Watling et al., 1979; Redburn et al., 1980), but radioligand binding of D_2 receptor antagonists has been reported (Magistretti and Schorderet, 1979; Redburn and Keyes, 1980; Schaeffer, 1980; Watling and Iversen, 1981; Makman et al., 1982). Three lines of evidence suggest the involvement of D_2 receptors in the inhibition of retinal NAT activity. First, D_2 receptors have a higher affinity for dopamine than do D_1 receptors (Kebabian and Calne, 1979), and dopamine inhibited the dark-dependent increase of NAT activity at concentrations below those required to stimulate retinal cyclic AMP accumulation (Iuvone, 1986a), a D_1 receptor-mediated response. Second, LY 171555 and bromocriptine, selective D_2 receptor agonists (Kebabian and Calne, 1979; Bach et al., 1980; Stoof and Kebabian, 1981; Tsuruta et al., 1981), inhibited the dark-dependent increase of NAT activity, while SKF 38393, a selective D_1 receptor agonist (Setler et al., 1978; Sibley et al., 1982), did not (Iuvone, 1986a; Iuvone and Besharse, 1986c). LY 171555 was both potent and efficacious as an inhibitor of the increase of NAT activity with an EC50 of approximately 75 nM and a maximal effect at 1 μM. Third, the inhibitory effects of dopamine and of LY 171555 on NAT activity were blocked by the D_2 receptor antagonists spiperone and metoclopramide, but not by the D_1 receptor antagonist SCH 23390 (Iuvone, 1986a).

Activation of the dopamine receptors involved in the regulation of NAT activity may result in an inhibition of adenylate cyclase. LY 171555 inhibited cyclic AMP accumulation in the presence of IBMX in dark-adapted retinas (Fig. 7). To test the hypothesis that the inhibitory effect of dopamine on NAT activity was mediated by an inhibition of adenylate cyclase, we examined the effect of dopamine on the increases of NAT activity in light elicited by IBMX and by dibutyryl cyclic AMP (Fig. 8). Dopamine inhibited the increase of NAT activity elicited by IBMX (Iuvone, 1986a), which appears to depend on endogenous cyclic AMP formation to increase NAT activity (Iuvone and Besharse, 1986a). In contrast, dopamine had no effect on the increase of NAT activity elicited by dibutyryl cyclic AMP, which does not require adenylate

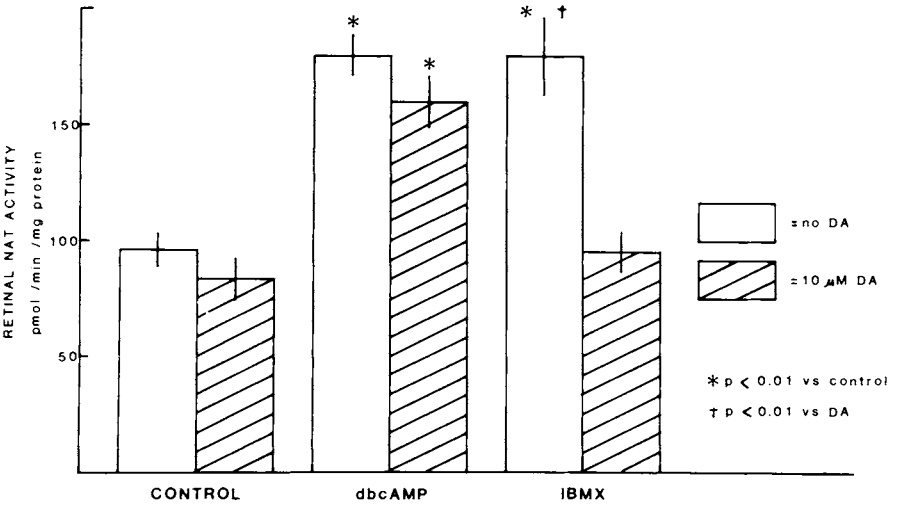


Figure 8. Effects of dopamine on the stimulation of NAT activity by dibutyryl cyclic AMP and IBMX. Eye cups were prepared at the time of light offset and were incubated in light for 6 hr. The concentration of dibutyryl cyclic AMP, IBMX, and dopamine were 2 mM, 0.3 mM, and 10 μM. N = 8-10/group. Reproduced from Iuvone (1986a), Life Sci. 38, 331-342, Pergamon, New York.

cyclase. The ability of dopamine to inhibit the IBMX-evoked increase of NAT activity was not diminished when Ca^{2+} was omitted from the culture medium, suggesting that Ca^{2+}-dependent synaptic transmission was not required to mediate the effect of dopamine. Based on these observations, we hypothesize that dopamine inhibits the increase of NAT activity by interacting with D_2 dopamine receptors that inhibit adenylate cyclase in the NAT-containing cells. Furthermore, the observation that dopamine inhibited both the dark-dependent increase of NAT activity and the increase elicited by IBMX in the light is consistent with the hypothesis that cyclic AMP mediates the dark-dependent increase of enzyme activity.

What then is the role of dopamine in the regulation of the circadian rhythm of NAT activity? Dopamine synthesis, release, and metabolism are stimulated by light, and both dopamine and light suppress the nocturnal increase of NAT activity. To test the hypothesis that dopamine mediates the inhibitory effect of light, we examined the effect of spiperone, a dopamine receptor antagonist, on the activity

of NAT in light-exposed retinas. Spiperone alone had no significant effect on NAT activity (Iuvone and Besharse, 1986c). However, spiperone in combination with a low concentration (50 μM) of IBMX, which alone had no significant effect, antagonized the light-evoked suppression of NAT activity (Iuvone, 1986b). Similar synergistic interactions were observed between spiperone and other phosphodiesterase inhibitors, Ro20-1724 and papaverine, and between IBMX and other dopamine receptor antagonists, including haloperidol, metoclopramide, α-flupenthixol and clozapine (Iuvone, in preparation). These results suggest that dopamine is partially responsible for the inhibitory effect of light on NAT activity, and raise the possibility that phosphodiesterase activation may play a role in the regulation of the enzyme.

The regulation of NAT activity in Xenopus retina, chicken pineal, and rat pineal have features in common, such as the stimulation of NAT activity by cyclic AMP and inhibition by protein synthesis inhibitors. However, some aspects of NAT regulation in Xenopus retina more closely resemble those in chicken pineal than those in rat pineal. For example, in the chicken pineal and Xenopus retina the circadian clock that regulates the rhythm of NAT activity is intrinsic to the tissue (Binkley et al., 1978; Deguchi, 1979; Besharse and Iuvone, 1983), while in the rat pineal the clock is localized in the suprachiasmatic nucleus of the brain (see Axelrod, 1974; Moore, 1974). Both chicken pineal and Xenopus retina contain photosensitive elements that influence NAT activity, while the rat pineal does not (Deguchi, 1981; Iuvone and Besharse, 1983). Furthermore, catecholamines inhibit NAT activity in chicken pineal and Xenopus retina (Deguchi, 1979; Riebman and Binkley, 1979; Iuvone and Besharse, 1986a), but stimulate NAT activity in rat pineal (Klein and Berg, 1970). It has been suggested that the role of norepinephrine in the regulation of chicken pineal NAT may be to synchronize the circadian oscillators in the pinealocytes (Cassone and Menaker, 1983). Perhaps dopamine serves a similar purpose in the retina.

VI. SUMMARY AND CONCLUSIONS

This paper has reviewed the evidence that NAT activity and, presumably, melatonin biosynthesis in the retina of Xenopus laevis is expressed as a circadian rhythm, and

that the clock that regulates this rhythm is intrinsic to the eye. Our studies on the regulation of this rhythm have focused on the molecular and cellular mechanisms responsible for the nocturnal increase of NAT activity and for the inhibitory effect of light on the activity of the enzyme. The nocturnal increase of NAT activity appears to involve both Ca^{2+}-dependent and cyclic AMP-dependent processes. The possibility that Ca^{2+} may be required for release of a transmitter which acts on the NAT-containing cells to increase enzyme activity has been considered, but candidates for such a transmitter have not yet been identified. The stimulatory influence of cyclic AMP on the expression of NAT activity appears to be mediated directly within the NAT-containing cell, but direct phosphorylation of the enzyme is probably not involved. The increase of NAT activity observed in darkness as well as that elicited by dibutyryl cyclic AMP in light is inhibited by protein synthesis inhibitors, suggesting that cyclic AMP may stimulate the synthesis of NAT or of an effector molecule.

Light inhibits the nocturnal increase of NAT activity. This inhibitory effect of light is mimicked by dopamine, a retinal neurotransmitter whose release is stimulated as a consequence of light exposure. The effect of dopamine appears to be mediated through a receptor with the pharmacological characteristics of a D_2 dopamine receptor. Activation of this receptor appears to decrease cyclic AMP synthesis and, consequently, to inhibit the nocturnal increase of NAT activity. Endogenous dopamine appears to play a physiological role in the light-evoked suppression of NAT activity, as evidenced by the ability of dopamine receptor blockers, in the presence of phosphodiesterase inhibitors, to antagonize the inhibitory effect of light. Thus, endogenous dopamine appears to play an inhibitory role in the regulation of retinal melatonin biosynthesis. Conversely, melatonin may play an inhibitory role in the regulation of retinal dopamine release. Melatonin inhibits the Ca^{2+}-dependent release of [^{3}H]dopamine from rabbit and chicken retinas (Dubocovich 1983,1985) and decreases the concentration of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in *Xenopus* retina (Pierce *et al.*, 1984). Thus, melatonin may contribute to the suppression of dopaminergic neuronal activity at night, and, thus, to the circadian rhythmicity of retinal dopamine turnover (Wirz-Justice *et al.*, 1984).

If melatonin is synthesized in photoreceptors, as indicated by the immunohistochemical localization of melatonin and of HIOMT (Bubenik *et al.*, 1974,1976; Vivien-Roels *et al.*, 1981; Wiechman *et al.*, 1985), then

these studies suggest that certain aspects of photoreceptor metabolism may be regulated by an inner retinal neurotransmitter, dopamine. This hypothesis is supported by the recent report of [^{3}H]spiperone binding sites (dopamine receptors) on bovine photoreceptor membranes (Brann and Jelsema, 1985) and by the observations that dopamine stimulates cone contraction and blocks dark-adaptive cone elongation in teleost and Xenopus retinas (Dearry and Burnside, 1985; Pierce and Besharse, 1985). Similar to the nocturnal increase of NAT activity, dark-adaptive cone elongation appears to occur by a cyclic AMP-dependent mechanism (Besharse et al., 1982; Burnside et al., 1982; Porello and Burnside, 1984), and dopamine may inhibit cone elongation through a D_2 receptor that decreases adenylate cyclase activity. In addition, melatonin has been shown to activate rod outer segment disc shedding (Besharse and Dunis, 1983; Besharse et al., 1984), and dopamine, by inhibiting melatonin synthesis, may influence the turnover of outer segment membranes.

These observations, coupled with the inhibitory effect of melatonin on retinal dopamine release and metabolism, suggest that melatonin and dopamine are components of a retinal feedback loop that regulates rhythmic metabolism in the photoreceptor-pigment epithelial complex. The existence of such of feedback loop may provide a cellular and molecular basis for the observations of Bubenik and Purtill (1980) that melatonin enhances light-induced photoreceptor degeneration, while bromocriptine, a D_2 dopamine receptor agonist, has a protective effect.

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MELATONIN AND DOPAMINE INTERACTIONS IN THE REGULATION OF RHYTHMIC PHOTORECEPTOR METABOLISM[1]

Mary E. Pierce
Joseph C. Besharse

Department of Anatomy and Cell Biology
Emory University School of Medicine
Atlanta, Georgia 30322

I. INTRODUCTION

Certain aspects of photoreceptor metabolism are regulated by the interaction of the light-dark cycle and an endogenous circadian clock (reviewed by Besharse, 1982; Besharse and Iuvone, 1983). Melatonin or a related indoleamine, synthesized locally, may play a role in the control of those rhythmic processes. The retina contains the enzyme systems for melatonin synthesis (Baker et al., 1965; Gern and Ralph, 1979; Binkley et al., 1980). Serotonin N-acetyltransferase (NAT) and hydroxy-indole-O-methyl transferase (HIOMT), the melatonin-synthesizing enzymes, both exhibit diurnal fluctuations in activity (Cardinali and Rosner, 1971; Hamm and Menaker, 1980; Iuvone and Besharse, 1983; Besharse and Iuvone, 1983) as do their products, N-acetylserotonin (Pang et al., 1981, 1983) and melatonin (Hamm and Menaker, 1980, Pang et al., 1985). The circadian rhythm of NAT activity persists in cultured Xenopus laevis eye cups and can be phase shifted suggesting that the eye contains both a self-sustaining clock and entrainment pathway (Besharse and Iuvone, 1983). Although the melatonin-synthesizing cell has not been unequivocally identified, melatonin-like and HIOMT-like immunoreactivity have been localized in the photoreceptor layer (Bubenik et al., 1974;

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Vivien-Roels et al., 1981; Wiechmann et al., 1985) making the photoreceptor a likely candidate.

The question which arises is what is the function of retinally synthesized methoxyindoles? Underwood et al. (1984) have suggested that the eye may release melatonin into the bloodstream. They have demonstrated in quail that the blood rhythm of melatonin concentration is the result of melatonin secretion from both the pineal gland and the eye. Within the retina, melatonin has been implicated in the regulation of photoreceptor membrane turnover (Besharse and Dunis, 1983a), cone photoreceptor movements (Pierce and Besharse, 1985a), pigment aggregation in the retinal, pigmented epithelium (Kraus-Ruppert and Lembeck, 1965; Chèze and Ali, 1976; Pang and Yew, 1979), and the regulation of retinal dopamine release and metabolism (Dubocovich, 1983; Pierce et al., 1984).

There is an interesting reciprocal relationship between melatonin and dopamine synthesis in the retina. Dopamine is synthesized by a subset of amacrine cells in most species (reviewed by Iuvone, 1986b), and by interplexiform cells of some teleosts and New World monkeys (Laties and Jacobowitz, 1966; Dowling and Ehinger, 1975). The activity of the rate limiting enzyme for dopamine synthesis, tyrosine hydroxylase, also occurs rhythmically with peak enzyme activity and dopamine release during the day (Iuvone et al., 1978; Reme et al., 1983, Wirz-Justice et al., 1984). NAT activity and melatonin release peak during subjective night, directly out of phase with the dopamine rhythm (Binkley et al, 1980; Hamm and Menaker, 1980; Pang et al., 1980; Iuvone and Besharse, 1983; Besharse and Iuvone, 1983). Dubocovich (1983, 1984) has demonstrated that melatonin inhibits the Ca^{2+} dependent release of dopamine from rabbit and chicken retinal slices. In Xenopus eye cups, melatonin decreases the level of 3,4-dihydroxyphenylacetic acid, a dopamine metabolite (Pierce et al., 1984). Dopamine also regulates melatonin biosynthesis: dopamine receptor agonists block the dark-induced rise in NAT activity (Besharse et al., 1984; Iuvone and Besharse 1986b; Iuvone, 1986ab; see Iuvone, this volume) and dopamine antagonists block the effect of dopamine (Iuvone and Besharse, 1986b; Iuvone, 1986ab). The dopamine receptor mediating effects on NAT activity is of the D_2 subtype (Iuvone and Besharse, 1986b; Iuvone, 1986a). These data have led to the suggestion that melatonin and dopamine may be important components of a regulatory mechanism that controls rhythmic photoreceptor metabolism (Iuvone and Besharse, 1986a,b). In this model melatonin would be a signal for darkness and dopamine a signal for light (Pierce and Besharse, 1985a).

Melatonin, Dopamine and Photoreceptor Disc Shedding

Photoreceptor outer segment membrane turnover is a complex process that involves the assembly of opsin-containing discs at the base of the outer segment, displacement of the discs distally, and shedding of the outer segment tip which is subsequently phagocytized by the pigment epithelium (reviewed by Young, 1976). The circadian nature of rod disc shedding has been demonstrated in some species (reviewed by Besharse, 1982). The observations that removal of several endocrine glands including the pineal had no effect on disc shedding (Currie et al., 1978; LaVail and Ward, 1978; Tamai et al., 1978), that disc shedding could be induced monocularly in intact animals (Hollyfield and Basinger, 1978; Tierstein, et al., 1980), and that rhythmic and light-evoked shedding would occur in in vitro culture systems (Flannery and Fisher, 1979, 1984; Besharse et al., 1980) has indicated that the principal locus of control for shedding is within the eye.

We have used eye cups from Xenopus laevis to study the regulation of rod shedding and cone retinomotor movement. Because this species exhibits a circadian component in its disc shedding rhythm (Besharse, et al., 1977) and because methoxyindoles are synthesized locally, we examined the effects of methoxyindoles on disc shedding (Besharse and Dunis, 1983b, Besharse et al., 1984). In culture conditions which were non-permissive for shedding (low bicarbonate concentration, Besharse et al., 1980; Besharse and Dunis, 1983a), melatonin activated rod shedding; that is melatonin treatment led to a light-evoked response. The activation required the presence of melatonin during a dark preincubation period before light onset. In addition, melatonin could be substituted for darkness as an activator of disc shedding in eye cups from constant light treated animals. This latter treatment abolished NAT rhythmicity (Iuvone and Besharse, 1983) and photoreceptor disc shedding (Besharse, 1980). This observation suggested that melatonin or a related indoleamine might play an important role in the dark-dependent process required for initiation of disc shedding (Besharse and Dunis, 1983b).

As seen in Table I, melatonin is a potent activator of disc shedding, and under the experimental conditions imposed is more effective than 5-methoxytryptophol, another methoxyindole reportedly synthesized in retina (Pévet et al., 1980) through a pathway that would not involve NAT activity (Wurtman and Ozaki, 1978). These data show that melatonin is effective at a lower concentration (0.5 μM) than reported in our original studies (0.5mM) where 5-methoxytryptophol

Table 1. Activation of disc shedding in non-permissive culture medium by melatonin and 5-methoxytryptophol

Condition[1]	Concentration	N	X±SE[2]	%Increase[3]
Preculture	0	5	7.4±2.3	--
Control	0	5	6.2±1.2	--
Melatonin[4]	0.5 μM	5	19.5±3.3	215*
Preculture	0	7	4.2±0.8	--
Control	0	10	9.9±2.2	--
5-methoxytryptophol[5]	5 μM	5	9.8±2.3	-1
	100 μM	5	17.9±3.9	80
	250 μM	5	26.0±7.8	163**
	500 μM	5	38.8±4.9	292*

[1]Eye cups were prepared in darkness just prior to light onset and either fixed immediately (Preculture) or cultured for 45 minutes in darkness followed by 2.5 hours in light in the presence or absence of (control) methoxyindole at the indicated concentrations. The low bicarbonate culture medium was identical to that used previously except that it contained 10μM ascorbic acid. Drugs were dissolved directly in medium without the aid of the carrier solvent, dimethyl sulfoxide.

[2]Mean phagosomes per mm RPE plus or minus standard error based on number of eye cups indicated under N.

[3]Average percent increase over control incubated in absence of the drug. Asterisks signify values significantly above control (*$p<.005$;**$p<.05$).

[4]The effect of 0.5μM melatonin seen here is comparable to that reported previously (Besharse, et al., 1984). The effect at this low concentration has been seen in three unpublished replicates of the experiment.

[5]The 5-methoxytryptophol data were derived from two successive experiments. The control groups were repeated for each of the two days and are reported here as pooled data. Three additional experiments during this period show no effect of 5-methoxytryptophol over the 0.005 to 5μM range. In one other experiment 100μM 5-methoxytryptophol gave an intermediate effect.

was actually found to be more potent than melatonin (Besharse and Dunis, 1983b). In those experiments we routinely used dimethyl sulfoxide to dissolve drugs. Subsequently, we conducted experiments at lower drug concentrations without the use of dimethly sulfoxide in a low

bicarbonate medium containing ascorbic acid (see Besharse et al. 1984). The higher potency of melatonin (Table I) may be related to the altered culture conditions or to the omission of dimethyl sulfoxide. Although melatonin appears more effective than 5-methoxytryptophol, the apparent dependence of melatonin potency on experimental conditions suggests that dose response data alone would be a poor index for judging which compound normally plays a physiological role.

Our data were derived from experiments on intact eye cups and therefore do not provide a basis for identifying the cell type directly affected by melatonin or 5-methoxytryptophol. Melatonin could effect pigment epithelium, photoreceptors, or other retinal elements. Ogino et. al. (1983) have reported that melatonin inhibits the phagocytosis of latex particles by cultured chick embryo pigment epithelial cells. This inhibition may represent a real difference between their experimental system and ours in which disc detachment and phagocytosis were activated by melatonin. However, since they were unable to distinguish between attached versus ingested latex spheres, and since the number of particles associated with pigment epithelium was very low (less than 1 per cell), their results should be interpreted with caution.

A role for dopamine in the control of rhythmic disc shedding in the rat has been suggested recently (Remé and Wirz-Justice, 1985). Intraperitoneal injection of clorgyline or metamphetamine caused a decrease in the peak of shedding observed after light onset. Both treatments would be expected to increase levels of dopamine but by different mechanisms (Cooper et al., 1982). In addition, both melatonin and dopamine have been implicated as modulators of photoreceptor degeneration in the rat (Bubenik and Purtill, 1980). It is interesting that both constant light treatment and dopamine treatment inhibit rod disc shedding in this system, and that constant light effectively blocks disc shedding in amphibian preparations as well (Besharse, 1980). Using eye cups from constant light treated Rana pipiens, we have recently found that apomorphine, a dopamine agonist, inhibits the large shedding response obtained following treatment with one hour of darkness followed by light exposure (Table II). Interestingly, dopamine seems to antagonize the dark-priming process necessary for subsequent stimulation of shedding by light (Currie, et al., 1978). As noted above, we have previously suggested (Besharse and Dunis, 1983b) that melatonin may be important in the dark process necessary for light-evoked shedding in Xenopus. One aspect of the dark process may be suppression of dopamine release as has been suggested for the control of dark-adaptive retinomotor movement (see below).

Table II. Inhibition of dark-dependent, L-evoked disc shedding in Rana pipiens eye cups by apomorphine

Condition[1]	Drug[2] Concentration	N	Phagosomes/mmRPE X±SE
Preculture	0	6	7.8±2.3
A. 1hr D → 1.5hr L	0	5	54.0±8.9
B. 1hr D → 1.5hr L	10 μM	6	21.0±4.8[3]
C. 1hr D → 1.5hr L Apo. in D only	10 μM	6	33.0±6.1[3]
D. 1hr D → 1.5hr L Apo. in L only	10 μM	6	60.2±11.7

[1]Animals were maintained in constant light for 4 days. Eye cups were prepared in room light and either fixed immediately (preculture) or following a treatment of 1 hr in darkness (D) followed by 1.5hr in light (L) using procedures like those in our prior studies of disc shedding (Besharse, et al., 1980).

[2]Apomorphine (Apo) at 10μM was added 15 minutes before beginning the dark treatment in B and C. In C eye cups were transferred to drug-free medium at the time of transfer from D to L. In D the drug was added to medium only at the time of transfer from D to L.

[3]Disc shedding measured as average phagosome content of pigment epithelium, was significantly ($p<.025$) inhibited compared to the drug free control (A) when apomorphine was present throughout the experiment (B) or when present in darkness only (C). Addition of apomorphine in light only (D) was without effect. This suggests that dark-priming rather than subsequent disc detachment and phagocytosis is blocked by apomorphine.

The mechanism for control of rhythmic rod disc shedding is complicated by two kinds of observations. First it is largely restricted temporally to a period just after light onset each day. Second, light has direct effects on disc shedding as well as effects on a circadian clock that influences shedding. Thus, light, darkness and a circadian oscillator may interact in complicated ways to influence both the amplitude and phase of the disc shedding rhythm. A

simplifying hypothesis is that direct effects of light and the effects of a circadian clock are mediated in different ways. This hypothesis comes from our recent work on light-evoked disc shedding in X. laevis and R. pipiens eye cups where we have obtained evidence that light directly triggers disc shedding through a mechanism that involves an excitatory amino acid neurotransmitter system (Greenberger and Besharse, 1985; Besharse, et al., 1986). Superimposed over such a direct triggering mechanism, we hypothesize that an additional pathway from a circadian clock also influences disc shedding. The relative balance between the two mechanisms may have been subject to evolutionary change. This would account for the observation that direct light effects predominate in R. pipiens (Basinger, et al., 1976) while control by a circadian clock may predominate in albino rats (LaVail, 1976). Disc shedding in X. laevis exhibits properties suggesting a strong direct influence by light as well as the influence of a circadian oscillator (Besharse, et al., 1977; Besharse, 1982). Circadian variations in melatonin and dopamine may be components of the clock input while an excitatory amino acid system may be fundamental to the direct triggering mechanism. In some species, strong interactions between the two systems may occur.

Melatonin, Dopamine and Retinomotor Movements

The retinas of some lower vertebrates adjust to changes in lighting conditions with movements of their photoreceptors. In the dark cones elongate, rods contract, and in those species which exhibit pigment migration, melanin pigment granules aggregate at the bases of the pigment epithelial cells. In the light, movements are reversed; cones contract, rods elongate and pigment granules move out into the long apical processes of the pigment epithelial cells. Photoreceptor movements may serve to position the outer segments for optimal exposure to incoming light while pigment dispersion may function to protect the retina from intense illumination (Ali,, 1975). Since the late 1800's many studies have described the effects of light and darkness on retinomotor movements (see reviews by Ali, 1975; Besharse, 1982; Burnside and Nagle, 1983), but relatively little is known about the mechanism by which the light cycle affects these movements. The circadian nature of retinomotor movements was first demonstrated in 1937 when Welsh and Osborne reported that cones continue to elongate and contract at subjective dusk and dawn in constant dark treated catfish. Similar phenomena have been described in other species of fish (reviewed by Besharse, 1982; Burnside and Nagle, 1983).

Insights into the mechanisms underlying the motile events have come from an analysis of reactivated movements in detergent-lysed teleost photoreceptor cells (reviewed by Burnside and Nagle, 1983). Elongation is a microtubule-dependent process in cones (Warren and Burnside, 1978) which may utilize a dynein-like ATPase as the force generator (Gilson et al., 1985). Cone contraction is mediated by an actomyosin system analogous to smooth muscle contraction (Burnside, 1978; Burnside et al., 1982b). Reactivated cone elongation requires high levels of cAMP and low levels of Ca^{2+} while contraction requires the reverse conditions (reviewed by Burnside and Nagle, 1983). In intact cells, the addition of cAMP analogues in the light stimulates all three dark-adaptive retinomotor movements which has led to the suggestion that night time increases in cAMP may serve as a general signal for darkness in the retina (Burnside et al., 1982a; Besharse et al., 1982).

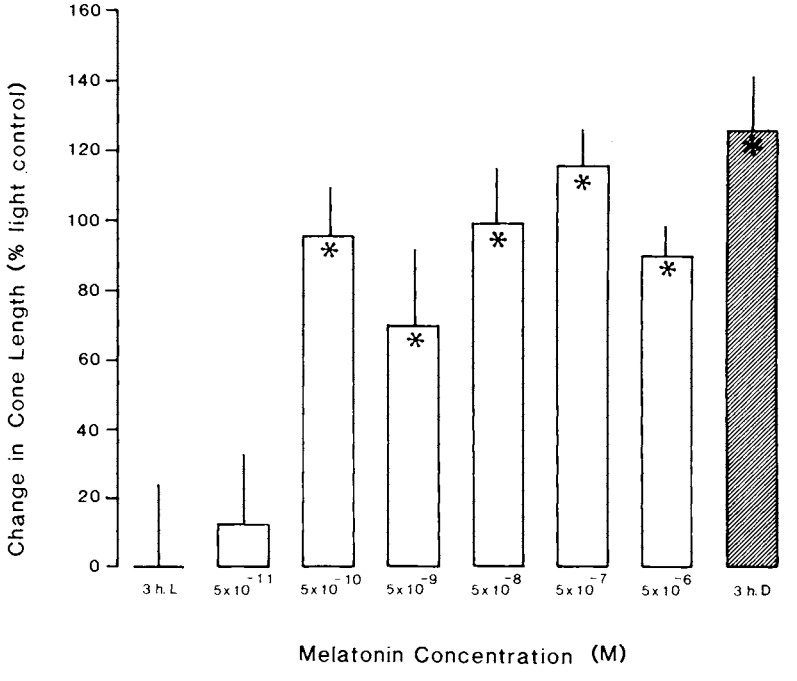

Fig. 1 Melatonin induces cone elongation in 4 day constant light treated X. laevis. Eye cups were cultured for 3 hrs. in light (open bars) or darkness (stippled bar) in the presence or absence of melatonin. *p.<.01 compared to light control (from Pierce and Besharse, 1985a).

Melatonin mimics the effects of darkness on retinomotor movements. There are several reports of melatonin-induced melanin pigment aggregation in the RPE of frogs (Kraus-Ruppert and Lembeck, 1965), trout (Cheze and Ali, 1976) and guinea pigs (Pang and Yew, 1979). Recently, we have demonstrated that melatonin causes dark-adaptive cone elongation (Fig. 1; Pierce and Besharse, 1985a) in X. laevis eye cups, an in vitro preparation that sustains light and dark adaptive cone movements (Besharse, 1982; Besharse et al., 1982) and changes in NAT activity (Besharse and Iuvone, 1983), as well as light-evoked disc shedding (see above). Cones were fully contracted after a three hour incubation in the light (Fig. 1) but were elongated when maintained in the dark or in the light with melatonin. Melatonin was very potent (0.0005-5μM) in stimulating cone elongation in eye cups prepared from constant-light treated Xenopus, a treatment known to block cone movement (Besharse, 1982) and NAT rhythmicity (Besharse and Iuvone, 1983). However, in our original study we were unable to obtain a reproducible effect of melatonin using eye cups prepared at the time of normal

Table III. Melatonin Induces Cone Elongation in Cyclic Light X. laevis at Low Light Intensity [1]

Treatment	Intensity (W/cm^2)	Melatonin (0.5μM)	N	Average Cone Length X (μm) ± SEM[2]
3 hr light	2×10^{-3}	absent	4	12.2±2.0
3 hr light	2×10^{-3}	present	4	9.15±2.3
3 hr light	7×10^{-8}	absent	5	9.2 ±0.9
3 hr light	7×10^{-8}	present	5	35.1±4.3 [3]
3 hr dark	--	absent	5	43.7±3.6 [4]

[1]Eye cups were prepared in the light near the time of normal light offset from X. laevis maintained on a 12 hour L: 12 D light schedule for 1 month.

[2]Cone length is the distance from the outer limiting membrane to the base of cone oil droplets. Twenty cones were measured for each eye cup and an average length obtained; N equals the number of eye cups.

[3]$p<.01$ compared to $7 \times 10^{-8} W/cm^2$ light control.

[4]$p<.01$ compared to either light control.

light offset from animals maintained on a 12 hour light: 12 hour dark cycle.

Recently, we have investigated the relationship among light intensity, cone position and melatonin's effect because in natural diurnal conditions melatonin's effect would probably occur at low light intensities or in the dark. We found that at the time of normal light offset cones maintained their contracted state at much lower light intensities than were used in our earlier experiments, (Table 3). At lower light intensities near the threshold for maintainance of the contracted state, melatonin stimulated cone elongation comparable to darkness. These results

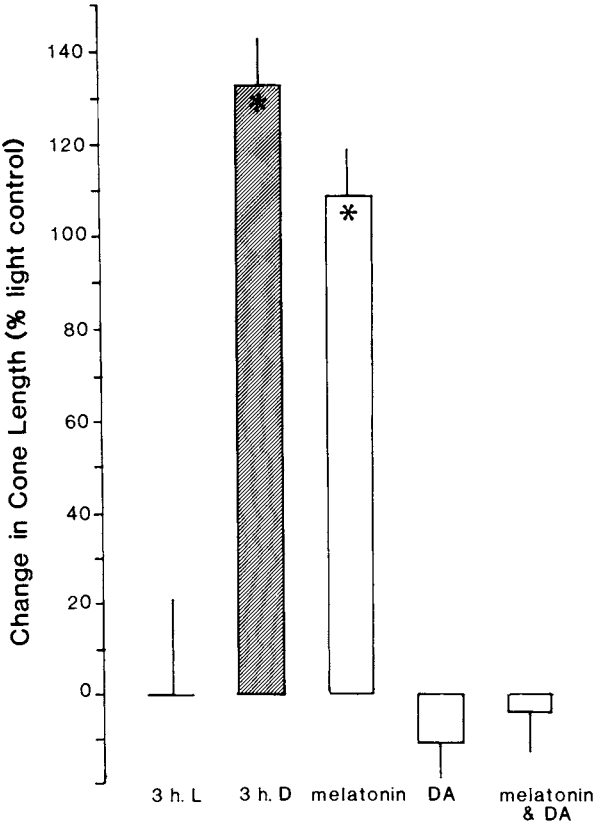

Fig. 2 Dopamine (50μM) inhibits melatonin (0.5μM) induced elongation in eye cups from constant light treated X. laevis. Open bars, light; stippled bar, dark. *p<.01 compared to light control (from Pierce & Besharse, 1985a)

suggest that melatonin is a key component of the nighttime signal for cone elongation. Furthermore, both melatonin-induced (Fig. 2) and dark-induced elongation were blocked by dopamine through a mechanism involving receptors of the D_2 subtype (Pierce and Besharse, 1985a). This suggests that one effect of melatonin may be to affect cone movement indirectly via modulation of dopamine.

These results also suggested to us that dopamine might be part of the light signal for cone contraction. We found that dopamine induced cone contraction in both cyclic and constant light treated animals (Fig. 3; Pierce and Besharse, 1985a). For these experiments, eye cups were preincubated in the dark for 2-3 hours. When dopamine was added in the subsequent dark incubation, cones contracted. The dopamine receptor antagonist spiroperidol blocked light-induced cone contraction implying that endogenous dopamine was involved in the response (Pierce and Besharse, 1985a). Spiroperidol also induced cone elongation in the light suggesting that

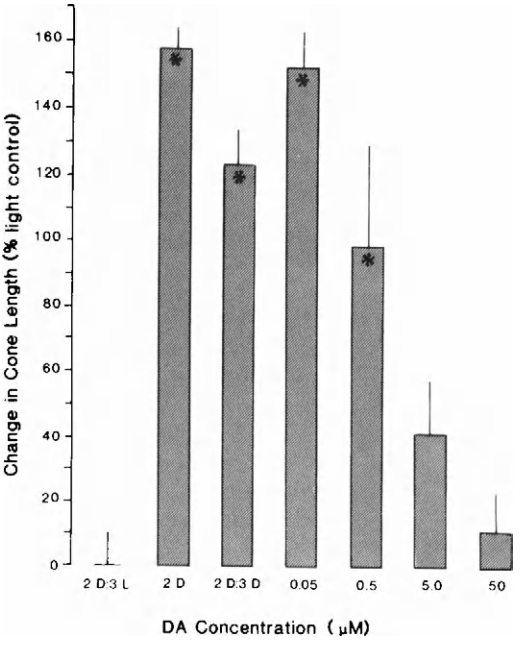

Fig. 3 Dopamine induces cone contraction. Eye cups were dark adapted for 2 hrs. In the subsequent incubation eye cups were returned to light (bar on left) or maintained in the dark with or without dopamine. *$p<.01$ compared to light control (from Pierce and Besharse, 1985a).

simply blocking dopamine receptors was sufficient to induce elongation. Detailed pharmacological analysis of the dopamine response indicated that cone contraction was mediated by a dopamine receptor specifically of the D_2 subtype (Pierce and Besharse, 1985a) which is believed to block or reduce adenylate cyclase activity and thus decrease levels of cAMP (Kebabian and Calne, 1979). D_2 receptors also mediate dopamine's inhibitory effect on NAT activity (Iuvone and Besharse, 1986b; Iuvone, 1986a), consistent with our prediction (Besharse et al., 1984; Iuvone and Besharse, 1986a) that catecholamine receptors that increase adenylate cyclase activity would not play a direct role in the suppression of night-time activity. Dearry and Burnside (1985) have also suggested that a D_2 receptor may be involved in the regulation of cone position in teleosts. They have demonstrated that dopamine blocks cone elongation induced by either the adenylate cyclase activator forskolin or the phosphodiesterase inhibitor, isobutylmethylxanthine.

Cone Movement as a Model for Control of Retinal Rhythmicity

The regulatory scheme for cone movement in X. laevis may prove useful as a model for understanding other aspects of photoreceptor rhythmicity. For example, melatonin's output in the retina is under circadian control (Besharse and Iuvone, 1983; Besharse, et al., 1984) and could provide a circadian signal which drives other circadian phenomena. Although the assumption is that melatonin's effects are receptor mediated, the nature of the receptor and second messenger remain unknown. It has been suggested that at high concentrations melatonin can effect microtubule assembly-disassembly (Cardinali, 1980). Since cone elongation is a microtubule-dependent process (Warren and Burnside, 1978) and the photoreceptor may be a melatonin synthesizing cell (Bubenik et al., 1974; Vivien-Roels et al., 1981; Wiechmann, 1985), it is possible that melatonin could effect motility directly. Melatonin could also directly or indirectly increase the level of cAMP which has been suggested to act as a general signal for darkness (Burnside et al., 1982a; Besharse et al., 1982). Another possibility is that melatonin effects cone movement indirectly by modulating dopamine release as discussed above (Dubocovich, 1983, 1984; Pierce et al., 1984; Pierce and Besharse, 1985a). Our observations on cone movement (Fig. 2, Pierce and Besharse, 1985a) and disc shedding (Table 2) suggest that the suppression of dopamine may be a necessary component of the dark process for both these phenomena.

Melatonin also interacts with another neurotransmitter, GABA, to promote cone elongation. In eye cups from constant light Xenopus the GABA agonist muscimol induces cone elongation which is blocked by dopamine, while the antagonist picrotoxin induces cone contraction (Pierce and Besharse, 1985b). At higher light intensities where neither melatonin nor muscimol will induce elongation in cyclic light animals, the combination of the two induces elongation (Pierce and Besharse, 1985b). It is known that GABA inhibits dopamine biosynthesis (reviewed by Iuvone, 1986b), and that GABA agonists can mimic the effects of darkness on NAT activity (see Iuvone, this volume). GABA has been localized to horizontal cells and to a subpopulation of amacrine cells in X. laevis retinas (Hollyfield et al., 1979). It is interesting to note that light microscopic autoradiography of ^{3}H-melatonin binding in R. pipiens shows specific binding in the outer plexiform layer (Wiechmann et al., 1986). Although the specific cell type labeled by melatonin has not been identified, this layer is known to contain GABAergic horizontal cells (Voaden et al., 1974, Hollyfield et al., 1979) in frogs.

Increasing dopamine levels in the light may be an important component of the signal for cone contraction. In X. laevis a high affinity dopamine uptake system is localized only in a subpopulation of amacrine cells (Sarthy et al., 1981; Besharse, unpublished). Assuming that this amacrine population represents the only source of endogenous dopamine, we envision two possible mechanisms for dopamine's effect. The effect could be mediated trans-synaptically utilizing other transmitter systems, or dopamine could diffuse through the retina to affect cones directly. Recent reports have suggested that dopamine receptors may be found on rod photoreceptor outer segments (Brann and Jelsema, 1985). Dearry and Burnside have developed a cone inner segment/outer segment preparation from dark-adapted teleost retinas that retain the elongated myoids (see Dearry and Burnside, this volume). Dopamine stimulates contraction of the cone myoid in this preparation suggesting that dopamine receptors may be localized on cones. It is interesting that cone contraction appears to be mediated by a D_2 receptor (Pierce and Besharse, 1985a; Dearry and Burnside, 1985) which if localized on the cone would decrease levels of cAMP, one of the factors needed for reactivated contraction in detergent-lysed teleost cones (Porello and Burnside, 1984).

In summary, the evidence reviewed in this paper suggests that the retina contains a circadian oscillator which may control local synthesis of melatonin. Melatonin

effects photoreceptors and dopaminergic neurons which in turn regulate melatonin biosynthesis. It may be the interaction between the latter which drives rhythmic photoreceptor metabolism. Perhaps evolutionarily melatonin's original function was to regulate retinomotor movements involved in light and dark adaptation in the retina (see Menaker, 1982). Melatonin and GABA which promote cone elongation may predominate in dim light and at night while dopamine, which promotes cone contraction may predominate in bright light and daytime. The analysis of a model for cone movement can provide us with fundamental insights about the temporal regulation of rhythmic events in the photoreceptor-pigment epithelial complex.

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MODULATION OF DOPAMINERGIC ACTIVITY BY MELATONIN IN RETINA[1]

Margarita L. Dubocovich

Department of Pharmacology
Northwestern University Medical School
Chicago, Illinois 60611, USA

I. INTRODUCTION

Dopamine fulfills most of the criteria for a functional neurotransmitter in the retina (Ehinger, 1983) and recent evidence also supports a neuromodulatory role for this catecholamine in retinal function. In the rabbit retina dopamine-containing neurons localized in the inner plexiform layer form only interamacrine cell synapses and have no direct contact with horizontal, bipolar, or ganglion neurons (Holmgren-Taylor, 1982). Dopamine biosynthesis occurs in the retina following activation of tyrosine hydroxylase, the rate limiting synthetic enzyme (Iuvone et al., 1978; Morgan, 1982). The mammalian retina also possesses the enzymes for the degradation of dopamine (Dubocovich and Weiner, 1981), as well as a high affinity uptake system for this biogenic amine (Thomas et al, 1978). Dopamine is released from rabbit retina in response to photic and electrical stimulation, and by potassium-induced depolarization (Bauer et al, 1980; Thomas et al., 1978; Dubocovich and Weiner, 1981).

In the retina, as in other areas of the central nervous system, dopamine activates two subtypes of dopamine receptors: the D-1 and the D-2 (Stoof and Kebabian, 1984;

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Dubocovich and Weiner, 1985). Biochemical, pharmacological and electrophysiological evidence suggest that dopamine influences physiological functions within the retina through activation of D-1 and D-2 dopamine receptor subtypes. The D-1 dopamine receptor of the mammalian retina is linked to the stimulation of adenylate cyclase (Dubocovich and Weiner, 1985). In rabbit retina the D-1 dopamine receptor has been pharmacologically characterized using measurements of adenylate cyclase activity (Dubocovich and Weiner, 1985), and the specific binding of the selective D-1 dopamine antagonist ^{3}H-SCH 23390 (Cotterell and Dubocovich, in preparation). Jensen and Daw (1984) reported that dopamine receptor antagonists by blocking D-1 dopamine receptors change the sustained ON response found in some ON-center ganglion cells and some ON-OFF directionally selective cells of rabbit retina into a sustained inhibition and suggested that these effects are mainly due to blockade of D-1 dopamine receptors. We have recently characterize a dopamine receptor in the rabbit retina, with the pharmacological and biochemical characteristics of a D-1 dopamine receptor, which mediates calcium-dependent release of acetylcholine possibly through a cyclic AMP dependent process (Hensler and Dubocovich, 1985, 1986). Selective D-1 dopamine receptor agonists elicited the calcium-dependent release of acetylcholine, an effect blocked by the selective D-1 dopamine receptor antagonists SCH 23390 (Hensler and Dubocovich, 1985, 1986). Direct synaptic contacts, however, between dopaminergic and cholinergic amacrine cells in rabbit retina have not been reported (Ehinger, 1983). Whether the D-1 dopamine receptor mediating acetylcholine release is located on cholinergic cells, or is located on a neuron postsynaptic to dopamine cells (i.e., A II amacrine glycinergic) (Pourcho 1980, 1982) that in turn contact cholinergic amacrine cells affecting acetylcholine release through the release of its transmitter (i.e., glycine) (Cunninghan and Neal, 1983) is not known.

The D-2 dopamine receptor of the vertebrate retina possibly linked to the inhibition of adenylate cyclase (Iuvone, 1985) has been pharmacologically characterized using the binding of ^{3}H-spiperone (Dubocovich and Zahniser 1982; Watling and Iversen, 1981) and several functional responses. The synthesis, metabolism and turnover of dopamine in the retina appear to be modulated by the administration <u>in vivo</u> of selective D-2 dopamine receptor agonists and antagonists (Cohen et at, 1981; Morgan, 1982). In the rabbit retina the calcium-dependent release of ^{3}H-dopamine is modulated through activation of stereoselective D-2 dopamine autoreceptors which are involved in a negative feed-back mechanism mediated by the neurotransmitter itself (Dubocovich and Weiner, 1981,

1985). The potency of dopamine agonists to decrease and dopamine antagonists to increase the electrical stimulation evoked release of dopamine depends on the endogenous concentration of neurotransmitter (Dubocovich and Weiner, 1981, 1985; Dubocovich and Hensler, 1986). When the concentration of dopamine in the synaptic gap is augmented (by increasing the frequency of stimulation at which the neurotransmitter is released or by inhibiting the neuronal uptake of dopamine) the inhibitory effect of D-2 dopamine agonists is reduced while the effect of D-2 dopamine dopamine antagonists is potentiated. These results suggest dopamine receptors may be involved in the physiological modulation of dopamine release from rabbit retina.

Recently D-2 dopamine receptors have been found on bovine rod outer segments (Brann and Jelsema, 1985; Cotterell, Hamm, Dubocovich, unpublished observations). Activation of these D-2 dopamine receptors appear to increase the activity of cyclic GMP phosphodiesterase in a manner analogous to light, suggesting coupling of D-2 dopamine receptors to a GTP binding protein in rod outer segments (Jelsema et al., 1985). In retinas of lower vertebrates D-2 dopamine receptors also appear to modulate physiological and biochemical processes. Activation of D-2 dopamine receptors inhibits the dark-induced increase in the activity of serotonin N-acetyltransferase in the Xenopus laevis retina (Iuvone, 1985), and induces light-adaptative cone contraction in retinas from green sun fish (Dearry and Burnside, 1985) and Xenopus laevis (Pierce and Besharse, 1985). The selective D-2 dopamine receptor antagonist metoclopramide (Dubocovich and Weiner, 1985) depresses the a wave of the human electroretinogram, suggesting a role for dopamine receptors in human retinal physiology (Jaffe et al., 1985).

In mammalian retina several types of receptors have been identified which mediate inhibition of dopaminergic activity. These include D-2 dopamine autoreceptors, alpha-adrenoceptors and GABA and opiate receptors (Dubocovich and Weiner, 1981, 1983, 1985; Dubocovich, 1984a; Morgan, 1982; Marshburn and Iuvone, 1981). Although the dopamine-amacrine cells are activated by light, the nature of the stimulatory input to these cells is not known (Ehinger, 1983). Melatonin, a hormone originally discovered in the pineal gland, has also been found in the retina of several vertebrate species (Yu et al., 1981; Reiter et al., 1983). The enzyme system for melatonin synthesis exists in the retina, where the activity of one such enzyme, (serotonin N- acetyltransferase) varies with changes in light intensity in a circadian pattern (Cardinali and Rosner, 1971; Iuvone and Besharse, 1983; Wiechmann et al., 1985). Since melatonin is synthesized in a diurnal rhythm in the retina, with peak levels during the

dark period (Iuvone and Besharse, 1983), several investigators have suggested that melatonin secreted by photoreceptors or other indoleamine-containing neurons may be involved in diurnal events that normally occur in the retina. Melatonin has been implicated in photoreceptor outer segment disc shedding and phagocytosis (Besharse and Dunnis, 1983; Ogino et al., 1983), melanosome aggregation in retinal pigment epithelium (Pang and Yew, 1979) and cone photoreceptor retinomotor movement (Pierce and Besharse, 1985).

As the activity of dopamine containing neurons (Iuvone et al., 1978; Morgan, 1982; Ehinger, 1983; Hadjiconstantinou and Neff, 1984) and the synthesis of melatonin (Iuvone and Besharse, 1983) in retina are influenced by changes in illumination it was of interest to determine the effect of melatonin on the release of dopamine from the rabbit retina in vitro and in vivo.

II. RESULTS AND DISCUSSION

A. *Modulation of Dopamine Release by Melatonin in vitro*

The efffect of melatonin and related indoleamines has been investigated on the spontaneous and stimulation evoked release of ^{3}H-dopamine from the rabbit retina in vitro. Melatonin (5-OCH3 N-acetyl tryptamine) at picomolar concentrations (1pM-1nM) inhibits the calcium-dependent release of ^{3}H-dopamine from rabbit retina, elicited by either by electrical (3Hz, 20mA, 2msec) (Fig. 1) or potassium (20 mM) (Dubocovich et al, 1985a) depolarization. The inhibitory effect of melatonin was fully reversed by superfusing the retina with melatonin-free medium. The spontaneous outflow of radioactivity, and the release elicited by the indirect amine tyramine, which are mediated through a calcium-independent process, are not modified by melatonin (Dubocovich, 1983; 1985). The inhibitory effect of melatonin on the calcium-dependent release of ^{3}H-dopamine is not mediated through an increase in the neuronal uptake of ^{3}H-dopamine, or by activation of monoamine or opiate receptor sites (Dubocovich, 1983, 1985). It is remarkable that melatonin is about 1,000 times more potent than D-2 dopamine, opiate, and alpha-2 adrenoceptor agonists to inhibit the calcium-dependent release of ^{3}H-dopamine from retina (Fig. 1).

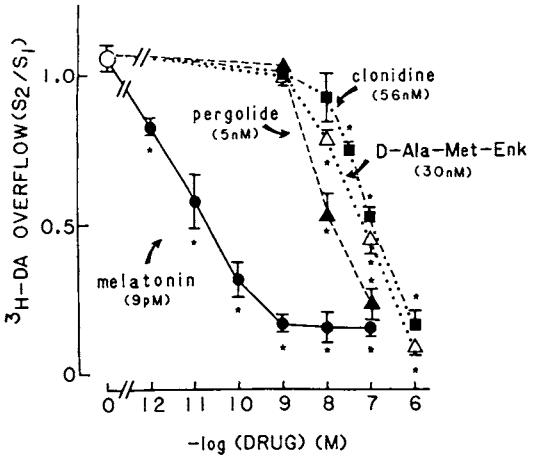

Fig. 1. Inhibitory effect of melatonin and dopamine, alpha-adrenoceptor and opiate agonists on the calcium- dependent release of dopamine from rabbit retina.

Ordinate: ^{3}H-DA overflow is the percentage of total tissue radioactivity released by electrical stimulation above the spontaneous levels of release. Results are expressed as the ratio obtained between the second (S_2) and the first (S_1) stimulation periods within the same experiment. The calcium-dependent release of dopamine was elicited by a 1-min period of electrical stimulation at 3 Hz (2 msec, 20 mA). In the controls, the percentage of the total tissue radioactivity release after the first min period of stimulation (S_1) was 1.49 ± 0.21 % (n=8). The radioactivity retained by the control tissue after 120 min superfusion was: 47.3 ± 4.8 nCi per chamber (n=8). Drugs were added 20 min before S_2. Only one concentration of each drug was tested per experiment.
○ , control; ● , melatonin; ▲ ,pergolide;
■ , clonidine; △ , D-Ala^2-Met^5-enkephalinamide.
Shown are mean values ± S.E.M.
* $p < 0.05$ when compared with the control.

The mechanism(s) involved in the inhibition of the calcium-dependent release of dopamine by melatonin is not known. Melatonin does not inhibit the calcium-dependent release of dopamine from dopaminergic nerve terminals in the

striatum or olfactory tubercle, which suggests a selective role for this hormone on the dopamine amacrine cells of retina (Dubocovich, 1985) and the dopamine interneurons of the hypothalamus (Zisapel and Laudon, 1982). In the brain, melatonin inhibits the depolarization-evoked calcium uptake and decreases cyclic AMP accumulation (Zisapel and Laudon, 1983; Vacas et al., 1981, 1984). It is possible that in the retina melatonin receptors are also linked to the inhibition of adenylate cyclase.

The involvement of calcium on the inhibitory effect of melatonin on ^{3}H-dopamine release from rabbit retina was investigated under experimental conditions known to change the availability of calcium for the secretory process (Dubocovich and Hensler, 1985; Dubocovich et al., 1985 a). In the retina the release of ^{3}H-dopamine elicited at 1, 3 or 6 Hz was of similar magnitude (2.63 ± 0.23 %, n=6) when the number of pulses was kept constant (360 pulses). Under these conditions the potency of melatonin to inhibit ^{3}H-dopamine release was of similar magnitude at all frequencies of stimulation (IC_{50}: 46 pM). The inhibitory effect of melatonin on ^{3}H-dopamine release elicited at 3Hz (20mA, 2msec) was more pronounced the lower the release of ^{3}H-dopamine (1.49 ± 0.21 %, n=8) elicited by a reduced number of pulses (IC_{50}: 9 pM for 180 pulses). Similarly, when the release of ^{3}H-dopamine was reduced by lowering the calcium concentration in the medium from 1.3 mM to 0.65 mM, the inhibitory effect of melatonin was potentiated at all frequencies (IC_{50}: 20 pM) (Dubocovich and Hensler, 1985). These data suggest that the inhibitory effect of melatonin is dependent on the external calcium concentration, and that activation of a melatonin receptor may decrease the availability of calcium for the secretory process (Dubocovich and Hensler, 1985; Dubocovich et al., 1985). It is unlikely that melatonin affects the secretory process directly since this hormone did not affect the calcium-dependent release of ^{3}H-dopamine from rabbit striatum or olfactory tubercle (Zisapel et al., 1982; Dubocovich, 1983, 1985).

Melatonin is about 1,000 times more potent than its precursor N-acetylserotonin in inhibiting the release of ^{3}H-dopamine from retina, while the putative neurotransmitter serotonin was inactive (Dubocovich, 1983). Figure 2 shows that the more active analogs of melatonin to inhibit ^{3}H-dopamine release were those possessing a 5-methoxy group on carbon 5 of the indole nucleus and an N-acetyl group on the same possition as in melatonin. The potencies of 5-methoxy indoles compounds was as follows (IC_{50}): melatonin (40 pM) = 6-chloromelatonin (40 pM) > 6-hydroxymelatonin (1.6 nM) > 6-methoxymelatonin (2 nM) > 5-methoxytryptamine (63 nM) > 5-methoxy-N,N-di-methyltryptamine (200 nM) >> 5-

methoxytryptophol (4 μM). N-Acetyl-5-OH-tryptamine was the only 5-OH indole found to inhibit the calcium-dependent release of ^{3}H-dopamine (Dubocovich, 1985). The structure activity relationships of melatonin and related indoles indicated that the efficacy of melatonin is determined by the moiety substituted on carbon 3 (i.e., ethyl N-acetyl group) of the indole nucleus (Dubocovich, 1985).

N-Acetyltryptamine, an indole with no substitution on carbon 5 of the indole nucleus, antagonizes the melatonin-induced lightening of the frog skin (Heward and Hadley, 1975). Similarly, in the chicken retina N-acetyltryptamine (10 nM-1 μM), although not inhibiting the calcium-dependent release of ^{3}H-dopamine, did antagonize competitively the melatonin-induced inhibition of ^{3}H-dopamine release (Dubocovich, 1984). In the rabbit retina, however, N-acetyltryptamine inhibited the calcium-dependent release of dopamine when tested alone. Nevertheless, increasing concentrations of this indole (10, 30 and 100 nM) shifted the

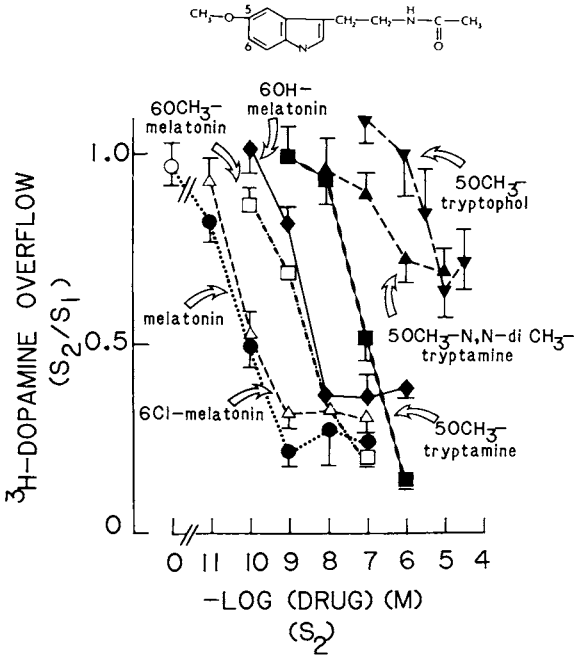

Fig. 2. Effect of 5-methoxy indoles on the calcium dependent release of ^{3}H-dopamine from rabbit retina.

For details see Fig. 1. The calcium-dependent release of ^{3}H-dopamine was elicited by a 2-min period of stimulation at 3Hz (20 mA, 2 msec). From Dubocovich, 1985.

concentration-effect curve for melatonin to the right. These results suggest that N-acetyltryptamine acts as a competitive antagonist in the chicken retina and as a partial agonist in the rabbit retina (Dubocovich 1984, 1985). Taken together these results suggest that melatonin selectively inhibits at picomolar concentrations the calcium-dependent release of ^{3}H-dopamine from rabbit retina through activation of a site possessing the pharmacological and functional characteristics of a receptor (Dubocovich 1983, 1984b, 1985).

B. *Inhibition of Dopamine Release by Melatonin in vivo*

The nature of the stimulatory input to the dopamine containing amacrine cells involved in the light-induced increase in dopaminergic activity is not known (Iuvone et al., 1978; Morgan, 1982; Wirzt-Justice et al., 1984; Parkinson and Rando, 1983). The potent inhibitory effect of melatonin on the release of dopamine from rabbit retina led to the suggestion that changes in illumination may modulate the activity of dopaminergic amacrine cells indirectly through changes in the release of melatonin, possibly by the photoreceptors (Dubocovich, 1983). Therefore we have investigated whether the light-dependent production of melatonin modulates dopamine release in vivo.

The effect of melatonin on dopamine release in vivo was studied indirectly by correlating specific ^{3}H-spiperone binding to D-2 dopamine receptors in retinas from rabbits kept under different light regimes with the melatonin levels in these tissues. ^{3}H-Spiperone labels sites with the pharmacological characteristics of D-2 dopamine receptors (Dubocovich and Zahniser, 1982). Scatchard plots were linear and Hill coefficients were one, indicating binding of ^{3}H-spiperone to a single site. Fig. 3 shows the effect of different light regimes on apomorphine (10 nM-100 uM) competition for binding sites labeled by a single concentration of ^{3}H-spiperone. The affinities of apomorphine for the dopamine site labeled by ^{3}H-spiperone in retinal membranes from rabbits sacrificed either at midnight or at noon were not significantly different. The specific binding of ^{3}H-spiperone in retinal membranes from rabbits exposed to constant light for one week was significantly reduced. Down regulation of D-2 dopamine receptors following constant light was also observed in the retina when receptors were labeled with a saturating concentration of ^{3}H-spiperone (Dubocovich et al., 1985b). The decrease in D-2 dopamine binding sites induced by constant light in retina appears to be associated with the activation of inhibitory melatonin receptors on dopaminergic neurons, since it is observed in retina, but not in striatum. In support of this hypothesis the light-induced

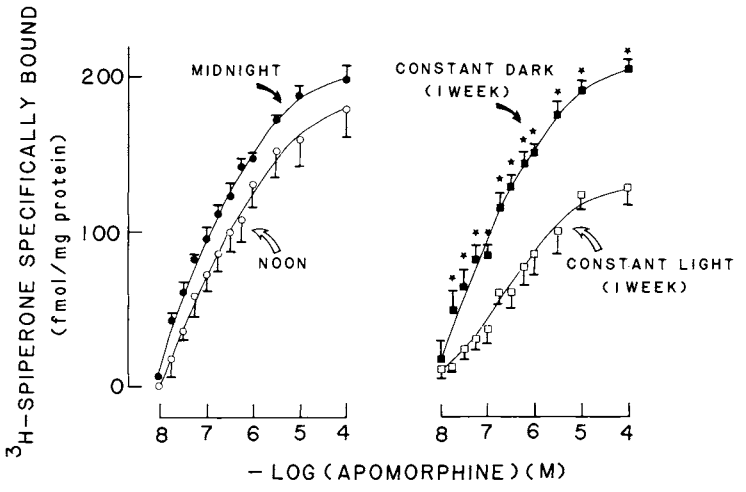

Fig. 3. Effects of light and dark on apomorphine competition for binding sites labeled by ^{3}H-spiperone in rabbit retina.

The ordinate represents the ^{3}H-spiperone (0.51 ± 0.01; n=6) specifically bound in the absence and presence of each concentration of apomorphine. Albino rabbits maintained on a 14:10 h light-dark cycle for one week were killed by decapitation at noon (○) or midnight (●). Rabbits maintained on constant light or constant dark for one week were killed by decapitation at noon (□) or midnight (■), respectively. Values shown are means ± S.E.M.
* $p < 0.05$ when compared with constant dark (Student's two-tailed t-test). Adapted from Dubocovich et al., 1985b.

down regulation of dopamine binding sites in retina was reversed by treatments that increase melatonin levels such as constant light and melatonin administration (Dubocovich et al, 1985). From these studies we concluded that the decrease in melatonin levels in constant light disinhibits the dopamine-containing retinal neurons in vivo, leading to elevated dopamine release and subsequent D-2 dopamine receptor down-regulation.

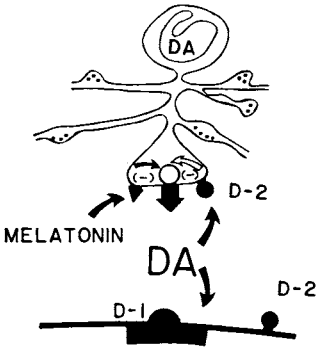

Fig. 4. Schematic representation of a dopamine-containing retinal amacrine cell.

Activation of D-2 dopamine autoreceptors and melatonin receptors inhibits the calcium-dependent release of dopamine from rabbit retina. Postsynaptic dopamine receptors (D-1 and D-2) may be located on other retinal neurons in the same or different synapse.

III. CONCLUDING REMARKS

In the rabbit retina inhibitory D-2 dopamine autoreceptors involved in the modulation of endogenously released dopamine are pharmacologically different from the D-1 dopamine receptors linked to the stimulation of adenylate cyclase.

Melatonin selectively inhibits dopamine release from rabbit retina through activation of a site with the functional and pharmacological characteristics of a receptor. The production of melatonin in vivo appears to modulate the release of dopamine as the D-2 dopamine receptor down regulation observed in retinas from rabbits exposed to constant light was reversed by treatments that elevate melatonin concentrations. Our results imply that changes in environmental light conditions may modulate retinal dopaminergic activity through changes in melatonin concentrations.

We conclude that the differences in the potencies of dopamine agonists and melatonin to inhibit dopamine release from retina suggest that in vivo, endogenous melatonin may be the more important modulator of dopamine release during

the dark period . Conversely, during the light period when melatonin levels in the retina are very low, modulation of dopamine release may be primarily mediated through activation of D-2 dopamine autoreceptors.

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THE INFLUENCE OF LIGHT IRRADIANCE AND WAVELENGTH ON PINEAL PHYSIOLOGY OF MAMMALS*

George C. Brainard
Patricia Podolin

Department of Neurology
Jefferson Medical College
Philadelphia, Pennsylvania 19107

Mark D. Rollag

Department of Anatomy
Uniformed Services University of Health Sciences
Bethesda, Maryland 20814

Bruce A. Northrup

Department of Neurosurgery
Jefferson Medical College
Philadelphia, Pennsylvania 19107

Curtis Cole

Center for Photobiology
Temple University
Philadelphia, Pennsylvania 19140

Felix M. Barker

Pennsylvania College of Optometry
Philadelphia, Pennsylvania 19141

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I. INTRODUCTION

For nearly all mammalian species, light is an important environmental stimulus. In addition to supporting the sensory capacity of vision, environmental light has a central regulatory influence on biological rhythms (1,2) and neuroendocrine physiology (3,4). The details of visual physiology have been much more clearly established than the mechanism underlying light's regulation of the neuroendocrine system. There appear to be at least four main physical parameters which determine whether or not a photic stimulus will elicit a neuroendocrine response: irradiance, wavelength, exposure duration and time of exposure (see Figure 1). The purpose of the following review is to summarize how these physical parameters of light influence a specific portion of the mammalian neuroendocrine system, the pineal gland.

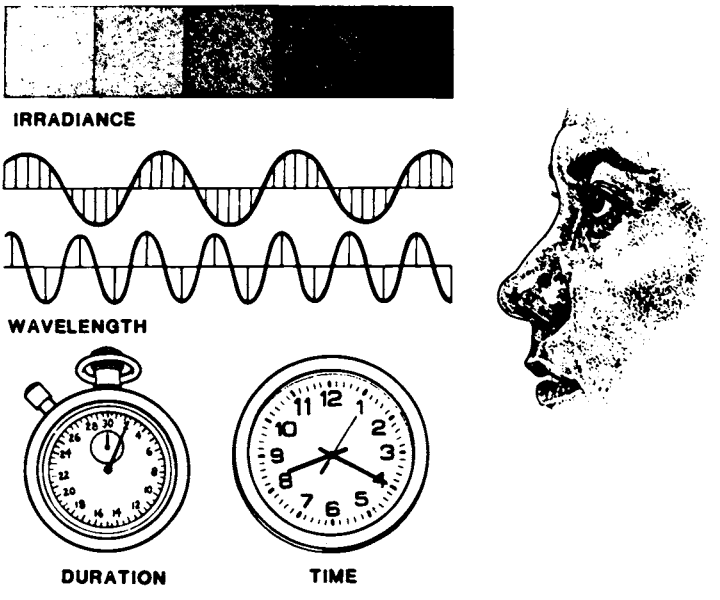

Fig. 1. The diagram above illustrates four principal physical parameters of photic input which regulate the pineal gland: irradiance, wavelength, time of exposure and exposure duration.

The mammalian pineal gland does not respond to direct photic stimulation. Instead, the pineal receives photic information via an indirect multisynaptic pathway, the retinohypothalamic tract. This pathway originates in the ganglion cell layer of the neural retina and projects through the optic nerves and chiasm to synapse in the suprachiasmatic nuclei (SCN) of the hypothalamus (5). The SCN have been identified as "endogenous oscillators" involved in regulating circadian rhythms (6). A multisynaptic pathway projects from the SCN to the pineal gland via the hypothalamus, the thoracic spinal cord intermediolateral cell column, and the superior cervical ganglia (7,8). Photic stimuli transduced along the retinohypothalamic pathway entrain circadian rhythms of pineal enzymes and indoles (7,8). In addition, the unexpected exposure of the eyes to light during the night can induce a rapid suppression of normally high nocturnal levels of serotonin-N-acetyltransferase activity (NAT) and pineal melatonin content. This light-induced suppression of pineal NAT and melatonin has been documented in a variety of mammalian species including rats (9,10), hamsters (11,12,13), ground squirrels (14), sheep (15,15a), monkeys (16) and humans (17,18,19,24). This response has consistent features among the different species whether they are nocturnal or diurnal. The magnitude of the response is relatively great, i.e., high nocturnal NAT and melatonin levels are reduced as much as 40%-96% depending on the species studied (11-18). The response is rapid, exhibiting a decay constant (t 1/2) between 2 and 30 minutes following exposure (11,13,15,18). Finally, the decay response appears to occur with similar characteristics regardless of the time of light exposure during the normal circadian peaks of NAT and melatonin (13,19). These characteristics of the light-induced suppression of melatonin and NAT make this response an ideal model for determining how the different physical parameters of light regulate at least this portion of the neuroendocrine system.

II. EFFECTS OF LIGHT IRRADIANCE AND WAVELENGTH ON PINEAL NAT AND MELATONIN

A. Irradiance

To characterize how light intensity or irradiance influences a given biological system, it is necessary to establish a fluence-response curve (dose-response relationship). A fluence-response curve can be used to

determine 1) the minimum light irradiance which induces any response; 2) the ED_{50}, or irradiance which induces a 50% response; and/or 3) the irradiance at which the response "saturates". Klein and Weller were the first to observe the light-induced reduction of NAT when rats were exposed to light at night (9). To date, fluence-response curves for the light-induced suppression of pineal NAT have been established only in rats (10) and hamsters (13). In albino rats, the 50% threshold for suppression of NAT is approximately 0.5 $\mu W/cm^2$ of white fluorescent light (10). In contrast, the ED_{50} for Syrian hamsters is 0.066 $\mu W/cm^2$ (12). With rats saturation was reached by 15 $\mu W/cm^2$ whereas hamsters require only 0.11 $\mu W/cm2$ to induce the maximum suppression of NAT. These data indicate a one log unit difference between the sensitivities of the hamster and the rat pineal melatonin rhythm generating systems. It is difficult to make exact comparisons of absolute threshold levels among different laboratories since animal housing conditions, light exposure systems, exposure chambers and experimental routines may influence the responsiveness of animals to a photic stimulus (14,20,21,22).

Fluence-response curves also have been established for the suppression of nocturnal melatonin in rats (23), hamsters (12) and humans (19,24). Figures 2 and 3 illustrate fluence-response data on the white light suppression of pineal melatonin in Syrian hamsters. The calculated ED_{50} for melatonin suppression, 0.058 $\mu W/cm^2$, was nearly identical to the ED_{50} for NAT suppression (12). In albino rats, the 50% suppression of both pineal and serum melatonin was between 0.11 and 0.22 $\mu W/cm^2$ (23). In that same study, pigmented Long Evans Hooded rats exhibited significant pineal and serum melatonin suppression in response to illumination as dim as 0.022 $\mu W/cm^2$. Hence, different strains of the same species can exhibit very different threshold sensitivities of the pineal to light. The role that eye pigmentation plays in the pineal response to light is unclear. In a different laboratory, intensities as low as 0.0005 $\mu W/cm^2$ suppressed pineal melatonin and NAT in albino rats in one experiment but not another (25). Perhaps pigmentation plays less of a role in modulating these responses than photic history and experimental techniques. Certainly, prior lighting conditions (21,26) and light-induced photoreceptor damage (25) can dramatically change the photic responsiveness of the rat pineal.

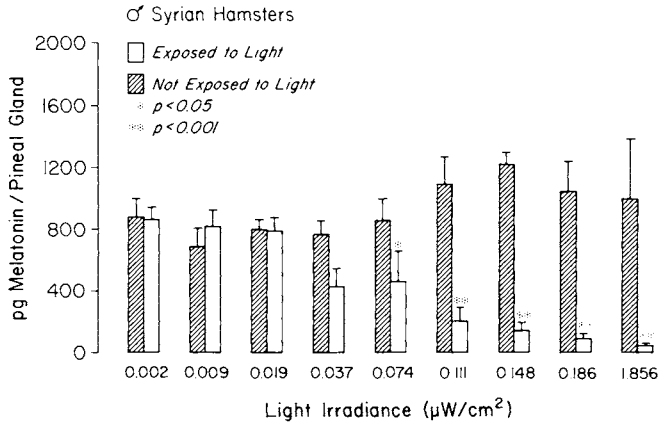

Fig. 2. Influence of different irradiances of white fluorescent light on nocturnal pineal melatonin content of adult male Syrian hamsters. Twenty minutes after turning the lights on, pineal glands were collected from exposed and unexposed animals. Bars indicate mean pineal melatonin content and lines on top of the bars indicate SEM. n = 8 for each group (From Brainard et al., Endocrinology 113, 1983).

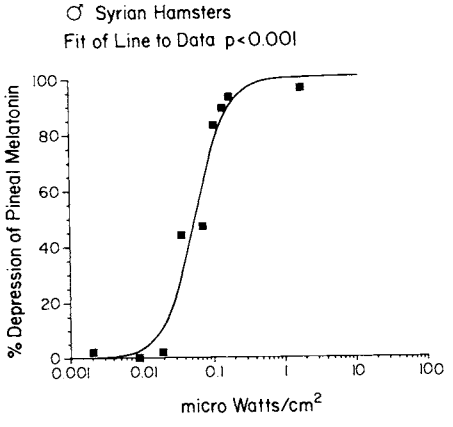

Fig. 3. Fluence response (dose-response) relationship between light irradiance and mean percent depression of melatonin content in male Syrian hamsters. The raw data for this graph are expressed in Figure 2. (From Brainard et al., Endocrinology 113, 1983).

Regardless of discrepancies in absolute thresholds between different laboratories, it is clear that the pineal glands of nocturnal rodents are very responsive to low light irradiances at night. In contrast, adult humans exhibit a much higher threshold for melatonin suppression by light (18). Although a complete fluence-response relationship has not been established for white light influence on human melatonin, approximately 200 $\mu W/cm^2$ caused no suppression, 600 $\mu W/cm^2$ caused partial suppression, and 1000 $\mu W/cm^2$ induced a 60% reduction in plasma melatonin of normal volunteers (18). In Figure 4, a fluence-response curve is illustrated for a single human volunteer (19). In that study 6 healthy males were exposed to varying irradiances of monochromatic light at 509 nm. Individual variations in threshold responses to monochromatic light were observed among the volunteers. Significant mean suppression of plasma melatonin was induced by 1.6 $\mu W/cm^2$ or greater. Melatonin suppression induced by a low irradiance such as this was probably due to the following: the volunteer's eyes were fully dilated with a mydriatic agent, the eyes were directed steadily and consistently at the light source, and the photic stimulus was adjusted to the peak sensitivity of human scotopic vision (19). At least 1,000 $\mu W/cm^2$ of white light is required to suppress melatonin in normal humans with undilated pupils. Psychiatric depression seems to reduce the threshold for light suppression of melatonin (17).

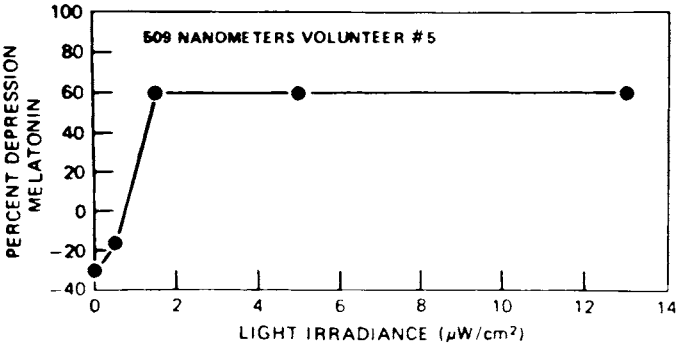

Fig. 4. The above graph illustrates a fluence-response curve for a single human volunteer. Data points indicate percent depression of this individual's plasma melatonin during a one hour exposure to patternless monochromatic light (λ max 509 nm) at night. The pupils of the volunteer's eyes were dilated with a mydriatic agent prior to light exposure. (From Brainard et al., Ann. N.Y. Acad. Sci. 453, 1985)

The great differences in sensitivities between humans and rodents cannot be explained solely on the basis of diurnal versus nocturnal behavior patterns. Some diurnal species such as Richardson's ground squirrels (14) and wild-captured Eastern chipmunks (26) require high irradiances (1850 and over 400 $\mu W/cm^2$, respectively) to perturb melatonin. However, diurnally active sheep and rhesus monkeys exhibit suppressed melatonin after exposure to ordinary room light irradiances (100-200 $\mu W/cm^2$). It has been argued that the photic regulation of the Rhesus monkey pineal is very different from that of the human (16). However studies on a different primate model, Cynomologus fasicularis, demonstrate that this species is very similar to humans in the light-induced suppression of melatonin. Preliminary data show that in the Cynomologus ordinary room light does not induce a nocturnal suppression of melatonin. In contrast, bright white light (2,000 $\mu W/cm^2$ or greater), or bright monochromatic light (13 $\mu W/cm^2$ of 500 nm), can cause a dramatic suppression of melatonin levels in primate CSF (See Figures 5 and 6).

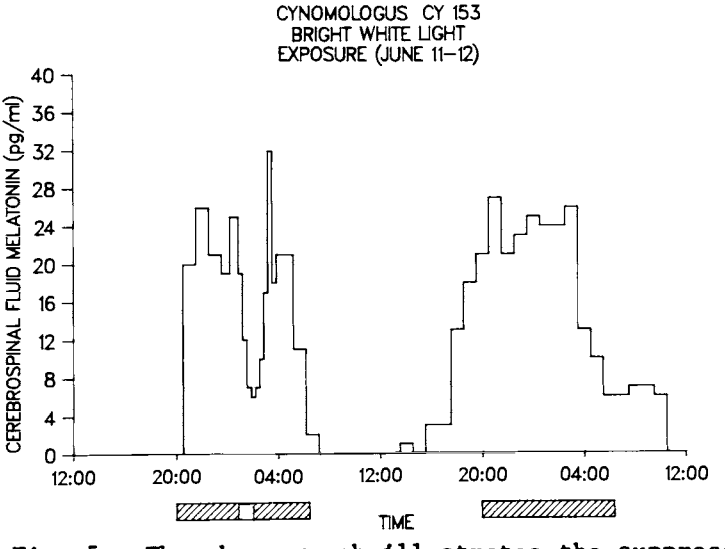

Fig. 5. The above graph illustrates the suppression of melatonin in the CSF of an adult male primate (Cynomologus fasicularis). The hatched bar at the base of the figure illustrates the normal period of darkness. The clear portion of this bar indicates a one hour exposure to bright white light (over 2000 $\mu W/cm^2$ exposure. The animals' eyes were not treated with a mydriatic agent during this study.

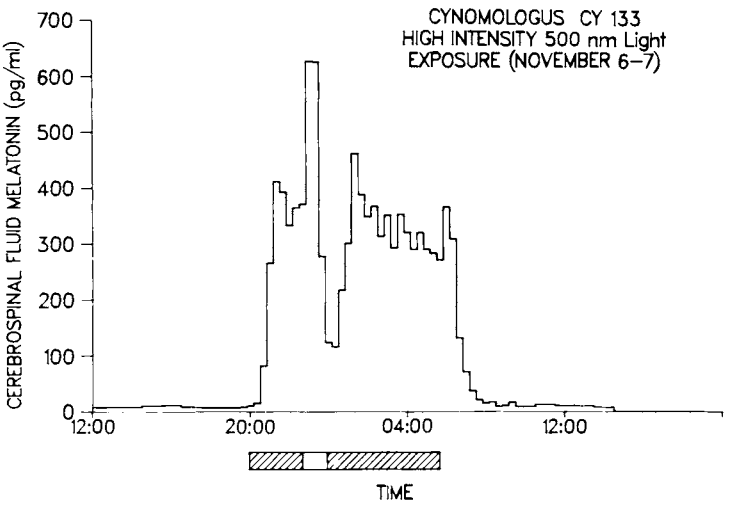

Fig. 6. This figure demonstrates the suppression of CSF melatonin by monochromatic light in a Cynomologus primate. The hatched bar at the base of the figure illustrates the normal period of darkness. The clear portion of this bar indicates a one hour exposure to monochromatic light ($\lambda_2$max 500 nm, 10 nm half peak bandwidth) at 13 $\mu W/cm^2$. The monkey's eyes were dilated with a mydriatic agent during this study.

Clearly, between species there is a wide variation in threshold intensities of light required to perturb melatonin levels. It is important to note that in both animals and humans, much more light is required to interfere with melatonin production than is needed for the sensory capacity of vision. The visual pathways are anatomically distinct from the retinohypothalamic tract and require far less illumination to elicit visual responses (27,28,29,30). An effective visual stimulus may not be an effective neuroendocrine or circadian stimulus. The physiological mechanism which determines whether the retinohypothalamic tract responds to a photic input remains to be elucidated.

B. Wavelength

All photobiological events are initiated by the absorption of a photon by a photopigment. The photopigment(s) responsible for relaying photic information

to the mammalian pineal gland have yet to be identified. Some investigators have hypothesized that rhodopsin is the primary photopigment for transducing light information to the neuroendocrine and circadian systems of rodents (31,32). While there is some evidence supporting this hypothesis, it is far from absolute confirmation.

One of the strongest lines of support linking a photopigment with a given biological effect is the establishment of an action spectrum that is comparable to the absorption spectrum of that photopigment. An action spectrum compares the relative effectiveness of different wavelengths for inducing a specific biological response. Cardinali and colleagues pioneered the field of testing the impact of different visible wavelengths on the pineal gland (31). In their study, rats were kept in darkness for 7 days and then exposed for 12-96 hours to broadband (greater than 100 nm bandwidths) red, yellow, green, blue or near-ultraviolet fluorescent light. They observed that the most rapid and complete suppression of the melatonin forming enzyme, hydroxyindole-O-methyltransferase (HIOMT), was produced by green light, followed by blue, yellow, near-ultraviolet and red light (31). In a later study (33), hamsters were exposed for 20 minutes during the night to equal irradiances of red, yellow, green, blue or near-ultraviolet fluorescent light (light sources were similar to those used in the rat study). Figures 7 and 8 illustrate the results of one experiment in this study. The combined data from this study demonstrated that the blue fluorescent light was the most efficient in suppressing pineal melatonin, followed by green, yellow, near-ultraviolet and red light (33). Perhaps these results differ from those of Cardinali and colleagues due to species differences (rats vs hamsters), protocol differences (chronic vs acute light exposures), or the measurement of different endpoints (HIOMT vs melatonin). The results of both experiments could be used to support the idea that rhodopsin mediates the transmission of photic information to the pineal. Both the green and blue fluorescent light sources contained wavelengths at 500 nm - the peak visible wavelength in the rhodopsin absorption spectrum. However, the hamster data are also consistent with the hypothesis that a blue-sensitive photopigment may mediate the pineal response to light (33). For example, cyanolabe, a blue-sensitive photopigment found in some mammalian cones, has a peak wavelength absorption approximately 440 nm (34). The rodent retina does contain cone photoreceptors and seems to have color sensitive photopigments other than rhodopsin (33,35). In a related study on hamster behavior, however,

an eloquent action spectrum for phase-shifted wheel-running activity seems to favor rhodopsin as the mediating photopiment (36). More detailed studies are necessary to clarify which photopigment(s) mediate the response of the rodent pineal to light.

Mammals exhibit tremendous diversity in retinal structure and function (34), and thus great caution should be exercised in assuming that different mammalian species will have identical photoreceptors and photopigments mediating pineal physiology. Furthermore, studies on light-induced responses in the pineal complexes of fish, amphibians and reptiles demonstrate a great diversity of responses to different light wavelengths (for review see 37).

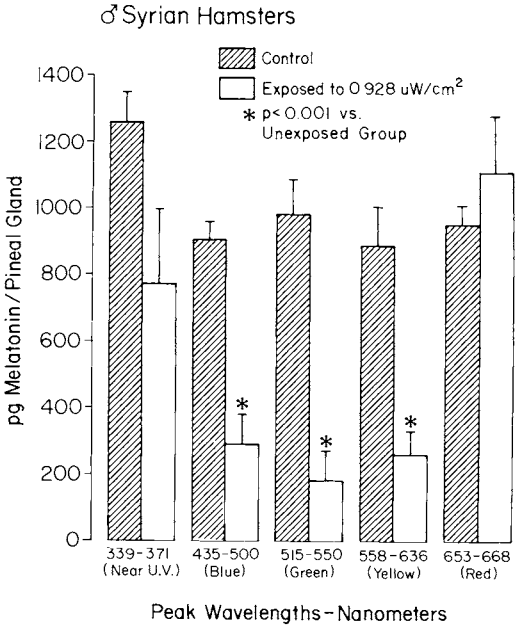

Fig. 7. Influence of different light spectra at 0.928 $\mu W/cm^2$ on pineal melatonin content of male Syrian hamsters. Numbers at the base of the graph refer to the half peak bandwidths of light sources. Pineal glands were collected from each group of light-exposed animals 20 minutes after lights were turned on. Pineal glands were collected from a corresponding group of unexposed animals at the same time. Bars indicate mean pineal melatonin contents and the lines on top of bars illustrate SEM. For each group of n = 8. (From Brainard et al., Brain Res. 294, 1984.)

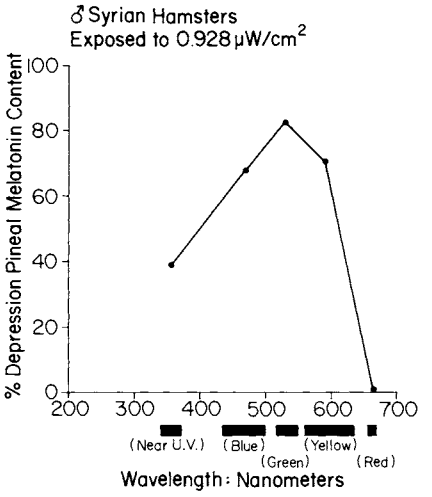

Fig 8. Data points in this graph illustrate mean percent depression of pineal melatonin by different light spectra at 0.928 $\mu W/cm^2$ in male Syrian hamsters. The raw data for this graph are expressed in Figure 7. (From Brainard et al., Brain Res. 294, 1984).

To date, there are relatively few data on the wavelength responsiveness of the pineal in higher mammals. Figure 9 illustrates some preliminary data on the light-induced suppression of plasma melatonin in human volunteers (19). These preliminary findings are provocative since they demonstrate that various wavelengths appear to induce differential responses in the human neuroendocrine system. However, it is important to stress that these data do not constitute a complete action spectrum and thus can not verify any specific photoreceptor or photopigment mediating human pineal responses.

III. EFFECTS OF LIGHT IRRADIANCE AND WAVELENGTH ON PINEAL-REPRODUCTIVE INTERACTIONS

A. Irradiance

Many investigators have addressed the issue of how light irradiance and wavelength regulate pineal biosynthesis and secretion of indoleamines. Few studies have examined how the physical parameters of light regulate pineal-reproductive interactions. Most mammalian species

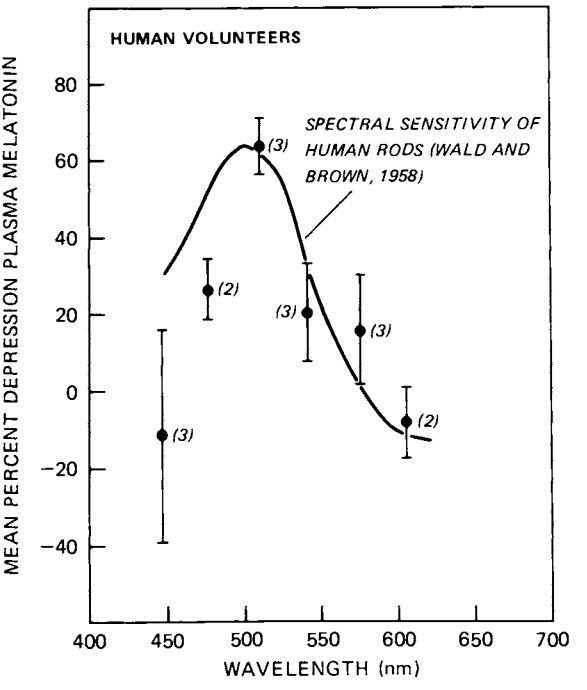

Fig. 9. The illustration above indicates mean ± SEM percent depression of plasma melatonin in male volunteers. The numbers in parenthesis indicate the number of subjects exposed at each wavelength. The curved line is adapted from Wald and Brown, Science 127, 1958. In the study, each volunteer was exposed to balanced quanta of each monochromatic wavelength. (From Brainard et al., Ann. N.Y. Acad. Sci. 453, 1985).

inhabiting temperate or polar regions exhibit annual reproductive rhythms (38). The further a habitat is from the equator, the more pronounced the seasonal changes are in day-length and climate. Accurate detection of seasonal change is critical to the survival of animals living in such regions (3,38). In some seasonally breeding species, the pineal gland responds to changes in ambient daylength (photoperiod) by altering its rhythm of melatonin secretion, which in turn regulates reproductive capacity (39,40).

Syrian hamsters undergo a regression of the reproductive axis when maintained in photoperiods comprising less than 12.5 hours of light (41). In one study, different groups of

hamsters were exposed to a long photoperiod (L:D 14:10), a short photoperiod (L:D 11:13) or a short photoperiod (L:D 11:13) extended by 3 hours of white light at irradiances of 20, 2, 0.2, 0.02 or 0.002 $\mu W/cm^2$ (22). After 12 weeks of exposure to these different conditions, the animals in the long photoperiod were reproductively active, whereas animals in the short photoperiod were reproductively regressed, as evidenced by decreased testicular weights, accessory sex organ weights, and pituitary prolactin levels. Hamsters kept in the short photoperiod extended by 3 hours of 20, 2, or 0.2 $\mu W/cm^2$ were reproductively competent, similar to animals kept in the long photoperiod. In contrast, animals kept in the short photoperiod extended by 3 hours of 0.02 or 0.002 $\mu W/cm^2$ exhibited significant reductions of gonadal weights and pituitary prolactin. These data demonstrate that light irradiance can have an important regulatory influence on the reproductive system of a seasonally breeding mammal. Melatonin is thought to mediate the short photoperiod-induced collapse of the hamster reproductive axis. The light irradiances capable of suppressing nocturnal melatonin in acute exposures (20,2,0.2 $\mu W/cm^2$) are the same irradiances which prevented the pineal-mediated reproductive collapse in the 12 week study above (12,22). Those irradiances which could not alter nocturnal melatonin (0.02 and 0.002 $\mu W/cm^2$) allowed the pineal-mediated reproductive atrophy to occur. These data indicate that light irradiance regulates pineal biochemistry and reproductive physiology in a parallel fashion. Furthermore, a study by Hoffman and Johnson suggested that the pineal gland may act as a "photodosimeter" (52). In their study, hamsters were born and raised in darkness, or in L:D 14:10 for four weeks. These animals were then placed in different chambers with L:D 14:10 at various low intensities of cool white light (0.001,0.008,0.015 scotopic footcandles) for 14 weeks. Analysis of testicular weights demonstrated that animals were significantly affected by both photic history and illuminance levels (52). Hence photic history is important to both pineal indoleamine production and pineal-reproductive interactions (21,26).

B. Wavelength

Two different studies have been done to examine how the hamster reproductive system responds to the photic parameter of wavelength (22,42). In the first experiment, groups of hamsters were exposed to a long photoperiod (L:D

11:13) extended by 3 hours of either red, yellow, green, blue or near-ultraviolet light at 0.2 $\mu W/cm^2$. After 12 weeks, animals in the long photoperiod were reproductively competent, whereas animals in the short photoperiod exhibited typical regression of reproductive parameters. Hamsters kept in short photoperiods extended by near ultraviolet, blue or green light were reproductively active, whereas animals in short photoperiods extended by red or yellow light underwent reproductive regression (22). These results demonstrated that the photic parameter of light wavelength can be an important determinant of reproductive status of a seasonally breeding animal. It was not expected, however, that the animals exposed to a short photoperiod extended by 3 hours of near-ultraviolet light would remain reproductively competent. Earlier studies demonstrated that near-ultraviolet light does not perturb pineal melatonin (33). Consequently, a second study was done on the influence of different wavelengths on hamster reproductive status (42).

As indicated above, hamsters maintained in daily photoperiods with less than 12.5 hours of light will undergo gonadal regression (41). However, there is one circumstance when exposure to short photoperiodic conditions does not induce reproductive atrophy. Hamsters in short photoperiods will remain reproductively competent if the dark period is interrupted by a pulse of light (43). Hence, a study was performed to determine the effectiveness of different wavelengths for blocking the short photoperiod-induced reproductive atrophy. Groups of hamsters were exposed to a long photoperiod (L:D 14:10), a short photoperiod (L:D 10:14), or a short photoperiod (L:D 10:14) with a mid-dark 1 hour pulse of either near-ultraviolet, blue, green, yellow or red light at an irradiance of 0.2 $\mu W/cm^2$. After 12 weeks of exposure to these photoperiods, hamsters maintained in the long photoperiod or short photoperiods with a near-ultraviolet, blue or green light pulse remained reproductively competent. In contrast, animals kept under the short photoperiod or short photoperiod with a red or yellow light pulse exhibited gonadal atrophy (see Figure 10). These data confirm that different wavelengths of light can have very different effects on the central and peripheral reproductive axis. In addition, this experiment confirms that near-ultraviolet light can alter the hamster neuroendocrine system.

Although restricted segments of the spectrum have differential impact on hamster reproduction, there is no firm evidence that the spectral distributions of commonly used lamps have specific reproductive consequences in

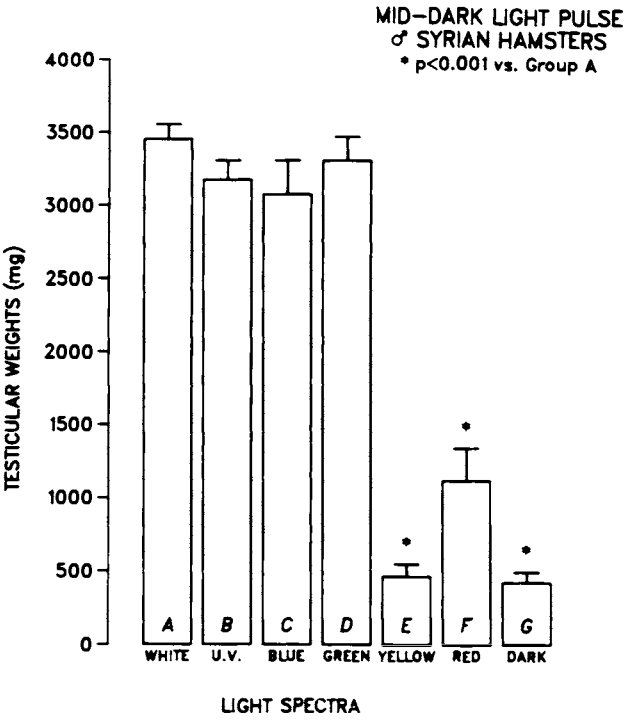

Fig. 10. Testicular weights (means + SEM) of hamsters maintained in different photoperiodic conditions. "White" indicates L:D 14:10 and "dark" indicates L:D 10:14. The other groups were exposed to L:D 10:14 with a one hour mid-dark light pulse from the same light sources described in Figure 7. (From Brainard et al., Adv. Biosci. 53, 1985).

rodents. In fact, one study has shown that when illuminance levels of three sources (cool white fluorescent, sunlight simulating fluorescent and high pressure sodium vapor lamp) are balanced to equal scotopic footcandle levels, the lamps produced no differences in hamster reproductive status (53).

IV. EFFECTS OF NEAR-ULTRAVIOLET LIGHT ON PINEAL MELATONIN

Ultraviolet radiation (200-400 nm) is generally considered to be outside the range of visible illumination for mammals. Furthermore, there has been little evidence that wavelengths outside the visible range (400-700 nm) are capable of regulating the circadian or neuroendocrine system. Specifically, at least three studies have shown that near-ultraviolet radiation (UVA, 315-400 nm) does not

influence pineal indoleamine synthesis (31, 33, 44). Thus it was surprising to observe that UVA did, in fact, block the short photoperiod-induced collapse of the hamster reproductive system in two separate experiments (22, 42). It is generally accepted that the effects of visible light in the photoperiodic regulation of the mammalian reproductive axis are mediated through the eye. To understand how UVA might influence the photoperiodic response, it was first necessary to determine if UVA reaches the hamster retina. This was important since the clear ocular media (cornea, aqueous humor, lens and vitreous humor) can limit the transmittance of short wavelength energy to the neural retina. Specifically, the chemical properties of the crystalline lens form a transmittance barrier to radiant energy of wavelengths shorter than a specific "cutoff" wavelength that is characteristic for each species (45,46). For example, the human lens cutoff point is reported to be 400 nm (46) and UVA has, therefore, not been considered to be an effective retinal stimulus for the human eye.

Since species differ in their cutoff point, the hamster eye was specifically measured for this study over the range of 200 nm-2500 nm with a Beckman model UV5240 spectrophotometer. This study showed that the absolute short wavelength cutoffs for hamster ocular tissues were as follows: cornea, 230 nm; aqueous humor, 230 nm; lens 300 nm; and vitreous humor, 220 nm. Figure 11 illustrates the transmission curve of a single hamster lens. Further details of the spectral transmittance of hamster ocular tissues are presented in depth elsewhere (47). Taken together, the transmittance values indicate that radiant energy wavelengths down to 300 nm do reach the retina of the hamster eye. Very recent studies have now confirmed that both broadband UVA (340-405 nm, λ max 365 nm) and monochromatic UVA (λ max 360 nm, 10 nm half-peak bandwidth) can suppress high nocturnal pineal melatonin content in the Syrian hamster (48). Data illustrated in Figure 12 show one experiment in which only 1.0 $\mu W/cm^2$ of monochromatic UVA (λ max 360 nm) suppressed hamster melatonin. In this experiment, animals were individually exposed for 5 minutes to ultraviolet radiation in a 12.5 x 10.5 x 14.5 cm box painted with flat black paint. Following exposure, animals were returned to darkness for 30 minutes, after which their pineal glands were removed, frozen, and later assayed for melatonin content (49). A group of control animals was handled similarly, but was not exposed to UVA. The control animals exhibited typically high mean nocturnal melatonin content of 389 $\pm$ 37 pg (mean $\pm$ SEM). In contrast, animals

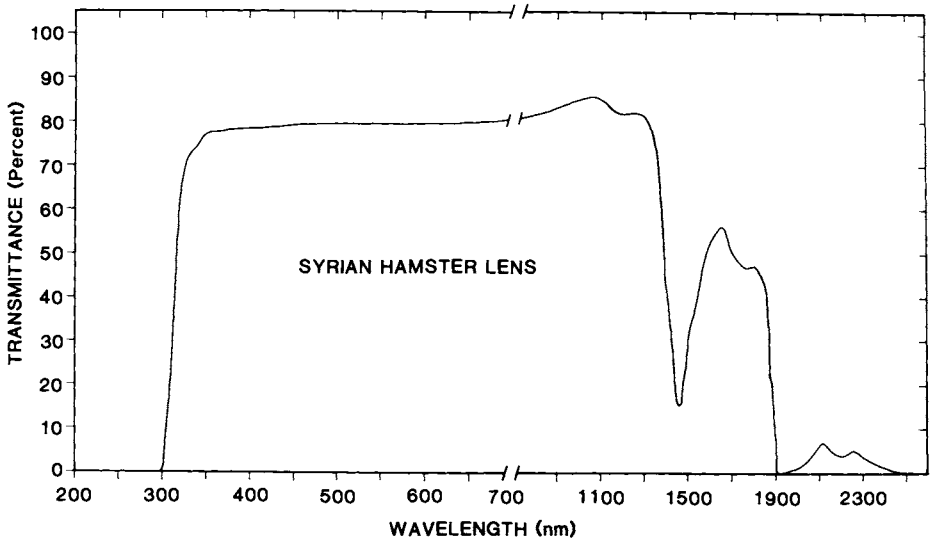

Fig. 11. Spectral transmittance characteristics of the ocular lens of an adult male Syrian hamster.

exposed to a low irradiance of monochromatic UVA had a significantly ($P<0.001$) reduced mean pineal melatonin content of 37 $\pm$ 6 pg. These data clearly demonstrate that UVA can suppress pineal melatonin. In working with ultraviolet radiaton, careful attention must be placed on the specifics of light exposure and measurement (48,51). In this study, the monochromatic light was produced by a tungsten bulb (500 W, General Electric ZY1 DAB). The light was collimated with condensing lenses and infrared radiation filtered out by 2 glass infrared filters. The light was further filtered with a glass, narrow band interference filter (Oriel, Model #53650) and glass neutral density filters (Oriel, Model #50630). UVA was measured with a UVA radiometer (Model #3D from the Solar Light Co.).

Depending on time of day, geographic location, and season, UVA at the surface of the earth can reach 3,000-5,000 $\mu W/cm^2$. Artificial lamps can emit wide ranges of UVA (0.1-80,000 $\mu W/cm^2$) depending on the type of lamp used (50). Thus, it seems likely that UVA may play a physiological role in neuroendocrine regulation in hamsters in natural habitats as well as in many laboratory facilities. Although it has been confirmed that the eyes must be intact for UVA to suppress melatonin (48), the

details of how UVA induces neuroendocrine changes are not yet known. The photopigment rhodopsin is capable of absorbing photons in the 300-400 nm range, although rhodopsin absorbs photons more effectively between 450-500 nm (34). Alternatively the hamster retina may contain other UVA sensitive photopigments. Interestingly, insects, birds and certain cold-blooded vertebrate species appear to have specific photoreceptor sensitivity to ultraviolet radiation (51).

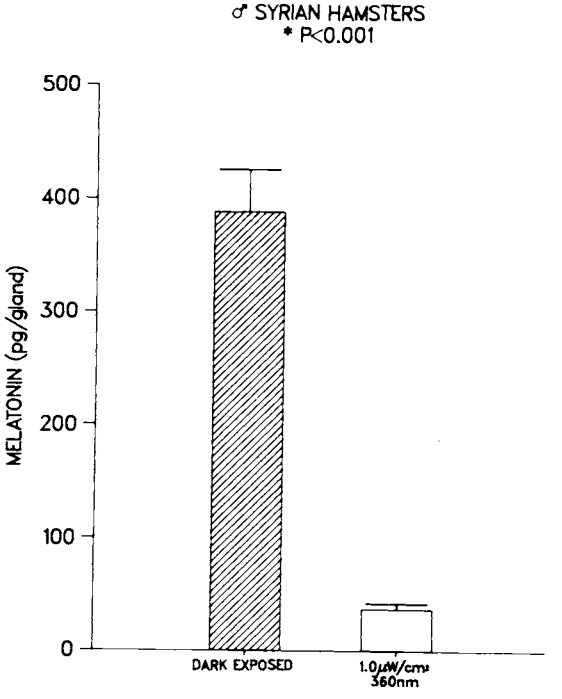

Fig. 12. This graph illustrates the effect of low intensity monochromatic ultraviolet radiation on nocturnal pineal melatonin in male Syrian hamsters. Hamsters were exposed to 360 nm radiation for 5 minutes and returned to darkness for 25 minutes before pineal glands were collected. Melatonin was significantly lower in the ultraviolet exposed group as compared to dark exposed controls ($P<0.001$).

V. SUMMARY

During the past 15 years, there has been increasing interest in the physiological impact of light on the pineal gland. In all mammalian species studied, environmental

light synchronizes circadian indoleamine rhythms and can rapidly suppress high nocturnal levels of melatonin synthesis. Such actinic effects of light are dependent on the physical parameters of wavelength and irradiance. Furthermore, different light irradiances and wavelengths have varying effects on pineal reproductive interactions. The photopigments and photoreceptor cells which transmit photic information to the pineal have yet to be defined in any mammalian species. Near-ultraviolet wavelengths typically considered to be outside the visible spectrum can also regulate melatonin and reproductive responses in at least one species, the Syrian hamster. The possiblity that some mammals can detect and respond to UVA is a significant departure from our current understanding of visual and neuroendocrine physiology.

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THE SUPRACHIASMATIC NUCLEUS (SCN) AS A SITE OF THE CIRCADIAN PACEMAKER IN MAMMALS

Shin-Ichi T. Inouye

Mitsubishi-Kasei Institute of Life Sciences
Tokyo, Japan
and
Department of Neurobiology and Physiology
Northwestern University
Evanston, Illinois

I. INTRODUCTION

The suprachiasmatic nucleus (SCN) of the hypothalamus plays a dominant role in regulation of circadian rhythms (1). Although the retina and the pineal organ are also involved, they are not required for the generation of circadian rhythms in mammals. Interestingly, the SCN is linked to the retina and pineal organ:

1. The SCN, retina and pineal organ are all involved in the vertebrate circadian system. The relative importance of the role played by these structures varies from species to species;
2. These structures are closely associated with light perception. The vertebrate retina and pineal organ in lower vertebrates are photosensitive. Although the SCN neurons in mammalian brain are not photosensitive, they are innervated by a small population of optic fibers arising from retinal ganglion cells;
3. All these structures are located along the third ventricle, suggesting a possible interaction among these at an early stage of development.

Therefore, it would not be surprising if the SCN shared some common cellular or biochemical mechanisms with the retina and the pineal, involved in circadian rhythm generation and the effects of light.

A. Anatomical Studies

Although the SCN had been described in old anatomical literatures, it was "rediscovered" when Moore & Eichler (2) and Stephan & Zucker (3) proposed it as a possible locus of a circadian pacemaker in rodents. This nucleus is bilaterally situated just on top of the optic chiasm and bordering the third ventricle and consists of about 20,000 neurons in the rat. The presence and boundaries of the SCN are evident from the simplest Nissl stained section, due to the high density of small neurons (Fig. 1). Several tracing studies (4) have demonstrated that a small fraction of optic fibers enter the ventro-lateral part of the SCN. This direct connection between the retina and the SCN is called the retino-hypothalamic tract (RHT) and provides the anatomical basis for the entrainment of circadian rhythms by light in mammals. The existence of an indirect connection between the retina and the SCN by the intergeniculate leaflet of the lateral geneculate body is also known. However, the intact RHT is sufficient for the entrainment of circadian rhythms to environmental light and dark cycles.

Utilizing modern tracing techniques, Moore (5) demonstrated the presence of the RHT and the SCN in five mammlian species. He noted there that the RHT and the SCN are phylogenetically the most stable structures in the mammalian visual systems.

B. Lesion and Biochemical Studies

Effects of lesions of the SCN on circadian rhythms have been extensively studied (6) using various functions of circadian rhythms. The list of circadian rhythms abolished by SCN lesions has grown and covers various behavioral and physiological rhythms from several mammalian species. The function of the SCN has also been explored biochemically. Schwartz et al. (7) have demonstrated that glucose uptake in the rat SCN showed a circadian rhythm, while other parts of the brain did not. Although the disappearance of circadian rhythms after SCN lesions strongly suggests an important role in circadian rhythm generation, this approach does not necessarily indicate a pacemaking function of the SCN. An alternate interpretation is that a large neuronal circuit including the SCN as a small componment is responsible for the generation or conveyance of circadian rhythmicity. To discriminate between these two possibilities, we undertook multiple unit activity recording from the SCN within hypothalamic islands, as described below.

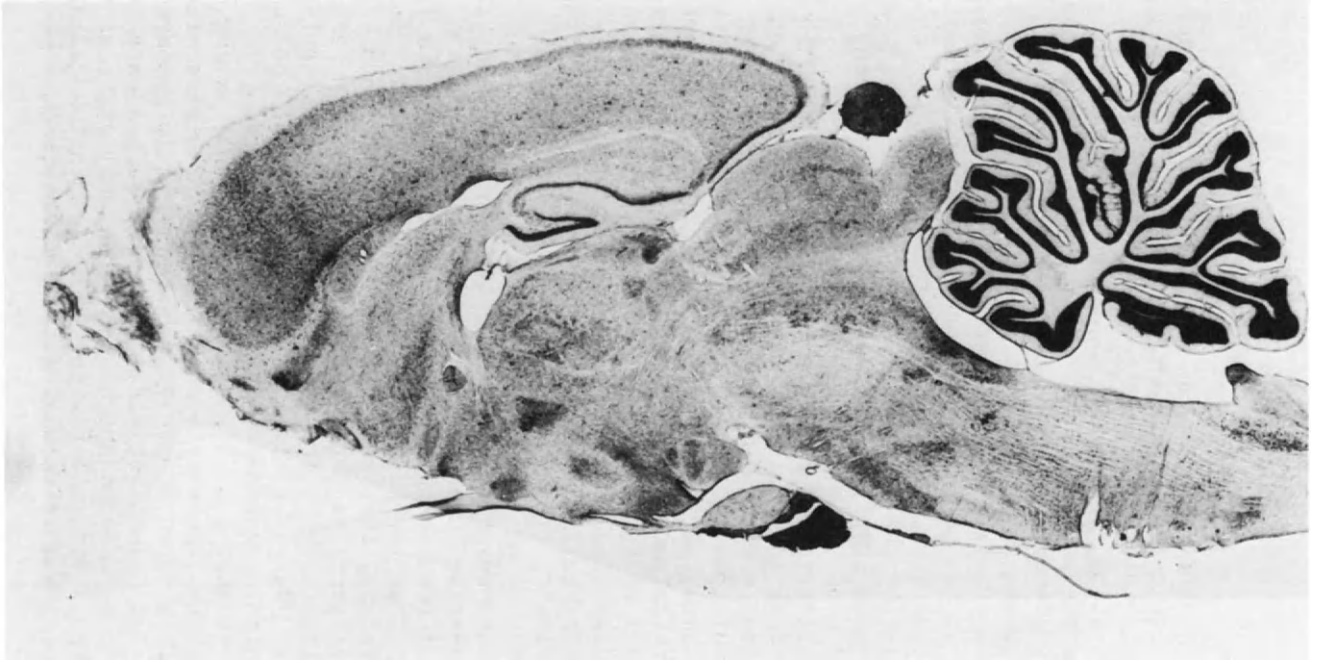

Fig. 1. Parasaggitalsection of the rat brain containing the SCN.
The SCN is seen at the root of the optic nerves on the ventral surface of the brain.

II. MULTIPLE UNIT ACTIVITY OF THE SCN

A. Intact Rats

Multiple unit activity (8) from the brain reflects a collective extracellular property of a group of cells. It employs an electrode with smaller impedance so that it detects activities of a number of neurons simultaneously from a distant site, as compared to single unit activity. Since the electrode is located some distance from the recording neurons, multiple unit recording enables us to record neural activities for a prolonged period of time. In our laboratory, continuous recording of neural activity from a freely-moving rat are made for longer than a month. A disadvantage of this approach is that the signal to noise ratio is low and precise identification of the neurons contributing a multiple unit is impossible.

The application of multiple unit recording technique revealed several interesting facets of the circadian rhythm in brain neural activity of the rat (9, 10). Circadian rhythms in multiple unit activity were found in almost every brain area, including the caudate, reticular formation and hypothalamus. This is not surprising considering that the level of behavioral activity in the rat is high during the nighttime and low during the daytime.

Circadian rhythms of the SCN in intact rats have three characteristics distinctive from those of other areas. The averaged frequencies of multiple unit activities in the dark period are never more than two times higher than that during the daytime in any areas except for the SCN, in which maximum frequencies sometimes reaches as high as ten times larger than the nighttime average. Thus, there is a difference in amplitude and phase. The rhythms found in non-SCN areas parallel behavioral rhythms, i.e. they are higher at night. The third property of SCN neural activity is stability. Neural activities recorded outside the SCN showed a variable dependence on sleep-wake stages. In contrast, frequencies of SCN activity during the waking states are indistinguishable from those in slow-wave sleep states or REM sleep states. This characteristic appears to ensure that the neural activity of the SCN varies only on a circadian basis, independent of the behavioral state of the animal. This is consistent with the notion that a reliable circadian clock should be unaffected by behavior.

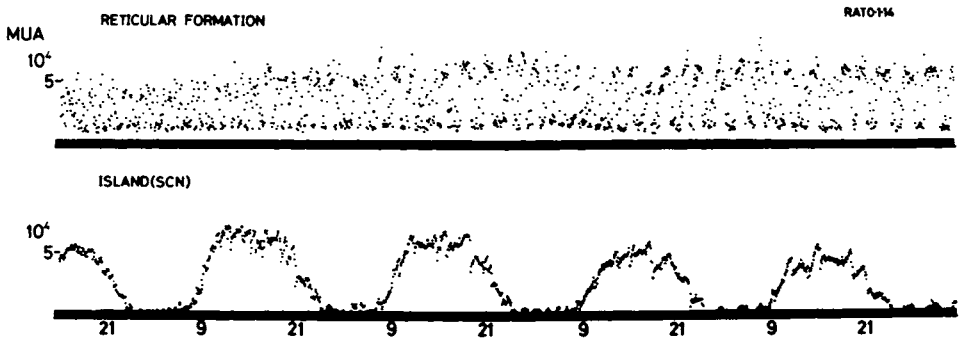

Fig. 2. Persistence of circadian rhythmicity in multiple unit activity of the SCN in the hypothalamic island. Note that the reticular formation outside the island is no longer rhythmic. Y axis is frequencies of multiple unit activity per 5 min and X axis is clock time in hours. The animal is kept in constant darkness.

B. Hypothalamic Island

We investigated whether the SCN contains a circadian rhythm generator or is just part of a larger circuit responsible for the generation of circadian rhythms. A microknife technique (a Halasz knife) was used to make a hypothalamic island around the rat SCN, transecting any input fibers to and output from the SCN. We then recorded neural activity of the SCN within the hypothalamic island (9, 10).

Animals in which the SCN was isolated in a hypothalamic island showed circadian arrythmicity in locomotor activity. Circadian rhythmicity in multiple unit activity of the areas outside the island was also undetectable in the animals bearing a hypothalamic island. Nevertheless, the SCN in the island had clear persistent circadian rhythms in multiple unit activity (Figs. 2 & 3).

The results of the experiments are summarized in Fig. 4. In an intact animal, any place in the brain except for the SCN show circadian rhythms in neural activity which peak at night. The rat SCN rhythms are characterized by a large amplitude, inverted phase relative to behavioral activities and remarkable stability to relative sleep stages. When the SCN was isolated in hypothalamic islands, circadian rhythms outside the island were eliminated. This result implies that circadian rhythmicity outside the island is dependent on the SCN.

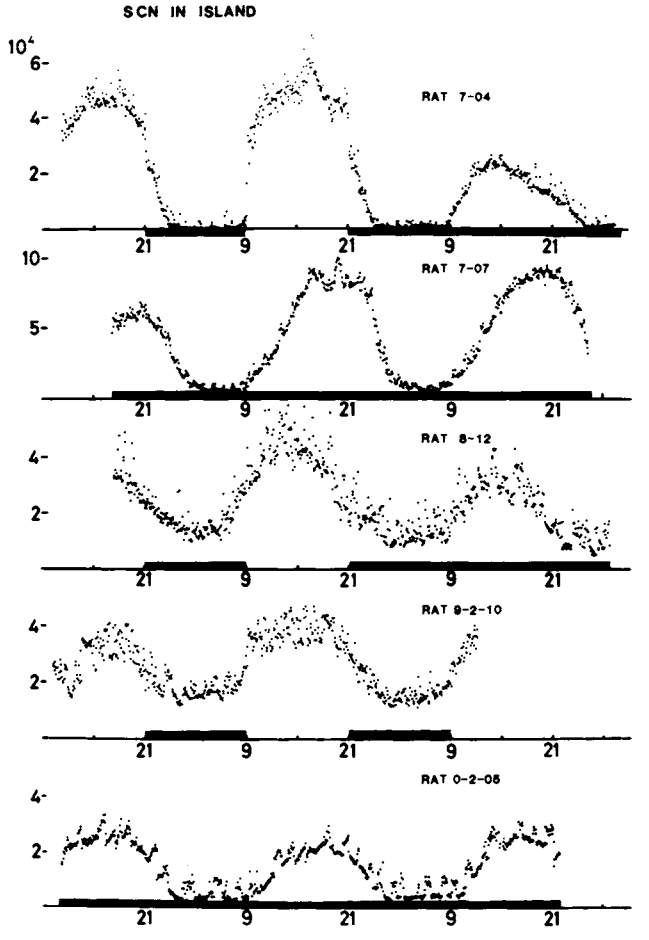

Fig. 3. Five examples of circadian rhythms of the SCN recorded in the animal in which the SCN is located in the island. Lighting conditions are represented by black bars.

On the other hand, circadian rhythms within the island clearly persist after transection of the fibers coursing out of and into the SCN. Thus, afferent fibers to the SCN are not necessary to sustain a circadian oscillation in the SCN. This indicate there is an autonomous circadian pacemaker in the island.

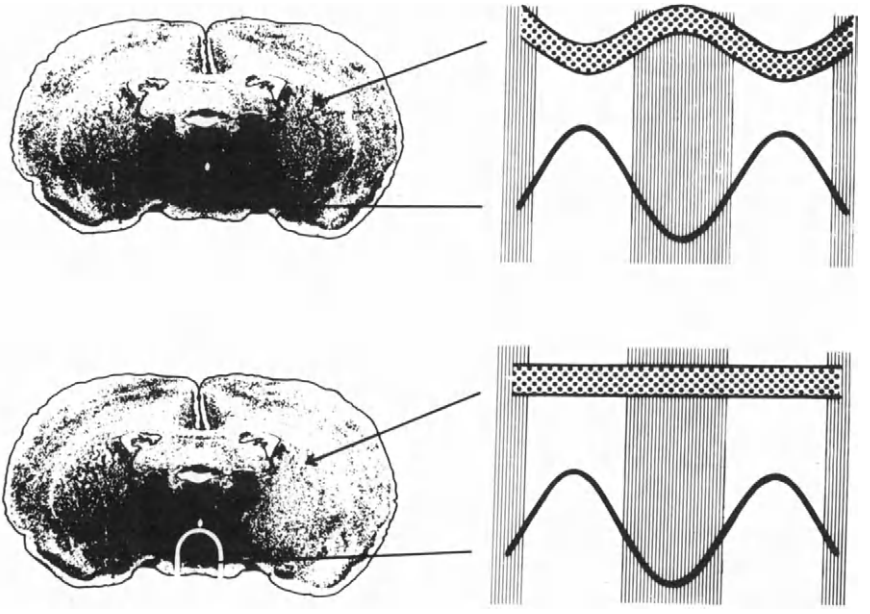

Fig. 4. Schematic drawing of the results of the multiple unit recording of the rat. In the upper panel, ultradian rhythms correlated with sleep-waking states, and circadian rhythms with moderate amplitudes and peaks at night are found in many sites in the intact rat brain except the SCN. Multiple unit activity recording of the SCN reveals unique circadian rhythms, characterized by a large amplitude, an inverted phase relative to other areas and behavior, and independence of sleep-waking states. In the lower panel, circadian rhythmicity outside the island is abolished in an animal with a hypothalamic island containing the SCN. Nevertheless, the SCN in the island continues to oscillate, despite the transection of all input fibers to the SCN.

C. Effects of Light

Light is the most potent entraining factor for circadian rhythms. Another factor is food availability. However, although exposure to attypical cycles of food availability can synchronized locomotor activity rhythms, circadian rhythms of multiple unit activity in the SCN are unaffected by this feeding schedule (11). This illustrates the dominant role of light in entrainment of circadian rhythms.

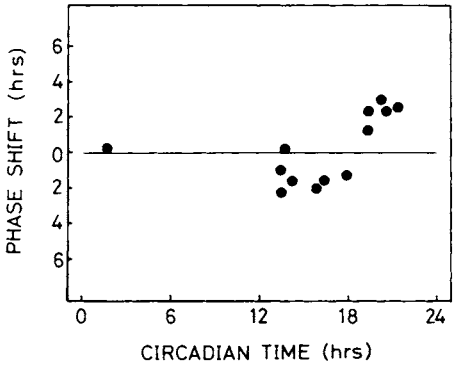

Fig. 5. Phase-response curve of 90 min light pulse to multiple unit activity of the SCN in animals bearing the hypothalamic island with the RHT spared.

Two neural connections between the eyes and the SCN have been established anatomically. One is the monosynaptic innervation of the optic nerves on the SCN, the RHT. The other is via the lateral geniculate body.

It is possible to selectively cut the latter pathway, leaving the former intact. This is done by pulling a knife 0.1 mm up when we cut the anterior edges of the hypothalamic island. This leaves a part of the RHT uncut so that the light information can reach the SCN in the island (12). Other connections between the retina and the SCN are transected when the hypothalamic island is made.

Circadian rhythms in multiple unit activity of the SCN in the hypothalamic island with the RHT spared were entrainable to the environmental 24 h light and dark cycle. Moreover, circadian rhythms in the SCN in the island revealed a phase shift in response to a light pulse in constant darkness. The phase-response curve obtained in this experiment (Fig. 5) was consistent with the phase-response curve for light on locomotor activity of intact animals (10). This indicates the entraining mechanism is in the SCN complex.

Environmental light was also found to induce a change in multiple unit activity of the SCN in the island with the RHT spared. While multiple unit activities from the optic chiasma showed a compound response to a light change, those of the SCN within the island with the RHT spared showed mainly tonic response to light. An exposure to short

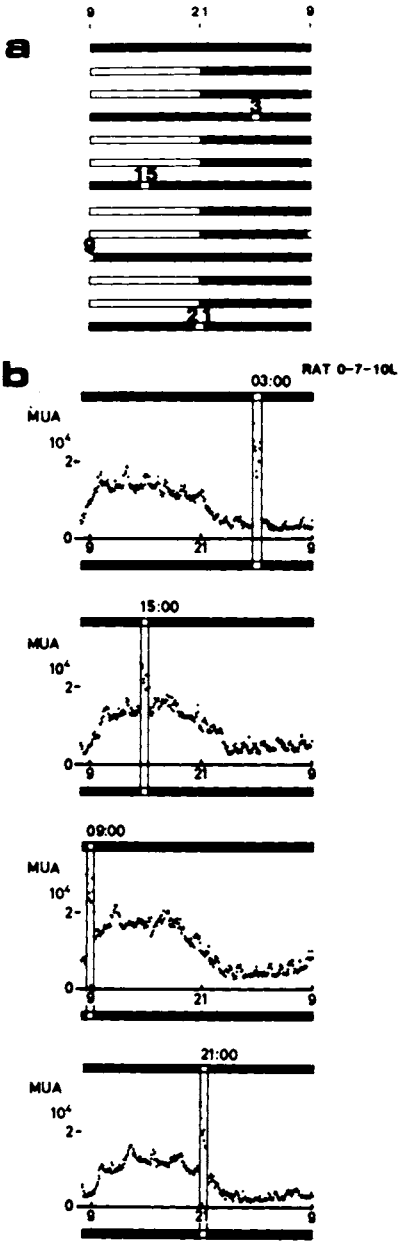

Fig. 6. Effect of light on the SCN multiple unit activity across a 24 h day. (a) Lighting protocol. (b) Increases in the SCN multiple unit activity at four time points of day from a single animal.

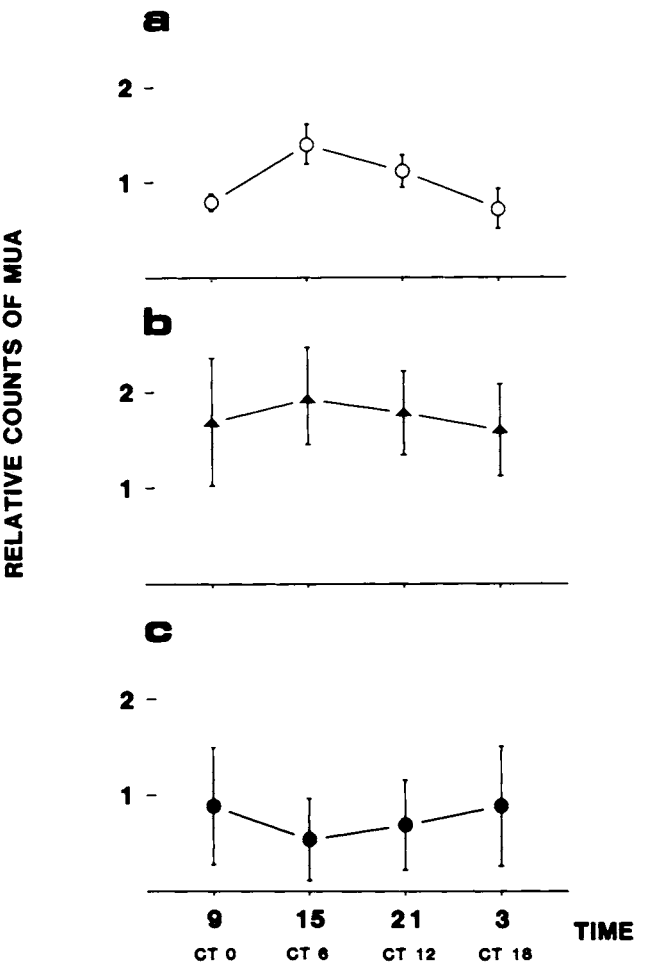

Fig. 7. Averaged light responsiveness of 5 multiple units of the SCN at 4 time points. (a) Average discharges in animals kept in constant darkness. Time of the day effects are clearly seen. (b) Averaged discharges during the period of light exposure in the animals. (c) Averaged increases in discharge rates of the SCN when the animals are exposed to light pulses.

periods of light induces excitement of multiple unit activity of the SCN, an example of which is shown in Fig. 6. This increase in frequencies was seen only in the animal in which the electrode was presumably placed in the

ventral part of the SCN. Since this increase lasted during the entire period of light exposure, averaged frequencies, absolute or relative to the base-line, during the light pulse at 4 different times of day were statistically analyzed to detect rhythmicity of the sensitivity of the SCN to light (Fig. 7). This analysis showed that the exciting effect of light on neural activity of the SCN does not vary as a function of time. As mentioned above, the same light pulse induced a phase-dependent phase-shift in subsequent circadian rhythms of multiple unit activity of the SCN. This fits very well with the concept that the SCN is generating the circadian rhythm, since the eventual shift in the SCN rhythm after light pulses is phase-dependent while the direct effect on the SCN is independent of the time of day.

III. STUDIES FOR THE FUTURE

As seen above, we believe we have found one major component of the circadian system in mammals, the SCN. Besides evidence described in detail in section II, persistence of circadian rhythms have been reported in single unit activity of the SCN neurons maintained in a brain slice preparation (13) and in levels of content of a neuroactive substance released from SCN tissue explants (14). These results support the idea that the SCN contains a self-sustained autonomic circadian oscillator in mammals. However, several fundamental problems in physiology and/or biochemistry of the circadian rhythm of the SCN remain to be solved in the future.

A. Generation of Circadian Rhythms in the SCN

We are generally ignorant of the mechanism through which circadian rhythms are generated in the SCN. For example, we do not know whether the capacity to generate circadian rhythms could be attributable to single cells in the SCN or is dependent on the interaction among neurons in the SCN. A large body of immunohistochemical studies (15) has provided information about the existence of neuroactive substances in the SCN and their localization within the SCN. Vasopressin is mainly localized in the dorsomedial part of the SCN, while VIP immunoreactivity is found only in ventrolateral part of the SCN. Bombesin is almost exclusively localized within the boundary of the SCN, while the GABA antibody stained cells are distributed both inside and outside the SCN within the anterior hypothalamus. In sum, predominant portions of the cells in the SCN seem

peptidergic and send their fibers to the cells within the SCN (intranuclear connections). However, the functional significance of these organizational characteristics of the SCN neurons remains unknown.

B. Entrainment to Light

Although it is established that the RHT is sufficient for light entrainment of the SCN, the circadian rhythm events involved in the light entraining process are largely unknown. A class of neurons in the SCN is shown to behave as luminance detectors (16). This finding is consistent with the overall effect of light, but a detailed description of the mechanism through which a change in neural activity induces a phase shift awaits further studies.

C. The SCN from a Comparative Standpoint

Although the SCN plays a predominant role in the mammalian circadian rhythm, the role of the SCN in non-mammalian vertebrates is not yet studied except for limited species of birds. In birds, effects of SCN lesions on the circadian rhythms varies depending on species (17). This might indicate that in non-mammalian vertebrates, the SCN, pineal organ and retina constitute a complex interacting system of circadian rhythm generators. The elucidation of the interrelationship among the SCN, pineal organ and retina in a different non-mammalian species may shed some light on the evolution of circadian systems. Knowledge about the interaction among the three loci may possibly be interesting in understanding the complicated phenomena also seen in mammals, such as splitting or dissociation of circadian rhythm.

Perhaps when one wants to understand biochemical events in the SCN, accumulated knowledge about the pineal and the retina in invertebrates and lower vertebrates might be useful in guiding future research.

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ADRENERGIC REGULATION OF CYCLIC AMP AND CYCLIC GMP IN RAT PINEALOCYTES

David Sugden
David C. Klein

Section on Neuroendocrinology
Laboratory of Developmental Neurobiology
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland 20892

I. INTRODUCTION

In the last 20 years the pineal gland has proven to be a remarkably useful model system for the study of adrenergic control mechanisms. Norepinephrine (NE), released from sympathetic nerve terminals innervating the pineal, regulates a large nocturnal elevation in melatonin synthesis by stimulating the activity of arylalkylamine N-acetyltransferase (NAT; EC.2.3.1.87) 30- to 50-fold (1,2). An essential step in both the induction and maintenance of high NAT activity is an increase in intracellular cyclic AMP (3,4). NE acts on a pinealocyte β-adrenoceptor (5) to increase intracellular cyclic AMP by activating adenylate cyclase (6). NE also produces large elevations in cyclic GMP, by a postsynaptic action and not presynaptically as was first thought (7,8). The pineal also has a high density of α_1-adrenoceptors (9,10) which mediate an increase in phosphatidylinositol turnover (11), but do not affect NAT activity when activated alone. However, recently it was discovered that the β-adrenergic stimulation of pineal NAT activity was markedly potentiated by activation of pineal α_1-adrenoceptors (12,13). This potentiation was evident both *in vivo* and *in vitro* and is thought to

reflect physiological activation because NE is a mixed α- and β-adrenergic agonist, which can bind to and activate both α_1- and β-adrenoceptors on pinealocytes. Recent data from our laboratory has revealed that α_1-adrenoceptor activation markedly potentiates β-adrenergic stimulation of both cyclic AMP and cyclic GMP (14), and that this mechanism, at least for cyclic AMP, involves the activation of a Ca^{2+}-, phospholipid-dependent protein kinase (protein kinase C) (15). These recent studies are reviewed here.

II. METHODS

Pinealocytes are prepared from 5 week old female Sprague-Dawley rats by trypsinization using published procedures (16). Cells are used 5 to 7 hours after trypsinization and are incubated (37°C, 95% O_2/5%CO_2) in suspension culture in Dulbecco's modified Eagle's Medium containing fetal calf serum (10% v/v). Samples of the cell suspension (0.5 ml, 10^5 cells) are treated as indicated, centrifuged (1000 x g, 2 min), the media aspirated and the cell pellet frozen immediately on dry ice. Samples are prepared for assay by sonication in perchloric acid (5% v/v), neutralized, then frozen (-30°C) until cyclic AMP and/or cyclic GMP are determined in duplicate by radioimmunoassay (17). Protein kinase C activity is measured by a radiochemical procedure following partial purification of the enzyme by anion exchange chromatography.

III. RESULTS AND DISCUSSION

A. Dose-Response Studies with Adrenergic Agonists (Figure 1)

NE stimulated pinealocyte cyclic AMP and cyclic GMP 60- and 400-fold respectively. The dose-response curve to isoproterenol (ISO) was biphasic for both nucleotides; a marked plateau was evident between 10^{-8} and 10^{-6}M. Such an 'atypical' response to ISO was noted previously for cyclic AMP in neonatal rat pinealocytes (18). The secondary increase occuring at high concentrations of ISO (>10^{-6}M) was completely blocked by prazosin, an α_1-antagonist, but the initial part of the response (ISO <10^{-6}M) was unaffected. Thus, the secondary rise

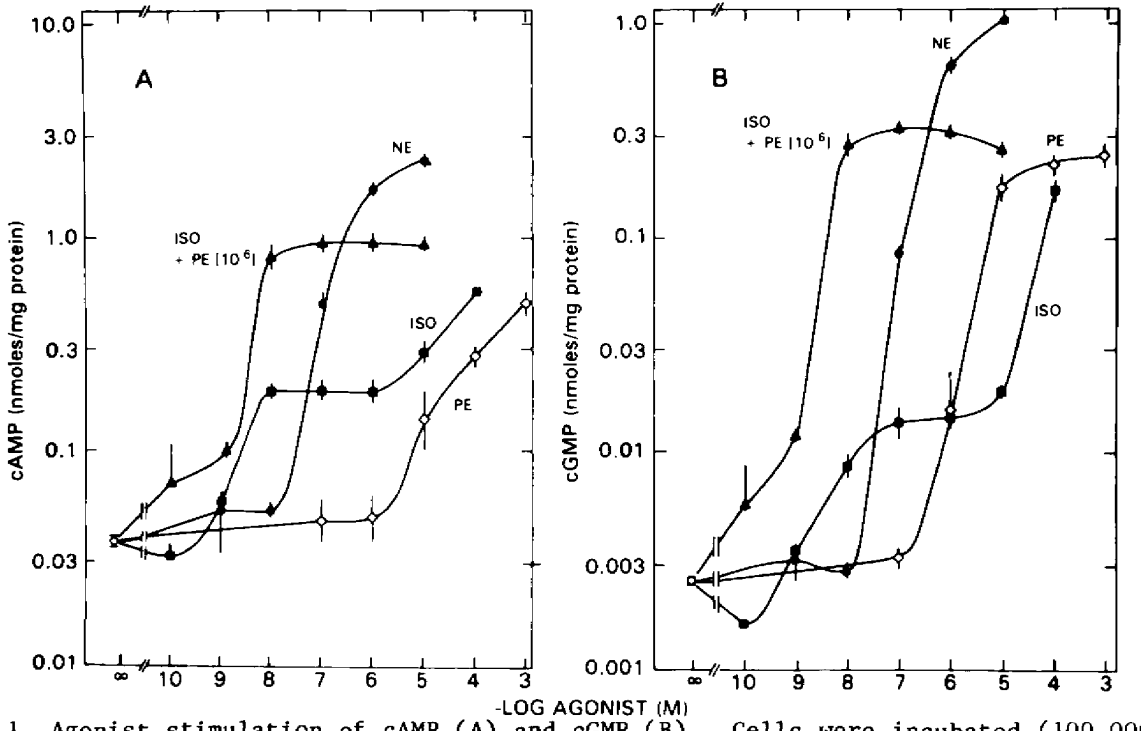

Fig. 1 Agonist stimulation of cAMP (A) and cGMP (B). Cells were incubated (100,000 cells, total volume 500 µl) for 15 min at 37° C with the indicated concentrations of (-) NE, PE, (-)ISO, or (-)ISO and PE (10^{-6}M). Each point is the mean of triplicate samples assayed in duplicate. Vertical lines represent the SEM; where absent, the SEM is smaller than the symbol.

represents a non-selective action of ISO on α_1-adrenoceptors. Phenylephrine (PE) increased cyclic AMP and cyclic GMP only at high concentrations ($>10^{-5}$M); these increases were completely blocked by a β-antagonist, propranolol, again indicating a non-selective β-adrenergic action of PE at high concentrations.

Interestingly, when the ISO dose-response curve was repeated in the presence of PE (10^{-6}M), a concentration producing no increase in cyclic AMP and only a slight elevation of cyclic GMP, the response was markedly potentiated. In addition, the dose-response curves were shifted to the left and lost their biphasic character.

B. Effects of α-adrenergic agonists (Figure 2).

ISO (10^{-5}M) produced a substantial increase in cyclic AMP (~ 10-fold) and cyclic GMP (~ 8-fold). Addition of various α-adrenergic agonists together with ISO produced a further dose-related increase in pinealocyte cyclic nucleotides. Alone, none of the α-agonists tested produced a propranolol-resistant elevation of cyclic nucleotides. Cirazoline and PE were approximately equipotent; methoxamine less potent and the α_2-selective agonists clonidine and UK 14304 failed to potentiate the ISO response.

C. Experiments with antagonists (Figure 3).

Stimulation of cyclic AMP and cyclic GMP produced by NE (10^{-5}M) was blocked by various α- and β-adrenergic antagonists. The α_1-selective antagonists, prazosin and HEAT were the most potent, while the α_2-selective antagonists RX 781094 and yohimbine were much weaker. Propranolol, a β-adrenergic antagonist, also blocked the NE response. None of the α-antagonists completely suppressed the NE response to control values; only propranolol was able to do this. The response to NE remaining in the presence of a high concentration of α-antagonist represents the β-adrenergic portion of the response to NE and was equal to what was seen at moderate concentrations of ISO (Figure 1).

These results strongly indicate that NE acts to regulate cyclic AMP and cyclic GMP in the pineal cell by an action on both α_1- and β-adrenoceptors. It would appear that β-adrenergic stimulation is an absolute

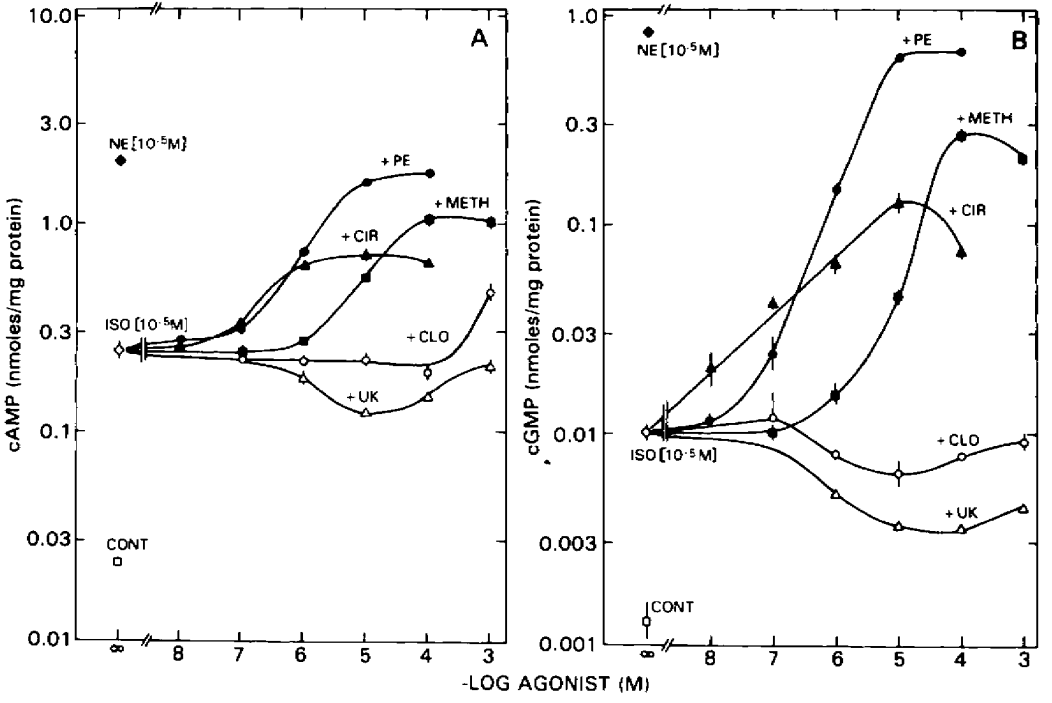

Fig. 2 α-Adrenergic agonist stimulation of cAMP (A) and cGMP (B) in the presence of (-) ISO (10^{-5}M). Cells were incubated for 15 min with the indicated concentrations of PE, cirazoline (CIR), methoxamine (METH), clonidine (CLO), and UK 14304 (UK) together with (-) ISO. See the legend to Fig. 1 for further details.

requirement as α_1-adrenoceptor activation alone cannot elevate either cyclic nucleotide. α_1-Adrenergic stimulation amplifies in some manner the effect of β-adrenergic stimulation. An earlier assessment of the cyclic GMP response to NE indicated wrongly that this was an "alpha-adrenergic like" response (7). Approximately 95% of the cGMP response to NE represents α_1-potentiation of a β-adrenergic response. Accordingly, the "alpha-adrenergic like" conclusion is understandable.

D. Mechanism of α_1-adrenoceptor potentiation

Recent data shed some light on the mechanisms involved in the α_1-adrenergic potentiation of cyclic AMP and cyclic GMP (15). The tumor promotors, 4β-phorbol 12-myristate, 13-acetate (PMA) and 4β-phorbol 12,13-dibutyrate (PDBu) mimic the action of α_1-agonists and potentiate the elevation in cyclic AMP produced by ISO. However, these agents did not potentiate the cyclic GMP response (Figure 4). The maximal rise in cyclic AMP was comparable to that seen with NE. The concentrations of PMA and PDBu producing a half-maximal potentiation of the response were approximately 5×10^{-9}M and 5×10^{-8}M respectively. These concentrations are very similar to the concentrations of these agents reported to half-maximally activate protein kinase C *in vitro* (19) Furthermore, 4α-phorbol 12,13-didecanoate (PDD), which does not activate protein kinase C, did not potentiate the cyclic AMP response. None of the phorbol esters elevated cyclic AMP or cyclic GMP when added alone. Interestingly, the amplification of the cyclic AMP response occured very rapidly. (Figure 5). An apparent small potentiation of the cyclic GMP response reflects a minor cross-reactivity of the cyclic GMP antisera with the large concentration of cyclic AMP generated in these cells.

The physiological activator of protein kinase C is thought to be diacylglycerol, generated in the plasma membrane following receptor-activated phosphatidyl-inositol hydrolysis (20). Activation of α_1-adrenoceptors results in an increase in phosphatidylinositol turnover in pinealocytes (21,22). A synthetic diacylglycerol, 1-oleoyl, 2-acetyl glycerol (OAG) can also activate protein kinase C *in vitro* (23) and was also found to potentiate the effects of ISO on cyclic AMP (Figure 6). Again a small effect of OAG plus ISO on cyclic GMP probably reflects a cross-reactivity of the antiserum.

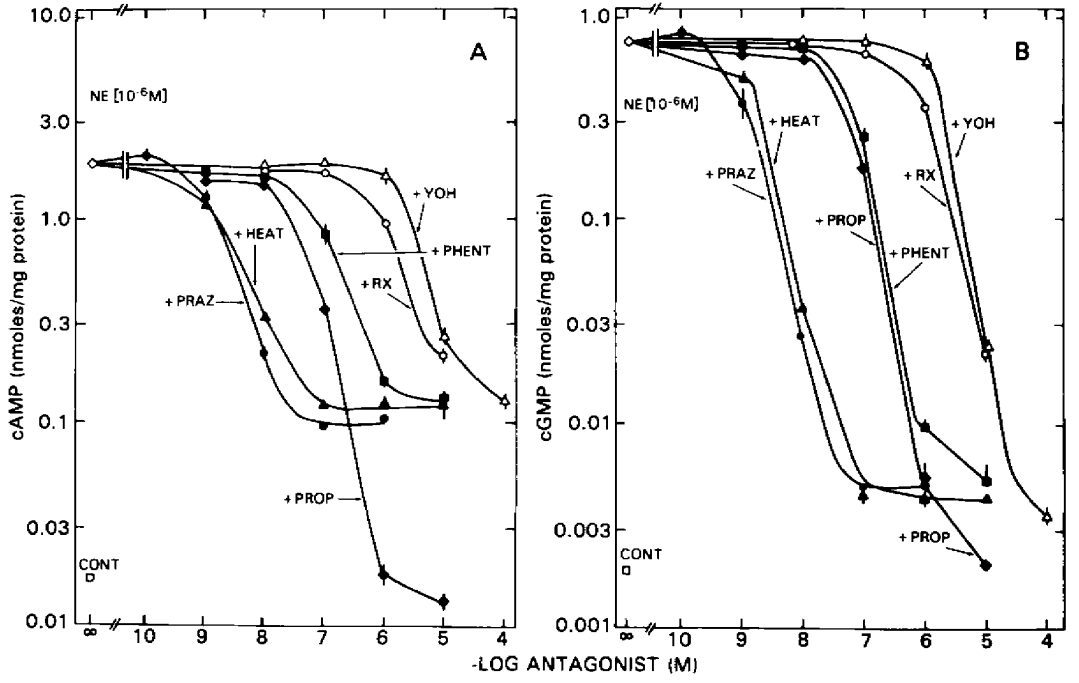

Fig. 3 Inhibition of NE-stimulated increase of cAMP (A) and cGMP (B) by antagonists. Cells were incubated for 15 min at 37° C with (-)NE (10^{-6}M) together with indicated concentrations of prazosin (PRAZ), HEAT, phentolamine (PHENT), Rx 781094 (RX), yohimbine (YOH), or (-) propranolol (PROP). See the legend to Fig. 1 for further details.

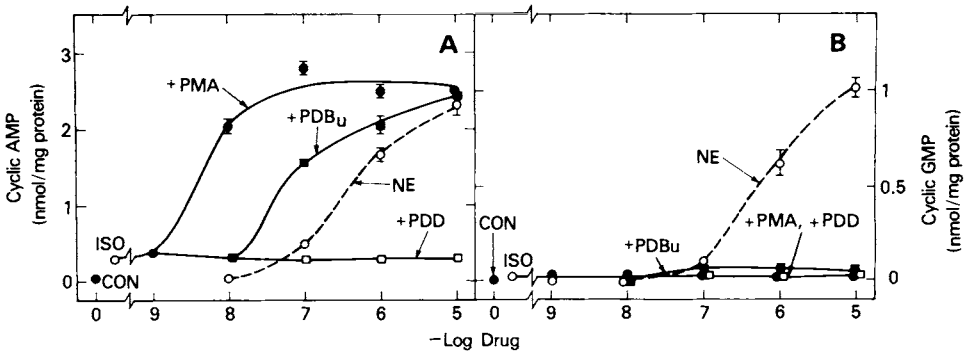

Fig. 4 Potentiation of β-adrenergic cyclic nucleotide responses by phorbol esters (A, cyclic AMP; B, cyclic GMP). Cells were treated for 15 min with isoproterenol (ISO; 10^{-6}M) either alone or in combination with 4β-phorbol 12-myristate 13-acetate (PMA), 4α-phorbol, 12,13-dibutyrate (PDBu) or 4β-phorbol 12,13-didecanoate (PDD). The dose-response curve to norepinephrine (NE) is shown for comparison.

Direct measurements of protein kinase C in pinealocytes revealed a very high activity (14 nmol of ^{32}P incorporated per min per mg protein) when compared to many other tissues (24,25). Interestingly, brief treatment of pinealocytes with PMA or PE, at concentrations demonstrating a marked potentiation of the cyclic AMP response, caused a decrease in cytosolic protein kinase C activity with a corresponding elevation in enzyme activity associated with the membrane fraction (Table 1). It has been suggested that this redistribution of protein kinase C activity reflects an activation of the enzyme, perhaps enabling it to gain access to its physiological substrate (26). Hormone-induced translocation of protein kinase C activity has been described in pituitary (27,28) and transformed cells (29,30).

These studies clearly show that activators of protein kinase C (phorbol esters and diacylglycerol) mimic the action of α_1-agonists and potentiate β-adrenergic stimulation of cyclic AMP accumulation in pinealocytes. In addition, protein kinase C activity is rapidly redistributed not only in response to PMA but also in response to the α_1-agonist PE. α_1-Adrenoceptor-mediated phosphatidylinositol hydrolysis by PE would elevate

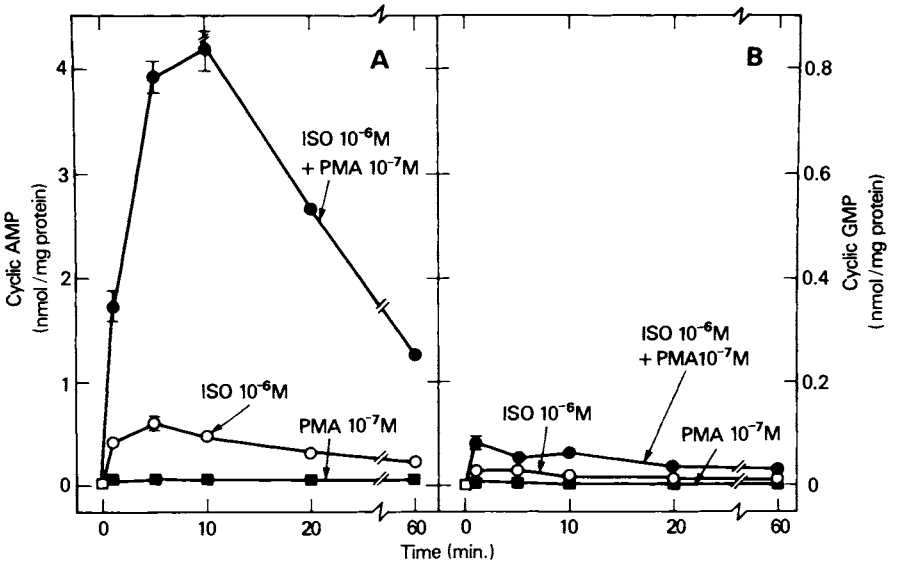

Fig. 5 Time-course of PMA potentiation of β-adrenergic cyclic nucleotide response (A,cyclic AMP; B, cyclic GMP).

diacylglycerol in the membrane of these cells. Thus, these data suggest that protein kinase C activation by diacylglycerol mediates the effects of α_1-agonists in amplifying β-adrenergic cyclic AMP accumulation. Presumably protein kinase C activation results in the rapid phosphorylation of some component of the system synthesizing cyclic AMP, perhaps the β-adrenoceptor, guanine nucleotide binding proteins, or the adenylate cyclase catalytic unit or of the system degrading cyclic AMP.

It appears that a different mechanism mediates α_1-potentiation of cyclic GMP accumulation. Considerable evidence suggests that hydrolysis of phosphatidyl-inositol generates at least two intracellular messages: diacylglycerol as described above, and 1,4,5-inositol trisphosphate (IP_3), which can mediate a release of intracellular Ca^{2+} (31). As the cyclic GMP response to NE in the pineal gland is known to be Ca^{2+}-dependent (32) perhaps an elevation in intracellular Ca^{2+} may mediate the effects of α_1-agonists. However, recent data suggests that little IP_3 is generated in the pineal

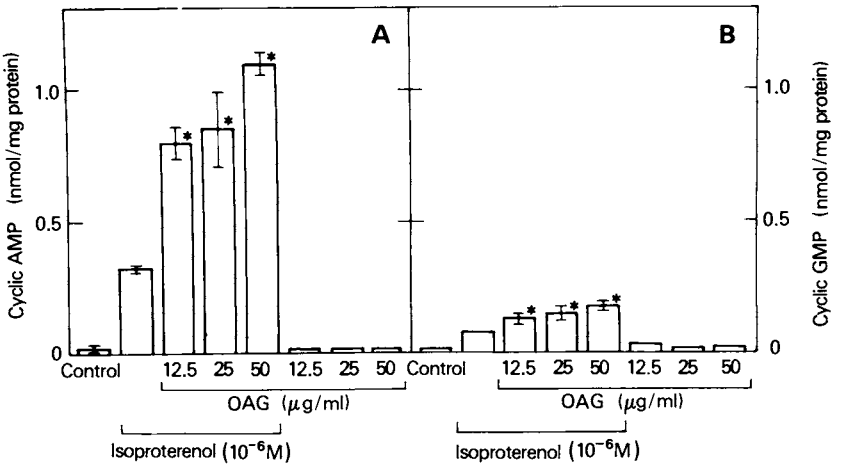

Fig. 6 Potentiation of β-adrenergic cyclic nucleotide responses (A, cyclic AMP; B, cyclic GMP) by 1-oleoyl, 2-acetyl glycerol (OAG). Cells were treated for 5 min with OAG alone or in combination with isoproterenol. OAG (10 mg/ml in chloroform) was dried under N_2, then suspended in dimethyl sulphoxide (1%) by brief, vigorous sonication on ice immediately before use.

following α_1-stimulation (33). An alternate mechanism involves phosphatidic acid, a metabolite of diacyl-glycerol, which has been reported to enhance Ca^{2+} influx (34). Conceivably, an increase in intracellular Ca^{2+} may be necessary to activate cyclic GMP production, perhaps indirectly by activating another enzyme. One possibility is that phospholipase A_2 may be involved. This is indicated by the finding that a low concentration of mepacrine (10^{-7}M), a phospholipase A_2 inhibitor, specifically blocks α_1-potentiation of cyclic GMP stimulation (35).

Finally, these studies are of particular interest because several other examples of interactions between intracellular signalling systems have recently been described. In some cell types activation of protein

Table 1 Effect of PHE or PMA treatment of pinealocytes on cytosolic and membrane-bound protein kinase C activity

Expt	Pretreatment	Protein kinase C activity (pmol ^{32}P incorporated per 3 min per 5 x 10^6 cells)	
		Cytosol	Membranes
1	Water	13,750	1,000
	PE (10^{-6}M)	8,725	5,160
2	Vehicle	13,248	1,395
	PMA (10^{-7}M)	6,286	3,164

Pinealocytes (5 x 10^6) were treated with PE (10^{-6}M) for 1 min or with PMA (10^{-7}M) for 15 min. Cells were pelleted (1,000g x 1 min), washed, then lysed in water (250 µl) containing $CaCl_2$ (10^{-5}M) by brief sonication (expt 1) or by repeated aspiration into a syringe through a fine (27-gauge) needle (expt 2). Membrane and cytosol were separated (12,000g, 1 min. at 4° C) and 0.5 ml of buffer (20 mM Tris-HCl, pH 7.5 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulphonylfluoride) was added. Membrane and cytosol protein kinase C activity was determined in triplicate (25).

kinase C results in reduction of cyclic AMP accumulation (36-38). Yet in other cell types, as in pinealocytes, potentiation of cyclic AMP responses follows protein kinase C activation. For example, PMA enhances β-adrenergic cyclic AMP accumulation in S49 lymphoma cells via a mechanism involving the guanine nucleotide binding proteins (39). PMA also enhances β-adrenergic and vasoactive intestinal peptide-induced cyclic AMP accumulation in cultured vascular smooth muscle cells (40). A similar effect on growth hormone releasing factor-induced cyclic AMP accumulation is seen in anterior pituitary cells (41). Clearly a better understanding of the mechanisms involved in these interactions will allow a greater insight into the way in which cells integrate diverse hormonal and/or neurotransmitter signals to regulate physiological responses.

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RETINAL S-ANTIGEN, AN OVERVIEW[1]

Waldon B. Wacker

Department of Ophthalmology
School of Medicine
University of Louisville
Louisville, Kentucky

Although the autoimmune theory of sympathetic ophthalmia dates from 1910,[1] it was not until 1965 that immunization with retina was found to produce an autoimmune uveitis experimentally.[2] The autoantigen was detected in retinal extract,[3-5] as well as in the rod outer segments.[6] Because of its ready solubility in aqueous media, it was simply termed soluble or S-antigen. It was shown to be tissue specific with species cross reactivity,[4,7] and was localized by immunofluorescence to the photoreceptor cells.[8] Guinea pigs,[3,6,9] rabbits,[3,10,11] and rats[12,13] were all shown to be highly susceptible to induction of this disease, termed experimental allergic or autoimmune uveitis (EAU).[14,15]

The retinal S-antigen was isolated in 1977[14,15] and many of its physicochemical properties, including animo acid composition, were determined. It is a protein with an apparent molecular weight of 50,000 daltons, and completely surrounds the photoreceptor cell by immunofluorescence as if associated with the plasma membrane. The antigen has been isolated from several mammalian species of retina,[14-18] although bovine is most commonly used because of the size and ready availability of such retinas.

Purified S-antigen has been used to produce and characterize the manifestations of EAU in guinea pigs,[14,19,20] rats,[21-23] rabbits[24] and monkeys.[25,26] In

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general, the disease is characterized by inflammation of the uveal tract and outer retina, by infiltration of the aqueous and vitreous humors, and by loss of photoreceptor cell structure and function. The exact manifestations in each species, however, may vary in part due to species variation in retinal vascularization, to the adjuvants used, and the dose of antigen employed.

Besides localization to the retinal photoreceptor cells, S-antigen has more recently been detected in the pineal gland of the guinea pig[27] and numerous other mammalian and non-mammalian species.[28] It was also shown that guinea pigs immunized with S-antigen develop inflammation in both the eyes and pineal gland[29] and, in addition, develop inflammation in both tissues when immunized with pineal gland extract.[30] Further, both pinealitis and EAU were observed in this species passively immunized with cultured peritoneal lymphocytes from donors immunized with S-antigen.[31] Similar findings have recently been reported[32] in rats both actively or passively sensitized to S-antigen. These observations demonstrating a relationship between the retina and pineal are consistent with the phylogenetic and ontological relationship of the pineal gland to photoreception.

Recent investigations with S-antigen have focused upon three areas of study; mechanisms of EAU, its possible relationship to human disease, and functional identity of the antigen. Although some lines of evidence have implicated immediate hypersensitivity in early stages of the disease,[33,34] EAU is considered to be T-cell dependent. This suggestion is supported by the capacity of antigen-stimulated lymphocytes from syngeneic donors sensitized to S-antigen[31] to transfer the disease to normal guinea pigs[35] and rats. The T-cell subsets capable of transferring the disease in rats were identified as helper/inducer cells. In addition, the development of EAU in immunized rats can be prevented by treatment with cyclosporine, an immunosuppressive agent selective for T-lymphocyte functions.[36,37,38]

The role of autoimmunity in human ocular diseases has been investigated for decades using crude extracts of various ocular tissues (for review see 39). The availability of purified S-antigen has led to several studies seeking evidence of sensitivity to this antigen in a wide range of such human disorders. Autoantibodies to S-antigen were detected by passive hemagglutination in a significant number of patients with panuveitis, chronic cyclitis, and chorioretinitis, as well as in Harada's disease, sympathetic ophthalmia and other disorders.[33] Similar findings were reported[40] using the Elisa test. Evidence of cell mediated immunity to S-antigen as detected by leukocyte migration

inhibition was found in about 50% of patients with panuveitis or posterior uveitis, as well as in Behcet's syndrome.[33] Cellular immune responsiveness to the antigen was also detected by lymphocyte stimulation in a significant number of patients with posterior uveitis.[41] Perhaps the largest percent of reactors occurs in the newly described entity, Birdshot Retinochoroidopathy, where over 90% of patients tested showed evidence of in vitro proliferative responses to S-antigen.[42] Although these findings all point to sensitivity to the antigen in diverse ocular disorders, it is not yet clear whether the relationship is causal or a result of the disease, or possibly an epiphenomenon.

The demonstration of sensitivity to S-antigen among uveitis patients, along with the observed effect of cyclosporine on development of EAU,[36-38] have led to clinical trials of this immunosuppresive agent. Pilot studies in patients with bilateral posterior uveitis, resistant to corticosteroid or cytotoxic therapy, showed improvement in visual acuity and diminution in ocular inflammatory activity in 7 of 8 cases on cyclosporine therapy.[43] Expansion of the study showed positive therapeutic responses in 15 of 16 patients on this therapy,[44,45] and a much larger group is now under investigation. These preliminary findings indicate considerable promise in the use of a new therapeutic agent in treatment of such disorders.

Finally, the functional identity of S-antigen has been the subject of speculation and investigation since its isolation eight years ago. In our initial study,[14] we found similarities in amino acid composition between S-antigen and a then recently isolated retinol-binding protein, suggesting it may play a role in transport of vitamin A. Other studies[19] raised the possibility of an identity with GMP-phosphodiesterase, based upon similarities in immunopathology induced by the antigen and the enzyme. However, in unpublished studies (Wacker and Chader), we were unable to detect either retinol binding or phosphodiesterase activity by purified S-antigen. More recently, several lines of evidence including immunologic, biochemical, and immunopathologic, have suggested a possible identity with rhodopsin kinase.[46-48] However, it has been reported[49] that rhodopsin kinase has a m.w. of 67,000, larger than S-antigen, and that S-antigen biosynthesized in the retina is not a maturation or degradation product of a larger protein,[50] thus casting doubt on this identity. Very recently, three reports[51,52,53] presented biochemical, immunologic, functional, and pathologic evidence identifying S-antigen as the 48K protein isolated by Kuhn.[49] This is a soluble protein from rod outer segments

which binds to photoexcited rhodopsin. It is involved in quenching of light-induced cGMP phosphodiesterase activity, a major step in the phototransduction process.

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PINEAL GLAND IN S ANTIGEN INDUCED EXPERIMENTAL AUTOIMMUNE UVEITIS[1]

Carolyn M. Kalsow

Department of Biology
Hope College
Holland, Michigan

Waldon B. Wacker

Department of Ophthalmology
University of Louisville School of Medicine
Louisville, Kentucky

I. CROSSREACTIVITY OF S ANTIGEN FROM RETINA AND PINEAL

Initial studies on tissue distribution of S antigen by complement fixation and immunodiffusion established a retinal specificity for this antigen (28). Because these techniques require the use of tissue extracts, any small discrete areas of tissue capable of crossreactivity were not detected as a result of dilution by homogenization procedures. When we reexamined tissue specificity of anti-S reactivity by immunofluorescence on ethanol fixed, paraffin embedded tissues, we found a very strong reactivity with guinea pig pineal gland (9). This reactivity was demonstrated with anti-retina extract (9), anti-pineal extract (11), and anti-partially purified S antigen sera (9). We have also found characteristic retinal (7) and pineal reactivity of sera of all 30 guinea pigs given 50, 25, 10, 5 or 1 μg of purified S antigen in complete Freund's adjuvant. The injection protocol and clinical and ocular histopathologic responses of these guinea pigs have been presented elsewhere (21).

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Antisera to other ocular antigens did not react in a similar manner with guinea pig pineal gland (9). None of 12 antisera produced to retina sediment (anti-P, probably anti-rhodopsin, sera [15,29]), showed any specific fluorescence in the pineal gland, although they did show characteristic reactivity in the outer segments of the retina (8). Of the anti-U (uveal homogenate) sera tested, which react in the area of Bruch's membrane (8), only one of 14 showed a very weak reaction with pineal gland. This probably resulted from contamination of uvea with some photoreceptor outer segments during dissection. None of five anti-lens sera tested showed any anti-pineal reactivity. Likewise, we have since tested four anti-optic nerve sera and eight anti-vitreous sera and found no detectable reactivity with guinea pig pineal gland. The production of these sera has been described elsewhere (28).

By immunodiffusion, we showed reactions of identity between pineal and retina extract with anti-pineal and anti-retina extract sera (10,11). We also used immunoelectrophoresis to demonstrate similar mobilities of retinal and pineal S antigen as precipitated with anti-retina extract or anti-pineal extract sera (11).

II. PINEAL INVOLVEMENT IN S-INDUCED EXPERIMENTAL AUTOIMMUNE UVEITIS

A single hind footpad injection of retina extract or purified S antigen produces a consistent, well defined experimental autoimmune uveitis (EAU) (25). In addition to the ocular aspects of this autoimmune response, these guinea pigs also have a distinct lymphocytic infiltration of the pineal gland (10; Table I). This pineal gland involvement is found in most injected guinea pigs, even in the absence of ocular inflammation. Furthermore, in a recent study, we

TABLE I. OCULAR AND PINEAL RESPONSE OF GUINEA PIGS TO S ANTIGEN PURIFIED FROM RETINAS OF VARIOUS SPECIES

	S ANTIGEN SOURCE		
	GUINEA PIG	BOVINE	RABBIT
OCULAR INFILTRATE	31/34*	32/41	4/15
PINEAL INFILTRATE	25/25	28/28	13/13

*Number of animals showing ocular or pineal infiltrate/ number examined.

have found pineal gland involvement in guinea pigs by 9 days postinjection (dpi) with 50 μg of purified bovine S antigen (Table II). In contrast, clinical and histopathologic signs of uveitis were detectable in only 1 of 4 guinea pigs at 9 dpi, in 4 of 5 at 13 dpi and in all thereafter (Table II). Additionally, in studies of S antigen purified from guinea pig, bovine or rabbit retina, we have found pineal gland involvement to be more consistent than the development of uveitis (Table I). The injection protocol and clinical and ocular histopathologic response of these guinea pigs to guinea pig S antigen have been previously reported (23). Adoptive transfer of EAU by peritoneal exudate cells or lymphocytes from donor guinea pigs sensitized to S antigen results in pineal infiltrate along with uveitis (27).

Guinea pigs injected with uveal homogenate develop a uveitis distinct from that of S antigen, most notably differentiated by the absence of anterior inflammation (22). In 18 guinea pigs so immunized with 1:3 (w/v) choroidal homogenate, all developed clinical and histopathologic signs of uveitis as well as serum antibodies showing characteristic immunofluorescent reactivity in the area of Bruch's membrane (8). However, there was no detectable infiltrate in the pineal gland of any of these animals.

To examine the effect of EAU associated pineal involvement on target organs of the pineal gland, tissues from several animals injected with retina (5 guinea pigs) or pineal (7 guinea pigs) extract and reinjected at 42 dpi were obtained at 52 dpi for histopathological examination. The injection protocol, and clinical, ocular and pineal histopathologic, and serologic responses of these animals have been described elsewhere (11). Examination of hematoxylin and eosin stained sections of ethanol fixed, paraffin embedded pituitary, spleen, ovary, adrenal, thymus, thyroid, superior cervical ganglion, uterus and hypothalamus

TABLE II. TIME COURSE OF OCULAR AND PINEAL INVOLVEMENT IN S-INDUCED EAU

	DAYS POSTINJECTION				
	5	9	13	17	21
OCULAR INFILTRATE	0/4*	1/4	4/5	4/4	4/4
PINEAL INFILTRATE	0/3	3/3	5/5	3/3	3/3

*Number of animals showing ocular or pineal infiltrate/ number examined.

revealed no overt evidence of histopathology as compared to the saline injected controls and one uninjected control guinea pig.

To study the effect of the pineal gland on EAU, several experiments were designed to compare the development of EAU in intact guinea pigs with those that had been pinealectomized or sham-pinealectomized. Two weeks prior to injection, female Hartley guinea pigs (300-400 gm) were pinealectomized according to the procedure of Kuszak (13). The protocol of Dyer et al. was used for anesthesia and postsurgical antibiotic therapy (5). Sham-operated animals were subjected to all steps of the surgery except the final aspiration of the pineal gland. In each of 4 trials development of EAU was compared across 3 groups of guinea pigs: pinealectomized, sham-operated, and intact. Each group consisted of 3 or 4 retina extract injected guinea pigs and 1 saline injected control animal. The guinea pigs were maintained in a 12/12 light/dark cycle. Procedures for injection, clinical observation, bleeding, sacrifice and preparation of tissues for observation of histopathology have been described elsewhere (11).

TABLE III. ONSET OF EAU IN PINEALECTOMIZED, SHAM-PINEALECTOMIZED, AND INTACT GUINEA PIGS

TREATMENT GROUP	DPI[a]	TRIAL A	B	C	D	TOTAL
PINEALECTOMIZED	10-11			1[b]	1	2/16[c]
	12		2			2/16
	13	2		1	2	5/16
	14	1	1		1	3/16
	15-21	1	1	1		3/16
SHAM-PINEALECTOMIZED	10-11	2	1	1	2	6/16
	12	2	1	1	2	6/16
	13		1			1/16
	14			2		2/16
	15-21		1			1/16
INTACT	10-11	2		1	1	4/13
	12	1	1		1	3/13
	13		2			2/13
	14			1	1	2/13
	15-21		1			1/13

[a]Days postinjection
[b]Number of guinea pigs showing onset of EAU
[c]Number of guinea pigs showing onset of EAU/number injected

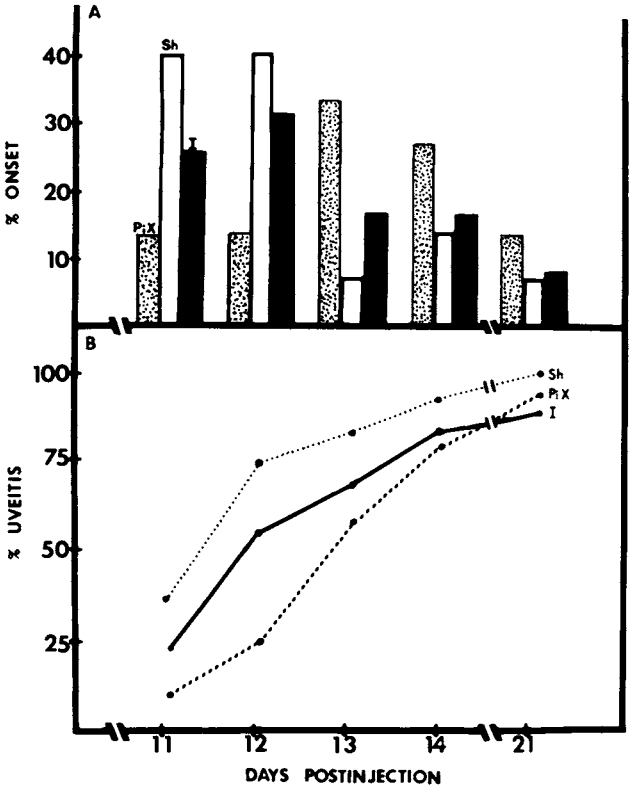

Figure 1. Incidence of uveitis in pinealectomized (PiX), sham-pinealectomized (Sh), and intact (I) guinea pigs. The data is presented as percent of experimental group showing onset of clinical signs of uveitis (A) or cumulative percent of guinea pigs with uveitis (B) as a function of days postinjection with homologous retina extract.

Comparison of the time of onset of clinical signs of uveitis in pinealectomized, sham-operated and intact guinea pigs (Table III and Figure 1) revealed a later onset of uveitis of pinealectomized guinea pigs. Only 4 of 16 pinealectomized guinea pigs showed onset of EAU before 13 dpi, but 12 of 16 sham-operated and 7 of 13 intact guinea pigs had developed EAU before 13 dpi. At 21 dpi there was no significant difference in the final degree of severity of clinical signs of uveitis in these animals. Since all animals were terminated at 21 dpi, no overt differences were observed in the histopathology of the eye or pineal gland or in the serological response to retina extract or S antigen as measured by complement fixation, immunodiffusion, or

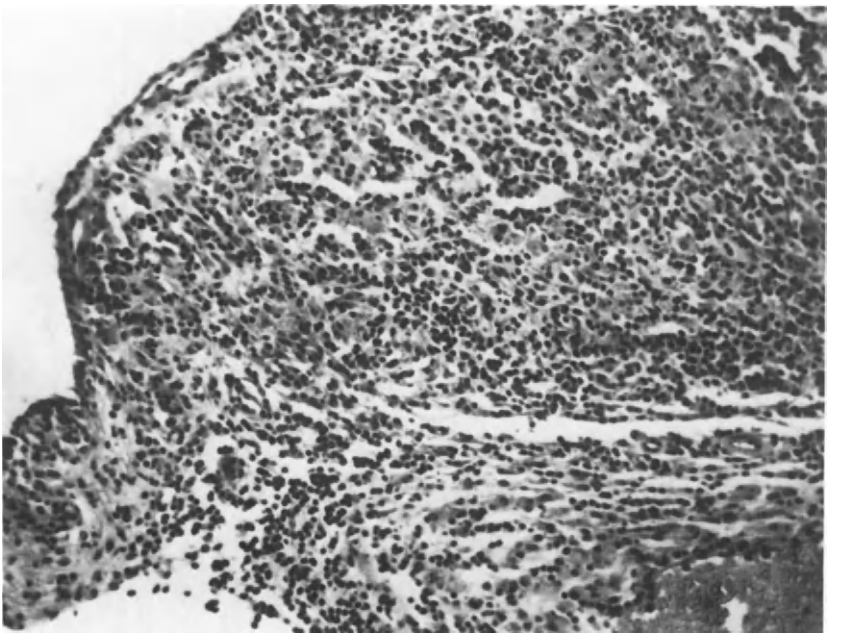

Figure 2. Lymphocytic infiltrate in residual deep pineal of a pinealectomized guinea pig exhibiting retinal extract induced experimental autoimmune uveitis. (Original magnification X 125, H+E.)

ELISA. In specimens from pinealectomized guinea pigs, lymphocytic infiltrate was seen in the residual deep pineal (Figure 2).

The absence of any differences in the final severity of uveitis in these animals suggests that the effect of the pineal gland was on an early phase of EAU development. It is possible that the surgical procedure, especially in the sham-pinealectomized animals, enhanced accessibility of S antigen in the pineal gland to immunocompetent cells thereby further stimulating the immune system to S antigen. Unfortunately, in the pinealectomized guinea pigs the residual deep pineal was given greater access to the immune system, thereby offsetting some of the effect of the removal of antigen. It is also possible that the effect of pinealectomy on these guinea pigs was a nonspecific effect of the immune system as has been shown in rats and mice (1,2). Adult-pinealectomized rats, but not neonatally pinealectomized, have been shown to have a decreased immunoresponsiveness, including decreased incidence of experimental allergic encephalomyelitis (6).

Further experiments into the role of the pineal gland in EAU involved the effect of environmental light along with pinealectomy on the induction of EAU. In several experiments using pinealectomized, sham-pinealectomized and intact guinea pigs that were housed in total light, total dark, or 12/12 light/dark cycle following the standard injection with retina extract, no overt differences were found in the EAU that developed in any of the experimental groups (Table IV). However, it must be noted that daily ophthalmoscopic observation of animals housed in total dark was not possible. In a larger experiment to test the effect of constant light on the development of EAU in intact guinea pigs, 27 guinea pigs were housed in 12/12 light/dark cycle and 27 were housed in total light beginning 3 days prior to injection with retina extract. With daily observation by direct ophthalmoscopy no difference was seen in the onset or severity of uveitis between these two groups (Figure 3).

TABLE IV. EFFECT OF ENVIRONMENTAL LIGHT ON DEVELOPMENT OF OCULAR AND PINEAL INVOLVEMENT IN S-INDUCED EAU

TREATMENT	TRIAL	ENVIRONMENTAL LIGHT					
		DARK		LIGHT		12/12	
		OCULAR	PINEAL	OCULAR	PINEAL	OCULAR	PINEAL
INTACT	A	7/7*	7/7	7/7	7/7		
	B			4/4	2/2	4/4	4/4
	C	4/4	3/3			2/2	2/2
PINEALECTOMIZED	A	4/4	3/3	2/2	1/1		
	B			4/4	1/1	4/4	1/1
	C	4/4				4/4	3/3
SHAM-PINEAL-ECTOMIZED	A	4/4	4/4	4/4	3/3		
	B			4/4	2/2	4/4	2/2
	C	4/4	3/3			4/4	4/4

*Number of animals showing ocular or pineal infiltrate/ number examined.

III. SPECIES SPECIFICITY OF S ANTIGEN IN PINEAL

This relationship of S antigen in pineal and retina is not totally guinea pig specific nor is it species independent. Using monoclonal antibodies to S antigen, Mirshahi et al. demonstrated reactivity in the pineal glands of eel, frog, lizard, bird, mouse and hamster (16). Using

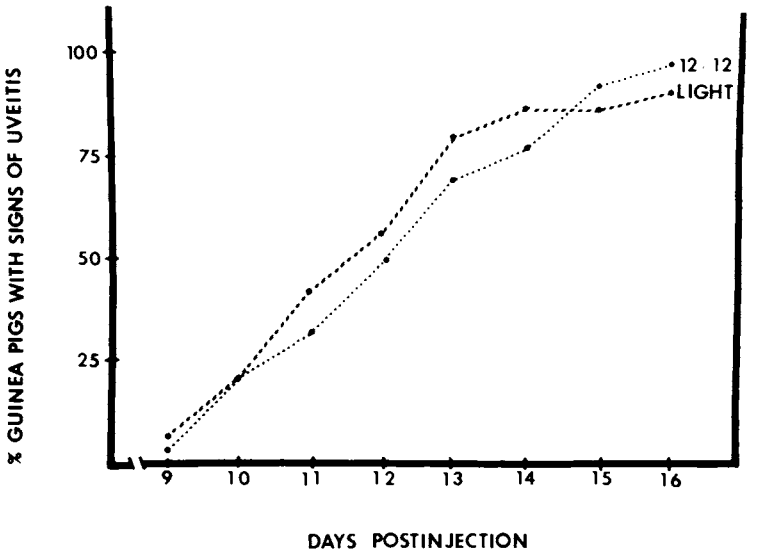

Figure 3. Incidence of clinical signs of uveitis in guinea pigs housed in constant light or 12/12 light/dark cycle. The data is presented as cumulative percent of guinea pigs with uveitis as a function of days postinjection with homologous retina extract.

polyclonal anti-S sera we have found reactivity in cat and lizard pineal glands. Using an extract of rabbit pineal glands (Pel-Freez Biologicals, Rogers, Ark.), we have not been able to demonstrate reactivity with anti-S sera by immunodiffusion. We have also not been able to demonstrate anti-S reactivity of several ethanol fixed, paraffin embedded pineal glands of New Zealand rabbits. Furthermore, although rabbits injected with S antigen purified from various species exhibited clinical signs of uveitis as described previously (30), only one of these rabbits showed any infiltrate of the pineal gland (12). These results would support an absence of S antigen in rabbit pineal gland.

Reactivity of polyclonal and monoclonal S antigen antibodies has been demonstrated on sections of pineal glands of Wistar and Lewis rats (3). In Wistar rats, which are not as susceptible to S-induced EAU (14,26), we have found uveitis and pinealitis in response to S antigen isolated from various species (Table V). However, in contrast to guinea pigs, ocular involvement was found

TABLE V. OCULAR AND PINEAL RESPONSE OF WISTAR RATS TO S ANTIGEN PURIFIED FROM RETINAS OF VARIOUS SPECIES

	S ANTIGEN SOURCE		
	GUINEA PIG	BOVINE	RABBIT
OCULAR INFILTRATE	2/6*	2/5	3/6
PINEAL INFILTRATE	1/5	2/5	1/6

*Number of animals showing ocular or pineal infiltrate/ number examined.

without pineal gland involvement and, the infiltrate in rat pineal was less intense and more restricted to the edge of the pineal (Figure 4). This has also been reported for the Lewis rat, as well as a more intense ocular and pineal response elicited with intravenous injection of pertussis along with the S-antigen in Freund's complete adjuvant (17). In our experience with Lewis rats, we have found pineal gland and ocular involvement to be independent responses to 25 or 50 μg of bovine S antigen in Freund's adjuvant without pertussis. With 50 μg of S antigen in complete Freund's adjuvant, pineal gland involvement was found as early as 5 dpi and again independent of uveitis (Table VI). This inconsistency of pineal involvement in S-induced EAU in the rat deters us from using rats in pinealectomy experiments, even though they are more readily pinealectomized than guinea pigs. Monoclonal antibodies to S antigen have also been used to demonstrate the development of S antigen in Lewis rat retina and pineal gland (3).

TABLE VI. TIME COURSE OF OCULAR AND PINEAL INVOLVEMENT IN LEWIS RATS IN S-INDUCED EAU.

	DAYS POSTINJECTION						
	5	9	11	13	15	17	21
TRIAL A							
OCULAR INFILTRATE	0/4*	0/5		3/5		3/5	4/4
PINEAL INFILTRATE	1/2	0/5		4/5		1/5	3/4
TRIAL B							
OCULAR INFILTRATE		0/4	2/5	3/5	5/5	3/5	
PINEAL INFILTRATE		0/4	3/5	3/5	3/5	3/3	

*Number of animals showing ocular or pineal infiltrate/ number examined.

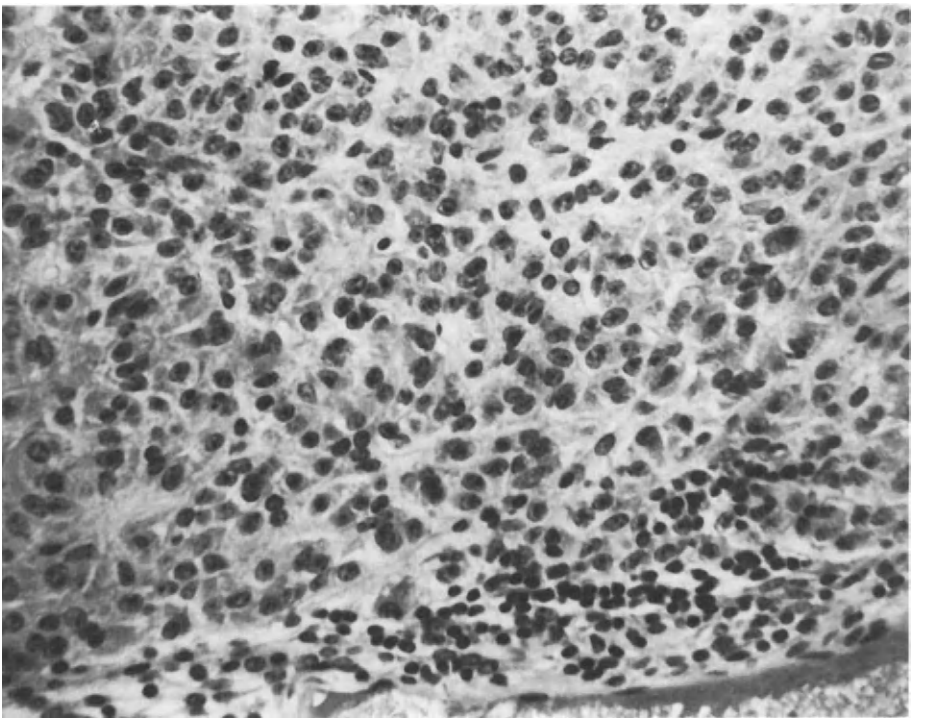

Figure 4. Lymphocytic infiltrate in pineal gland of a rat exhibiting S antigen induced experimental autoimmune uveitis. (Original magnification X 312, H+E.)

Ethanol fixed, paraffin embedded pineal glands of cynmologous monkeys (gift of Dr. Ted Lawwill, University of Louisville) showed reactivity of isolated cells by indirect immunohistochemistry (9) using a high titered pool of anti-S sera (7) in the first layer and fluorescein conjugated IgG fraction of goat anti-guinea pig IgG (Cappel Laboratories, Dowington, PA) or horseradish peroxidase conjugated Protein A (Polysciences, Inc., Warrington, PA) for the second layer (Figure 5). Dr. Robert Nussenblatt (NEI) has supplied us with pineal gland of a cynmologous monkey injected with S antigen in complete Freund's adjuvant. The ocular response of this monkey has been reported previously (18). The pineal gland of this monkey exhibited significant lymphocytic infiltrate (Figure 6).

Figure 5. Immunoperoxidase demonstration of anti-S reactivity of a normal monkey pineal gland. (Original magnification X 125.)

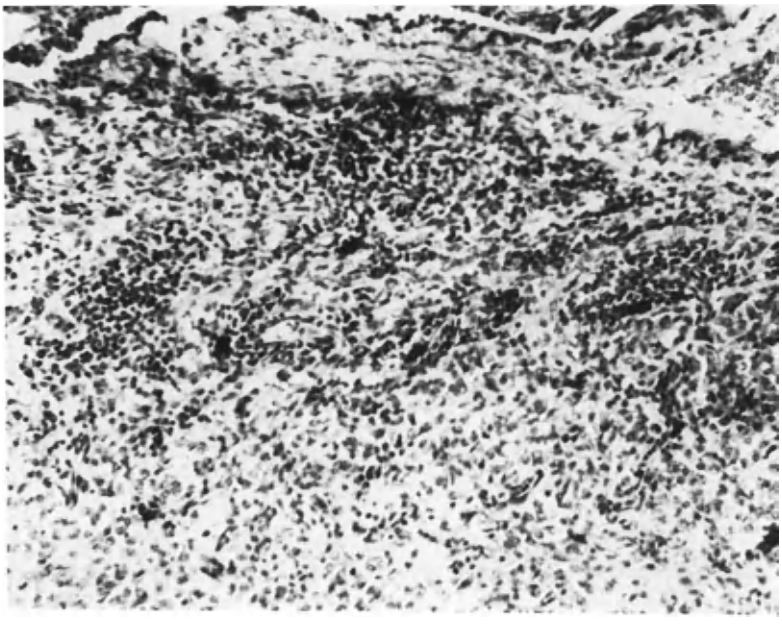

Figure 6. Lymphocytic infiltrate in pineal gland of a monkey exhibiting S antigen induced experimental autoimmune uveitis. (Original magnification X 125, H+E.)

S antigen is not the only antigen shared by retina and pineal gland. As reported above, rabbits do not develop a pineal infiltrate with S-induced EAU nor do they show a reactivity of pineal gland with anti-S sera. However, they do show a uveitis and pinealitis when injected with retina extract or fractions of retina extract not containing S antigen (12). Antisera of these rabbits have been shown to react with rabbit retina and pineal gland as well as guinea pig retina but not with guinea pig pineal gland. Serological and chromatographic evidence indicates that this autoantigen is not S antigen, but another uveitogenic autoantigen shared by retina and pineal gland.

Crossreactivity of a retinoblastoma and pinealoma has also been demonstrated with a monoclonal antibody to S antigen (4).

IV. CONCLUSION

The animal model of S-induced EAU was developed to test the hypothesis that some human uveitides have an autoimmune etiology. Such a model can also have significance in areas that are not directly related to the clinical disease process under study. It is important to our basic understanding of the ocular immune response to determine the mechanism by which an antigen introduced into the footpad of an animal can initiate a specific autoimmune response in the eye and in the brain. Studies involving pinealectomy and/or enucleation along with the effects of environmental light will help us understand some of these relationships.

The studies of the effect of environmental light on the development of EAU are also significant in understanding the relationship of the antigenic determinants of S antigen to the conformation of the molecule in response to light or dark i.e., in relating the functional nature of the molecule known as S antigen to its autoimmunologic role. Such a change in structure of S antigen is suggested by the possibility that it is the "48 K" molecule which binds to photoexcited rhodopsin and is intimately involved in photoreception (19,20).

Cross reactivity of S antigen and other antigens of retina and pineal in various species is significant in that it further underscores the phylogenetic relationship of the mammalian pineal gland to the photoreceptor pineal body of lower vertebrates, as well as the ontological relationship of morphological similarity between developing pinealocytes and retinal photoreceptors in mammals.

Furthermore, the presence of S antigen in the primate pineal gland and the development of uveitis and pinealitis in monkeys in response to S antigen supports the clinical relevance of this model and suggests that the pineal gland may be involved in some uveitides seen in humans. Although there is yet no direct evidence of pineal gland involvement in human uveitis, the diverse activities of the pineal gland might well explain disease manifestations in other tissues that may accompany some uveitides.

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Isolation of cDNAs for Bovine S-Antigen

Toshimichi Shinohara[1], Cheryl M. Craft[3], Paul Stein[2], J. Samuel Zigler, Jr.[2], Graeme Wistow[1], Albine Katial[1], Igal Gery[2], and David Klein[3]

[1] Laboratory of Molecular and Developmental Biology, NEI
[2] Laboratory of Vision Research, NEI, and [3] Laboratory of Developmental Neurobiology, NICHD,
National Institutes of Health,
Bethesda, Maryland 20892

I. INTRODUCTION

S-Antigen is a soluble protein of 42-50 kilodaltons (1-3) which was originally isolated from bovine retina (2,4). S-Antigen in photoreceptor cells binds specifically to photoexcited and phosphorylated rhodopsin and inhibits cyclic-GMP hydrolysis, a major step in the phototransduction process (5).

S-Antigen is of special interest because it is capable of inducing experimental uveitis when injected with an adjuvant into certain animals (2,6-8). Uveitis is responsible for 10% of blindness in the human population, making the experimental uveitis model potentially of great clinical importance (7,9).

S-Antigen is also found in abundance in the pineal gland (10-13), which is thought to have evolved from the parietal eye. Proteins involved in phototransduction in retinal rod cells such as rhodopsin kinase (14) and interphotoreceptor retinoid-binding protein (IRBP) (15), have been found in pinealocytes. Some proteins, however, such as rhodopsin (16) and Gα-transducin (17) are found only in minute amounts or

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are absent from mammalian pinealocytes, although they are present in the pineal of birds, reptiles, amphibians and fish (18,19,20). The role of S-antigen in the retina and pineal may be different in different species. However, a process similar to retinal phototransduction may play a role in the modulation of pineal activity in lower animals. To approach the questions of the function and structure of S-antigen in both tissues, we have isolated and characterized bovine S-antigen cDNA clones from retina cDNA libraries. This report describes the isolation and characterization of S-antigen cDNA clones. Evidence is provided which indicates that multiple S-antigen epitopes are present within the polypeptides encoded in these cDNAs. The cDNA hybridizes to retina and pineal messenger RNA (mRNA) of similar size (1,900 $\pm$ 300 nucleotides), which is appropriate to encode S-antigen.

Materials and Methods

Materials

A λgtll bovine retinal cDNA library was the gift of Dr. Daniel D. Oprian (Department of Chemistry, MIT, Cambridge, MA). A λgtlO bovine retinal cDNA library was supplied by Dr. Jeremy Nathans (Stanford University School of Medicine, Stanford, CA). Restriction enzymes, isopropylthio-β-D-galactoside (IPTG) and nick translation kit were purchased from BRL (Gaithersburg, MD). The deoxycytidine 5'-[α^{32}P] triphosphate (dCTP) (3000 Ci/mmol) was from Amersham Corp. (Arlington, IL). Goat antirabbit and rabbit antimouse horseradish peroxidase (HRP) antibodies and 4-chloro-1-napthol were purchased from BioRad (Rockville Center, NY). PIPES, cyanogen bromide activated Sepharose 4B, and lysozyme were purchased from Sigma (St. Louis, MO).

S-antigen was purified by high performance liquid chromatography (HPLC) (22). The partial amino acid sequence was determined by Applied Biosystems Inc. (Foster City, CA). The oligopeptide 17-mer was chemically synthesized in the peptide synthesis facility of the Children's Hospital at Boston.

Library Screening

A λgtll bovine retina cDNA library was screened using antibovine S-antigen rabbit antiserum (23-25). Phage were purified on cesium chloride step gradients, and the DNA was isolated (26). The inserted cDNAs were cleaved by EcoRl restriction enzyme and isolated on vertical 5% polyacrylamide gels (27). The cDNAs were labelled with ^{32}P-dCTP using a nick translation kit and this probe was used for hybridization studies. All hybridization reactions were

carried out using prehybridization mixture (10 mM Tris-HCl pH 7.5, 1M NaCl, 1mM EDTA, 1% SDS) for 2 hrs followed by hybridization with probe in of 6X SSC (90 mM sodium citrate, 0.9 M sodium chloride), 4 X Denhardt's (0.2 mM Ficoll, 0.02 mM polyvinyl pyrrolidone, 0.15 mM BSA), and 0.1 mM EDTA. The filters were incubated at 65°C overnight with the ^{32}P-labelled probe with gentle shaking, and washed twice in 2 X SSC with 1% SDS, twice in 1 X SSC with 0.1% SDS at room temperature for 10 to 20 minutes, and finally twice in 0.1 X SSC with 0.01% SDS at 60°C for 1 hr. Afterwards, the filters were autoradiographed with Kodak X AR5 X-ray film.

Production and preparation of antibodies

The monoclonal antiserum G5 was the gift of Dr. L.A. Donoso (Wills Eye Hospital, Philadelphia, PA) (13). The monoclonal antisera IVD was produced by established hybridoma procedures utilizing NS1 myeloma cells fused with polyetheylene glycol to spleen cells derived from mice immunized with S-antigen (28). Both monoclonals were derived from ascites fluid and used at 1:2,000 dilution. Established procedures were used to produce the polyclonal S-antigen antiserum and antiserum to the 17-mer polypeptide linked to keyhole limpet hemocyanin (KLH).

All antisera were absorbed using a cyanogen bromide-activated Sepharose 4B column linked with a lysate of E. coli strain BNN 97 to remove antisera against solubilized bacterial and bacterial phage antibodies (25). This preparation, at a dilution of 1:600, was then used to screen the library and in immunoblotting procedures.

Characterization of bacterial lysates by immunoblotting

The E. coli Y1090 containing S-antigen cDNA clones and controls were cultured for 6-8 hours in 1 liter of LB broth with ampicillin (40 µg/ml), tetracycline (40 µg/ml) and IPTG (1 µm). The bacteria were harvested by centrifugation and suspended in PBS. After treatment with lysozyme, the polypeptides derived from the bacterial lysates were separated on a 10% SDS polyacrylamide gel. The separated proteins were electrophoretically transferred from the polyacrylamide gels to nitrocellulose sheets (29). The transferred proteins were probed with monoclonal, polyclonal or normal ascites and serum controls (1:2,000 dilution in blocking buffer) (29). After incubation with the second antibody (1:2,000 dilution goat antimouse IgG-Peroxidase conjugate), the membranes were developed with 4-chloro-1 naphthol peroxidase substrate.

Northern Blot

Bovine RNA was prepared in the presence of 4 M guanidinium thiocyanate (26). Samples were electrophoresed on an 0.8% agarose gel containing 2.2 M formaldehyde, and 0.2M MOPS and the RNAs were transferred to nitrocellulose filters (26). After heating (80°C, 2 hr) at reduced pressure, the filters were soaked in prehybridization mixture for at least 2 hrs (50% formamide, 20 mM PIPES pH 6.4, 10% SDS, 50 µg/ml salmon sperm DNA and 5 X Denhardt's) and hybridized overnight at 43°C in hybridization mixture (50% formamide, 6 X SSC, 250 mM sodium phosphate, 50 µg/ml salmon sperm DNA and 5 X Denhardt's) with ^{32}P-dCTP labelled probe.

The filters were washed twice at room temperature with 2 X SSC with 1% SDS for 10 minutes, washed twice with 1 X SSC with 0.1% SDS for 20 minutes. After washing twice with 0.1 X SSC and 0.01% SDS for 1 hr, the filters were then dried and autoradiographed.

RESULTS

S-Antigen cDNA screening

A bovine retina λgt11 cDNA library was screened using bovine anti S-antigen antiserum (23-25). Initially, six million phage were screened and 139 positive clones were found (Fig. 1). Sixty of these were isolated and further purified. Ten purified putative S-antigen clones from these were characterized. The largest cDNA insertion was 420 base pairs long (BSC 500) and the other 9 clones had 100-300 base pair insertions. To further verify that these 10 clones contained cDNAs encoding S-antigen, we tested their immunochemical properties with two different mouse monoclonal antibodies (G5 and IVD). One monoclonal antibody (G5) positively reacted with polypeptides of all the 10 clones. In contrast, the other antibody (IVD) reacted only with the polypeptide of the largest cDNA clone (BSC 500) and did not react with polypeptides from the other 9 clones.

In order to find a larger or full size cDNA, another cDNA library was screened by DNA-DNA hybridization using the largest available cDNA (BSC 500) as a screening probe. Approximately 100 positive clones were isolated from the bovine retina λgt10 library (30) and 20 of these were purified. The largest cDNA insertion from this screening was 880 nucleotides long (BSC 900). Restriction enzyme analysis revealed that the two largest clones (BSC 500 and BSC 900) have Bam HI and Sst I sites at the same relative positions (Fig. 2) indicating that these two clones share overlapping

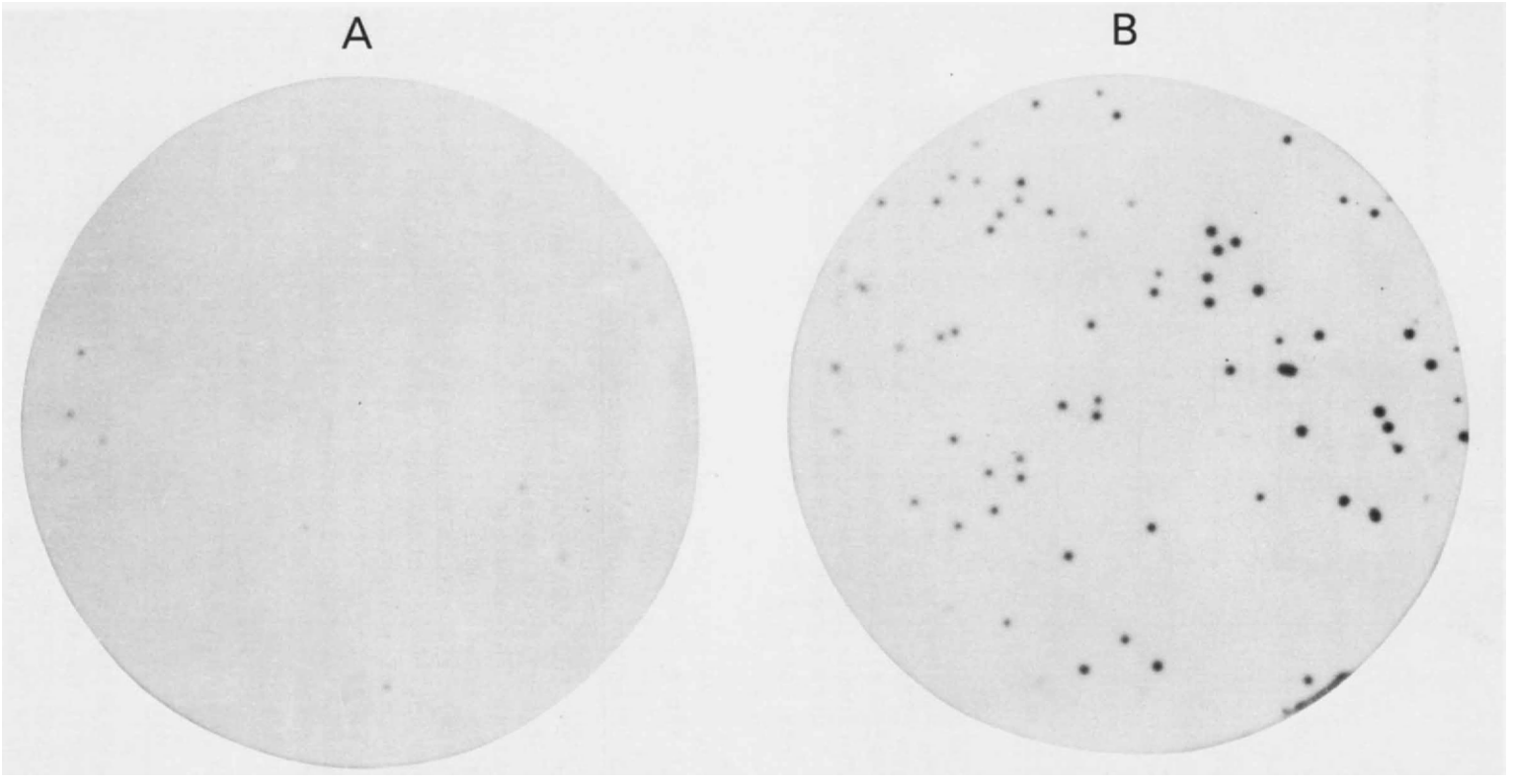

Fig. 1. Immunogenic reaction of S-antigen polyclonal antibody. Serial screening was performed until all phage showed positive reaction with antibovine S-antigen antibody. A: Initial screening of 1 million phage on one nitrocellulose filter from retina λgt11 library. B: Final screening. All plaques were recognized by the S-antigen polyclonal antibody.

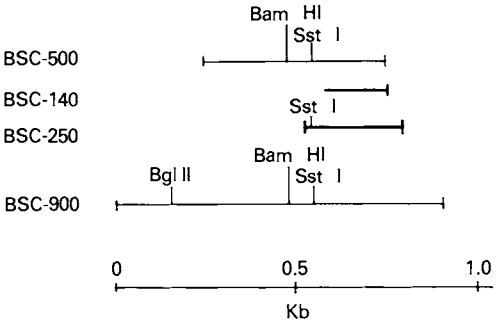

Fig. 2. Partial restriction maps of four cloned S-antigen cDNAs. The insert cDNAs were prepared by using EcoRI restriction endonuclease. The cDNAs were then analyzed with other restriction enzymes. Overlapping sites of Bam HI and Sst I were detected in the BSC 500 and BSC-900. No Bgl II site was found in BSC 500.

sequences. Neither cDNA had Hind III, Sma I, Mbo I, Sau 3A or Bgl I sites.

Analysis of polypeptide synthesized by cDNA clone

The BSC 500 cDNA clone in λgtll produced a fusion protein of β-galactosidase and the partial S-antigen polypeptide. The expressed product was analyzed by SDS-PAGE followed by immunoblotting (Fig. 3). A single peptide, approximately 130 kilodaltons, was observed which reacted with both anti-β-galactosidase and anti-S-antigen antibody (Fig. 3 B and E), suggesting that it was the expected fusion protein. Since the molecular weight of β-galactosidase is 116 kilodaltons, the fused S-antigen polypeptide was about 14 kilodaltons or about 130 amino acid residues. BSC 500 cDNA contains 420 base pairs which could code for 140 amino acids. Therefore, BSC 500 cDNA is almost entirely a coding sequence. This has now been verified by sequencing (in preparation).

Northern blot hybridization

The S-antigen probe (BSC 500) was hybridized to mRNA from the retina and the pineal gland (Fig. 4); the size of mRNA in both tissues was identical (1,900 $\pm$ 300 nucleotides long). Hybridization was not observed with mRNAs from cerebrum, liver and cerebellum. Since the molecular weight

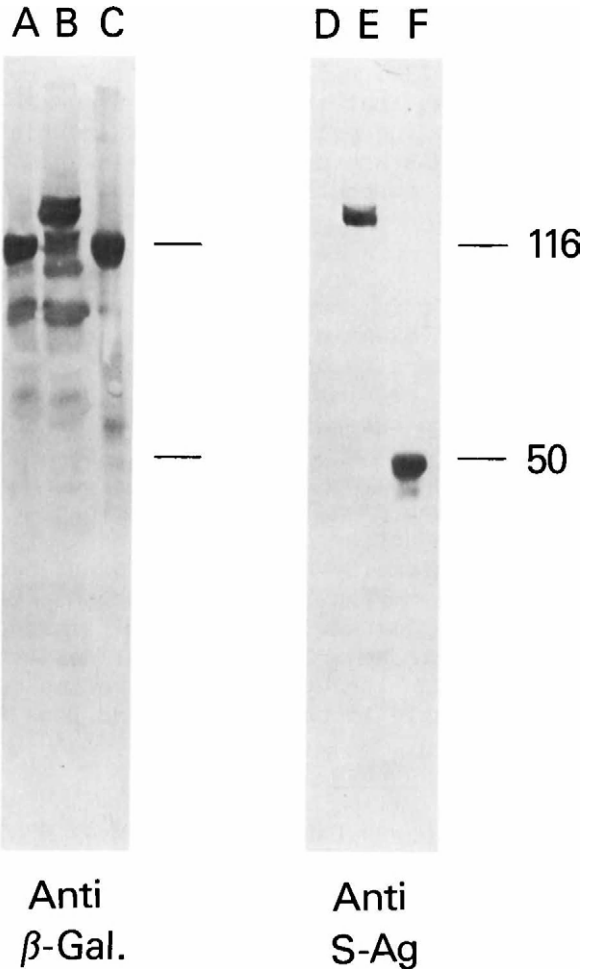

Fig. 3. Immunoblot of 10% SDS-PAGE of S-antigen (S-AG) fusion polypeptide with anti β-galactosidase (β-Gal) and anti-S-antigen antisera. Bacterial lysates were mixed with SDS/2-Mercapto-ethanol electrophoresis buffer and heated (100° C, 2 min). Thirty microliter samples (200 ug) were loaded into appropriate lanes and electrophoresed overnight at constant voltage. Subsequently, the separated proteins were transferred to nitrocellulose membranes and treated with PBS/BSA/Tween 20 and probed with antiserum. Lanes ABC are conjugated with anti-β-Gal antibody and lanes DEF with anti-S-AG antibody. Lanes A & D: Control λgtll without inserts; lanes B and E: clone BSC-500; lane C: purified bacterial β-galatosidase; and lane F: purified retinal S-antigen. The fusion protein produced by BSC 500 (E) is larger than the controls of λgtll or the pure β-Gal (C).

of S-antigen is 42-50 kilodaltons or 360-440 amino acid residues, the protein coding sequence of S-antigen mRNA is expected to be 1000-1320 nucleotides. A 1.9 K nucleotide mRNA, then, indicates that the noncoding sequence is 600-900 nucleotides. The evidence that mRNAs which hybridize to our cDNAs are present in retina and pineal but not in other tissues supports the idea that the cDNA encodes S-antigen.

Immunoblot

Since S-antigen mRNA is of similar size in the retina and pineal gland, the size of the S-antigen protein in both tissues is likely to be similar. We have examined freshly homogenized tissue to determine the size of S-antigen in both tissues (Fig. 5).

A 50 kilodalton polypeptide was observed in homogenates from bovine retina and pineal gland which reacted with monoclonal S-antigen antiserum. Interestingly, highly purified S-antigen normally has a single band, but occasionally two immunoreactive bands were observed on SDS-PAGE (Fig. 5A). Immunoblot analysis indicated that these two bands reacted with an S-antigen monoclonal antibody (G5 or IVD). The protein extracted from the retina or pineal contained only a single immunoreactive band (see Fig. 5B, C).

Partial amino acid sequence

Bovine S-antigen was further purified by HPLC (22), giving one band on SDS-PAGE. This was subjected to amino acid sequence determination (Applied Biosystems) and the following 19 amino acid sequence was obtained:
Asn-Lys-Pro-Ala-Pro-Asn-His-Val-Ile-Phe-Lys-Lys-Ile-Ser-Arg-Asp-Lys-Ser-Val.

The recovery of amino acid residues during sequence analysis was approximately 20%. The recovered amino acid residues in each sequencing step gave a single major peak, which established the purity of the S-antigen preparation. To further establish that this sequence is from S-antigen, 17-mer polypeptides (from $^{1}_{Asn}$ to $^{17}_{Lys}$) were prepared by chemical synthesis. The 17-mer polypeptide was linked to KLH and injected into Lewis rats to raise specific anti-17-mer antibody. The antibody cross-reacted with S-antigen denatured with SDS or urea but not with the native protein. Immunoblot analysis with this antibody gave results similar to those obtained above with authentic anti-S-antigen.

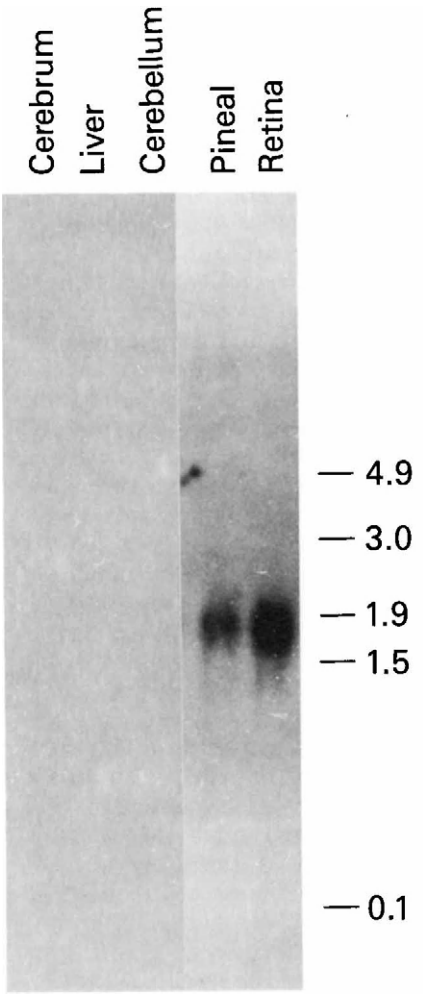

Fig. 4

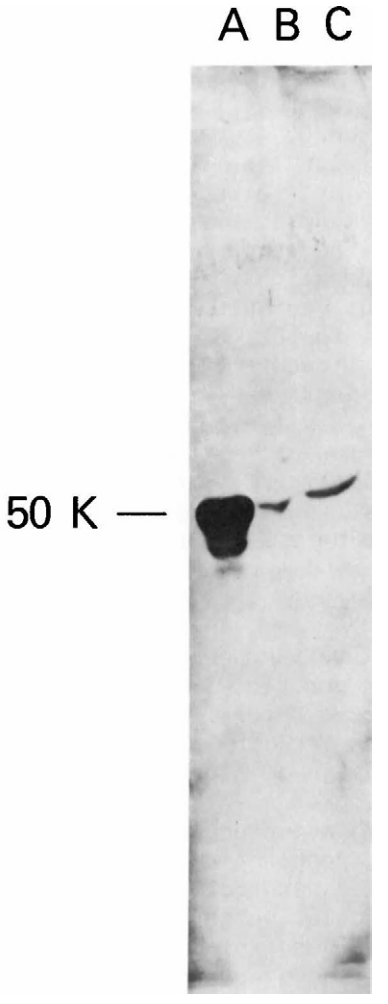

Fig. 5

Fig. 4. Northern blot analysis of S-antigen messenger RNAs. Twenty micrograms of total RNA from bovine retina, pineal gland, cortex, cerebellum and liver were subjected to electrophoresis in a 0.8% agarose denaturation gel, transferred to nitrocellulose filter, and hybridized to isotope labelled BSC 500 cDNA probe (10 million cpm). The RNA markers were 28S (4.9 kilobases Kb), 18S (1.9 Kb), 23S (3.0 Kb), 16S (1.5 Kb) and 4S (0.1 Kb).

Fig. 5. Immunoblot of a 10% SDS-PAGE of proteins from bovine retina and bovine pineal gland. The samples (approximately 100 µg of protein) were electrophoresed. A: purified S-antigen (1 µg); B: the retina sample; and C: the pineal. The 50 kilodaltons size was determined by using standard markers.

Discussion

The cDNAs isolated from two retinal libraries are S-antigen by the following criteria: a) expressed products of the cDNAs have been identified using pure S-antigen polyclonal antibody and two monoclonal antibodies; b) the cDNA hydridized only with mRNA from retina and pineal gland; c) amino acid sequence deduced from cDNA (BSC 900 and BSC 500) sequence matches that of authentic S-antigen (in preparation). The largest cDNA clones we have isolated are about one-half of the full size of S-antigen cDNA and could code for 2/3 of the full size S-antigen protein; however, at least two immunogenic epitopes are present. Fusion polypeptides (with β-galactosidase) encoded by the S-antigen cDNA smaller than 300 nucleotides were recognized by one of the monoclonal (G5) antibodies for S-antigen. In contrast, the polypeptide encoded by the cDNA BSC 500, 420 nucleotides long, was recognized by both monoclonal antibodies. Two out of nine cDNA clones smaller than 300 nucleotides (BSC 140, BSC 250) were also sequenced showing that the two clones overlapped the larger cDNAs (BSC 500 and BSC 900, see Fig. 1).

We were able to determine a partial amino acid sequence from purified S-antigen despite the fact that it has a blocked N-terminus (31). The rather low recovery of amino acids from the sequencing would be consistent with a specific cleavage of a part of the sample which would expose a new unblocked amino terminal residue (Asn). Such a cleavage could occur during the purification process, however, SDS electrophoretic analysis indicated by silver staining a single band following HPLC (22). This band had a calculated molecular weight of 50 kilodaltons and comigrated with the major band which was present in the preparation of S-antigen purified by established methods. It is unlikely that the sequence obtained is the result of a contaminating polypeptide. The same sequence has been obtained independently by another laboratory (private communication, Dr. Donoso) as part of a cyanogen bromide peptide from S-antigen purified by different methodology. Furthermore, the anti-17-mer antiserum clearly reacts with denatured S-antigen. Since this amino acid sequence is not present in the C-terminal 237 residues of S-antigen (in preparation), it must be near the amino terminal portion of the molecule. It is interesting and perhaps physiologically significant that S-antigen may have a small amino terminal peptide which is susceptible to cleavage. The anti-17-mer antibody did not bind to native S-antigen, but it did react with the denatured

protein. This result also suggests that the 17 mer sequence in native S-antigen is not accessible to antibody.

The mRNA in retina and in pineal are the same size and hybridize strongly to retinal S-antigen cDNA. S-antigen proteins in pineal and retina are also similar in size. This is consistent with the apparent evolutionary relationship between retina and pineal. The cDNAs can be used as probes in a number of studies to detect S-antigen mRNA in cells by in situ hybridization. The S-antigen cDNA probe can also be used to isolate the full size cDNA and gene, which would allow more on detailed studies of the structure and expression of this protein. Our cDNAs will also allow further investigation of experimental uveitus and the physiological role that S-antigen plays in retinal visual transduction and pineal neuroendocrine modulation.

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S-ANTIGEN IMMUNOCYTOCHEMISTRY

Horst-W. Korf, Andreas Oksche

Department of Anatomy and Cytobiology,
Justus Liebig University of Giessen,
Giessen, Federal Republic of Germany

Peter Ekström, Theo van Veen

Department of Zoology, University of
Lund, Lund, Sweden

J. Samuel Zigler, Igal Gery, Paul Stein

National Eye Institute, National Institutes
of Health, Bethesda, Maryland, U.S.A

David. C. Klein

Section on Neuroendocrinology, National
Institute of Child Health and Human Development,
National Institutes of Health, Bethesda, Maryland

I. INTRODUCTION

The retina and the pineal complex of vertebrates share several common characteristics: 1) Both organs have developed as diencephalic evaginations (see von Frisch, 1911). 2) In poikilothermic vertebrates the pineal complex is endowed with sensory cells which display outer and inner segments and ultrastructurally resemble retinal photoreceptors (Eakin and Westfall, 1959; Oksche and von Harnack, 1963; Oksche, 1971; Collin, 1971). 3) Like retinal rods pineal photoreceptors

contain, at least in their majority, immunoreactive opsin (Vigh and Vigh-Teichmann, 1981; Vigh- Teichmann et al., 1982, 1983).

According to structural and ultrastructural criteria these similarities between the retina and the pineal organ are less conspicuous in mammals. Mammalian pinealocytes lack inner and outer segments typical of retinal and pineal photoreceptors. Therefore, in the literature, they have been regarded as entirely secretory (neuroendocrine) elements. However, the immunocytochemical demonstration of retinal S-antigen in the retina (Kalsow and Wacker, 1973) and in the pineal organ of the guinea pig (Kalsow and Wacker, 1977) indicates that mammalian pinealocytes have retained certain features of photoreceptor cells. Furthermore, immunization with retinal S-antigen induces not only "experimental autoimmune uveoretinitis" (Wacker and Lipton, 1968; Wacker and Kalsow, 1973; Wacker, 1981; Faure, 1980; Gery et al., 1985), but also inflammatory reactions in the pineal organ (Kalsow and Wacker, 1978; Gery et al., 1985).

These results must be viewed in context with the concept that, during evolution, pineal photoreceptors of poikilothermic vertebrates gradually changed into modified pineal photoreceptor cells of sauropsids and secretory pinealocytes of mammals (Oksche, 1965, 1971; Collin, 1971, Collin and Oksche, 1981).

S-antigen immunocytochemistry appears to be a valuable tool (i) to extend this concept to the molecular level and (ii) to establish similarities between the retina and the pineal organ on a representative comparative basis.

II. MATERIALS AND METHODS

A. Animals

1. Pisces, Teleostei
   Phoxinus phoxinus, Salmo gairdneri
2. Amphibia, Anura
   Rana temporaria, Rana esculenta, Bufo bufo
3. Reptilia, Lacertilia
   Lacerta sicula, Tupinambis nigropunctatus
4. Aves
   Passer domesticus, Anas platyrhynchos, Coturnix coturnix

5. Mammalia
   Didelphis virginiana, Erinaceus europaeus, Phodopus dsungorus, Mesocricetus auratus, Rattus norvegicus, Meriones unguiculatus, Oryctolagus cuniculus, Felis catus, Perodicticus potto, Lagothrix lagothrix, Aotes trivirgatus, Cebus, Macaca mulatta, Pongo pygmaeus, Homo sapiens

B. Tissue preparation

The animals were sacrificed either by perfusion of a fixative or by decapitation with subsequent immersion fixation of the dissected retinae and brains including the pineal organ. Human pineals were obtained from autopsies (the age of the subjects investigated varied from 2 days post partum to 89 years). Human retinae were obtained after surgical removal of one eye from 5 patients suffering from intraocular melanomas (2 patients) or perforating traumatization of the eye bulb (courtesy of Prof. W. Jacobi, Dept. of Ophthalmology, Giessen). Biopsy material of pineal tumors was provided by Prof. H.-W. Pia, Dept. of Neurosurgery, and Prof. W. Schachenmayr, Dept. of Neuropathology, Giessen. The following fixatives were applied: 1) SUSA fixative according to Heidenhain, 2) Bouin's fluid, 3) Carnoy's fluid, 4) 4% buffered paraformaldehyde, 5) 10 % formalin, 6) 2.5% buffered glutaraldehyde. Paraffin sections (5 to 7 µm thick) or frozen sections (25 µm thick) of the tissues were prepared for immunocytochemical analysis.

C. Immunocytochemical procedures

Immunoreactive S-antigen was detected by use of (i) a polyclonal and (ii) a monoclonal antibody against highly purified bovine S-antigen. The polyclonal antibody was raised in rabbits (for details, see Korf et al., 1985a). The monoclonal antibody was obtained by immunizing BALB/c mice with highly purified bovine retinal S-antigen and fusion of spleen cells with NS 1 myeloma cell lines. Binding of the antibodies was visualized by means of the peroxidase-antiperoxidase method of Sternberger (1979). Depending on the species and the fixation procedures, the dilution of the primary antibody varied from 1:400 to 1:4000. Immunocytochemical controls were performed by (i) replacing the polyclonal antibody with normal non-immune rabbit serum and the monoclonal antibody by ascites fluid of non-immunized mice, and (ii) incubating the sections with the diluted antibodies to which 100 nMol of purified bovine S-antigen (Zigler et al., 1984) was added.

III. RESULTS

All fixatives tested, with the exception of glutaraldehyde, were suitable for detection of S-antigen-immunoreactive material. Positive immunoreaction was observed in paraffin and frozen sections. However, the dilution of the antibody could be considerably increased (up to 1:4000) when frozen sections were used. On the other hand, the preservation of the structures was best in paraffin sections.

Generally, the polyclonal antiserum and the monoclonal antibody revealed similar results; however, the monoclonal antibody did not show immunoreactive S-antigen in the pineal organ of the opossum, Didelphis virginiana, and in retinae and pineal organs of all the primates examined, including man. Preabsorption of the primary antibodies with the purified antigen prevented the immunoreaction, thus indicating the specificity of the reaction.

Immunoreactive S-antigen exclusively occurred in the retina and the pineal organ. In contrast, other parts of the brain were immunonegative.

In the retina of the majority of species investigated the immunoreaction was restricted to the photoreceptor layer; other cells and layers were immunonegative (Fig. 1-4). However, in some species (e.g., the European minnow and the rat) positive immunoreaction was also found in individual cells scattered in the inner nuclear layer; these cells were very distinctly marked with the use of the monoclonal antibody. Particularly in primates some photoreceptor cells remained unlabeled (Fig. 3).

Immunoreactive S-antigen may occur in all portions of the retinal photoreceptor cells. Strong S-antigen immunoreaction was seen in the outer and inner segments. With interspecific variation immunoreactive S-antigen could also be visualized in the perikarya and the basal pedicles of these cells (Fig. 1, 3). In the human retina, S-antigen immunoreaction was primarily localized to outer segments of retinal photoreceptors (Fig. 4).

S-antigen-immunoreactive material was observed in the pineal organs of all species investigated with the exception of Passer domesticus, Aotes trivirgatus and Cebus.

In fishes and amphibians immunolabeled cells exhibited the characteristic features of true pineal photoreceptor cells. Immunoreactive outer and inner segments of these cells often protruded into the pineal lumen or the third ventricle (Figs.

5, 6). The intensity of the immunoreaction varied among individual cells; in the Carnoy-fixed material the immunoreaction was very strong in the perinuclear and supranuclear region of pineal photoreceptor cells (Fig. 6).

In the lacertilian and avian pineal organs immunoreactive cells resembled the "modified pineal photoreceptors (Fig. 7; for definition, see Collin and Oksche, 1981). Again, strongly immunoreactive cells were distinguished from cells displaying distinctly weaker immunoreaction or immunonegative elements. Occasionally, immunolabeled basal processes were traced toward the basal lamina enclosing the pineal follicles (Fig. 7).

In mammals the intensity of the immunoreaction and the number of immunoreactive cells differs considerably among the species. According to their size and shape, the immunoreactive cells in the mammalian pineal organ correspond to pinealocytes. In the opossum, very few immunoreactive pinealocytes were intermingled with immunonegative cells (Fig. 8). Several of the stained pinealocytes exhibited labeled processes directed toward the pineal lumen of the pineal recess of the third ventricle where they terminated with a bulbous swelling (Fig. 8).

The vast majority of pinealocytes was strongly immunoreactive in the cat (Fig. 9) and the rodents studied. In the latter, immunoreactive pinealocytes extended in rostral direction and were scattered in the medial habenular complex. S-antigen immunoreactive processes originating from pinealocytes in the deep portion of the pineal organ penetrated deeply into the medial habenular complex, the region of the posterior and the pretectal area. These processes were very conspicuous in the Djungarian hamster.

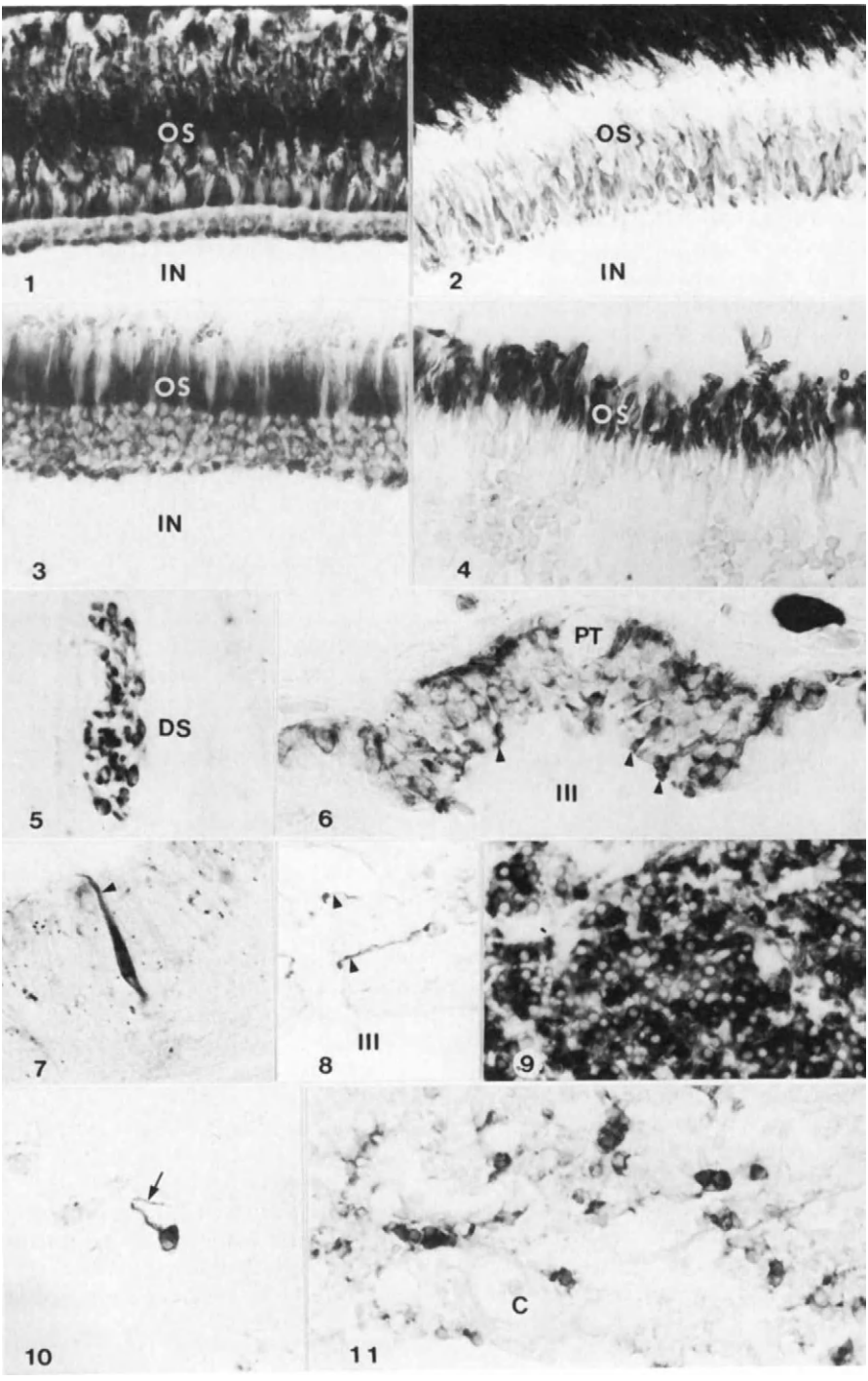
No immunoreactive cells were found in the pineal organs of Aotes trivirgatus and Cebus; other primates including man exhibited only very few labeled pinealocytes between non-immunoreactive elements (Fig. 10). Occasionally, processes of these cells were stained by the immunoreaction.

S-antigen immunocytochemistry was also applied to certain tumors of man (1 retinoblastoma, 1 pineocytoma, 1 pineoblastoma, four embryonic tumors of the pineal region). Immunoreactive neoplastic cells were present in the retinoblastoma and the pineocytoma (Fig. 11); the other tumors were completely devoid of immunoreaction.

IV. DISCUSSION

By means of immunocytochemistry, S-antigen has been demonstrated in a representative number of vertebrate species, including man. Positive immunoreactions were exclusively observed in the retina and the pineal organ. In contrast, other parts of the brain were immunonegative. Immunoreactive S-antigen was located in retinal photoreceptors and the different structural variants of the pinealocytes, i.e., pineal photoreceptors of poikilothermic vertebrates, modified pineal photoreceptors of sauropsids and pinealocytes of mammals (see also Kalsow and Wacker, 1973, 1977; Faure et al, 1984; Mirshahi et al., 1984; Korf et al., 1985a) These results are of significance for several reasons: 1) They indicate similarities between retinal photoreceptors and pinealocytes in all classes of vertebrates. 2) They support the concept that the different structural variants of pinealocytes belong to a common cell line of the "receptor type" (Collin, 1971; Oksche, 1971; Collin and Oksche, 1981). The selective expression of S-antigen in retinal photoreceptors and pinealocytes speaks in favor of the hypothesis that these cells are closely related in genetical terms and have developed from a common neuronal precursor probably located in the anlage of the diencephalon (see von Frisch, 1911).

Figs. 1-11. S-antigen immunocytochemistry. **1.** Phoxinus phoxinus, retina, frozen section; fixative: 4% paraformaldehyde (PF). **2.** Salmo gairdneri, retina, paraffin section; fixative: SUSA (S). **3.** Aotes trivirgatus, retina, paraffin section, fixative: Bouin's fluid (B). **4.** Human retina, paraffin section, fixative: 10% formalin (F). **5.** Phoxinus phoxinus, pineal organ, paraffin section, fixative: S. **6.** Rana esculenta, pineal organ, paraffin section, fixative: Carnoy's fluid (C). **7.** Tupinambis nigropunctatus, pineal organ, paraffin section, fixative: B. **8.** Didelphis virginiana, pineal organ, paraffin section, fixative: B. **9.** Felis catus, pineal organ, paraffin section, fixative: B. **10.** Macaca mulatta, pineal organ, paraffin section, fixative: B. **11.** Pineocytoma of a 49-year-old patient, paraffin section, fixative: F. OS outer segments, IN inner nuclear layer, DS dorsal sac, PT pineal tract, III third ventricle, C capillary, arrowheads pinealocyte processes directed toward the pineal lumen or third ventricle, arrow basal process of pinealocyte. **Figs. 1-4, 6, 9, 11** x280; **Figs. 5, 8** x180; **Figs. 7, 10** x320.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Immunoreactive S-antigen may occur in all portions of retinal photoreceptors and pinealocytes including their basal processes. As these processes are devoid of granular endoplasmic reticulum we assume that the S-antigen, like other proteins, is transported to the basal processes via axoplasmic flow.

The polyclonal antibody used in the present study allowed immunocytochemical demonstration of retinal S-antigen in all species investigated, including man. This suggests that several antigenetic determinants of the S-antigen molecule are well preserved during phylogeny and widely distributed in vertebrates. On the other hand, the opossum and all primates investigated including man apparently lack epitopes recognized by the monoclonal antibody. Our observations conform to findings of Faure et al. (1984) and Mirshahi et al. (1985) showing that the S-antigen is composed of species-specific and non-species-specific epitopes.Some of the non-species-specific epitopes were found even in photoreceptors of certain invertebrates (Mirshahi et al., 1985).

These findings indicate (i) the stability of the S-antigen molecule during phylogeny and (ii) its specific relation to photoreceptor cells and photoreceptive mechanisms. The precise functional role of the S-antigen has been a matter of dispute. Recent studies with the retina have provided evidence that the S-antigen is identical to the "48 K protein" (Pfister et al., 1984; Buzdygon et al., 1985). This protein responds to light signals by binding to rod outer segment membranes (Kühn, 1978, 1984); it mediates rhodopsin-catalyzed ATP-binding and quenching of cyclic GMP-PDE activation (Buzdygon et al., 1985).

The demonstration of a photoreceptor-specific protein in all types of pinealocytes is closely related to the functional role of these cells. It is well established that pineal photoreceptors of poikilotherms are capable of perceiving light stimuli and transmitting them to secondary intrapineal neurons by means of a synaptic mechanism (Dodt, 1973; see also Meissl, this volume). Modified pineal photoreceptors of sauropsids do not establish conspicuous synaptic contacts with intrapineal neurons; however, as shown by biochemical in-vitro experiments these cells have retained direct sensitivity to light and are capable of transformation of light stimuli into a neuroendocrine response (Deguchi, 1981). Thus, the modified pinealocytes may be classified as photoneuroendocrine cells (Oksche, 1971, 1983; see also Oksche and Hartwig, 1979) that respond to light stimuli by means of a neuroendocrine reaction at the cellular level. Mammalian pinealocytes have been considered as entirely secretory elements that have lost their

photosensitivity. However, the immunocytochemical demonstration of the S-antigen in pinealocytes of numerous mammals may cast new light on the functional significance of this cell type.

Considering the the phylogenetic stability of the S-antigen and its specific relation to photoreceptor mechanisms one may conclude that this protein serves similar functions in pinealocytes. This might indicate that mammalian pinealocytes still possess characteristics of photoreceptor cells as is also suggested by the observations that (i) the pineal organ of the rat contains high amounts of rhodopsin kinase (Somers and Klein, 1985) and (ii) mammalian pinealocytes may also display other photoreceptor-specific markers such as immunoreactive Interphotoreceptor Binding Protein (IRBP) (Chader and Wiggert, this volume) and immunoreactive opsin (Korf et al., 1985b).

Two additional results obtained with S-antigen immunocytochemistry may provide further insight into the function ofmammalian pinealocytes. 1) In the atypical pineal organ of the opossum a number of immunoreactive pinealocytes displayed processes directed into the third ventricle or into the pineal recess. Such an intimate relationship between immunoreactive pinealocytes and the cerebrospinal fluid (CSF) is also present in the deep portion of the pineal organ of the hamster and the Mongolian gerbil (see also Korf et al., 1985a). When protruding into the pineal recess or the third ventricle these cells closely resemble S-antigen-immunoreactive pineal photoreceptors of poikilothermic vertebrates (compare Fig. 6 and 8) or, in more general terms, CSF-contacting neurons, which are regarded as receptor elements (Vigh-Teichmann and Vigh, 1983). This structural pattern may reflect a receptive capacity of certain pinealocytes (see also Quay, 1984). 2) It was very obvious in the Djungarian hamster that S-antigen-immunoreactive pinealocytes give rise to immunoreactive processes of beaded appearance, which leave the deep pineal organ and penetrate deeply into the medial habenular nucleus, the region of the posterior commissure and the pretectal area. This suggests that the mammalian pineal organ may not act exclusively via neuroendocrine mechanisms (i.e., by the release of melatonin), but also by means of direct projections into the brain. Also this finding suggests that mammalian pinealocytes have not completely lost neuronal features characteristic of retinal and pineal photoreceptor cells.

Interestingly, the number of S-antigen-immunoreactive pinealocytes varied among different species as did the intensity of the immunoreaction among individual pinealocytes. In rodents and in the cat most of the pinealocytes contained immunoreactive S-antigen; in primates and man, however, only a very limited number of pinealocytes displayed a positive immunoreaction. In man, proteolytic processes might account for a postmortal loss of the immunoreactivity. This possibility can be excluded for the non-human primates, which were fixed by perfusion. In further studies it must be established whether (i) these differences in immunostaining reflect different functional stages of a single cell type or (ii) they depend on the existence of two or more types of pinealocytes. In several mammalian species intensely and weakly stained pinealocytes are arranged in a peculiar topographical pattern. Similar patterns were also observed when the serotonin content of individual pinealocytes was investigated by use of the histofluorescence technique (cf. Vollrath, 1981). These findings may speak in favor of the existence of two or more types of pinealocytes.

Like in the pineal organ, intensely stained elements could be distinguished from immunonegative cells in the retina. Based on investigations of retinae of Tamias striatus and the rhesus monkey, Broekhuyse and Winkens (1985) suggested that immunoreactive S-antigen is restricted to retinal rods; however, in other vertebrate species immunoreactive S-antigen was found in retinal rods and cones (Faure et al., 1984; Mirshahi et al., 1984). Systematic immunocytochemical studies at the ultrastructural level are required to elucidate these differences in immunostaining among individual photoreceptor cells. These investigations may also help to clarify whether the S-antigen-immunoreactive elements scattered in the inner nuclear layer of the retina in some species represent displaced photoreceptors or Landolt's clubs.

Finally, the relevance of S-antigen immunocytochemistry for neuropathological investiagtions should be discussed. The finding that immunoreactive S-antigen is a selective marker of retinal photoreceptors and pinealocytes suggests that S-antigen immunocytochemistry may become a valuable tool in pathohistological investigations of brain tumors of man that are related to derivatives of a photoreceptor-producing primordium (i.e., the retina and the pineal organ).

Immunoreactive S-antigen occurs in certain neoplastic cells of retinoblastomas in the G0/G1 phase of the cell cycle (Donoso et al., 1985). We were able to confirm these results in our laboratory. Furthermore, we investigated several tumors of the pineal region (1 pineocytoma, 1 pineoblastoma, 1

germinoma II-III, 2 teratomas, 1 embryonic tumor). S-antigen-immunoreactive cells were exclusively found in the pineocytoma (Korf et al., 1985c). These preliminary results indicate that S-antigen immunocytochemistry may supplement conventional neuropathological investigations and, thus, help to characterize tumors in the pineal region of man more precisely.

Recently we have also started to analyze medulloblastomas, which occasionally resemble retinoblastomas in their histological features. Three tumors out of nine investigated to date contained S-antigen-immunoreactive cells. These results are of considerable importance for the classification of medulloblastomas; they may indicate that at least certain types of these tumors might have some kind of relationship to the photoreceptor-producing primordium of the brain. Studies are in progress to prove the validity of this hypothesis.

V. SUMMARY

The immunocytochemical demonstration of S-antigen reveals that this protein is exclusively located in retinal photoreceptors and pinealocytes of different vertebrates. This suggests that both cell types have developed from a common diencephalic precursor. The results support the concept that mammalian pinealocytes are derivatives of pineal photoreceptor cells of poikilothermic vertebrates. Furthermore, they indicate that mammalian pinealocytes still bear characteristics of photoreceptor cells. S-antigen immunocytochemistry may become a valuable tool in the neuropathological diagnosis of brain tumors of man related to the retina or the pineal organ (retinoblastomas, pineocytomas, medulloblastomas).

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Effects of Continuous Light Exposure on Pinealitis Induced by Retinal Soluble Antigen

Manabu Mochizuki
Toichiro Kuwabara
Igal Gery

Laboratories of Vision Research and Ophthalmic Pathology, National Eye Institute, NIH, Bethesda, MD, USA

I. INTRODUCTION

The pineal gland of lower vertebrates has anatomic features, as well as functional capacities, similar to the retina (1,2). Although the pineal gland in the mammals has become a secretory organ, its unique relationship to the retina is retained partially (3,4). Kalsow and Wacker have demonstrated that the pineal gland in guinea pigs contains the retinal specific soluble antigen (S-antigen) (5), which is an uveitopathogenic autoantigen isolated from the photoreceptor cell layer of the retina (6). Furthermore, it has been demonstrated that guinea pigs (7) and rats (8) immunized with S-antigen develop inflammatory changes in both the eye and the pineal gland. The present study was aimed at examining the inflammation in the eye and pineal gland following exposure to strong continuous light. This treatment has been shown to cause severe degenerative changes in the retina, particularly in the photoreceptor cell layer (9,10).

II. MATERIALS AND METHODS

Animals: Rats of the inbred Lewis strain were purchased from M.A. Bioproducts (Walkersville, MD). Male rats between 12 and 16 weeks of age were used.

Immunization: S-antigen was prepared using frozen bovine retinas according to the method of Dorey et al (11). The antigen was emulsified (1:1) in complete Freund's adjuvant (Difco, Detroit, MI), containing Mycobacterium tuberculosis H 37 Ra at a concentration of 2.0 mg/ml. A total of 0.1 ml/rat, containing 30 μg of S-antigen, was injected into one footpad. An additional bacterial adjuvant, Bordetella pertussis, 10^{10} bacteria per rat, was injected intravenously along with the aforementioned immunization.

Treatment of rats with continuous light exposure: Rats were kept individually in a 40 cm diameter round cage surrounded by ring-shaped fluorescent lamps. The intensity of the light was 400 footcandles at the center of the cage.

Experimental schedule: Rats were treated with continuous light exposure for 10 days and rested for 10 days under normal light condition, 12 hours light and 12 hours dark. Then, the animals were immunized with S-antigen. Both eyes of some rats were removed before the initiation of the continuous light treatment, to study the influence of light signal through the eye on pinealitis induced by S-antigen.

Evaluation of EAU and pinealitis: Clinical signs of EAU were monitored daily, with or without a slit lamp microscope. Rats were killed on day 16 following the immunization with S-antigen. Eyes and pineal glands were examined histologically as described previously (8).

III. RESULTS

Table 1 summarizes the results. First of all, 12 healthy naive rats were histologically examined if they had any pathologic changes in the eye and the pineal gland. No inflammatory changes were found in both the eye and the pineal gland of the naive rats. In contrast to the naive rat eye, rat eyes treated with continuous light exposure lost almost all photoreceptor cells, indicating that the light treatment caused intense degenerative changes in the retina, particularly in the photoreceptor cell layer where S-antigen is located. On the other hand, the pineal glands

of the rats treated with continuous light exposure showed minimal histologic changes, i.e. very mild edematous changes in the parenchyma cells of the gland.

Eight rats were immunized with S-antigen without any light pretreatment and all these rats exhibited severe clinical signs of EAU between 12 and 14 days postimmunization. Histologically, all the rats in this group showed severe inflammatory changes in both the eye and the pineal gland. However, the cells infiltrating in the two organs were different: cells infiltrating in the eye were mainly polymorphonuclear leukocytes (PMNs) whereas those in the pineal gland were mononuclear cells. Thirteen rats were treated with continuous light exposure and immunized with S-antigen. Only 3 animals in this group developed clinical signs of EAU, while histological evidence of ocular inflammation was observed in all animals. However, the intensity of ocular inflammation was much milder than that observed in rats not treated with continuous light exposure before the immunization with S-antigen. In addition, pinealitis was found histologically in only one half of animals in this group and the intensity of inflammation in the pineal gland was much milder than that found in rats not pretreated with the continuous light exposure.

Five rats were enucleated prior to the initiation of the light treatment. All rats in this group developed pinealitis following the immunization with S-antigen and the intensity of the pinealitis was as severe as that found in the control group of rats which were not pretreated with continuous light prior to immunization with S-antigen.

IV. DISCUSSION

Exposure of rats to continuous strong light was found to affect the pattern of inflammation in the eye and pineal gland following immunization with S-antigen. The effect of continuous light on the structure of the retina was investigated in mice (9) and rats (10). Light treatment was found to induce severe degenerative changes in the retina of these animals, particularly in the photoreceptor cell layer. Since S-antigen localizes in this cell layer (6), the treatment with light is assumed to markedly reduce the amount of S-antigen in retinas of exposed animals. It is conceivable, therefore, that the reduction in retinal inflammation in light exposed rats was mainly due to the low levels of available S-antigen in retinas of these animals.

Table 1

Light Treatment	Immunization with S-antigen	Ocular Inflammation		Pinealitis (Severity)
		Clinical	Histological (Severity)	
No	No	0/12	0/12 (0)	0/12 (0)
No	Yes	8/8	8/8 (3.0)	8/8 (2.3 ± 0.3)
Yes	Yes	3/13	13/13 (1.2 ± 0.1)	7/13 (1.1 ± 0.1)
Yes*	Yes	-	-	5/5 (2.6 ± 0.2)

*Both eyes of rats in this group were removed prior to the initiation of the light treatment (see the text).

The data reported here also provide new evidence to support the close relationship between the retina and pineal gland. Earlier studies have shown the localization of the retinal S-antigen in the pineal gland (5) and the involvement of the pineal in the immunopathogenic process provoked by immunization with this antigen (7,8). Slight changes were found in the structure of pineal glands in rats exposed to continuous light, which could be related to the unusual stimulation of this tissue which is involved in secondary photic transduction. More significant, however, was the effect of light exposure on the pineal inflammatory changes following immunization with S-antigen: treated rats showed reduction in both frequency and severity of pineal changes. The mechanism of decrease in pineal involvement in the light-treated rats is not known and could be attributed to at least three hypothetical effects of the exposure to light: (a) immunosuppression, (b) reduction of the available S-antigen in the pineal gland and (c) a partial specific unresponsiveness to S-antigen following its release from the retina.

The notion that the exposure to light is immunosuppressive derives from the well known effects of ultraviolet light on the immune system (e.g., ref 12). In the present study, however, the immunopathogenic response to S-antigen in the pineal gland was not affected by the light exposure, since rats enucleated before exposure developed pinealitis similar to the untreated control animals (Table 1).

The possible decrease in available S-antigen in pineals of light-treated rats is hypothetical and more studies are needed to examine this possibility, by comparing the amounts of S-antigen in treated and control groups of animals.

The third possibility, that the S-antigen released from light damaged retinas induces specific unresponsiveness in treated animals, is supported by experimental data. Animals injected with protein antigens in water solution or in incomplete Freund's adjuvant (IFA) may develop specific unresponsiveness toward these antigens (13). Furthermore, treatment with tissue specific antigens was found capable of rendering animals unresponsive toward immunopathogenic processes which develop when the same antigens are injected in emulsion with bacteria containing CFA. The tissue specific antigens used in these experiments include the encephalitogenic myelin basic protein (14), thyroiditis inducing thyroglobulin (15) as well as the uveitogenic retinal extract (16). Additional studies are under way, aiming at examining the possible suppression of EAU by pretreatment of naive rats with various amounts of S-antigen in water solution.

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Interphotoreceptor Retinoid-Binding Protein: A Link Between Retinal Rod Photoreceptor Cells and Pineal Gland

Gerald J. Chader, Barbara Wiggert, Igal Gery, Ling Lee and T. Michael Redmond

Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, National Institutes of Health, Bethesda, MD, 20892

Toichiro Kuwabara and Merlyn M. Rodrigues

Laboratory of Ocular Pathology, National Eye Institute, National Institutes of Health, Bethesda, MD 20892

I. INTRODUCTION

Photosensitive pigments are widespread throughout the plant and animal kingdoms. Bacteriorhodopsin, for example, has many structural and functional similarities to mammalian rhodopsin (Ovchinnikov, 1982). Even Chlamydomonas, a unicellular alga, uses a type of rhodopsin for phototaxis (Foster et al. 1984). In higher animals, photopigments are thought to be almost exclusively found in specialized photoreceptor cells of the retina. These are rod and cone cells which mainly function in scotopic and photopic vision respectively. Pineal cells are also involved in light responses in many organisms although, in higher vertebrates and mammals, the response is complex and thought to be an

indirect process mediated through the primary ocular photoreceptors. The relationship between the three cell types, i.e. rods, cones and pinealocytes, is yet not well understood, particularly with regard to the possibility that similar photic mechanisms are present in the cells and that these processes could be mediated by similar complements of specialized proteins.

Some years ago, we described a large, soluble 7S protein in the mammalian eye which readily bound 3H-retinol and which was distinct from the 2S cellular retinol-binding protein, CRBP, (Wiggert et al, 1976; Wiggert et al, 1977). Work from several laboratories now indicates that this species is a lipoglycoprotein and is found predominantly in the interphotoreceptor space between the external limiting membrane of the neural retina and the pigment epithelial cell layer (Bunt Milam and Saari, 1983; Adler and Evans, 1983; Pfeffer et al, 1983). The protein is now referred to as the Interphotoreceptor (Interstitial) Retinoid-Binding Protein (IRBP) and has been isolated and characterized from cow (Fong et al., 1984a; Saari et al., 1985; Adler et al., 1985; Redmond et al., 1985) and from the monkey (Redmond et al., 1985).

In the monkey, IRBP is readily extracted from the subretinal space by a gentle cannulation technique (Pfeffer et al., 1983). It comprises up to 70% of the soluble protein and thus appears to be the major glycoprotein of the retinal extracellular matrix. More importantly, it is the only retinoid-binding protein in the subretinal space. IRBP has long been known to bind endogenous retinol (Wiggert et al., 1977). Differential binding in light and dark has been demonstrated with ^{3}H-retinol in frog (Wiggert et al., 1979), rabbit (Lai et al., 1982) and, with endogenous retinoids in the cow (Adler and Martin, 1982; Liou et al., 1982). Thus, the extracellular location of IRBP between the pigment epithelium and neural retina and the unique retinoid-binding characteristics of the protein make it a prime candidate for a retinoid transport vehicle between the two tissues. Of some interest as well is the fact that IRBP carries a high concentration of fatty acid, bound both covalently and non-covalently (Bazan et al, 1985). This could also indicate that IRBP is involved in the movement of lower molecular weight hydrophobic molecules besides retinoids.

It has now been well established that it is the neural retina which synthesizes and secretes IRBP as demonstrated in incubation studies in vitro with retina of the monkey (Wiggert et al, 1984) and human (Fong et al., 1984) and in human retinoblastoma cells (Kyritsis et al., 1985). In a

similar vein, Hollyfield et al.(1985) have excellent indirect evidence that IRBP is synthesized by retinal rod photoreceptor cells. However, the intracellular localization of IRBP has yet to be firmly established as to retinal cell type. Also, the possibility that IRBP could be present in cell types that are related to retinal photoreceptors (e.g. pinealocytes) has not been addressed.

Using immunological and cytochemical techniques, we have recently found that IRBP can be readily detected in monkey rod photoreceptor cells but that little if any is present in other retinal cell types (Rodrigues et al., 1986). Moreover, biochemical, cytochemical and immunological results strongly indicate the presence of IRBP in the pineal gland (Wiggert et al,1986; Rodrigues et al., 1986). In the present paper, we review some of our recent work on IRBP which we feel demonstrates a new and potentially important biochemical link between this organ and the retina.

II. EXPERIMENTAL PROCEDURES

A. Antibody Preparation

At NIH, we have the opportunity of obtaining Rhesus monkey eyes (Macacca mulatta) through the In Vitro Vaccine Testing Program of the FDA. IRBP was purified according to the procedure of Redmond et al. (1985). Primary antiserum to the purified IRBP was raised using standard immunological techniques in rabbit or goat. The antiserum was purified using glutaraldehyde cross-linked IRBP Sepharose immunosorbent as described by Kapoor and Cho-Chung (1983). For specificity experiments, absorption of the antibody was accomplished by addition of an excess of the purified IRBP antigen, incubation at 4°C for 24 hrs and removal of the antibody-antigen complex by centrifugation.

B. Microscopy Techniques

Immunocytochemical staining of the tissues was performed by light microscopy using indirect immunofluorescent staining or immunoperoxidase staining with the avidin-biotin complex, ABC, (Hsu et al., 1981). Affinity-purified antibody

was used at a dilution of 1:100 or 1:200. Fluorescein-labeled goat anti-rabbit IgG was used at a dilution of 1:20 to 1:50.

For immunoelectron microscopy, eyes were injected with 0.5-1.0% phosphate-buffered glutaraldehyde, incubated for 1 hr and frozen prior to sectioning and embedding in Lowicryl. Colloidal gold (15-20 nm) linked to goat anti-rabbit IgG or protein A was then used according to the method of Roth et al. (1981). In all cases, controls were performed using non-immune serum and antibodies absorbed with purified IRBP.

C. Biochemical Techniques

Tissue samples were analyzed for IRBP using an enzyme-linked immunosorbent assay (ELISA) developed in our laboratory that is specific for monkey IRBP (Wiggert et al., 1986). Appropriate controls omitting either the primary or secondary antibodies were included in each assay.

In some cases, tissue samples were subjected to Western blotting (Wiggert et al, 1986). For this purpose, SDS-polyacrylamide gel electrophoresis was first performed. Then, one-half of each gel was fixed and stained and the other half used for electrophoretic transfer to nitrocellulose paper by the method outlined by Towbin et al. (1979). The primary antiserum used was goat anti-monkey IRBP (1:200 dilution); the secondary antibody was horseradish peroxidase conjugated to rabbit anti-goat $F(ab^1)2$ fragment (1:1500 dilution).

D. Immunological Procedures

Lewis rats were used in the immunization studies on the induction of uveoretinitis and pinealitis in rats. In these studies, purified bovine IRBP (Redmond et al, 1985), emulsified in CFA containing 2 mg/ml of M. tuberculosis (H37 RA), was injected into the footpad (16µg/animal). In addition, the rats were injected with B. pertussis (10^{10} organisms/ rat). In the monkey studies, Rhesus (M. mulatta) and Cynomologus (M. fascicularis) animals were used. Bovine IRBP was emulsified in CFA containing 0.5 mg Mycobacteria/ml and injected intra- and subcutaneously in multiple sites at the nape of the neck. A total of 100 µg antigen/kg was injected into each monkey at two intervals, two weeks apart. In addition, the monkeys were injected intravenously with B. pertussis bacteria ($50x10^9$/kg).

III. RESULTS

As demonstrated by Bunt-Milam and Saari (1983) in the cow, the bulk of IRBP is localized extracellularly in the monkey interphotoreceptor space (Fig 1A). Cone inner segments are strikingly non-reactive. Other retinal areas (e.g. Muller cells) and the retinal pigment epithelium also demonstrate little if any reactivity. Figure 1B illustrates the distribution pattern obtained using the immunoperoxidase staining technique. As with immunofluorescence, IRBP is seen to predominate in the interphotoreceptor matrix. The presence of IRBP is delimited to the area of the subretinal space in that a sharp transition zone is observed at the ora serrata (Fig. 1C). Staining is present in the peripheral retinal area but not in the ciliary epithelium. When antibody is used that had been previously absorbed with the IRBP antigen, no staining is found (Fig. 1D).

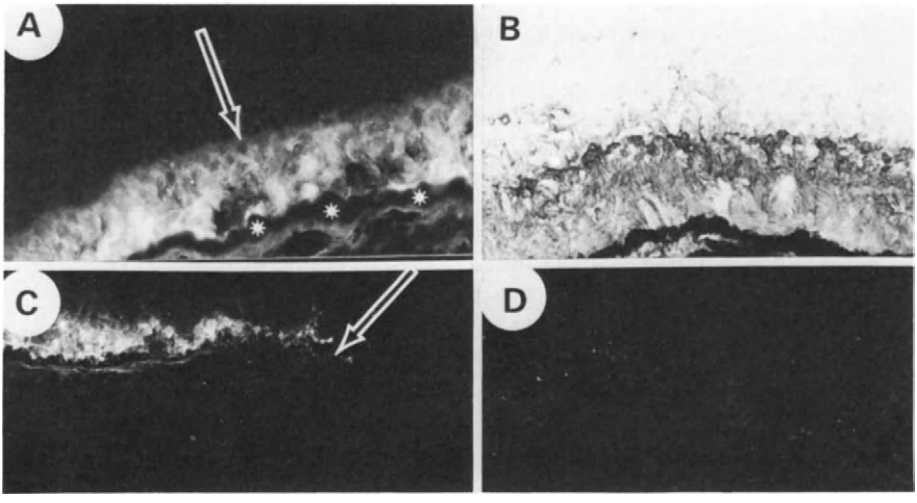

Fig.1. A. Immunofluorescent staining of interphotoreceptor matrix area of monkey retina with anti-IRBP affinity-purified antibody. (arrow): cone inner segments; (*): retinal pigment epithelium (x64). B. Immunoperoxidase staining of interphotoreceptor matrix area of monkey retina (x330). C. IRBP staining in area of ora serrata (arrow) (x40). D. Control section demonstrating lack of staining using antibody previously absorbed with IRBP (x64). From Rodrigues et al. Invest. Ophthalmol. Vis. Sci. (in press).

Figure 2 shows a typical staining pattern observed with immunoelectron microscopy of Lowicryl-embedded peripheral monkey retina reacted with anti-IRBP antibodies linked to colloidal gold-goat anti-rabbit IgG. Rod inner segments display marked reactivity. A higher magnification of a portion of the inner segment of a rod cell demonstrates the same pattern (Fig. 2A insert). Rod outer segments show much less staining, while cone cells show minimal or no reactivity. Muller cells appear to contain no IRBP. The retinal pigment epithelium demonstrates sparse reactivity with staining somewhat localized along the apical processes. No staining is observed in control sections treated with anti-IRBP antibody absorbed with purified IRBP followed by colloidal gold staining (Fig. 2B).

The primate retina contains a specialized, high-resolution area called the macula which primarily contains cone cells rather than rod cells. We felt it of interest to examine this area of the retina for IRBP content since there appeared to be such a substantial rod/cone difference in IRBP concentration in the peripheral retina (Fig. 2). Figure 3 shows immunoelectron microscopy of a Lowicryl-embedded portion of the monkey macula reacted with anti-IRBP antibodies linked to colloidal gold-goat anti-rabbit IgG. An almost complete lack of staining of both cone inner and outer segments is observed. Residual extracellular matrix, however, did show sparse granular staining.

Figure 4A shows the staining pattern of monkey pineal gland with anti-IRBP antibodies using the avidin-biotin-peroxidase method. Substantial staining is observed in what appeared to be both intracellular and extracellular locations. As in the retinal samples depicted in Figs. 1-3, staining is absent with antibody previously absorbed with antigen (Fig. 4B). Figure 5 shows the distribution pattern of IRBP in the monkey pineal observed with immunoelectron microscopy. Reactivity is found within intracytoplasmic vesicles as well as scattered in the extracellular matrix. IRBP has also been found to be present in pineals of species other than the monkey. Human pineal, for example, also exhibits specific IRBP immunochemical staining (Fig. 6A,B). Similarly, rat pineal (Wiggert et al., 1986) exhibits significant staining with IRBP antibody (Fig. 6C) but none with antibody preabsorbed with IRBP antigen (Fig. 6D).

Western blotting is a technique by which antigenic specificity of a protein can be assessed along with information concerning the molecular size of the protein in question. As seen in Figure 7, the soluble fraction of human pineal exhibits substantial cross-reactivity with

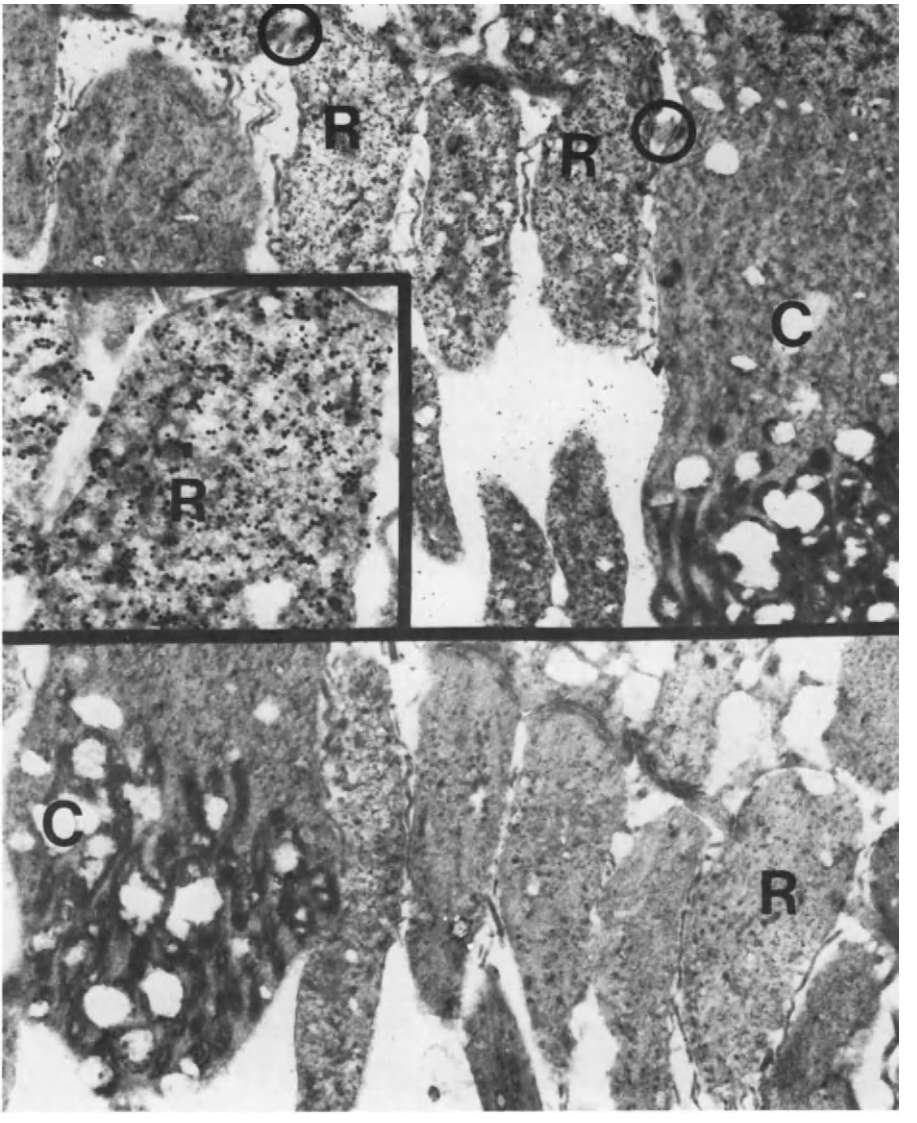

Fig. 2. A. Localization of IRBP in the peripheral monkey retina as assessed by immunoelectron microscopy. (R): rod inner segments; (C): cone cell; (circles): external limiting membrane (x15,840). The insert shows a higher magnification (x45,000) of a portion of an inner segment of a rod cell (R). B. Control section demonstrating lack of staining using antibody previously absorbed with IRBP (x16,500). From Rodrigues et al. Invest. Ophthalmol. Vis. Sci. (in press).

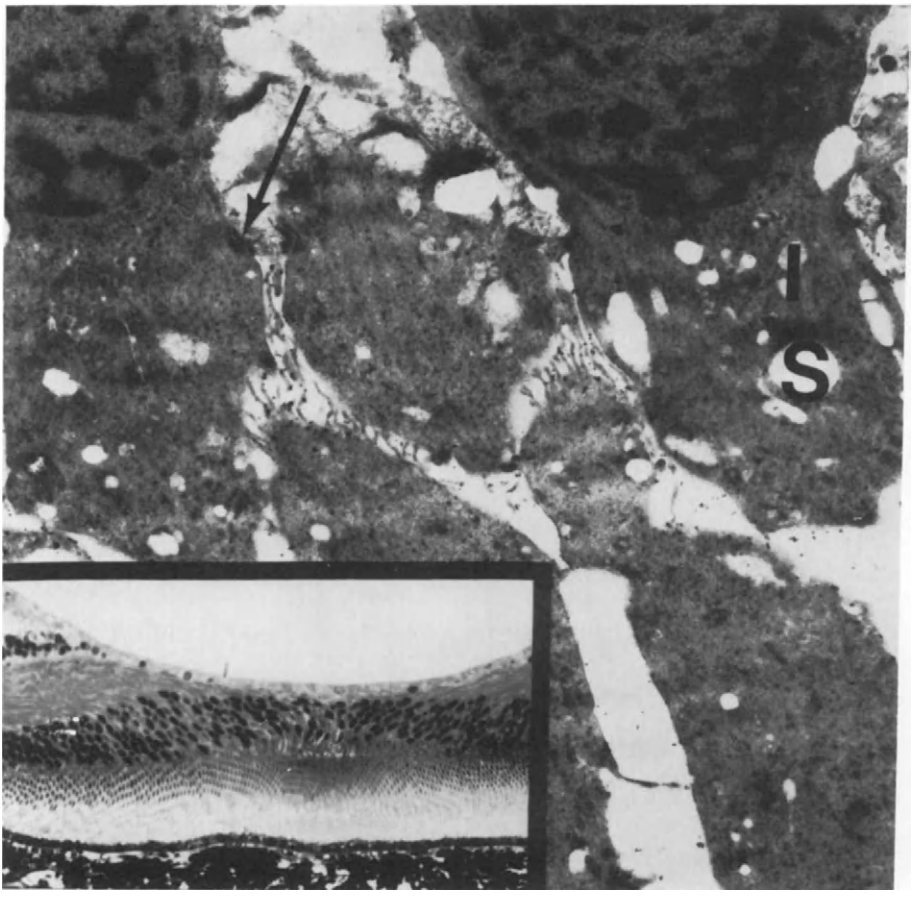

Fig. 3. Distribution of IRBP in the macular region of the monkey retina as assessed by immunoelectron microscopy (IS): foveal cone inner segments in tangential section; (arrow): external limiting membrane (x12,900). The insert shows a light micrograph of the same tissue stained with Toluidine blue (x220). From Rodrigues et al. Invest. Ophthalmol. Vis. Sci. (in press).

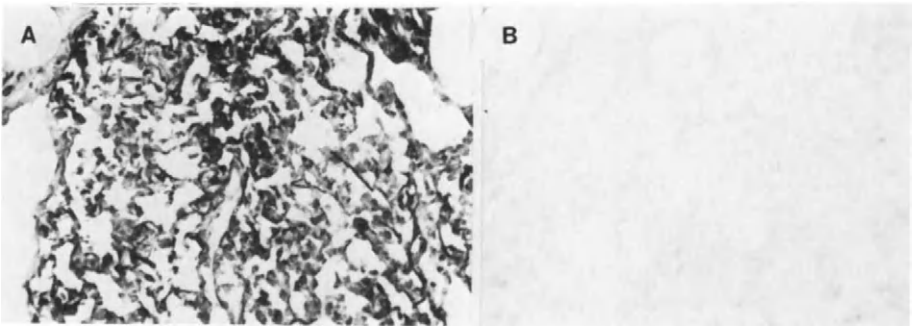

Fig. 4. A. Localization of IRBP in the monkey pineal gland using the ABC staining method (x220). B. Control section demonstrating lack of staining using antibody previously absorbed with IRBP (x220). From Rodrigues et al. Invest. Ophthalmol. Vis. Sci. (in press).

anti-monkey IRBP. It is also apparent that the molecular size of the human IRBP is indistinguishable from that of the purified monkey protein (Wiggert et al, 1986).

Because at least one retinal protein, the S-antigen, has been shown to induce autoimmune pinealitis (Kalsow and Wacker, 1978) as well as uveoretinitis (Wacker et al., 1977), we felt it of interest to determine if immunization of animals with IRBP would result in a parallel syndrome affecting both eye and pineal. Figure 8A shows the normal posterior segment of a control rat 10 days after immunization with bovine serum albumin. In contrast to this normal situation, immunization with IRBP under identical conditions results in marked pathological changes (Fig. 8B). The retina is detached and is markedly edematous. The photoreceptor layer demonstrates marked degeneration. Polymorphonuclear and mononuclear leukocyte infiltration is observed in both retina and subretinal space. The vitreous and subretinal space contain serous exudate. By 21 days after immunization with IRBP, the entire photoreceptor layer has degenerated and is missing; retinal thinning and attachment to the choroid has occurred (Fig. 8C). The vitreous is clear and no inflammatory cells are present in the retina. Figure 9 demonstrates the effects on the pineal gland 10 days after immunization with IRBP. Focal infiltration with mononuclear cells is observed in the peripheral area with an accumulation of inflammatory cells in the stalk region. The effect of IRBP is not unique to the rat;

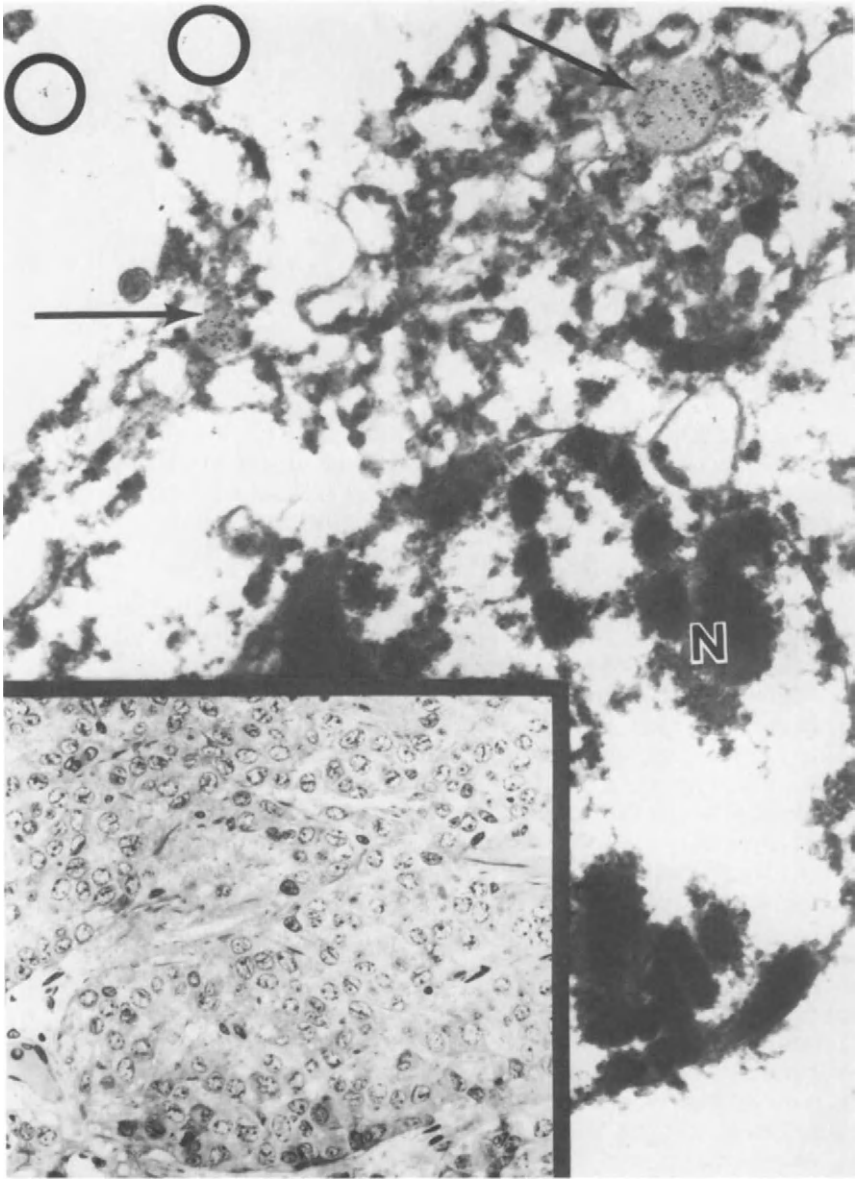

Fig. 5. Distribution of IRBP in the monkey pineal as assessed by immunoelectron miscroscopy. (arrows): intracytoplasmic vesicles; (circles): extracellular material; (N): nucleus (x21,000). The insert shows a light micrograph of typical pineal lobules stained with Toluidine blue (x330).

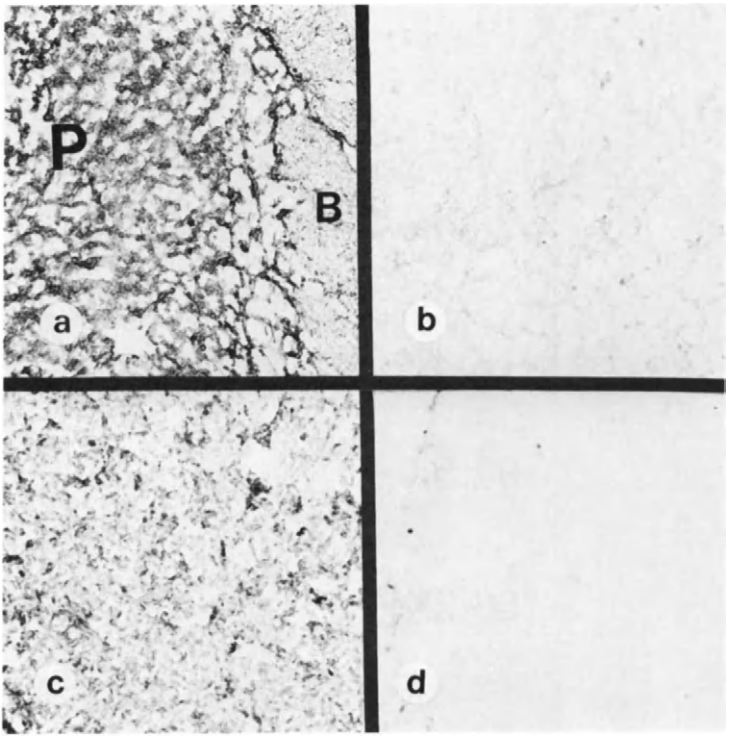

Fig. 6 A. Localization of IRBP in the human pineal gland using the immunoperoxidase (ABC) method (x280). B. Control section demonstrating absence of staining using antigen-absorbed antibody (x220). C. Localization of IRBP in the rat pineal using the ABC method (X130). D. Control section demonstrating absence of staining using antigen-absorbed antibody (130). P - pineal; B - brain

immunization of monkeys with IRBP also results in inflammation and degenerative changes in monkey eye and pineal. Figure 10A shows the normal structure of the retina and choroid in a control monkey eye, while the pathological changes in these tissues following immunization with IRBP are demonstrated in Figure 10B. Characteristic changes include choroidal thickening with massive focal and diffuse infiltration (mainly with mononuclear leukocytes) extending into the retina. Retinas are less affected, with occasional focal infiltration and thinning of photoreceptor outer segments (Fig. 10B). Pathological changes were also found in the

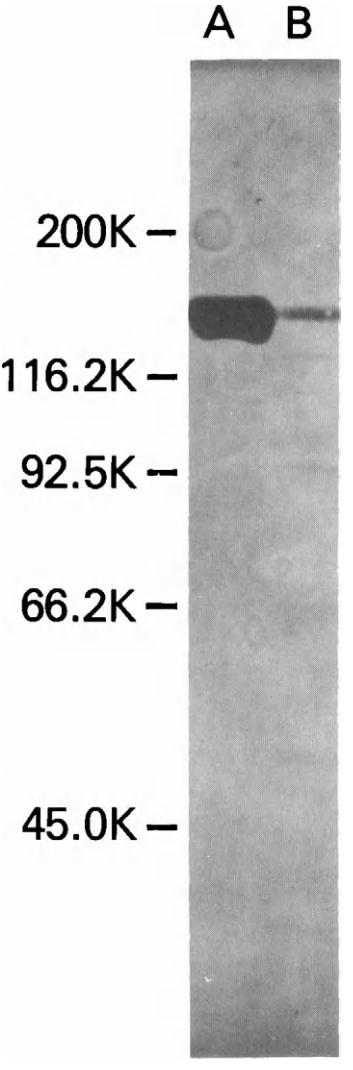

Fig. 7. Western blot analysis of soluble proteins in human pineal cytosol using goat anti-monkey IRBP (1:200 dilution). A. purified monkey IRBP. B. Human pineal cytosol. From Wiggert et al. (submitted for publication).

pineal glands of monkeys immunized with IRBP (Fig. 11). The most prominent change was found to be the infiltration of mononuclear leukocytes, usually surrounding blood vessels.

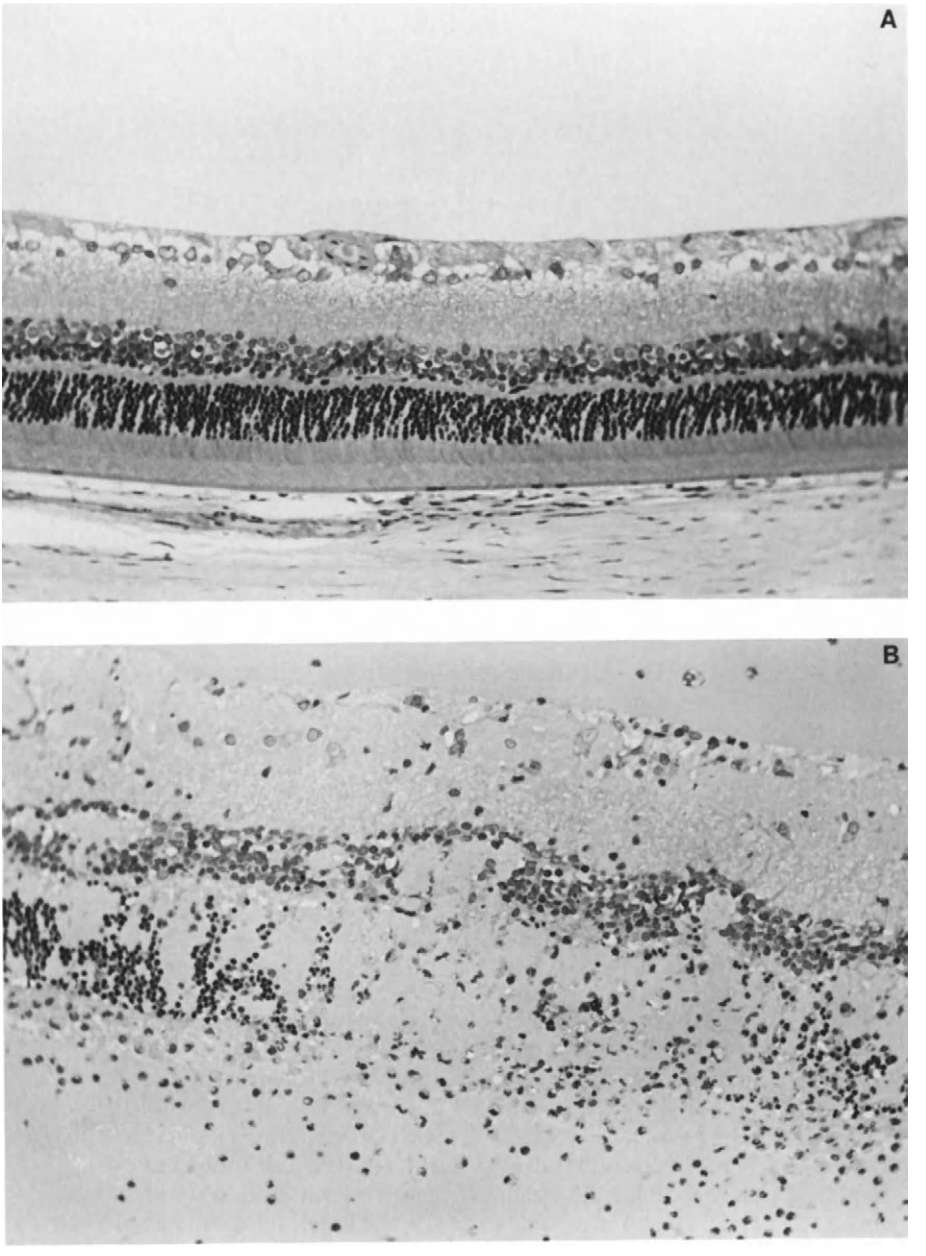

Fig. 8A,B. Histopathological changes in the posterior segment of rats immunized with bovine IRBP. A. The normal structure in a control rat immunized with bovine serum albumin. B. Ten days after immunization with 16 µg IRBP. From Gery et al. (submitted for publication).

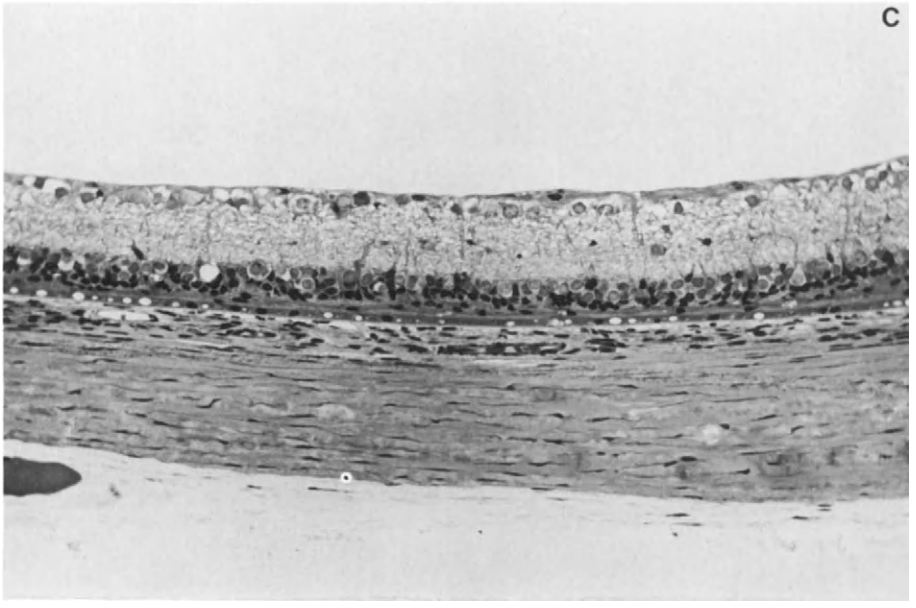

Fig. 8C. Histopathological changes in the posterior segment of rats immunized with bovine IRBP. C. Twenty-one days after immunization with IRBP. (H and E stain, x150). From Gery et al. (submitted for publication).

IV. DISCUSSION

In addition to membrane-bound rhodopsin, four soluble retinoid-binding proteins are now known to be associated with the retina-pigment epithelium complex. Three of these are intracellular proteins: the cellular retinol-binding protein (CRBP), cellular retinoic acid-binding protein (CRABP) and the cellular retinaldehyde-binding protein (CRALBP). Of these, CRBP and CRABP are found in many organs besides the eye while CRALBP seems to be localized in the pigment epithelium and retinal glial (Muller) cells (Bunt-Milam and Saari, 1983). The fourth and perhaps most novel binding protein, IRBP, is localized predominantly in the interphotoreceptor matrix (Bunt-Milam and Saari, 1983) where, biochemically, it appears to be the major soluble protein and the only retinoid-binding protein (Pfeffer et al., 1983). Since this protein could be of importance in

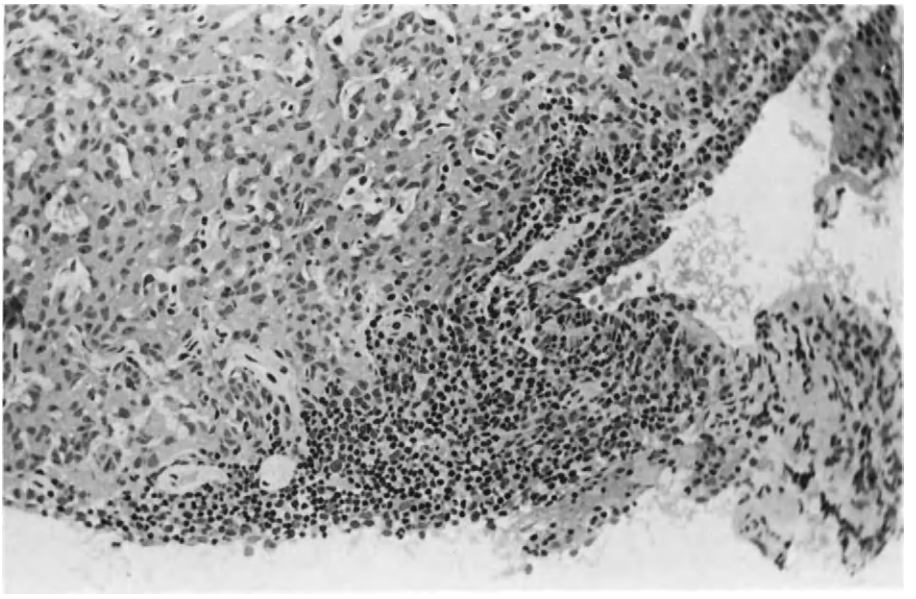

Fig. 9. Histopathological changes in the pineal gland of a rat 10 days after immunization with IRBP. (H and E stain, x150). From Gery et al. (submitted for publication).

intercellular retinoid transport, we felt it of some interest to determine the precise intracellular localization of IRBP and perhaps gain information on its cellular origin.

Four cell types border the interphotoreceptor space: pigment epithelial cells, Muller cells and rod and cone photoreceptor cells. We have previously excluded the pigment epithelium as the site of IRBP synthesis in the monkey retina (Wiggert et al., 1984). The same appears to be true for the human (Fong et al., 1984). These biochemical synthesis studies are in agreement with our present findings since, immunocytochemically, pigment epithelial cells contain only scant amounts of IRBP. This could be due to uptake from the interphotoreceptor space. The retinal site of synthesis has been unclear, however, although Hollyfield and his collaborators (1985) have published compelling albeit indirect evidence that IRBP is synthesized in rod cells of the human retina. In parallel with this, we now directly demonstrate that intracellular IRBP is located mainly in rod photoreceptor cells of the monkey retina. The outer segments of these cells show much less reactivity compared with the inner segments and the cytoplasm. This is reasonable if one assumes that, after synthesis in the inner

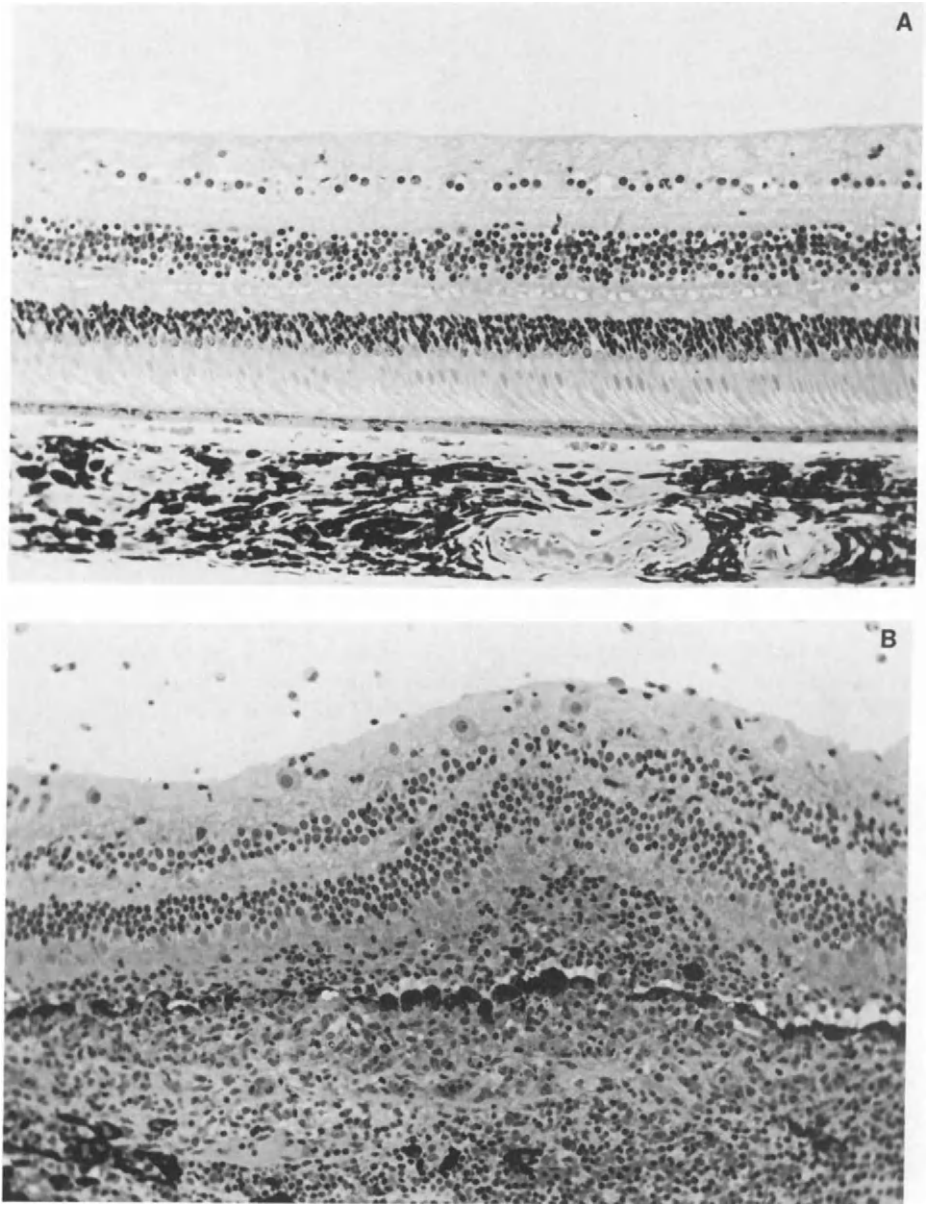

Fig. 10. Histopathological changes in the choroid and retina of a monkey immunized with IRBP. A. The normal structure in a control monkey injected with phosphate-buffered saline in CFA. B. Inflammatory changes in a monkey eye three weeks after the second immunization with IRBP. (H and E stain, x150). From Gery et al. (submitted for publication).

segment, IRBP would be processed and packaged in the Golgi apparatus (with rhodopsin?) and secreted at the cilial junction (perhaps as rhodopsin is incorporated into nascent disc membranes). Minimal reactivity was found in photoreceptor cone cells, either in the inner or outer segments. This is especially true in the macula where we find no staining in the cone cells, which make up the vast bulk of the photoreceptor cells, and diminished extracellular staining between the retina and pigment epithelium. Virtually no IRBP was demonstrable in Muller cells. Thus, intracellularly, IRBP appears to be localized mainly in monkey rod photoreceptor cells with these cells most probably being its site of synthesis. Since we recently have isolated a peptide-matched cDNA clone for IRBP (Barrett et al., 1985), it will soon become possible to use it for in situ hybridization studies which will definitively establish the cellular site(s) of IRBP synthesis.

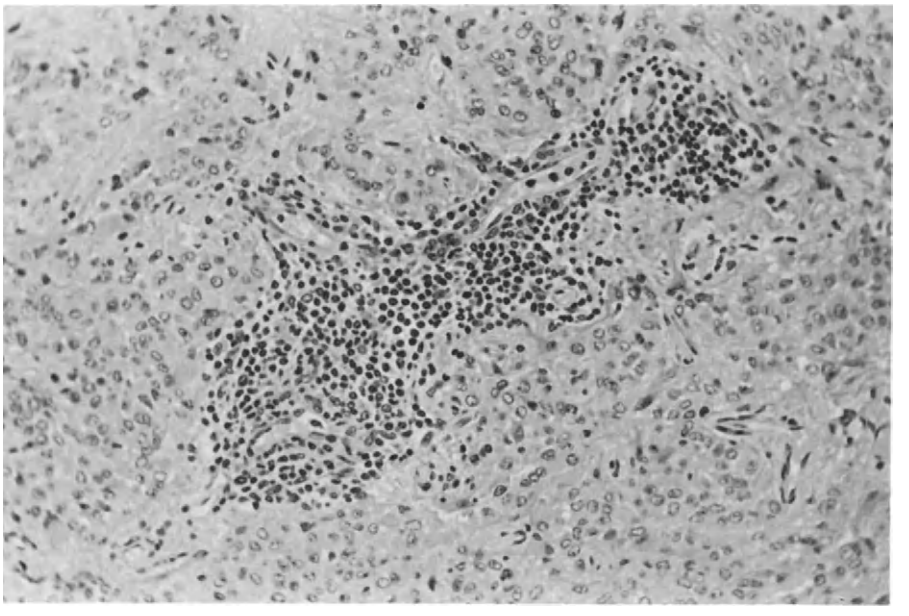

Fig. 11. Histopathological changes in the pineal gland of a monkey four weeks after the second immunization with IRBP. (H and E stain, x150). From Gery et al. (submitted for publication).

Of perhaps equal importance is our finding of significant levels of IRBP in monkey and rat pineal gland. Similarities between retina and pineal have long been known. Zimmerman and Tso (1975) have described a transient photoreceptor-like differentiation of rat pinealocytes peaking in the early postnatal period which mimics retinal photoreceptor differentiation. It is characterized by the appearance of highly elongated, polarized pinealocytes, prominent inner segments, synaptic ribbons, cilia with a 9 + 0 arrangement and vesicular membranes at the cilial tips. Biochemically, evidence is now also becoming available as to this similarity in the adult animal. The mammalian pineal gland has recently been shown to contain high levels of rhodopsin kinase, an enzyme thought to play a role in the visual process (Somers and Klein, 1984). Wiechmann and Bok (1984) have also demonstrated the histochemical localization of the hydroxyindole-O-methyl transferase (HIOMT) enzyme in mammalian photoreceptor cells, indicating that melatonin could be synthesized by retinal photoreceptor cells as it is by cells of the pineal. On the other hand, we have recently stained fresh frozen human, monkey, cow and rat pineal glands with anti-rhodopsin antibody (a kind gift of Dr. P. Hargrave) and found no evidence of reactivity using the immunoperoxidase (ABC) method.

Immunological evidence also links the mammalian eye and the pineal gland in that the immune response towards the intracellular retinal protein "S-antigen" is known to be present in mammalian pineal as well as in the retina (Kalsow and Wacker, 1977). Evidence given here and more thoroughly elsewhere (Gery et al.,1986) indicates that a second retinal antigen, IRBP, is also recognized in both pineal and retina producing inflammation and degenerative changes although in a different manner from those seen with the S-antigen. It is interesting to note that significant IRBP-like immunoreactivity is found both intracellularly and extracellularly in the pineal perhaps reflecting the evolutionary transformation of the pineal from a true photoreceptor to a neurosecretory organ involved in secondary photic signal transduction. In any event, our work significantly extends the evolutionary, developmental and functional analogy between pineal and retinal cells. Since neither retinoids nor their receptors have yet been reported in pineal, our work constitutes the first report of a retinoid-binding protein in this organ. Although the function of IRBP is not fully understood in retina or pineal, its presence in the pineal could indicate a previously unsuspected role for

retinoids in pineal physiology. It will also be of interest to determine if IRBP binds fatty acids and other lipids to the same high degree in the pineal as in the interphoto-receptor matrix.

In summary, IRBP has been found to be specific for rod photoreceptor cells in the retina and could thus perhaps be thought of as a "rod-specific tag" at least in the primate. The similarity of primate rods and pinealocytes and their possible evolutionary divergence from cones may thus be indicated.

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INTERSTITIAL RETINOL-BINDING PROTEIN (IRBP)[1] IN RAT AND BOVINE PINEAL ORGANS: EVOLUTIONARY VESTIGE OR FUNCTIONAL MOLECULE?

C.D.B. Bridges
R.A. Landers
S.-L. Fong
G. I. Liou

Cullen Eye Institute
Department of Ophthalmology
Baylor College of Medicine
Houston, Texas

I. INTRODUCTION

It is generally accepted that a major population of mammalian pinealocytes evolved from the pineal photoreceptor cells characteristic of lower vertebrates (Quay, 1984; Karasek, 1983; Pévet, 1983; Oksche, 1984; Collin & Oksche, 1981). The pattern of evolution appears to have involved a progressive transformation of clearly differentiated photoreceptors with outer segments into endocrine secretory cells. However, it has recently been shown that mammalian pinealocytes and retinal photoreceptors still have many biochemical markers in common (Mirshahi et al., 1984; Korf et al., 1985a,b; Somers & Klein, 1985). These include the 48k protein or S-antigen (Kühn, 1980; Pfister et al., 1985), rhodopsin kinase (Kühn & Dreyer, 1972; Bownds et al., 1972) and opsin.

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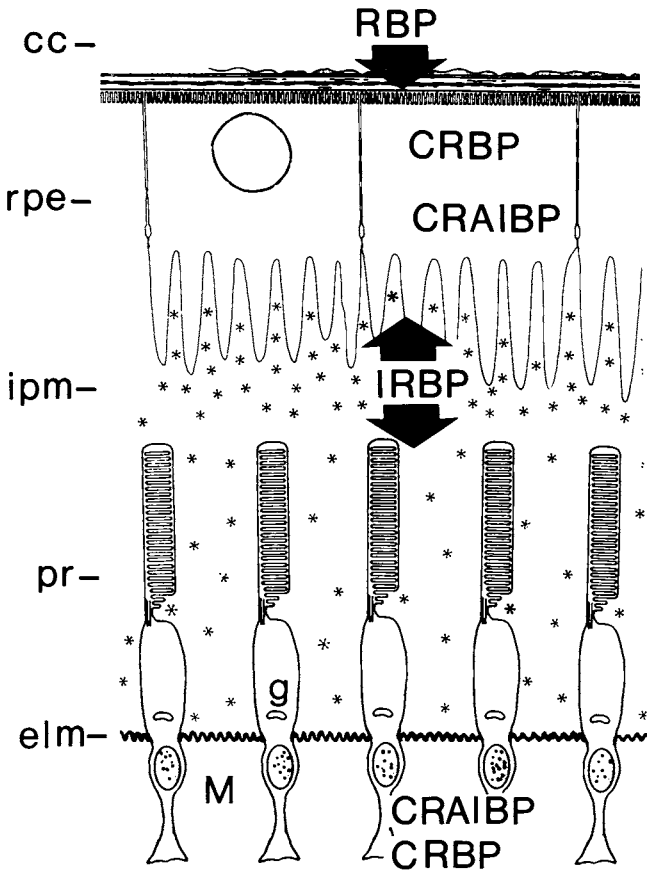

Fig. 1 Retinoid-binding proteins and the visual cycle. Endogenous ligands shown below in parentheses. RBP, plasma retinol-binding protein (all-trans retinol); CRBP, cellular retinol-binding protein (all-trans retinol); IRBP, interstitial retinol-binding protein (all-trans and 11-cis retinol; traces of isomers of retinaldehyde); CRAlBP, cellular retinaldehyde-binding protein (11-cis retinol and 11-cis retinaldehyde). CRABP, cellular retinoic acid-binding protein, is not shown. Retinoic acid is not convertible to retinol or retinaldehyde and hence cannot participate directly in the visual cycle. cc, choriocapillaris; rpe, retinal pigment epithelium; pr, photoreceptors; elm, external limiting membrane; g, Golgi; M, Müller cell. The interphotoreceptor matrix is indicated with asterisks.

In retinal photoreceptors, the rhodopsin kinase and 48k protein may be implicated in the "ATP-dependent quench" of photolyzed rhodopsin's ability to activate cGMP phosphodiesterase via the intermediary G-protein or transducin (Zuckerman et al., 1985; for review, see Pugh & Cobbs, 1986). The pinealocytes of fish, amphibians and reptiles are electrophysiologically responsive to direct illumination (Meissl & Dodt, 1981), but functional photoreception has not yet been demonstrated in any mammalian pineal. Coupled with the possible presence of rhodopsin, the occurrence of these elements of the cGMP cascade in mammalian pinealocytes may imply that photochemical mechanisms have persisted in the absence of specialized membrane assemblies.

Rhodopsin regeneration and biosynthesis require a supply of 11-cis retinaldehyde and its retinoid precursors. In the eye retinoid-binding proteins appear to have an important function in this process. Their properties and occurrence will be discussed in the present paper, which also documents some recent experimental findings demonstrating that IRBP (interstitial retinol-binding protein) is present in rat and bovine pineal organs. IRBP is an extracellular retinoid-binding protein that is synthesized and secreted by the retinal photoreceptors: the present observations therefore extend the list of biochemical links between these cells and the pinealocytes.

II. RETINOID-BINDING PROTEINS AND THE EYE

A. Serum Retinol-Binding Protein

The major route by which vitamin A is delivered to the eye is via the choroidal circulation. In the bloodstream, all-trans retinol is carried by a 21,000 dalton protein known as serum retinol-binding protein (RBP; for review, see Goodman, 1984). This protein circulates as a 1:1 complex with transthyretin (prealbumin). RBP solubilizes retinol, protects it from oxidative degradation and provides the means for targeting its delivery to cells that have RBP receptors on their plasma membranes (Fig. 1). Receptors for RBP have been found on the surfaces of intestinal mucosa, testicular and corneal epithelial cells, as well as on the basement membranes of the pigment epithelium (RPE) cells (Rask & Peterson, 1976; Bhat & Cama, 1979; Bok & Heller, 1976; McGuire et al., 1979; Rask et al., 1980).

B. Cellular Retinoid-Binding Proteins

When retinol passes from the pigment epithelium plasma membrane to its intracellular sites of esterification and utilization, it apparently becomes bound to an intracellular protein, cellular

retinol-binding protein (CRBP; Ong & Chytil, 1978). CRBP is distinct from RBP in its immunological reactions, spectroscopic properties, lower molecular weight (15,000 daltons) and failure to complex with transthyretin. CRBP occurs in many tissues (for review, see Chytil & Ong, 1984), but most of the CRBP in the eye is found in the RPE (Fig. 1). A small quantity is associated with the cells of the neural retina (Bok et al., 1984; Saari et al., 1978). Its endogenous ligand is exclusively all-trans retinol (Saari et al., 1982).

Binding proteins for other retinoids are also present in the retina and RPE (Fig. 1). They include cellular retinoic acid binding protein (CRABP; Saari et al., 1978) and cellular retinal-binding protein (CRALBP; Stubbs et al., 1979). CRABP occurs in many tissues as well as the retina (Chytil & Ong, 1984). It is absent from the RPE. Its endogenous ligand consists of all-trans retinoic acid. CRALBP is restricted to the RPE and to the Müller cells of the retina (Bunt-Milam & Saari, 1983). Its endogenous ligands consist of 11-cis retinaldehyde and 11-cis retinol (Saari et al., 1982).

C. Interstitial Retinol-Binding Protein

1. Function

The all-trans retinol that is generated when rhodopsin is bleached flows outward from the rod outer segments (ROS) and enters the RPE cells through their apical membranes. It accumulates in the form of all-trans retinyl esters (Dowling, 1960; Bridges, 1976). The transfer of retinol between the ROS and the RPE entails passage through the interphotoreceptor matrix, which contains interstitial retinol-binding protein (IRBP; Liou et al., 1982a,b), a glycoprotein that in cattle has been found to carry endogenous all-trans and 11-cis retinol (Adler & Martin, 1982; Liou et al., 1982a,b). IRBP is a major component of the interphotoreceptor matrix (Fig. 1); in cattle it is present at a concentration of 30-100 μM (Fong et al., 1984b).

Figure 2 illustrates the immunohistochemical localization of IRBP in the interphotoreceptor matrix of the rat. Fixed, frozen 16 μm sections were incubated with rabbit antibovine IRBP antibodies followed by a fluorescein isothiocyanate conjugated goat-antirabbit second antibody. Immunospecific fluorescence is observed between the apical surface of the RPE (slanted arrow) and the external limiting membrane (horizontal arrow). Fluorescence is intensely localized in a thin band adjacent to the RPE, is less intense over the remainder of the rod outer segment layer and is comparatively faint in the inner segment region (cf. Schneider et al., 1986).

It has been suggested that IRBP is the transport vehicle that shuttles retinol between the cells of the retina and the RPE during the visual cycle (Fig. 1; Adler & Martin, 1982; Liou et al., 1982a,b; Lai et al., 1982; Pfeffer et al., 1983; Fong et al., 1984a,b; Bridges,

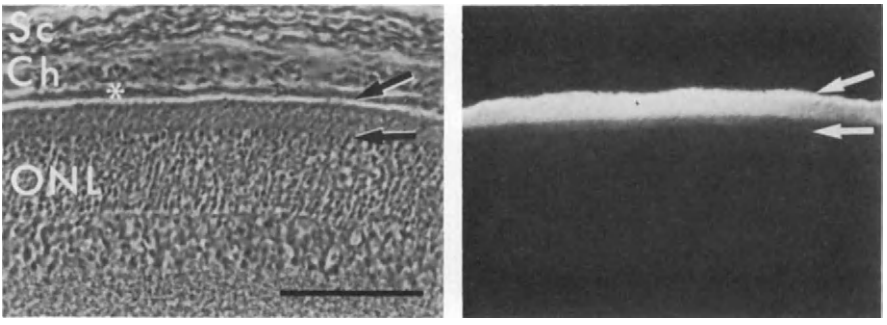

Fig. 2. Immunocytochemical localization of IRBP in rat retina. The antigen was visualized with rabbit antibovine IRBP antibodies and fluorescein isothiocyanate labeled goat antirabbit IgG. Area depicted is near the posterior pole. Left - phase-contrast; right - fluorescence. Slanted arrow - apical surface of RPE cells; horizontal arrow - position of external limiting membrane. A line of more intense fluorescence is visible adjacent to the RPE. Sc, sclera; Ch, choroid; ONL, outer nuclear layer; asterisk, RPE. Bar, 100 μm (methods of Gonzalez-Fernandez et al., 1984).

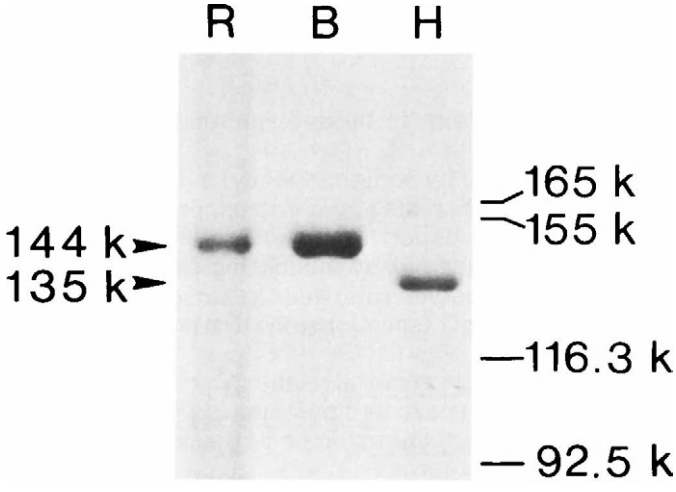

Fig. 3 Rat, bovine and human IRBP's: comparison by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

R, rat IRBP (molecular weight = 144k); B, bovine IRBP (molecular weight = 144k); H, human IRBP (molecular weight = 135k). Molecular weights of standard proteins run in the same gel are shown on right. Conditions as described by Fong et al. (1984a,b).

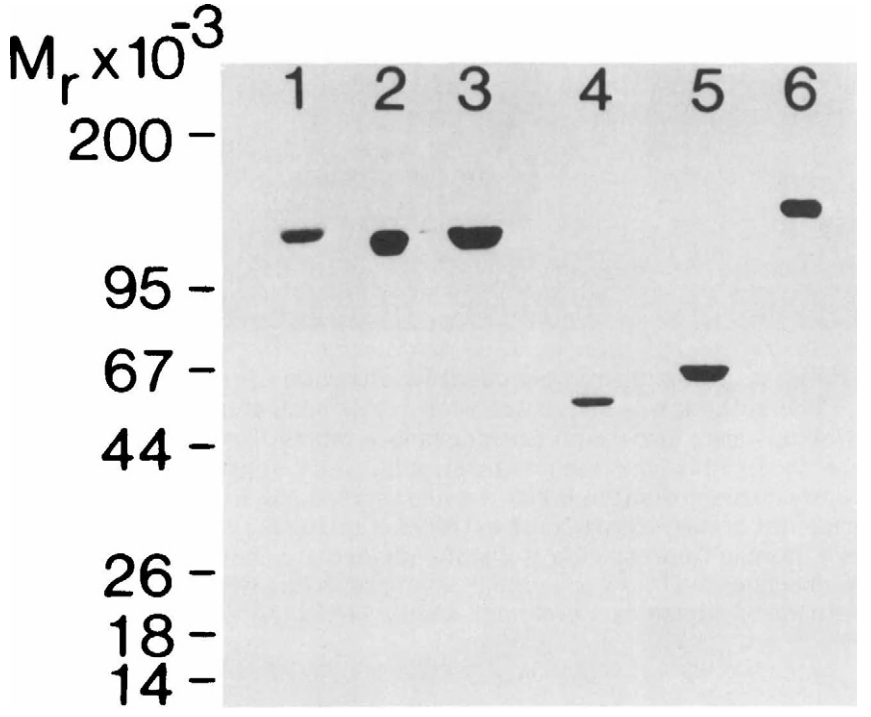

Fig. 4 IRBP-like proteins in the interphotoreceptor matrix of five vertebrate species.

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose paper. Immunological visualization of the reactive bands was carried out by incubating the paper with rabbit antibovine IRBP antibodies followed by horseradish peroxidase-conjugated goat antirabbit IgG (see Gonzalez-Fernandez et al., 1984, for methods).

Lane 1, turtle (Pseudemys scripta); lane 2, adult frog (R. pipiens); lane 3, tiger salamander (Ambystoma tigrinum); lane 4, gizzard shad (Dorosoma cepedianum); lane 5, black drum (Pogonias cromis); lane 6, cattle (Bos taurus).

1984; Bridges et al., 1984; Saari et al., 1984). It is therefore significant that its first appearance during development has been noted just prior to the time when rhodopsin and 11-cis retinyl esters are first detectable in the eye (Carter-Dawson et al., 1986).

2. Properties

IRBP has been purified and characterized from human, bovine, monkey and rat eyes (Fong et al., 1984a,b; Adler et al., 1985; Gonzalez-Fernandez et al., 1985a; Redmond et al., 1985; Saari et al., 1985). Bovine IRBP has an apparent molecular weight of 144,000 daltons on sodium dodecyl sulfate polyacrylamide gels (Fig. 3), binds about 2 molecules of all-trans retinol, and has 4-5 carbohydrate chains that appear to consist of fucosylated hybrid- and complex-type oligosaccharides (Fong et al., 1984a, 1985a; Taniguchi et al., 1986). It exhibits an anomalously high molecular weight of 250,000 daltons on gel-filtration columns, apparently because it is an elongated molecule with an axial ratio of about 8:1 (Adler et al., 1985; Saari et al., 1985). As illustrated in Fig. 3, rat IRBP (lane R) has the same molecular weight as bovine IRBP (lane B) but human IRBP has a lower molecular weight (lane H). "Native" human IRBP also has a lower apparent molecular weight than bovine IRBP on gel-filtration columns (Fong et al., 1984a).

3. Distribution in Vertebrates

As might be expected from its suggested role in the visual cycle, IRBP is universally distributed in the IPM of the major vertebrate classes, which include the Mammalia, Aves, Reptilia, Osteichthyes, Chondrichthyes and Amphibia (Bridges et al., 1984; Fong et al., 1985b; Bridges et al., 1986a). This was demonstrated by electrophoretically transferring the IPM proteins from sodium dodecyl sulfate polyacrylamide gels to nitrocellulose sheets, then incubating the sheets with rabbit antibovine IRBP immunoglobulins followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. An immunoreactive protein was observed in all IPM preparations. In most of these animals, immunoreactivity was observed in a protein with molecular weight of $134{,}200 \pm 8{,}600$ ($n = 17$). Four examples (one reptile, two amphibians, one mammal) are shown in lanes 1,2,3 and 6 in Fig. 4. In the Osteichthyes (two examples are shown in lanes 4 and 5, Fig. 4), the molecular weight was about half of this value ($67{,}600 \pm 2{,}700$, $n = 8$), leading to the suggestion that there may have been at least one gene duplication in the evolution of this protein. Such an event, perhaps involving the gene coding for a primordial retinoid-binding protein, might explain the ability of bovine IRBP to bind two molecules of retinol (Fong et al., 1984b; Saari et al., 1985), possibly in discrete binding sites.

4. Biosynthesis by the Retina in vitro

IRBP is synthesized by the retina, not by the RPE. This has been demonstrated in experiments where isolated retinas from cattle, rats, humans, monkeys, ranid frogs and Xenopus have been incubated with labeled sugar (fucose, glucosamine) or amino acid precursors (Bridges et al., 1983; Fong et al., 1984a,b; Wiggert et al., 1984; Rayborn et al., 1984; Gonzalez-Fernandez et al., 1984, 1985a,b,c; Hollyfield et al., 1985). In each case, labeled protein with an electrophoretic mobility identical with that of IRBP and immunoprecipitable with rabbit antibovine IRBP immunoglobulin was secreted into the incubation medium. Secretion was not prevented by tunicamycin (Fong et al., 1984b, 1985a), an antibiotic that inhibits the assembly of oligosaccharides linked N-glycosidically to proteins. Secretion is also not affected by castanospermine and swainsonine, agents that interfere with oligosaccharide processing (Fong et al., 1985a).

In similar experiments, no biosynthesis or secretion of IRBP by the pigment epithelium cells was detected.

5. Evidence that Photoreceptors Synthesize and Secrete IRBP

Of the retinal cells that border the subretinal space, the photoreceptors rather than the Müller (glial) cells appear to be the source of IRBP. These cells are therefore involved in the biosynthesis of two major glycoproteins that have an important role in the biochemistry of the visual process, namely rhodopsin and IRBP.

The evidence that the photoreceptors produce IRBP has been previously discussed by Bridges (1985), and is summarized below.

(a) In mutant rats and mice that progressively lose their retinal photoreceptors during postnatal development, there is a corresponding reduction of IRBP in the interphotoreceptor matrix and in the ability of the retinas to synthesize IRBP in vitro (Gonzalez-Fernandez et al., 1984; Carter-Dawson et al., 1986; Fig. 5). IRBP persists longer when photoreceptor degeneration is retarded in mutants reared in darkness (Gonzalez-Fernandez et al., 1985b).

(b) Cultures of adult human retinal cells secrete IRBP into the medium only when neuronal cells are present (Oka et al., 1985). Some of these neuronal cells are probably photoreceptors. Cultures containing retinal glial cells do not synthesize IRBP.

(c) Pulse-chase autoradiography of isolated human retinas incubated with (^{3}H)-fucose demonstrates that a fucosylated compound is synthesized and secreted by the photoreceptors (cf. Feeney, 1973). In these experiments, (^{3}H)-IRBP was subsequently recovered from the medium (Hollyfield et al., 1985).

The search for IRBP mRNA within the photoreceptor inner segments is now being carried out with cDNA probes derived from the coding region of the IRBP gene and should provide an unequivocal answer to this question (Liou et al., in preparation).

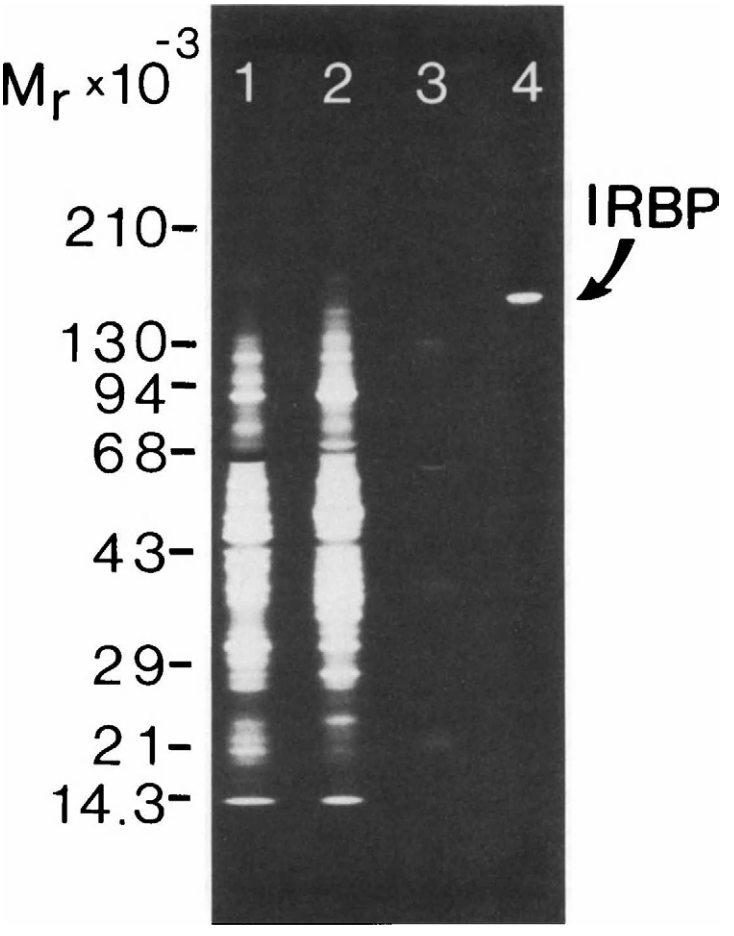

Fig. 5 Evidence that retinal photoreceptors synthesize and secrete IRBP.

Retinas from Royal College of Surgeons ca. 50 day-old retinal dystrophic rats and controls were incubated with (^{3}H)-leucine. After 4 hrs, media and retinas were collected separately. The retinas were homogenized, and the cytosol collected after centrifuging at 100,000 g. Media and cytosol samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and fluorography (Gonzalez-Fernandez et al., 1984).

Lanes 1,2 - fluorogram of cytosols from dystrophic and control retinas, respectively. Lanes 3,4 - fluorograms of media from dystrophic and control retinas respectively.

Labeled IRBP has been secreted into the medium by the control retinas (lane 4) but not by the retinas that had lost their photoreceptors (lane 3).

6. Expression of IRBP during Retinal Differentiation and by the Neoplastic Cells of Retinoblastoma

IRBP is first detected in the developing mouse retina at the stage when photoreceptor inner segments start to differentiate (Carter-Dawson et al., 1986), but it should be noted that large amounts of IRBP have been observed in undifferentiated retinoblastoma tumors. It has also been shown that in vitro the cells from these tumors are able to synthesize IRBP from radiolabeled precursors and secrete it. These findings have led to the suggestion that regulation of the IRBP gene in this neuroectodermal neoplasm may be defective (Bridges et al., 1985).

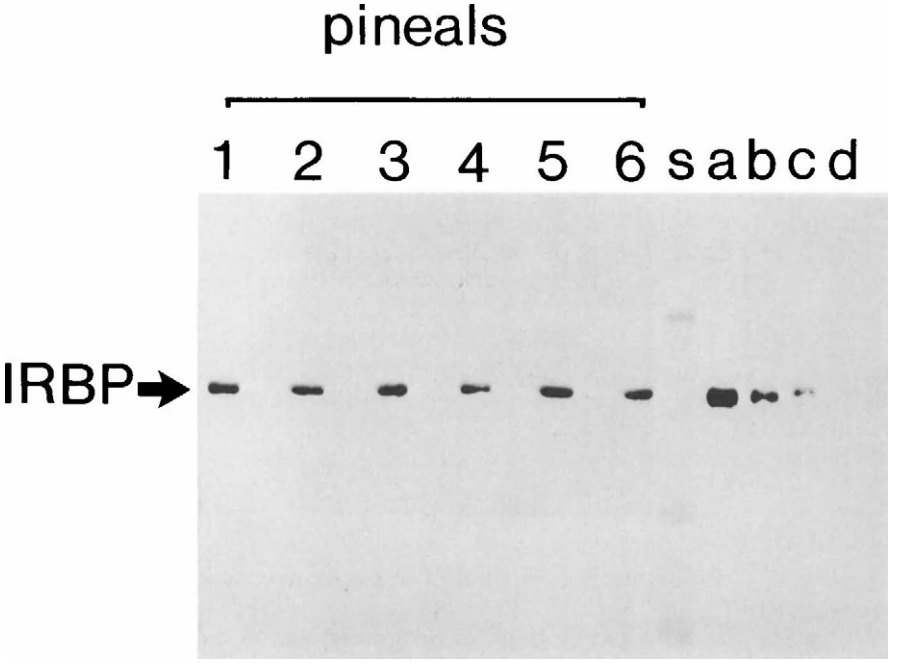

Fig. 6 IRBP in bovine pineal organ.

Soluble pineal proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose sheet. The blot was probed with rabbit antibovine IRBP antibodies as described in Fig. 4.

Lanes 1 through 6 - 100 µg soluble protein from six individual bovine pineal organs; lane S - prestained protein markers (myosin, 200k; phosphorylase B, 97k; bovine serum albumin, 67k); lanes a,b,c,d - 10, 5, 2.5, 1.0 ng of pure bovine retinal IRBP, respectively.

III. INTERSTITIAL RETINOL-BINDING PROTEIN IN THE PINEAL ORGAN

Since IRBP is normally expressed by differentiating photoreceptors, it is of interest to examine various pineal organs. Rudimentary photoreceptors occur in the Japanese quail pineal. In a recent report, Bridges et al. (1986b) used antibovine IRBP antibodies to demonstrate immunoreactivity over quail pinealocyte outer segments. A less intense reaction was observed in the follicles. This finding complements the work of H.W. Korf, R.G. Foster and J. J. Schalken (in preparation), who have demonstrated rhodopsin, S-antigen (= 48k protein, see below) and alpha-transducin in the quail pineal.

The mammalian pineal, which does not contain differentiated photoreceptors, was also examined. Individual bovine pineal organs were homogenized in phosphate-buffered saline containing phenylmethylsulfonyl fluoride as a protease inhibitor (150 mM NaCl, 5 mM Na phosphate, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). The soluble proteins were prepared by centrifuging at 100,000 g for 1 hr, and were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (cf. Liou et al., 1982b). After electrophoretic transfer to nitrocellulose paper, these proteins were tested for immunoreactivity with rabbit antibovine IRBP antibodies (Gonzalez-Fernandez et al., 1984).

The results from 6 pineals are illustrated in Fig. 6. Lanes 1 - 6 respectively, represent 100 µg of soluble protein from six pineal organs. An immunoreactive band of the same molecular weight as authentic bovine IRBP (lanes a - d) is visible in all six lanes. The similar intensities of these bands indicate that these preparations contained comparable levels of IRBP, the abundance of which was estimated to be 32 $\pm$ 7 ng IRBP/mg soluble protein. This corresponded to an average of 80 ng/pineal, or about 0.02% of that present in a bovine eye with a 25 cm^2 retina (Fong et al., 1984b).

A similar experiment was carried out with the pineal organs of adult Sprague-Dawley rats. In Fig. 7, lane 1 illustrates the result obtained from soluble protein equivalent to two rat pineals. A clear band of immunoreactivity is visible at the same molecular weight as bovine IRBP (lanes a - d). It is concluded that this is rat IRBP. As illustrated in Fig. 3, it has the same electrophoretic mobility as bovine IRBP on sodium dodecyl sulfate polyacrylamide gels. The significance of immunoreactive proteins at lower molecular weights in the soluble proteins from other brain regions (lanes 2,3,4) is not known at this time.

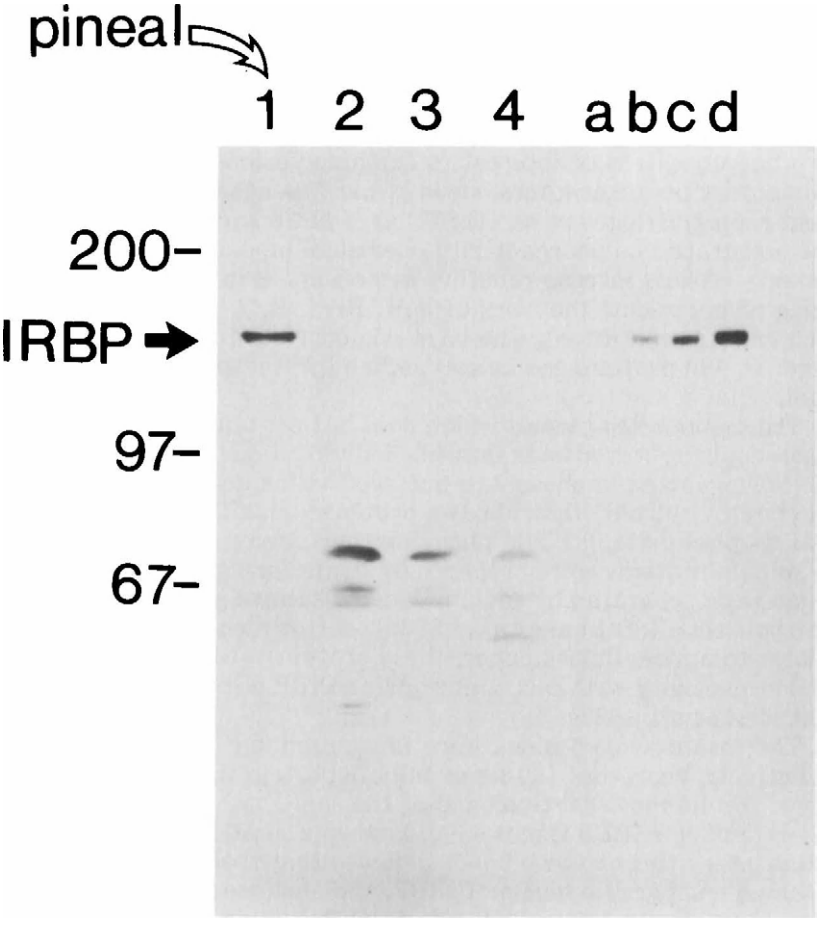

Fig. 7 IRBP in rat pineal organ.

Soluble rat pineal and brain proteins (200 µg portions) were treated as described in Fig. 6.

Lane 1 - soluble proteins equivalent to two rat pineal organs; lanes 2,3,4 - soluble proteins from brainstem, cerebellum and cerebral cortex, respectively; lanes a,b,c,d - 0.5, 1.25, 2.5, 5 ng pure bovine retinal IRBP.

IV. CONCLUSION

The presence of IRBP in the pineal organ is perhaps less surprising than the occurrence of opsin, 48k protein and rhodopsin kinase, all of which are associated with outer segment photoreceptor processes. In the eye, IRBP is a putative intercellular shuttle vehicle for retinoids during the visual cycle. If opsin immunoreactivity means that rhodopsin is present in mammalian pinealocytes, there may exist an analogous cycle of bleaching and regeneration that would similarly require an extracellular retinoid transport protein. Rather than representing an interesting but irrelevant vestige of the time when these pinealocytes were recognizably differentiated photoreceptors, IRBP could therefore have retained its functional role in pineal photochemistry.

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ELECTRON MICROSCOPIC LOCALIZATION OF IMMUNOREACTIVE OPSIN IN THE PINEAL ORGAN

Ingeborg Vigh-Teichmann
Béla Vigh

2nd Department of Anatomy, Semmelweis University
Medical School Budapest, Hungary

I. INTRODUCTION

Our studies of retina and pineal organ started from the investigation of the cerebrospinal fluid (CSF)-contacting neuronal system (20, 21). CSF-contacting neurons are located in the walls of the brain ventricles and central canal of all vertebrate classes. The diencephalic CSF-contacting neurons exhibit intraventricular dendrite terminals with 9x2+0 cilia, a feature resembling primitive chemoreceptors and/or developing/regressing photoreceptors. Morphologically, certain populations of the CSF-contacting nerve cells may represent sensory elements corresponding to the chemo-, thermo-, mechano- and so-called extraretinal deep encephalic photo-receptors supposed to occur in the ventricular walls by neurophysiological experiments (lit. see 20, 21, 23).

Since the question of a photoreceptive function of the CSF-contacting neurons concerned us mostly, we compared their cytology with that of the photoreceptors of the lateral eye and pineal complex (lit. see 13, 14, 15, 16, 17). Our comparative light and electron microscopic (EM) work resulted in the recognition that - in a broad sense - the retinal and pineal photoreceptors are components of the CSF-contacting neuronal system by their location, polarization,

principal cytological similarities and development from neuronal elements of the central nervous system (cf. 20). Moreover, our cytological data on the structure and different types of pineal photoreceptors (13, 16, 17, 20, 21) and on the presence of regular photoreceptor outer segments in the avian pineal (14, 19) influenced the concepts evolved in the earlier literature (lit. see 29).

Photoreceptors can only perform light perception in the presence of visual pigments and photoreceptor-specific proteins like S-antigen playing a role in photochemical transduction (lit. see 11, 12, 28). The peptide component *opsin* of rhodopsin can be demonstrated by immunocytochemistry. This method proved to be useful in locating rhodopsin/porphyropsin in photoreceptors of the pineal, parapineal and frontal organs of various submammalian species and in certain retinal and "extraretinal" photoreceptors at the light and EM levels (10, 13, 15, 16, 17, 18, 24, 25, 26, 27, 28). In addition, different types of photoreceptors were found by their cytology and antigenicity to antibovine rhodopsin antisera (17, 18, 20,21) as well as to monoclonal antisera raised against photoreceptor membranes of chicken retina, presumably their opsins (8, 9, 18, 21). Quite recently, the occurrence of rhodopsin was found to be correlated with that of S-antigen by immuno-EM of resin-embedded material (18, 28). In this paper, we summarize our results on the immunocytochemical multiplicity of the pineal photoreceptors.

II. RESULTS

A. Rhodopsin-Immunoreactive And Rhodopsin-Immunonegative Photoreceptors

By means of antibovine rhodopsin antisera *strong immunoreaction* was found in a large number of - morphologically cone - outer segments (Fig. 1, 3) of photoreceptors of the pineal organ from cyclostomes to birds (13, 15, 16, 17, 24, 25, 26, 28). It was striking that in the European minnow, toad, green and tiger frogs a small-numbered population of outer segments displayed *weak opsin immunoreaction*. This finding may be explained by the presence of another kind

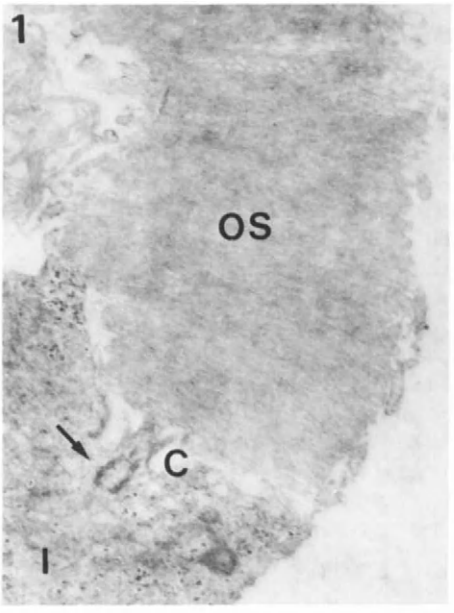
of opsin (iodopsin in amphibians?) with minor cross-reactivity to the antibovine rhodopsin antisera used (18, 28).

In *comparative studies*, strongly opsin-immunoreactive photoreceptors were observed in the pineal organ of lamprey, numerous freshwater fishes and amphibians (10, 13, 15, 18, 20, 21, 24, 25, 26, 28). This result presents evidence of the presence of rhodopsin and/or porphyropsin both being closely related in their epitopes and chemical composition. Our results are in accord with data on light absorption maxima of the pineal organ (lit. see 4, 18, 20).

With regard to the reptilian pineal organ, the rhodopsin immunoreaction was positive in turtles and moderately positive or negative in Lacertilians (13, 15, 18, 24). In turtles, the opsin-immunoreactive outer segments were scattered in the pineal lumen. Obviously, they contain rhodopsin/porphyropsin-like photopigment. Electrical recordings revealed light absorption maxima at 570 nm for Lacertilians and of 620 nm for the red-eared turtle (4). The absorption maximum at 620 nm is typical of a cyanoopsin as found in retinal red cones. The latter do not cross-react with the rhodopsin antiserum used, in contrast to the porphyropsin-containing rods and single green cones (12). In some inner segments of pineal photoreceptors we found oil droplets (18). Therefore, studies are in progress to clarify whether the chelonian pineal is composed of two types of photoreceptors: porphyropsin-containing and cyanoopsin-containing ones.

Among the avian species studied, the opsin immunoreaction was relatively intense in the pineal outer segments of pigeon and chicken (13, 15) indicating the presence of rhodopsin. Recently, electrical responses were obtained to photic stimuli in the pigeon (7) and in vitro evidence of photoreception was found in chicken (3). Moreover, the presence of photoreceptors with regularly lamellated outer segments, of intrinsic neurons, a synaptic neuropil (14, 15, 18), further of photoreceptor-specific proteins (11) prove that the avian pineal organ has the capacity to detect light directly.

In the mammalian pinealocytes, no clear-cut opsin immunoreaction could be found in opossum, bat, hedgehog, rat, cat, rabbit (13) and cattle by the antibovine rhodopsin antisera used. The question needs further study whether the mammalian pinealocyte contains photopigment(s). By photoreceptor-specific S-antigen immuno-EM three kinds of pinealocytes were observed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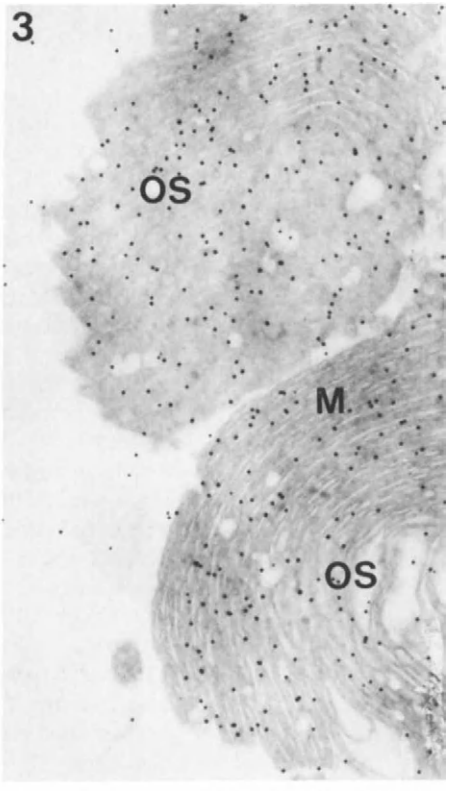
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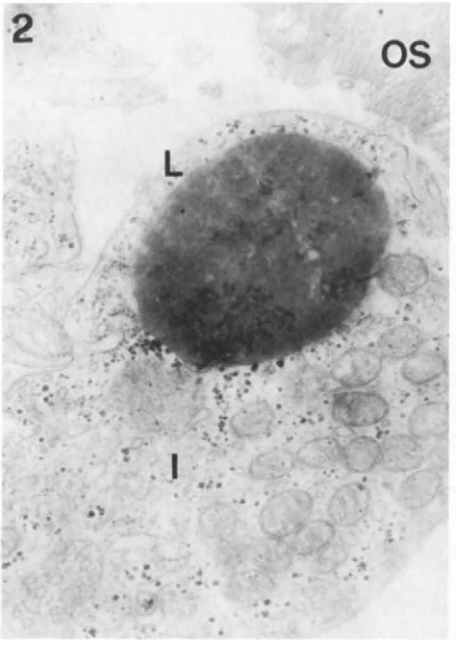
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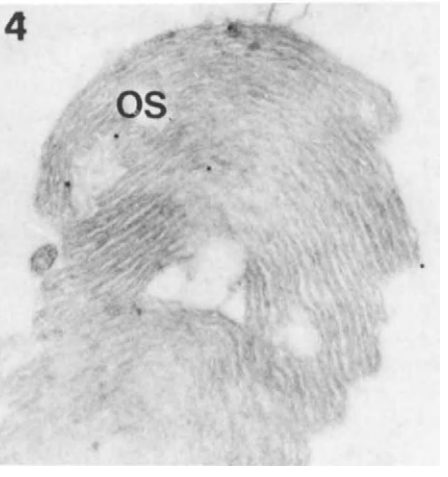
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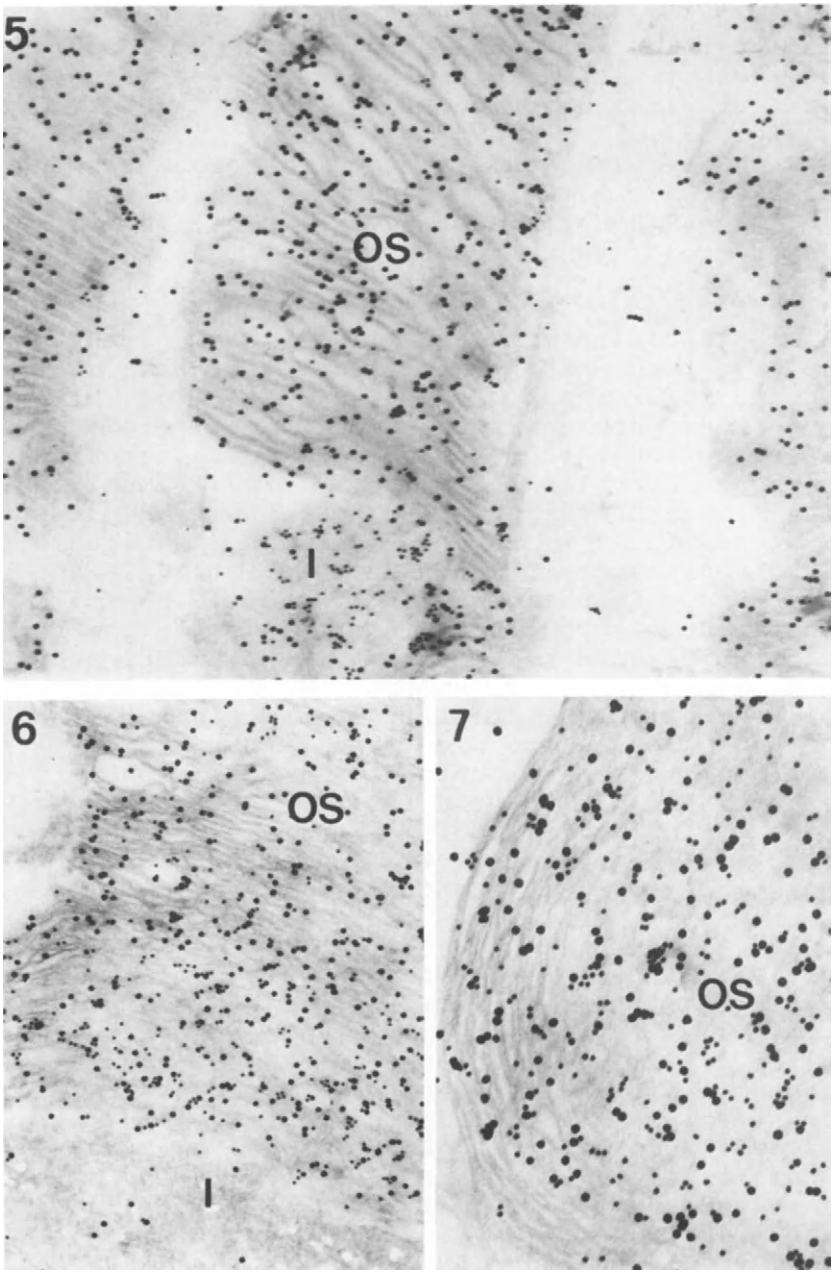
in hedgehog and bat: either strongly or weakly S-antigen-positive and -immunonegative ones (18, 28). We suppose that similarly as in lower vertebrates, the mammalian pineal is composed of three types of pinealocytes (22).

Rhodopsin-immunonegative outer segments of photoreceptors have been found in small number among the majority of *rhodopsin-positive* elements in the pineal organ of goldfish and green frog as well as in the parapineal organ of lamprey (16, 17, 20, 21) by use of the ABC and/or protein A-gold methods (Fig. 4). Similar rhodopsin-immunonegative pineal photoreceptors have been demonstrated in the European minnow, toad and tiger frog (18, 28).

In *Rana esculenta*, the immunonegative outer segments were continuous with inner segments containing a large *oil droplet* (Fig. 2) (17, 21). Thus, in the green frog, three types of pineal photoreceptors can be distinguished by their cytology and antigenicity to the antirhodopsin antisera used: strongly and weakely rhodopsin-positive, and rhodopsin-negative ones with oil droplet in their inner segment. The functional significance and nature of the photopigments of the latter two photoreceptors is open to discussion (see Conclusions). Electrical recordings from the frog pineal organ revealed a spectrosensitivity corresponding to the absorption maxima of rhodopsin in the dark- and of iodopsin in the light-adapted state of the organ (2), a finding providing ground to speculate that we have detected iodopsin in one of these populations of photoreceptors.

Figs 1-4. Details of photoreceptors of the pineal organ in the frogs Rana tigrina (1) and R. esculenta (2, 3, 4). 1) Morphologically, the outer segments (OS) of pineal photoreceptors are cone-like in the arrangement of their membrane lamellae. Arrow: basal body, C: ciliary connecting piece, I: inner segment. Osmicated section. x 22760. 2) Inner segment (I) containing an oil/lipid droplet (L). OS: outer segment. Osmicated section. x 25000. 3) Details of strongly rhodopsin-immunoreactive outer segments (OS). M: photoreceptor membranes. Protein A-gold method. x 32360. 4) Rhodopsin-immunonegative outer segment (OS). A few protein A-gold particles correspond to background labeling. x 32360



Figs 5-7. Immunoelectron microscopy of outer segments of the hedgehog retina (5) and of the pineal organ of the tiger frog (6, 7). Double labeling with antibovine rhodopsin and anti-S-antigen antisera.

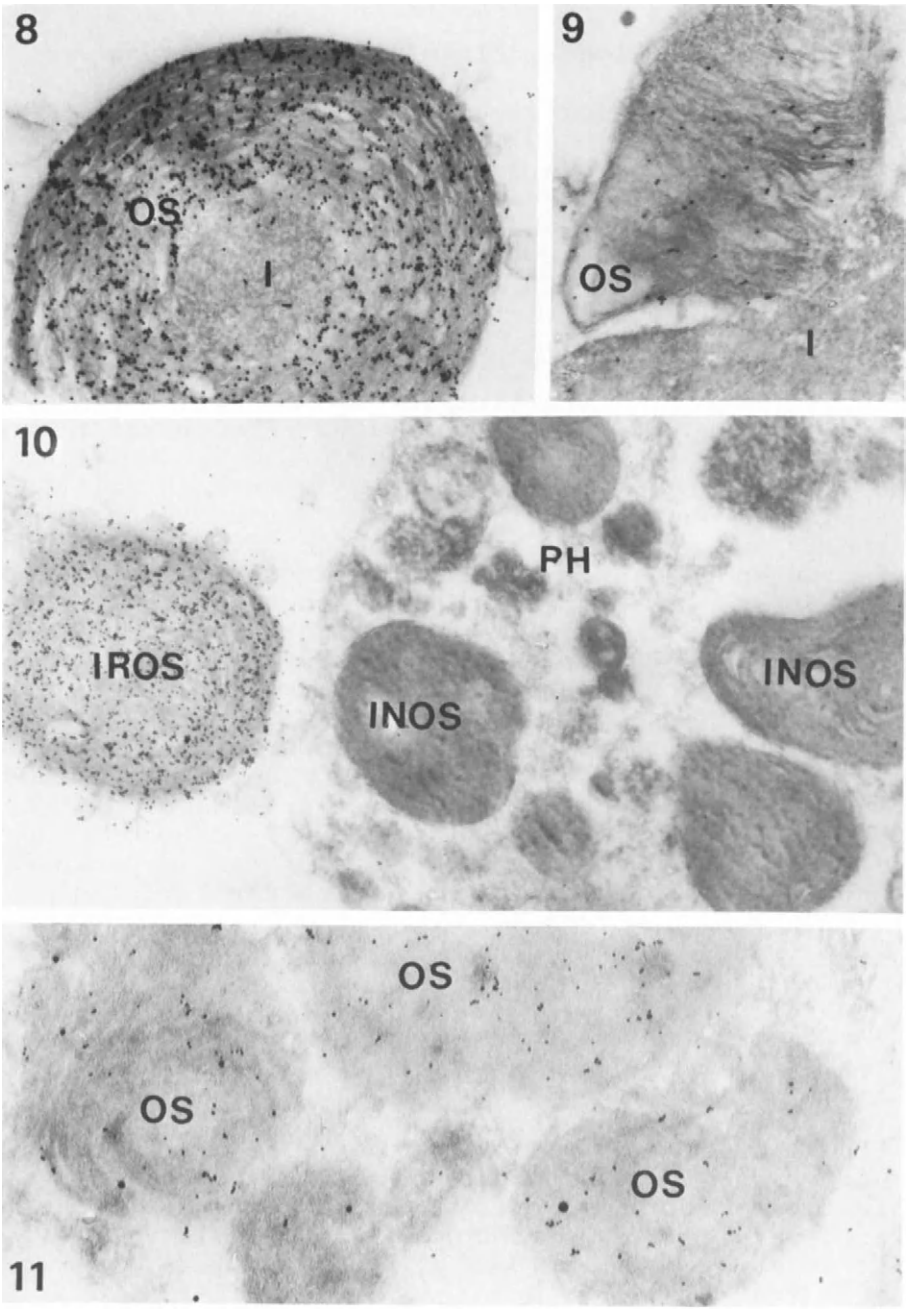
B. *Comparison Of Antirhodopsin And S-antigen Immunoreaction In Pineal Organ and Retina*

S-antigen binds specifically to bleached phosphorylated rhodopsin and quenches cGMP phosphodiesterase (6). The comparison of the immunocytochemical distribution of S-antigen with the occurrence of the visual pigment is, therefore, a first step toward the elucidation of cytological aspects of photochemical transduction (lit. see 11, 12, 28).

Immuno-EM labeling of the pineal organ of toad and tiger frog revealed either strongly or weakly S-antigen-positive and few -immunonegative outer segments (Fig. 6). Similarly as in the retina (Fig. 5) the proximal portion of the outer segment was stronger labeled than the distal one (Fig. 7) (migration of S-antigen?). Double labeling with both antibovine S-antigen and rhodopsin antisera demonstrated either strong or weak dual immunoreaction (Fig. 6), while few outer segments were immunonegative. Thus, in the amphibian pineal organ, the presence of S-antigen is correlated with that of rhodopsin (18, 28).

As already mentioned, in the pineal organ of hedgehog and bat, either strongly or weakly S-antigen-positive and -negative pinealocytes were observed by immunogold-labeling (18, 28). However, the rhodopsin immunoreaction was negative by means of rat and sheep antibovine rhodopsin antisera. Our data of different types of mammalian pinealocytes by S-antigen immunoelectron microscopy and the role of this protein in photochemical transduction inspire further studies on a possible direct light sensitivity of the mammalian pineal organ.

5) Strongly rhodopsin-immunoreactive rod outer segments (OS) labeled by 15 nm protein A-gold particles. S-antigen immunoreactivity (10 nm IgG-gold particles) is stronger in the inner segment (I) than in the outer segment. x 38400. 6) Strongly immunoreactive proximal portion of a pineal outer segment (OS) double-labeled by antibodies to both S-antigen (10 nm gold particles) and rhodopsin (15 nm gold particles). I: inner segment. x 38400. 7) Higher magnification of a pineal outer segment (OS) exhibiting strong rhodopsin (15 nm gold particles) and S-antigen (10 nm gold particles) immunoreactions. x 62400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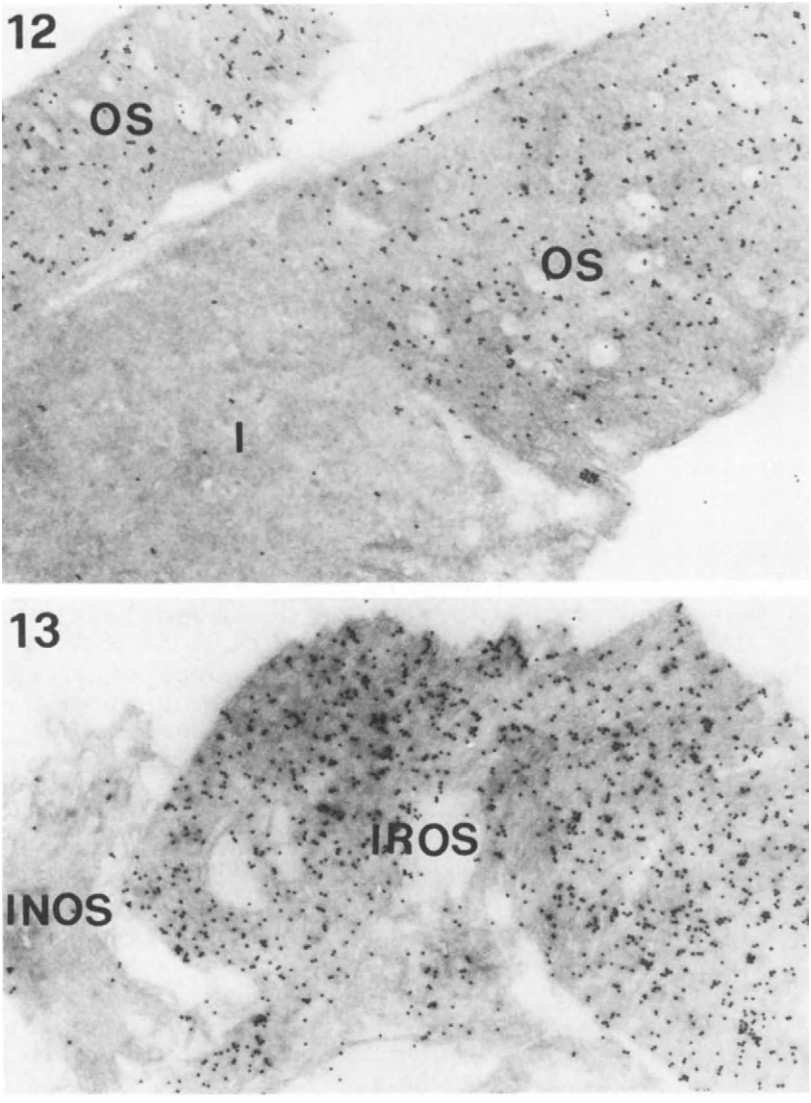
C. Pineal Photoreceptors As Revealed By Monoclonal Antichicken Opsin Antisera

The chicken retina is known for its content of rhodopsin, iodopsin, chicken blue and violet photopigments. Recently, we have applied monoclonal antisera raised against chick photoreceptor membranes, presumably their opsins (8, 9). At the light microscopic level, the hybridoma supernatant (S) A1 and D6 stained few photoreceptor outer segments in the pineal organ of lamprey (S-A1) and European minnow (S-A1, S-D6) while the majority of the outer segments was immunonegative (18, 21). On the other hand, the latter were immunoreactive by use of antibovine rhodopsin antisera (26, 26). Apparently, the pineal organ of *Phoxinus phoxinus* contains three types of photoreceptors, the rhodopsin/porphyropsin-containing ones and those immunoreactive with the two different anticone opsin antisera (18).

In an immuno-EM study in the European minnow, toad and tiger frog, we applied the monoclonal antiserum RET-2 and S-21/21 (corresponding to S-A1, which stains retinal double cones and one type of single cones; 8,9). By means of S-21/21 either strongly or weakly immunoreactive outer segments were found (Fig. 8, 9) together with few immunonegative photoreceptors. Outer segments incorporated into phagocytes of the pineal lumen (Fig. 10) could not be immunogold-labeled by S-21/21 although they were immunoreactive with the antibovine rhodopsin antiserum (Fig. 11). Apparently, the epitopes detected by the two antisera differ in their stability to phagocytotic events.

Figs 8-11. Details of photoreceptor outer segments of the pineal organ immunoreacted with the hybridoma supernatant S-21/21 raised against photoreceptor membranes of chicken retina (8, 9, 10) and with antibovine rhodopsin antiserum (11). 8) Strongly immunoreactive outer segment (OS). Rana tigrina. x 31460. 9) Weakly immunoreactive outer segment (OS). Its proximal portion is stronger labeled than its distal portion. I: inner segment. x 31460. 10) Note unlabeled outer segment (INOS) inside, and strongly immunoreactive outer segment (IROS) outside the phagocyte (PH). x 21620. 11) The phagocytized outer segments (OS) are rhodopsin-immunoreactive (5 nm gold-labeling). x 73650



Figs 12-13. Details of photoreceptor outer segments of the retina of the toad, Bufo bufo (12) and of the pineal organ of the tiger frog, Rana tigrina (13) immunoreacted with the monoclonal antibody RET-2 raised against isolated photoreceptor membranes of chicken retina. 12) Medium immunoreactive outer segments (OS) of retinal double cone. I: inner segment. x 39700. 13) Strongly immunoreactive (IROS) and weakly positive or negative (INOS) pineal outer segments. x 31270

The monoclonal antiserum RET-2 stained rods, single cones and double cones in the retina of toad and tiger frog (Fig. 12). The immunoreaction of the double cones was medium intense. In the pineal organ, also this antiserum distinguished between either strongly or weakly immunopositive, and immunonegative outer segments (Fig. 13). Results of immunoblotting appear to indicate that the S-21/21 and RET-2 antibodies were raised to opsin (9). Obviously, the antisera recognize three kinds of pineal photoreceptors. Immunoelectron microscopic studies are in progress to correlate these elements with the rhodopsin/S-antigen-immunoreactive and -immunonegative photoreceptors of the pineal organ.

III. CONCLUSIONS

Our results show that the ultrastructural organization of a pineal (and retinal) cone outer segment is not always correlated with an antigenicity typical of a cone photopigment (17). These observations are in accord with results of Dodt (1) who found rhodopsin in cone-type retinas.

By means of the antibovine rhodopsin and S-antigen antisera three types of photoreceptors could be distinguished in the pineal organ: either strongly or weakly rhodopsin/S-antigen-immunoreactive and immunonegative ones. The strongly immunoreactive ones contain rhodopsin **or** porphyropsin accompanied by S-antigen, while the weakly immunoreactive ones are thought to represent elements with a less cross-reactive photopigment, presumably an iodopsin in toad as well as green and tiger frogs (28). Electrophysiological recordings also revealed light absorption maxima of similar photopigments (λ500 nm and λ560 nm) in the pineal organ of the green frog, *Rana esculenta* (2).

The rhodopsin-immunonegative photoreceptors may elaborate some other photopigment, e.g., percepting in the violet, blue or red range of the light spectrum, since electrical recordings revealed light absorption maxima at λ355 nm, λ500 nm and λ570 nm in the pineal organ of the bull frog, *Rana catesbeiana* (5) and of λ355 nm, λ530 nm and λ620 nm in that of the pike, *Esox lucius* (4). Apparently, the different

types of pineal photoreceptors enable the animal to detect light of special ranges of the light spectrum, their quality obviously depending on the environmental life conditions.

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STRUCTURE OF TRANSDUCIN AND ITS RELATIONSHIP TO CELLULAR GUANINE NUCLEOTIDE-BINDING PROTEINS

Bernard Kwok-Keung Fung
Samuel E. Navon

Jules Stein Eye Institute
University of California
Los Angeles, California

I. INTRODUCTION

Visual transduction in vertebrate retinal rods has been proposed to involve a sequence of first-order processes with which a signal generated at the disk membrane of the outer segment (ROS) is amplified and then conveyed to the plasma membrane (Baylor et al., 1979; Matthews and Baylor, 1981). An attractive scheme that can account for such a mechanism is outlined in Fig. 1. The primary event is the photo-isomerization of the 11-cis-retinal chromophore of rhodopsin to all-trans-retinal. A series of protein conformational changes then occurs, resulting in the formation of an active intermediate of rhodopsin (R^*) at the disk membrane. The first active state of rhodopsin is most probably Metarhodopsin II (Emeis, et al., 1982; Pfister, et al., 1983). It transiently interacts with transducin (T), a GTP-binding regulatory protein associated with the disk membrane surface (Fung, et al., 1981; Kuhn, 1980). As a result, hundreds of transducin-GTP complexes are formed in a catalyzed GDP-GTP exchange reaction (Fung and Stryer, 1980). Transducin-GTP then switches on a cGMP-specific phosphodiesterase (PDE) by counteracting the action of an inhibitory subunit (Fung, et al., 1981; Hurley and Stryer, 1982). Hence, signal amplification is achieved in two stages: a first stage gain of 10^2 due to the formation of hundreds of activated PDE, and a second stage gain of 10^3 due

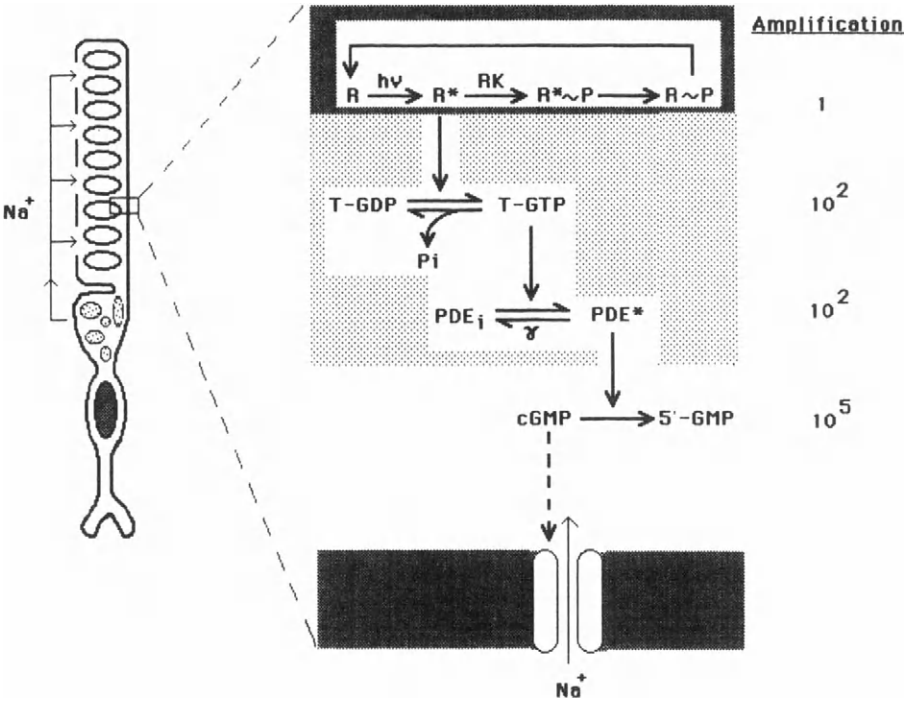

Fig. 1. A summary of the cGMP cascade of vision. Solid areas at the top and bottom of the diagram represent the disk membrane and the plasma membrane, respectively. The dotted area denotes disk membrane surface. The broken arrows designate biochemical pathways that have not yet been elucidated. Abbreviation: R, rhodopsin; R*, photolyzed rhodopsin; R~P, phosphorylated rhodopsin; T, transducin; PDE, phosphodiesterase; γ, phosphodiesterase inhibitor; RK, rhodopsin kinase.

to the rapid hydrolysis of thousands of cGMP per sec by an activated PDE enzyme (Baehr, et al., 1979; Yee and Liebman, 1978). cGMP is the proposed internal transmitter (reviewed by Hubbell and Bownds, 1979). The mechanism by which cGMP controls the Na^+ permeability of the plasma membrane, however, is still not clear. Recent evidence has suggested that it may directly regulate an ion channel located in the plasma membranes of ROS (Fesenko, et al., 1985; Koch and Kaupp, 1985; Yau and Nakatani, 1985).

Three processes are known to terminate the PDE activity and restore the cGMP concentration to that of the dark level. First, PDE is switched off when GTP bound to transducin is

hydrolyzed to GDP (Fung, et al., 1981). Second, the active form of the photolyzed rhodopsin is inactivated by phosphorylation (Liebman and Pugh, Jr., 1980). This inhibitory regulation is most probably mediated by the binding of a 48-kDa protein to phosphorylated opsin (Kuhn, et al., 1984; Pfister, et al., 1985). Finally, the system is reset to the dark-adapted state when photolyzed rhodopsin is regenerated by recombining with 11-cis-retinal chromophore, and when the depleted cGMP is replenished by the action of a guanyl cyclase.

Our interest in the light-activation of PDE is further stimulated by the finding that transducin is structurally related to other guanine nucleotide-binding proteins, including N proteins of the adenylate cyclases, ras proteins, the elongation factors EF-Tu and EF-G, and the initiation factor IF2 (Hurley, et al., 1984; Medynski, et al., 1985; Tanabe, et al., 1985; Yatsunami and Khorana, 1985). The available data suggest that they are members of a family of regulatory proteins and may have evolved from a common ancestral protein. Hence, a considerable amount of our effort has been directed at determining the structure of transducin and its relationship to other GTP-binding regulatory proteins. Structural comparisons of these proteins with transducin may help to elucidate their functions in mediating signal coupling. Here, we describe briefly the subunit composition of transducin, focusing on evidence that establishes the locations of its functional domains. We then present our results on the immunological and functional similarities between transducin and the guanine nucleotide-binding proteins of the adenylate cyclases.

II. FUNCTIONAL DOMAINS OF TRANSDUCIN

A. Subunit Composition

Transducin is the first signal-carrying intermediary protein in the cGMP cascade (see Figure 1). It is composed of two distinct subunits: T_α and $T_{\beta\gamma}$. The T_α subunit of transducin (Mr=39,000) binds GTP and GDP, and in its GTP form acts as an activator of the phosphodiesterase (Fung, et al., 1981). It contains sites for cholera toxin-catalyzed (Abood, et al., 1982; Navon and Fung, 1984) and pertussis toxin-catalyzed ADP-ribosylation (Manning, et al., 1984; Van Dop, et al., 1984a), and cysteine residues that are sensitive

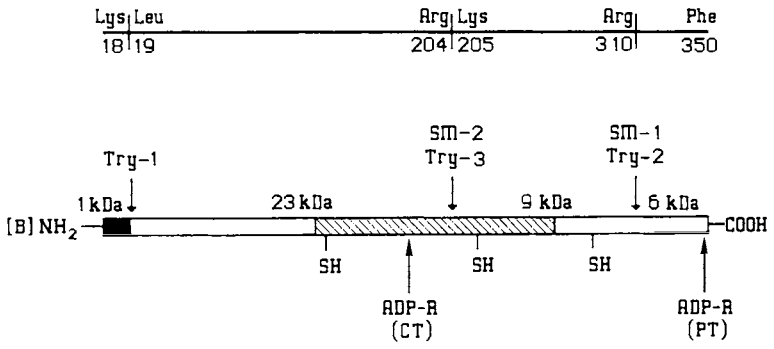

Fig. 2. Schematic diagram of T_α. Alignment of the four proteolytic fragments generated by tryptic digestion (try) and Submaxillarus protease (sm) are indicated. Their amino and carboxyl terminal residues are given above. Sites accessible to ADP-ribosylation by cholera toxin (C.T.) and pertussis toxin (P.T.) are designated by the bottom arrows. Accessible sulfhydryl groups are represented by SH. The hatched and dark areas denote domains postulated to interact with guanine nucleotide and $T_{\beta\gamma}$, respectively.

to sulfhydryl reagents (Ho and Fung, 1984a). Labeling of these sites has been shown to markedly alter the GTPase activity of transducin. More detailed information on the locations of these sites will be described in the next section.

The $T_{\beta\gamma}$ subunit of transducin is a heterodimer consisting of two polypeptides (Mr = 36,000 and 8,000). Its does not directly participate in GTP hydrolysis or phosphodiesterase activation, but is required for the binding of T_α to photolyzed rhodopsin (Fung, 1983). In this regard, it probably serves to modulate the T_α activity in ROS. The amino acid sequence of the T_γ polypeptide has been determined by the Edman procedure (McConnell, et al., 1984; Ovchinnikov, et al., 1984) and from the nucleotide sequence of cDNA clones (Hurley, et al., 1984a; Van Dop, et al., 1984c). It is 73 amino acid residues long and contains an unusually high proportion of charged residues. There are 3 residues of cysteine. Only one is accessible to chemical modification under denaturing conditions (unpublished observation), suggesting T_γ contains a disulfide.
Little is known about the structure or the amino acid sequence of the T_β polypeptide. There are five sulfhydryl groups, of which only two are accessible to modification

under nondenaturing conditions (Ho and Fung, 1984). Trypsin splits T_β into two tightly associated fragments (Fung and Nash, 1983); each contains an accessible sulfhydryl group. The activity of $T_{\beta\gamma}$ is not affected by the covalent modification of its sulfhydryl groups, nor by tryptic digestion of the T_β polypeptide (Ho and Fung, 1984).

B. Alignment of the Tryptic Fragments

Complementary DNA encoding the T_α subunit of transducin has been cloned and sequenced (Medynski, et al., 1985; Tanabe, et al., 1985; Yatsunami and Khorana, 1985). The deduced polypeptide sequence consists of 350 amino acid residues and has a molecular weight of 39,971. Our earlier experiments have shown that trypsin cleaves T_α at three specific sites and generates four proteolytic fragments of apparent molecular weights of 1, 6, 9, and 23 kDa (Fung and Nash, 1983). Comparison of the known partial amino acid sequences of the 6, 9 and 23 kDa-fragments (Hurley, et al., 1984b) with the amino acid sequence deduced from the cDNA indicates that tryptic sites are at positions Lys-18, Arg-204, and Arg-310. We have also arrived at this same conclusion based on N- and C-terminal analyses of the individual tryptic fragments, and from additional limited digestion with Submaxillarus protease (Ho and Fung, 1984b). The alignment of the tryptic fragments is shown schematically in Fig. 2. This information, together with the results obtained from our previous labeling studies, allows us to identify several possible functional domains within T_α.

C. Guanine Nucleotide Binding Domain

T_α contains several regions that exhibit significant homology to sequences of other known guanine nucleotide-binding proteins such as ras proteins, elongation factors EF-Tu and EF-G, and initiation factor IF (Medynski, et al., 1985; Tanabe, et al., 1985; Yatsunami and Khorana, 1985). The most significant homology exists between residues 31 to 49 near the the N-terminus (region 1), residues 208 to 222 (region 2), and residues 259 to 269 (region 3). Region 1 is located in the 23-kDa fragment at 12 amino acid residues from the first trypsin-sensitive site, and regions 2 and 3 are located in the 9-kDa fragment (Fig. 2). Levinson and coworkers (1984) have demonstrated that the substitution of

valine for glycine at position 12 of c-Ha-ras1 (corresponding to the glycine residue at position 38 of T_α) markedly inhibits the ability of the ras protein to hydrolyze bound GTP. Thus this region of T_α, like that of ras protein, probably controls the hydrolysis of bound GTP. An earlier study showing that bound Gpp(NH)p, (a nonhydrolyzable analog of GTP), alters the rate of tryptic digestion at Lys-18 near this region (Fung and Nash, 1983) has provided additional support for this assignment. The other two regions (2 and 3) in the 9-kDa fragment most likely participate in guanine nucleotide binding since the corresponding regions in EF-Tu, EF-G, and IF-2 have been shown to form part of the guanine nucleotide-binding site (Leberman and Egner, 1984). There is also additional biochemical evidence of support. For example, it is known that the tryptic site (position 204) adjacent to region 2 and a sulfhydryl group in the 9-kDa fragment are blocked by bound Gpp(NH)p (Fung and Nash, 1983; Ho and Fung, 1984a). Furthermore, the 9-kDa fragment has been specifically labeled by bound 8-azido-GTP, a photoaffinity analog of GTP with the reactive nitrene at the purine moiety (Ho and Fung, 1984b).

D. The Reactive Sulfhydryl Groups

T_α contains three accessible cysteine residues which have been shown to play an important role in transducin activation (Ho and Fung, 1984a). Proteolytic analysis of the labeled protein indicates that two are in the 9-kDa fragment and one is in the 23-kDa fragment (Ho and Fung, 1984b). Bound Gpp(NH)p blocks two sulfhydryl groups from chemical modification; of these one is in the 9-kDa fragment, the other in the 23-kDa fragment (Fig. 2).

E. The ADP-ribosylation Sites

Both cholera toxin and pertussis toxin catalyze the transfer of ADP-ribose from NAD to T_α. The modified protein exhibits a markedly reduced GTPase activity (Abood, et al., 1982; Navon and Fung, 1984). The ADP-ribose inserted by cholera toxin is covalently linked to Arg-174 located in the 23-kDa fragment (Navon and Fung, 1984; Van Dop, et al., 1984b). In contrast, the target for ADP-ribosylation by pertussis toxin is within the 6-kDa fragment at a region near the carboxyl terminus (Ho and Fung, 1984b; Manning, et al.,

1984). The amino acid residue that is ADP-ribosylated has not been definitively identified. One report indicated that it was an asparagine residue corresponding to position 346 (Manning, et al., 1984). However, the cDNA sequence predicts an aspartic acid residue at that position (Medynski, et al., 1985; Tanabe, et al., 1985; Yatsunami and Khorana, 1985). It seems that Cys-347, which was not previously detected, is likely to be the modified residue.

F. The $T_{\beta\gamma}$ Binding Domain

ADP-ribosylation of T_α by pertussis toxin requires a stoichiometric amount of $T_{\beta\gamma}$ (see Fig. 5). This interesting observation enables us to identify a region of T_α that may be involved in $T_{\beta\gamma}$ binding. We showed that tryptic digestion of T_α rapidly removed a fragment from the N-terminal region (Fung and Nash, 1983). The remaining 38-kDa fragment cannot serve as a pertussis toxin substrate even in the presence of $T_{\beta\gamma}$ (unpublished observation). Since the pertussis toxin ADP-ribosylation site is at the carboxyl terminal region, our result suggests that the N-terminal region of T_α may participate in $T_{\beta\gamma}$ binding. There is other evidence to substantiate our assignment of this region to $T_{\beta\gamma}$ binding. Thus, for example, the 38-kDa fragment cannot bind photolyzed rhodopsin in the presence of $T_{\beta\gamma}$ (Fung and Nash, 1983), suggesting the absence of subunit interaction. Furthermore, the binding of GTP to a GTPase site nearby (region 1) leads to the dissociation of $T_{\beta\gamma}$ from T_α (Fung, 1983). Interestingly, the N-terminal region of T_α contains a strongly hydrophilic segment (residue position 8 to 28) with alternating sets of positively and negatively charged residues extending into the GTPase site (see Medynski, et al. for the amino acid sequence). It seems possible that the local conformation of this region could be altered by the binding of a negatively charged GTP (as evidenced by a change in susceptibility to trypsin), which then leads to a decrease in subunit interaction. In this context, it is worthy to note that the ras proteins, which lack the charged terminal region, are not ADP-ribosylated by pertussis toxin even in the presence of $T_{\beta\gamma}$ (personal communication). In contrast, the α subunit of N_o which contains an almost identical charged terminal region (Hurley, et al., 1984b), exhibits a similar requirement for $T_{\beta\gamma}$ in pertussis toxin-catalyzed ADP-ribosylation (see Fig 5).

G. Other Domains

At present, available information does not permit the positive identification of regions in T that interact with PDE or rhodopsin. It is known that the loss of both N- and C-terminal regions does not significantly affect T_α's ability to activate PDE (Fung and Nash, 1983), suggesting the PDE binding site resides within the mid-region of the molecule. Also, very little is known about the rhodopsin binding site. We have previously shown that the cooperative interaction between T_α and $T_{\beta\gamma}$ is required for the binding of transducin to photolyzed rhodopsin (Fung, 1983). This result implies that T_α may contain only part of a rhodopsin binding site. Significant advances in defining the rhodopsin-binding domain may have to await information on the amino acid sequence of T_β, which is now being determined.

III. SIMILARITIES BETWEEN TRANSDUCIN AND GTP-BINDING PROTEINS OF ADENYLATE CYCLASES

A. GTP-binding Regulatory Proteins of Adenylate Cyclases

The protein architecture and signaling mechanism of the light-stimulated phosphodiesterase system in retinal rods are remarkably similar to that of the hormone-regulated adenylate cyclase system (Bitensky, et al, 1981). In both systems, the signal generated by the interaction of a ligand to a receptor is amplified and then transferred to a catalytic enzyme via a GTP-binding regulatory protein. However, unlike PDE which is always activated by light in the presence of GTP, adenylate cyclases are under both positive and negative control by hormones and GTP (Rodbell, 1980). Such opposing regulations appear to be mediated via stimulatory (N_s) and inhibitory (N_i) GTP-binding proteins which are linked to distinct classes of hormone receptors (Gilman, 1984). More recently, Sternweiss and Robishaw (1984), and Neer, et al. (1984) have independently isolated from bovine brain a third GTP-binding protein termed N_o. Its function, however, remains to be elucidated.

Similar to transducin, the N proteins of the adenylate cyclases are heterotrimers consisting of α, β, and γ polypeptides (reviewed by Gilman, 1984). The structure of the β subunit of these proteins is very similar, if not

identical to that of T_β (Manning and Gilman, 1984). The GTP-binding α subunits, although similar in many ways, do differ in size, amino acid composition, and function (Gilman, 1984). $N_{s\,\alpha}$ is heterogeneous in size with molecular weights ranging from 42 to 52 kDa (Ross and Gilman, 1980). It contains sites for cholera toxin-catalyzed ADP-ribosylation, and this covalent modification markedly reduced its ability to hydrolyze bound GTP. Stimulation of cAMP synthesis appears to be due to a direct interaction of the active $N_{s\alpha}$-GTP complex with the catalytic subunit of adenylate cyclase. In contrast, $N_{i\,\alpha}$ and $N_{o\,\alpha}$ have molecular weights of

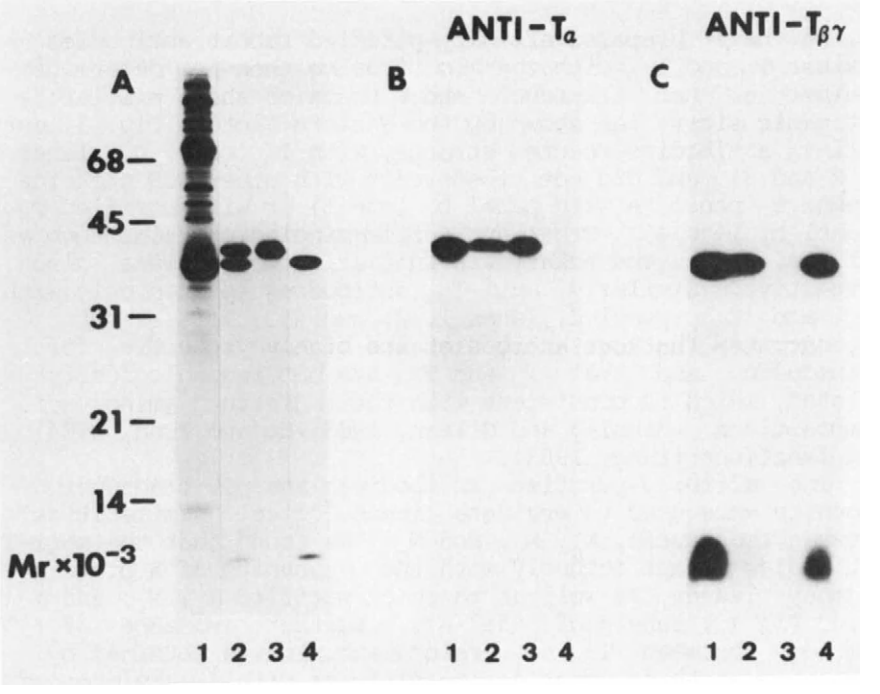

Fig 3. Specificity of affinity purified anti-T_α and anti-$T_{\beta\gamma}$ antibodies. ROS membrane proteins (lane 1), transducin (lane 2), T_α (lane 3), and $T_{\beta\gamma}$ (lane 4) were separated by SDS-polyacrylamide gel electrophoresis and visualized by staining with coomassie blue (panel A). The amount of proteins applied to lanes 1, 2, 3, and 4 were 50, 20, 12, and 12 μg, respectively. Immunoreactive polypeptides were identified by western blotting of these samples with anti-T_α (panel B) or anti-$T_{\beta\gamma}$ (panel C) followed by ^{125}I-protein A. The amount of proteins applied onto the gel shown in panel B and C were 200-fold less then those shown in panel A.

41 and 39 kDa, respectively (Katada, et al., 1984a and b; Sternweiss and Robishaw, 1984; Neer, et al., 1984). Both are substrates of ADP-ribosylation catalyzed by pertussis toxin. Inhibition of adenylate cyclase activity is thought to be due to a dissociation of the $N_{i\alpha}$-GTP complex from the β subunit, which then confers an inhibitory effect on $N_{s\alpha}$ (Gilman, 1984).

B. Immunological Similarity between Transducin and N-protein

We have prepared affinity-purified rabbit antibodies against T_α and $T_{\beta\gamma}$ with the aim of using them to determine whether or not transducin and N proteins share similar antigenic sites. As shown by the Western blot in Fig. 3, our anti-T_α antibodies reacted strongly with T_α (panel b, lanes 1, 2 and 3), and did not cross-react with other ROS proteins (compare panel A with panel b, lane 1) or with purified $T_{\beta\gamma}$ (panel b, lane 4). Other nucleotide-binding proteins such as tubulin, actin, and eukaryotic initiation factor were also unreactive. Similarly, anti-$T_{\beta\gamma}$ antibodies reacted only with T_β and T_γ (panel C, lanes 1, 2, and 4). This result demonstrates that our antibodies are highly specific for transducin, and that T_α and $T_{\beta\gamma}$ are not immunologically related, which is consistent with their distinct amino acid compositions (Manning and Gilman, 1983; Ho and Fung, 1984) and functions (Fung, 1983).

The affinity-purified antibodies against transducin subunits were used to evaluate immunological similarities between transducin, N_s, N_i, and N_o. We found that the anti-T antibodies react strongly with the β subunits of N proteins in many tissues, as well as those of purified N_s, N_i, and N_o (e.g. Fig 4., lane 3 of panel A). Further evidence of homology between T_β and brain β subunit was obtained by subjecting both polypeptides to cleavage with Staphylococcus aureus V8 protease and by analyzing the proteolytic peptides with Western blotting. The antibodies were found to recognize the same proteolytic fragments, indicating that T_β and brain β subunit contain very similar, if not identical, antigenic determinants. This observation is consistent with that reported by Manning and Gilman (1984), who found that the peptide maps of transducin and N_i are almost identical.

Our anti-T_α antibodies fail to recognize liver $N_{s\alpha}$, brain $N_{s\alpha}$ or liver $N_{i\alpha}$ (data not shown), but readily react with brain N_o (Fig. 4, lane 4 of panel A). $N_{o\alpha}$ is found to have properties very similar to T_α. It contains a region

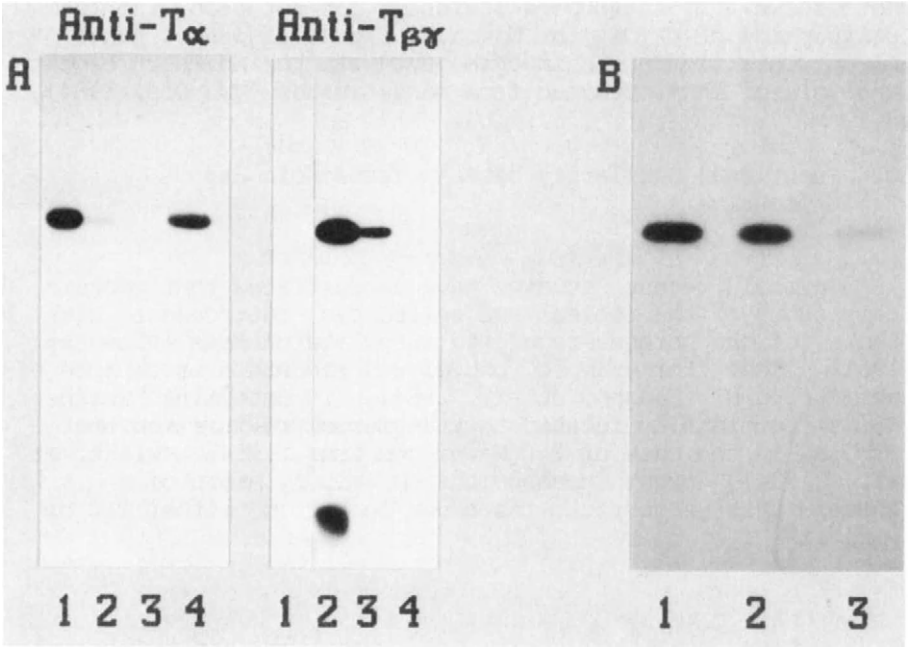

Fig 4. Recognition of $N_{0\alpha}$ and $N_{0\beta}$ by anti-transducin antibodies. Panel A: Immunoblot analysis of T_α (lane 1), $T_{\beta\gamma}$ (lane 2), $N_{0\beta}$ (lane 3), and $N_{0\alpha}$ (lane 4) with anti- T_α antibodies (left panel) and anti- $T_{\beta\gamma}$ (right panel). Panel B: Immunoprecipitation of ADP-ribosylated T_α (lane 1) and $N_{0\alpha}$ (lane 2). Method: Transducin and N_0 were ADP-ribosylated with pertussis toxin in the presence of [^{32}P]NAD. The reaction was terminated by adding an excess amount of NAD. The labeled proteins were then incubated with affinity purified anti-T_α antibodies (1 µg/ml in phosphate buffered saline containing 2% bovine serum albumin) at 23° C for 1 h, and then immunoprecipited with 100 µl of 10% pansorbin. Lane 3 represents a control sample containing 50 µg/ml of pre-immune Ig and labeled transducin.

homologous to T_α (Hurley, et al, 1984), exhibits GTPase activity, and is a substrate of ADP-ribosylation catalyzed by pertussis toxin (Sternweiss and Robishaw, 1984; Neer, et al., 1984). Moreover, the ADP-ribosylated $N_{0\alpha}$ can be immunoprecipitated by anti-T_α antibodies, but not by nonimmune serum (panel B of Fig. 4).

In contrast to the widespread occurence of T_β-like and T_α-like proteins in other tissues, we found that T_γ is specifically localized to the outer segment of the rod (data

not shown). Antibodies against T_γ do not recognize the γ polypeptide of N_s, N_i, or N_o (e.g. Fig 4, lane 3 of panel A). This finding is in agreement with the Northern blot analysis of mRNA obtained from many tissues (Van Dop, 1984).

C. Functional Similarity between Transducin and N-proteins

Several recent studies have demonstrated that protein components of the retinal PDE system can interchange with those of the hormone-regulated adenylate cyclase (Bitensky, 1981). Thus, for example, photolyzed rhodopsin is capable of activating N_i (Kanaho, et al., 1984). To determine whether N_o is functionally related to transducin, we surmised that $T_{\beta\gamma}$, as in the case of T_α (Navon and Fung, 1984; Hewlett, et al., 1984), might promote the ADP-ribosylation of $N_{o\,\alpha}$. Indeed, this is correct. As shown in Fig. 5, either $T_{\beta\gamma}$ or

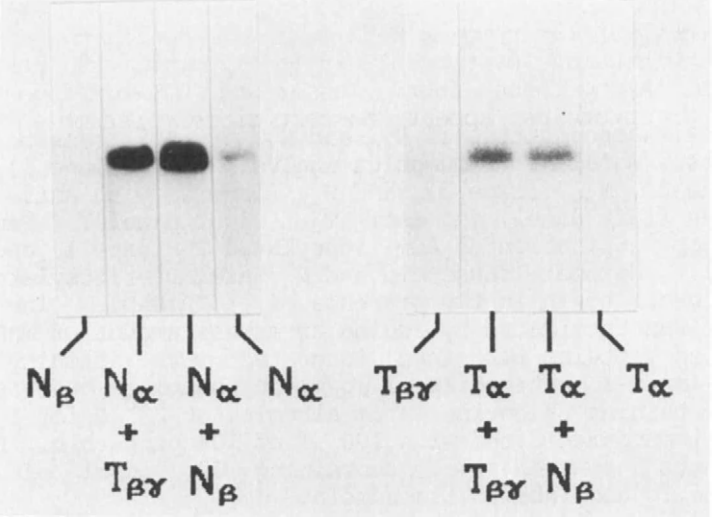

Fig. 5. Pertussis toxin-catalyzed ADP-ribosylation of reconstituted subunits of transducin and N_o. Purified subunits of transducin and N_o were mixed and ADP-ribosylated with pertussis toxin in the presence of [^{32}P]NAD. The labeled proteins were then subjected to SDS-polyacylamide gel electrophoresis followed by autoradiography.

brain $N_{o\beta}$ can enhance the ADP-ribosylation of both $N_{o\alpha}$ and T_{α}. This result suggests that, at least in terms of pertussis toxin-catalyzed ADP-ribosylation, the subunits of transducin and N_o are functionally interchangable.

IV. SUMMARY

Several lines of evidence have suggested that transducin belongs to a super-family of GTP-binding regulatory proteins known to consist of ras proteins, elongation factors, and N proteins of the adenylate cyclases. Here we describe two sets of studies which provide additional insight into structures that are common to these classes of proteins. A comparison of their amino acid sequences and functional domains indicates that they share a strikingly similar guanine nucleotide-binding region. However, significant differences are found in other regions of the molecules. These regions most likely are not directly involved in nucleotide binding, but might reflect the specificity of various GTP-binding proteins towards different cellular components. Among these four classes of GTP-binding proteins, N proteins appear to most closely resemble transducin. They share an almost identical β subunit, contain similar functional domains, and both serve as information-carrier proteins. In this context, the results of our immunological study and reconstitution experiment imply that N_o, by analogy with transducin, may also serve in signal coupling.

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LIGHT ACTIVATION OF PHOSPHOLIPASE C IN FROG ROD OUTER SEGMENTS[1]

Abdallah J. Ghalayini
Robert E. Anderson

Cullen Eye Institute
Baylor College of Medicine
Houston, Texas

Receptor-activated phosphoinositide turnover is a process associated with a variety of cellular responses mediated by a wide variety of cell-surface receptors (1,2,3). A common feature of these receptors is that they elicit their respective intracellular responses through calcium as a second messenger. Several lines of evidence have demonstrated that one of the early events associated with receptor activation is the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3) (4,5,6). Thus, occupation of a receptor by its appropriate ligand is thought to cause the activation of phosphoinositide-specific phospholipase C. The hydrolysis products generated by phospholipase C action on PIP_2, DG and IP_3, have potent intracellular effects. The former can activate a calcium- and phospholipid-dependent protein kinase, while the latter can mobilize calcium from non-mitochondrial intracellular stores (7,8).

Hagins and Yoshikami (9) postulated that calcium is an internal messenger in vertebrate photoreceptors responsible for hyperpolarization of the plasma membrane following photon capture. Although evidence now strongly implicates cyclic GMP in this response (10), changes in photoreceptor calcium levels are still thought to occur following photon capture. Acute changes in photoreceptor calcium

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levels may be associated with metabolic events other than transduction, such as control of protein phosphorylation and guanylate cyclase activity. Thus, we were curious to determine if the reported light-driven changes in calcium concentration in photoreceptor outer segments were mediated via the phosphoinositide effect.

In an attempt to study the effect of light on phosphoinositides in ROS, a series of experiments was conducted using dark-adapted frog retinas. Retinas were prelabeled in Ringer's bicarbonate-pyruvate buffer with either ^{3}H-myo-inositol or ^{32}P-orthophosphate for 2.5-4 hrs in the dark. After washing the retinas with fresh, unlabeled Ringer's buffer, they were exposed to 5 or 15 second flashes of light. The retinas were quenched with 20% trichloroacetic acid and were shaken in a vortex mixer for 1 minute. The supernatants obtained from such preparations following a low speed spin contained predominantly rod outer segments as judged by phase contrast microscopy. Upon lipid extraction and quantitation of radioactivity in the ROS, a significant decrease in the amount of label in PIP_2 relative to total lipid radioactivity was found in light-exposed retinas when compared to the dark controls (Tables I & II). Such an observation (11) is indicative of the presence of a light-activated PIP_2-specific phospholipase C in frog ROS. In other experiments (Ghalayini and Anderson, unpublished results) using inner segment-outer segments (IS-OS) prepared by gentle shaking of ^{32}P-prelabeled retinas in buffer, the effect of light on PIP_2

TABLE I. Effect of a Five Second Light Flash on Retinal Phospholipids Prelabeled with [^{32}P]PO_4

Lipid Class	Ratio		P-Value
	Dark (n)	Light (n)	
PIP_2/Total Lipid	0.352 $\pm$ .059 (14)	0.308 $\pm$ .040 (14)	$<$0.025
PIP/Total Lipid	0.145 $\pm$ .034 (18)	0.151 $\pm$ .043 (18)	$>$0.30
PI/Total Lipid	0.273 $\pm$ .057 (18)	0.267 $\pm$ .037 (18)	$>$0.35
PA/Total Lipid	0.101 $\pm$ .032 (18)	0.106 $\pm$ .028 (18)	$>$0.30
PC + PE/ Total Lipid	0.143 $\pm$ .038 (14)	0.152 $\pm$ .038 (14)	$>$0.30

Ratios given represent the mean $\pm$ S.D. of three experiments conducted as described in Methods.

TABLE II. Effect of a Fifteen Second Light Flash on Retinal Phosphoinositides Prelabeled with myo-[2-^{3}H] Inositol

Lipid Class	Ratio		P-Value
	Dark (n)	Light (n)	
PIP_2/PI	0.260 ± .082 (9)	0.164 ± .051 (9)	< 0.01
PIP/PI	0.236 ± .056 (13)	0.230 ± .047 (12)	> 0.4

Ratios given represent the mean ± S.D. of two experiments conducted as described in Methods.

radioactivity was not always found. The inability to consistently observe a light effect may be due to leakiness of the (IS-OS) preparations, allowing for rapid changes in intracellular concentrations of Ca^{+2}, nucleotide triphosphates, cyclic nucleotides, or other water soluble components. Some of these components can have pronounced effects on both the dark current and light sensitivity (12,13).

Using frog ROS prepared by sucrose flotation and subsequently labeled with γ-^{32}P-ATP in the presence of exogenously added PIP, Hayashi and Amakawa (14) reported the reduction of label in PIP_2 following a 5 sec light flash. The other two lipids labeled under these conditions, PIP and PA, were not changed by this light flash. Miller and Hawthorne (15) reported a similar observation using bovine ROS preparations.

The observed activation of PIP_2-specific phospholipase C is indicative that this enzyme may play an important role in coupling the excitatory light stimulus to the intracellular response. The question of whether this coupling encompasses an intracellular effect of IP_3 on Ca^{+2} mobilization or of DG on protein kinase C is virtually unknown for the vertebrate photoreceptor. While the function of PIP_2 hydrolysis is still open to speculation, a mechanism for the regulation of phospholipase C comes to mind which is not entirely speculative. Recent evidence (17,18,19,20) suggests that GTP-binding proteins may be involved in the activation of a phosphoinositide-specific phospholipase C in several cell types exhibiting enhanced phosphoinositide turnover upon stimulation. ROS contain transducin, a very well studied regulatory GTP-binding protein. A great deal is known about the regulation of c-GMP phosphodiesterase by transducin and its resolved subunits (21,22). However, information on the possible interaction of transducin and phospholipase C is completely lacking. In light of evidence presented here and recent

reports on the regulation of phospholipase C by GTP-binding proteins, phospholipase C in the ROS may be a candidate for regulation by transducin or another GTP-binding protein.

Recent efforts in our laboratory have focused on characterization and regulation of phospholipase C. These studies indicate that the enzyme is present in purified ROS from both frog and bovine retina (16). Moreover, the enzyme shows a high degree of specificity for PIP_2 and PIP as substrates (16). A soluble form of the enzyme has been obtained from ROS by hypotonic and isotonic washes (23). Studies of this soluble form of phospholipase C may help elucidate any function the enzyme might play in ROS.

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