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CELL PHYSIOLOGY OF THE PINEAL BODY

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INTRODUCTION

In the last few years this laboratory developed a research program to identify electrophysiologically functional analogies between pineal and retinal photoreceptors in fishes (see also 47). In the first part of this report we shall briefly describe the results of these studies, already published elsewhere (41-43,46), while in the following section we introduce some new concepts consequential to our studies and indicating extremely interesting research paths.

Pineal receptor cells share both embriological and fundamental ultrastructural parallelism (Fig. 1) with retinal photoreceptors, but develop in two disparate regions of the head, each characterized by distinct visual properties: photoreceptors in the retina are part of a cellular mechanism subserving contrast detection with a high degree of visual acuity; pineal receptors on the other hand are submerged in a totally opaque medium which should restrict their activity to luminosity detection. Thus the principal question we posed was whether pineal receptor cells had developed special transduction properties to match their specific functional role.

It was also important to consider that pineal receptor cells produce an hormon, melatonin (MLT), with circadian rhythmicity (58) which, at least in fish ought to be regulated by the pineal photoreceptor responses to illumination (cfr. 4). Furthermore, various authors indicate MLT as an intracellular calcium regulator and a potential antioxidant agent (65). Here we discuss how these MLT influences could be advantageously experimented on retinal photoreceptors to elucidate their mechanism of action.

I. *Cell electrophysiology.*

We observed that the responses of pineal cells to brief flashes are similar to those recorded from retinal cone cells of the same species, except for a slower time course (Fig. 2). The average membrane potential recorded from dark adapted pineal photoreceptors was -23 mV. Brief light flashes produced hyperpolarizing responses of increasing amplitude with increasing intensities. The relation between amplitude response and light intensity is described by a hyperbolic function, a modified form of the Michaelis-Menten equation:

$$\frac{V}{V\,\max} = \frac{I^n}{I^n + I_o^n} \qquad (1)$$

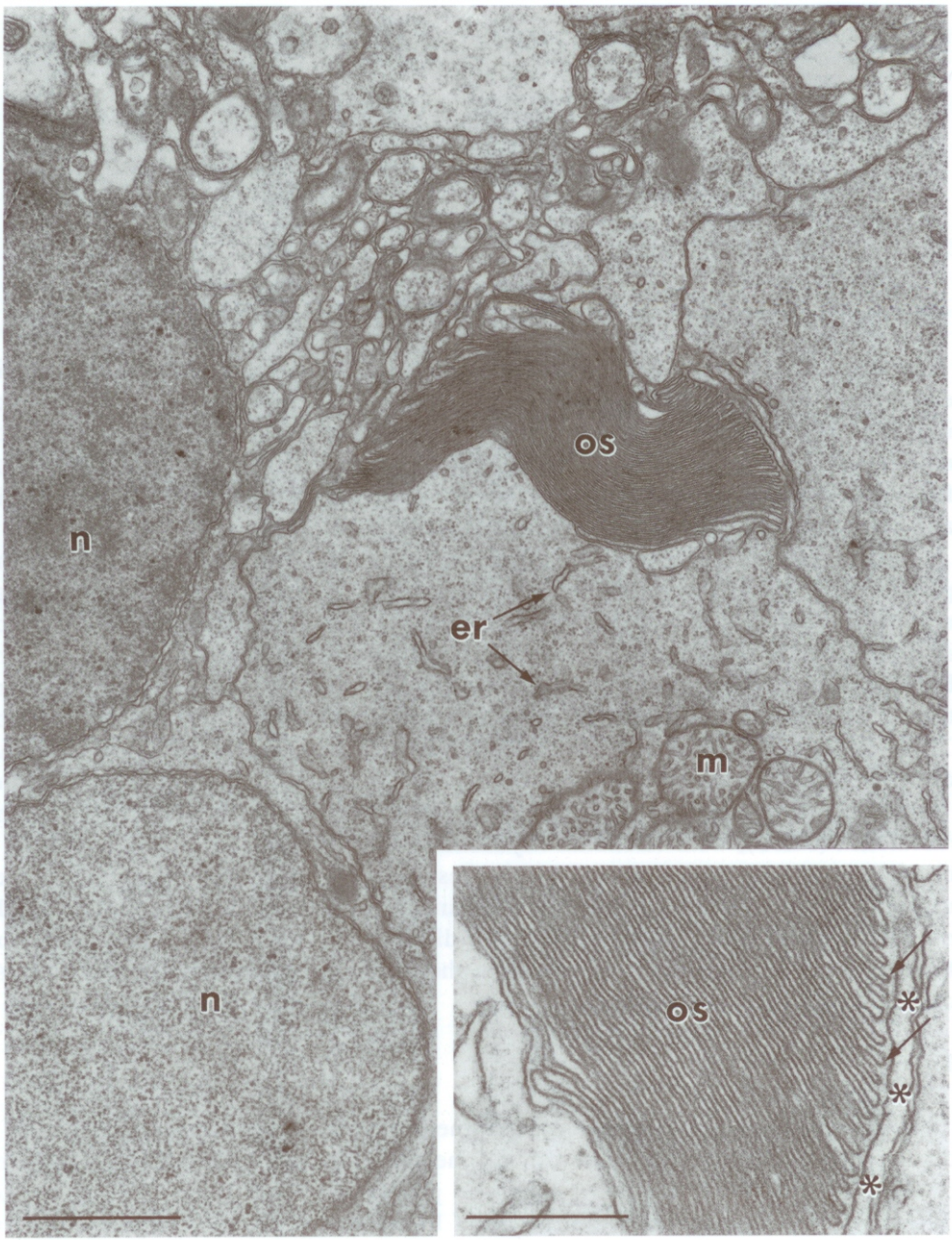

Fig. 1. - *Electron micrograph illustrating the fine structure of pineal photoreceptor cells.*

Eache cell is characterized by the presence of a short, multilamellar outer segment (os) connected to a region rich in endoplasmic reticulum (er), ribosomes and mitochondria (m). N: nuclei of adjacent photoreceptors. Bar is one micrometer. Inset: outer segment lamellae are opened to the extracellular space (arrows), like in retinal cones photoreceptors. Asterisks mark caliceal process. Bar is 350 nanometers.

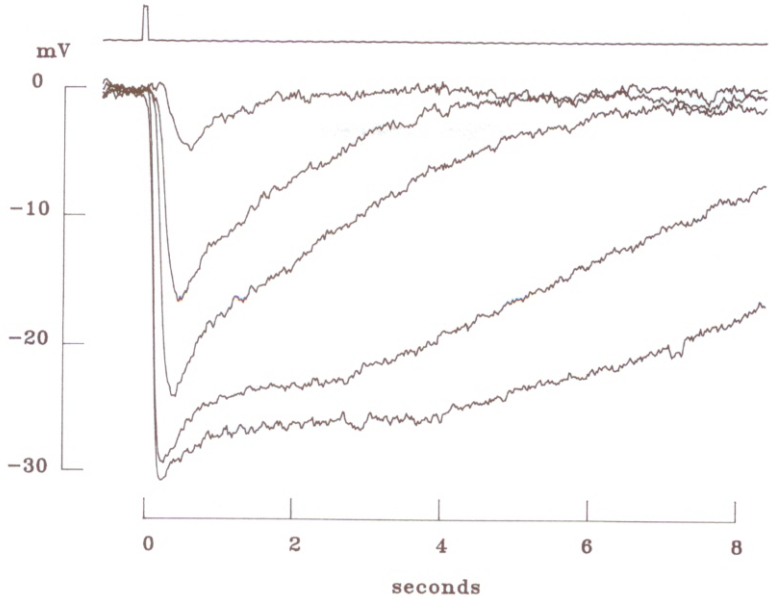

Fig. 2. - *Superimposed voltage responses of an identified pineal photoreceptor to 50 ms white flashes of increasing intensity ($2.4x10^4$, $1x10^5$, $3.4x10^5$, $6x10^6$ and $8x10^6$ ph μm^{-2} sec^{-1}).*

Zero in the ordinate represents the value of dark membrane potential. The upper line indicates the time of illumination.

where V is the amplitude in mV with maximum V_{max}, I the flash intensity and I_0 the half saturating intensity. The exponent n is a sign of cell sensitivity. Data are best fitted by a n value ranging from 0.7 to 0.85, indicating that the dynamic range of pineal photoreceptors spreads over more than four logarithmic units of light intensity (Fig. 3), a value about twice as wide as that of cones.

A significant difference between pineal and retinal receptor cells appears when comparing the time to peak of their respective photoresponses. Thus, the pineal response to near threshold light intensities shows time to peak up to 1.2 sec, decreasing to about 0.25 sec for saturating responses (Fig. 4). These values are greater than for retinal cones, which show values ranging from about 200 ms to 80 ms going from threshold to saturating intensities, respectively (5). Light induced membrane conductance changes recorded under voltage clamp indicate a conductance decrease of 187 pS during illumination. This value amounts to about 10% of the absolute membrane conductance calculated from the dark current just before the test flash. The extrapolated potential at which the photocurrent reverses is about 60 mV above the dark potential, indicating that an ionic mechanism similar to that in retinal photoreceptors may be involved (12, 44, 61). Further analogies with retinal phototransductive mechanism are also observed by adding 50 mM 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, to the bathing solution (11). This produces an about twofold increase in amplitude of the

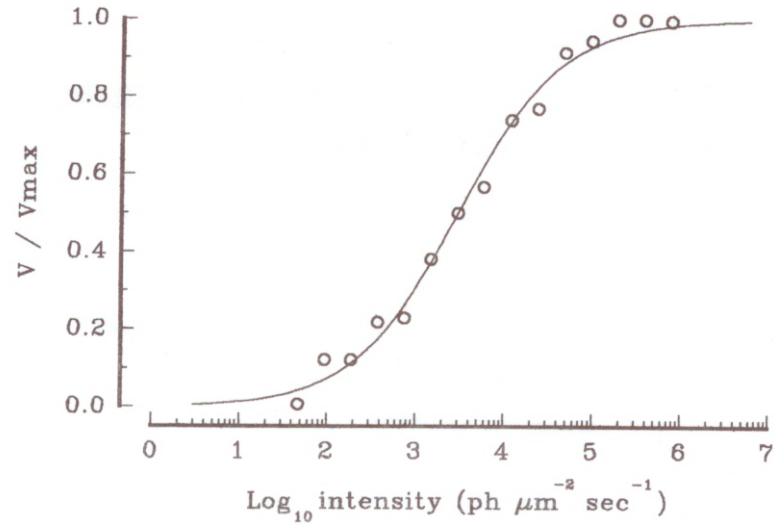

Fig. 3. - *Stimulus-response relation of pineal photoreceptors.*

Data were obtained by averaging the normalized peak responses from 31 cells (empty circles). The continuous line represents the equation (1). Absciassae indicate logarithmic units of light intensity (photons/area time).

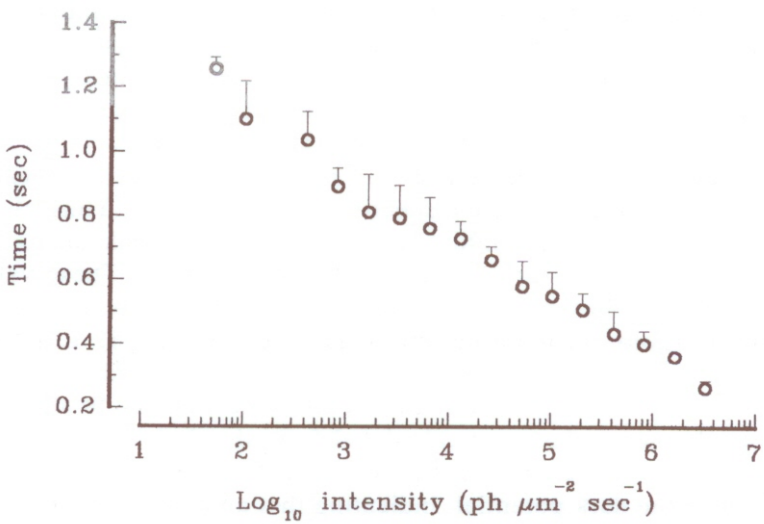

Fig. 4. - *Time to peak of pineal responses to 50 ms flashes of increasing intensity.*

Empty circles indicate the averaged data from 11 cells. Bars plotted upward are s.d. values. Abscissae indicate logarithmic units of light intensity (photons/area time).

photoresponse associated to a 25% increase of the time to peak. These results are qualitatively comparable with those observed in amphibian retina (11), thus supporting the hypothesis that cGMP regulates the pineal photoreceptor membrane permeability to Na^+ ions, as in the retina (28, 29, 33, 73).

A typical feature of pineal photoreceptors consists in their responses to prolonged illumination which does maintain the same amplitude throughout the whole period of stimulation, independently of the light intensity used (Fig. 5). Thus, these cells do not show the typical time dependent changes in cell sensitivity observed in retinal photoreceptors (13, 27). Similarly, the amplitude and kinetics of the superimposed flash pineal responses, obviously depressed as function of the background light, are not modified for the whole period of background illumination.

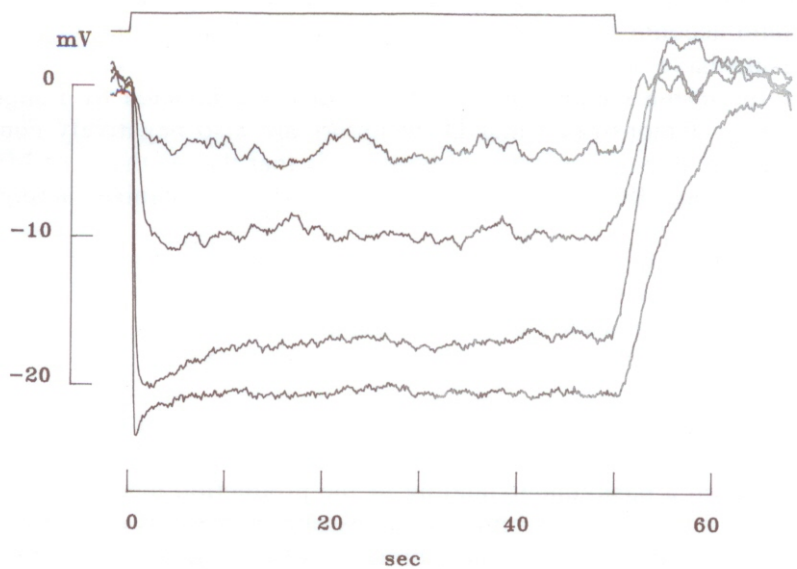

Fig. 5. - *Superimposted pineal photorespones to prolonged illumination of increasing intensities ($5x10^2$, $1.1x10^3$, $2x10^4$ and $3.2x10^4$ ph μm^{-2} sec^{-1}).*

Zero in the ordinate indicates the dark membrane potential. The upper line represents the time of illumination.

The fact that pineal receptor cells maintain the same response amplitude during sustained illumination has an important functional implication with respect to their secretory role, which is obviously dominated by complex sets of voltage sensitive processes. The fix relationship between the level of ambient illumination and the receptor cell membrane potential, typical of a luminance detector, thus reveals a fine control of the hormone synthesis by absolute levels of light intensity.

II. *The light dependent control of pineal MLT.*

The previous set of results indicated that photoreceptors activity is adequate to control the process of MLT synthesis within a wide range of slowly changing illumination, from threshold to saturating light.

The obvious next target was to investigate the mechanism linking the light-induced variations of membrane potential to the biochemistry of MLT inside the photoreceptor. MLT derives from 5HT reacting in the presence of two regulatory enzymes, N-acetyl-transferase (NAT) and 5-hydroxy-O-metiltransferase (5-HIOMT) whose activation is known to depend on cAMP (see 71). Thus blockage of cyclic-AMP (cAMP) formation would clearly lead to halting MLT synthesis. Since the D_2 class of dopamine receptors are known to be negatively coupled to adenylate-cyclase (see 71, 72), a project was then initiated by one of us (B.L.) to verify the existance of this class of receptors in the photoreceptor cell membrane. Light is known to stimulate dopaminergic retinal cells which trough the activation of D_2 receptors could account for a drop of cAMP during illumination (35).

Recent results have shown the presence of D_4 receptors, a D_2 subtype, across the photoreceptors plasma membrane (16). Experiments conducted by Longoni and others (45) have demonstrated that D_4 receptors are also negatively coupled to adenylate-cyclase, thus supporting the hypothesis that the synthesis of MLT may be inhibited, during illumination by a dopaminergic mechanism acting on D_4 receptors.

In darkness however dopaminergic cells are no more activated, so that the inhibition of cyclase would be released leading to synthesis of MLT, a process potentiated by the increased Ca^{++} entry through light sensitive channels. Once produced, MLT may inhibit further dopamine syntesis (8).

III. *Prospective studies of mlt functions in retinal photoreceptors.*

Having achieved a basic understanding of the production mechanism of MLT, the action of this molecule upon biological substrates represents our next question. Indeed, MLT has been attributed many biological functions (2, 3, 25, 58, 60, 63, 64), however it still appears difficult to identify a common mechanism of action, perhaps due to the existence of at least two types of melatoninergic receptors bound to nervous membranes modulating adenylate cyclase (20), which may be activated concomitantly to a passive diffusion of the highly lipophilic MLT molecule across cell membranes.

More recent studies on MLT consisted in testing its reported "in vitro" antioxidant properties (53, 54, 66), on artificially induced cellular damages "in vivo" (1, 3, 48, 59). It has been shown that MLT exerts a very potent protective role against cellular damages induced by oxygen derived free radicals, among which the peroxyl and hydroxyl radicals play a major role as initiators of lipid peroxidation. Such an autocatalytic oxidative reaction plays a crucial role in membrane degeneration through a progressive fragmentation of the unsaturated fatty acid chains of

phospholipids with subsequent functional impairment of membrane proteins (26, 57, 69), eventually leading to the cell death. Since lipid peroxidation is often considered as a major contributor to neurodegenerative diseases (24, 30, 32, 36, 51, 54, 55, 67, 70), the finding of a potential, new endogenous antioxidant like MLT, devoid of appreciable side effects, should not be underestimated.

It would be restrictive to classify MLT just as an antioxidant agent, even if such property may be of prime importance for therapeutical use. Indeed, other functions of MLT have been described to be potentially important for the cell homeostasis, like those affecting the intracellular calcium concentration (68). MLT affects several cell components which play a key role in the regulation of internal free calcium turnover, like Ca^{++}-ATPase and calmodulin (14, 15). MLT shows high affinity binding to calmodulin (7) and, as other analogous substances do, it competes with calcium, thus preventing the Ca-calmodulin dependent activation of many enzymes, including phosphodiesterase. As a calmodulin antagonist, MLT also interferes negatively with microtubules assembly (6, 34) which spontaneously occurs during the continuous cytoskeletal rearrangement observed under physiological conditions. Moreover, the hypothesis may be advanced that both the activity of calcium as a second messenger and the calcium dependent direction of calcium flux across both inositol-1, 4, 5-trisphosphate (IP_3) and ryanodine-sensitive intracellular calcium stores (62) could be influenced by MLT.

By contributing to keep a low cytosolic calcium concentration, MLT negatively affects enzymatic Ca-dependent activities, including the Ca-calmodulin dependent Nitric Oxide Synthase (NOS) (9, 10, 39). A decrease production of NO induced by MLT has been recently described (56). In this context, MLT may be involved in reducing neurotoxicity induced by NMDA receptors (17, 18), since the latter activate NOS by increasing intracellular calcium concentration (50). An excess of NO, a very active free radical reacting with superoxide, produces peroxynitrates which may be fatally toxic to nerve cells (19). The concentration boundary where NO starts to be toxic is still ill defined, and further studies will be needed to clarify at what range of activity NOS may be kept under the influence of MLT.

It is surprising that neither of the two above mentioned activities of MLT, i.e. the antioxidant and that of calcium regulation, have not yet been examined in the cells of origin, the photoreceptors. This seems to us an interesting work to do, since photoreceptors combine their neuroendocrine indolaminergic function with phototransduction, a process involving both a great amount of oxidative phosphorylation and fundamental calcium dependent activities. In both pineal and retinal photoreceptors, the volumetric ratio between inner segment mitochondria and the remaining cellular organelles is as high as in no other nerve cell (43), suggesting a great number of oxidative processes generating oxygen derived free radicals, such as superoxide, hydroxyl and hydrogen peroxide (31). So the presence of an additional radical scavenger, like MLT added to other endogenous enzymes, like superoxide dismutase (SOD), catalase and gluthatione peroxidase may be beneficial for optimizing the nerve cell survival. Thus photoreceptors may result being equipped with a self-contained mechanism to produce and metabolize MLT (58), whose antioxidant

action seems particularly useful at night when the energetic demand for active transport may become more pressing (19 bis).

It would be particularly interesting to verify, on retinal photoreceptors, the ability of MLT to modulate calcium concentration. In physiological conditions, intracellular calcium acts upon guanylate cyclase whereby cGMP production is reduced at night, as calcium enters through the light sensitive channels, but it increases during illumination, as the intracellular calcium lowers, thus becoming a major factor for entraining a state of light adaptation (37, 38). Thus, by indirectly modulating the cGMP dependent membrane conductances MLT could be an important regulator of photoreceptor sensitivity to light.

A further mechanism by which MLT may influence photoreceptor activity is by suppressing production of NO which is known to modulate photoreceptor ion channels (40). In particular, MLT is expected to suppress the NO induced increase both of the diltiazem sensitive current and of the voltage dependent Ca conductance, the latter being further associated to facilitation of synaptic transmission between photoreceptors and second order neurons.

In conclusion, the vast knowledge on photoreceptors properties would clearly facilitate the interpretation of the possible changes induced by artificially varying the levels of MLT and to identify its mechanism of action (49). Indeed, the possible effects produced by MLT on photoreceptors may be due to some of its general properties potentially applicable to other cell types as well, if one considers that MLT receptors are distributed over numerous regions of the CNS (21-23) and in cells of other tissues (52).

SUMMARY

The results from recent experiments on the cellular physiology of the trout pineal photoreceptors are briefly reviewed. The arguments are mainly concerned with pineal phototransduction. These studies have stimulated further research on melatonin, a molecule produced in pineal as well as in retinal photoreceptors. A discussion follows on our actual research object, that is a study of the influences of endogenous melatonin upon retinal receptor cells activities.

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