Neuroendocrinology Letters ISSN 0172–780X

ORIGINAL ARTICLE

Wavelength dependency of light-induced changes in rhythmic melatonin secretion from chicken pineal gland in vitro.

Valér Csernus, Péter Becher & Béla Mess

Department of Anatomy, University Medical School Pécs, H-7624 Pécs, Hungary

Correspondence to: Valér Csernus, M.D., Ph.D., Department of Anatomy, University Medical School Pécs, H-7643 Pécs, Hungary
TEL: +36 72 326222 ext. 2053; FAX: +36 72 326244
E-mail: csvaler@apacs.pote.hu

Submitted: *July 25, 1999*
Accepted: *August 9, 1999*

Key words: **chicken pineal; in vitro; melatonin; light wavelength; circadian rhythm; perifusion**

Neuroendocrinology Letters 1999; ***20****:299–304 pii: NEL200599A02*

Abstract Avian pineals show rhythmic, circadian melatonin secretion pattern also *in vitro*. The phase of this rhythm can be modified by changing the illumination. Reversed *in vitro* illumination reverses the phase of the circadian melatonin rhythm in two days. In the present study the effect of the wavelength on the speed of the phase shift of the melatonin rhythm of the explanted chicken pineals was investigated in a dynamic *in vitro* bioassay. Chicken pineals were placed into perifusion columns and the tissue was exposed to reversed illumination through various light filters. Changes in the melatonin release during 4 day long experiments were studied. Clear differences in the speed of the reversal of the melatonin rhythm were observed as a function of the transmission spectrum of the light filters. The shorter the transmitted wavelength, the more rapid the phase shift was found. These data are in good agreement with earlier studies showing that the chicken pineal photo-pigment, pinopsin, is a blue light sensitive molecule. Our observation reveals that the blue light sensitive pigment is not only present but also fully functional in controlling the circadian biological clock in the chicken pineal gland.

Introduction

In the last decades a large number of publications have reported the importance of light in the maintenance and regulation of pineal function in a variety of species. The interrelationship between light and the pineal is one of the most unequivocally confirmed facts in pineal physiology. However, very limited data are available on the wavelength of the light to which the pineal gland is the most sensitive. Most authors agree that only the visible portion of the light spectrum can influence pineal activity. Within this range, the pineal-influencing activity of the light decreases with the increase of wavelength: blue light is the most potent, while red light has no effect on the pineal gland. Cardinali et al. [1, 2] reported the above sequence of effectivity of the different wavelength of light on the Hydroxy indole-O-Methyl Transferase (HIOMT) activity in the pineal gland of rats. Similarly, N-Acetyl-Transferase (NAT) activity and melatonin production of the rat, hamster and mouse pineal gland also showed parallel decrease with the increase of wavelength [3]. In night peak of melatonin with blue, green and yellow light or "near ultraviolet" light provoked a 40% decrease, while red light was ineffective [4, 5]. Blue light proved to be 20-25% more potent than the green light [3]. Similar findings were reported on human volunteers [6]. Also, an evoked (EEG-like) activity was also elicited in the pineal gland of the rat by blue, green and yellow lights, but not by red [7, 8].

All the above experiments were performed *in vivo* on mammals. Nelson and Zucker [9] and Gross and van der Kooy [10] stated that there are no functioning extra-retinal photoreceptors in mammals. The light control of the circadian rhythm of the mammalian pineal gland is mediated exclusively by the retina. This observation explains why only visible light influences the activity of the mammalian pineal gland.

On the other hand, light receptors were demonstrated by morphological methods in the pineal gland of a variety of sub-mammalian species from the teleost up to the birds [11, 12, 13, 14, 15]. The photosensitive pigment in the avian pineals was named pinopsin [16, 17].

Our previous studies revealed that the chicken pineal photoreceptors are functionally coupled to the intracellular melatonin synthesizing machinery in the chicken pineal gland. The circadian rhythm of melatonin secretion of the explanted, perifused pineal gland can be influenced by changes in the lighting schedule [18, 19, 20]. These experiments were performed with neutral, white light. The sensitivity of these pineal light receptors to the different wavelengths of light, however, has not been investigated. The aim of the present study is to show the role of the different wavelengths of light in the regulation of melatonin secretion and circadian rhythm of the perifused chicken pineal gland. This might be a direct approach of the differential light sensitivity of the avian pineal photoreceptors. Since *in vitro* reversed illumination of the chicken pineals was found to be the most suitable model to study the effects of the light on the rhythmic melatonin secretion, to pursue our goals, explanted chicken pineals were exposed to reversed illumination through various light filters.

Materials and methods

Chemicals and instruments.

Organic and inorganic chemicals, including Medium 199 (M-5017), BSA (A-7888), Gentamycin (G-3632) and Sephadex G-10 (G-10-120), were purchased from Sigma Chemical Co. For light filters, household cellophane foils were stained with microscopic stains: Victoria blue (Gurr. Co.), Trypanrot and Smaragdgrün (both from Dr. Gübler & Co.). To increase the stain-absorbency, the cellophane foils were coated with gelatine; then they were soaked into the stains dissolved in 40% aqueous ethanol. After drying them at room temperature, the light transparency spectrum of the filters was determined with a Beckman DU-64 spectrophotometer. The intensity of the light on the surface of the perifusion columns was measured with a PU150 Luxmeter (Metra Blansko Co.).

Perifusion.

Four to five-week-old white Leghorn chicken were used for our experiments. The animals were kept in our facilities for at least two weeks before the experiment under controlled light (light on from 6 A.M. to 8 P.M.). Food (wheat and corn) and water were available ad libitum. The chicken were sacrificed with decapitation on Mondays at 1 P.M. Their pineal glands were removed, cut into 4 pieces and placed into perifusion columns. Perifusion experiments were carried out in a system similar to that described earlier [21, 22]. Briefly: Pieces of the pineal gland of one chicken, mixed with Sephadex G-10, were transferred onto superfusion columns. The glass perifusion columns were placed in a dark room and illuminated with a 18 W white fluorescent light during the light periods. Medium-199-based tissue culture medium, supplemented with 1 g/l BSA and 50 mg/l Gentamycin, was passed through the columns at

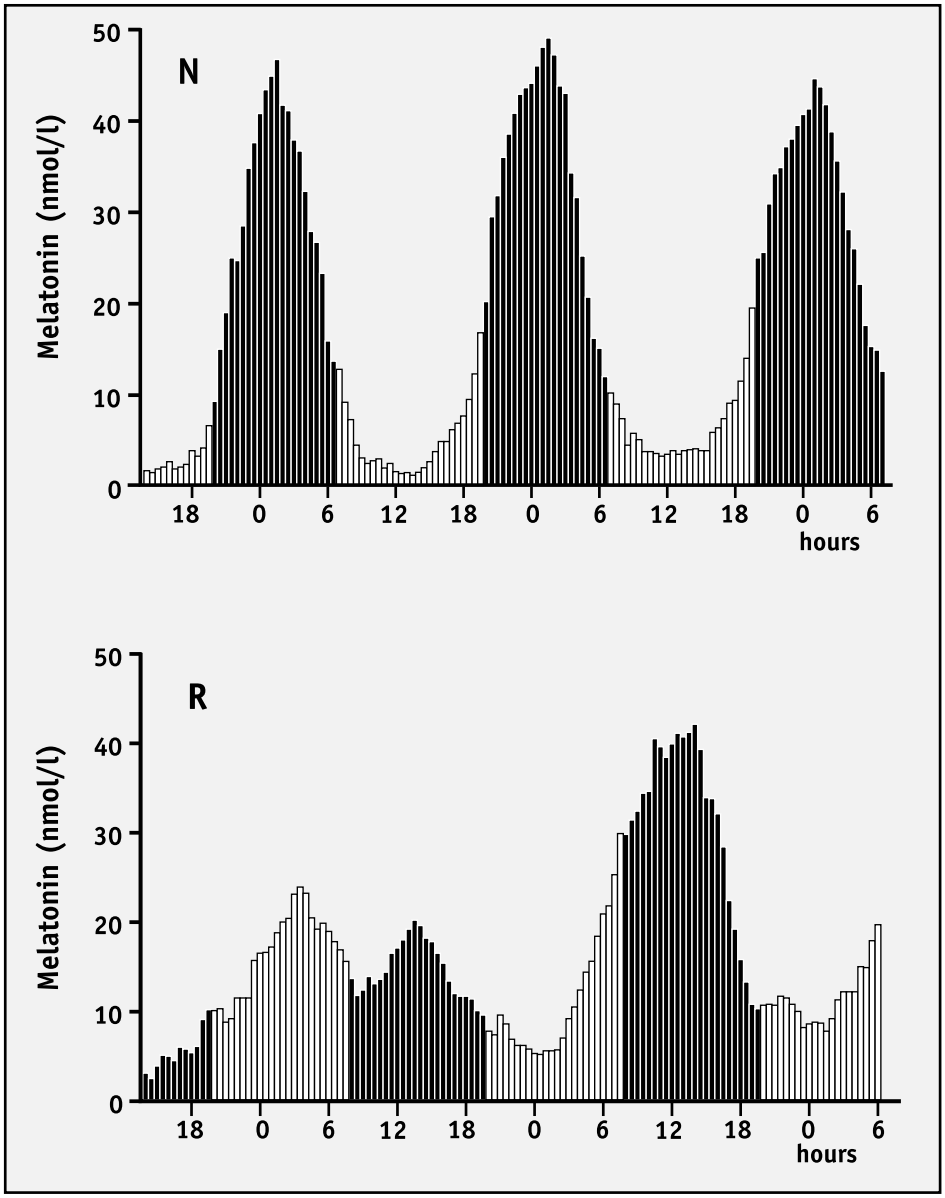

Fig. 1. Melatonin release from explanted chicken pineals exposed to normal (**N**, environmental light, on between 7 A.M. and 8 P.M.) or reversed (**R**, 18 W flourescent light, 1200 lux, on between 8 P.M. and 8 A.M.) illuminations. The columns on the plots represent melatonin concentration of consecutive 30 minute samples. On the horizontal axis, the real time-of the-day is indicated. Black columns show the time of the dark periods.

a rate of 6 ml/h. Starting immediately after the column preparation, 3 ml (30 minutes) fractions were collected from the effluent media for four days. In each experiment, 2 columns (parallel experiments) were used and 180 fractions were collected from each column. Melatonin contents of the fractions were determined by RIA as described earlier [22]. Washout studies indicated that melatonin passes rapidly through the column; more than 90% passes within 5 minutes.

RIA results were further analyzed with a dedicated computer program [21]. In the figures, representative examples of 2 to 4 identical experiments, with visually the same results, are shown.

Results

The effects of reversed illumination on melatonin secretion from explanted chicken pineals.

Chicken pineals were placed into perifusion columns and exposed to normal (Fig. 1. N) or reversed (Fig. 1. R) illumination during 4 day experiments. Normal *in vitro* illumination was served by natural, environmental light through the laboratory window facing north. Lights on (more than 10 lux on the surface of the glass columns) was between 7 A.M. and 8 P.M. at the time of these experiments. For reversed *in vitro* illumination, the columns were covered with a thick, black cloth during the daytime and illuminated with a 18 W white fluorescent light between 8 P.M.

and 8 A.M. The light-intensity on the surface of the columns was 1200 lux.

Under normal, daily light rhythm, the explanted chicken pineals released melatonin in an evident circadian rhythm with the zenith in the middle of the dark period. The increase of the melatonin level, however, apparently started already around the middle of the light period. Similarly, the fall of the melatonin level began still in the dark (Fig. 1 N). Reversed *in vitro* illumination deeply modified the melatonin rhythm. During the first day, in spite of the illumination, the melatonin concentration started to increase, although the peak was well below the dark level of the controls. In the middle of the second day, the dark period already resulted in a secondary peak. On the third day, an apparently complete reversal of the melatonin rhythm was observed; at noon, in the middle of the dark period, a large melatonin peak was obtained similar in size to that in the middle of the dark period of the normal *in vitro* cycle.

Light-transparency spectrum of the filters

The relative light-transparency spectrum of the homemade cellophane filters are plotted on Figure 2. Victoria-blue was transparent in the 420-560 nm range, Smaragdgrün in the range of 480-620 nm and Trypanrot in the range of 690 to 800 nm. The transparency window of the three filters span well the visible light spectrum.

The effects of the filtered light on the reversal of the in vitro melatonin rhythm of the explanted chicken pineal glands.

Explanted chicken pineals were exposed to reversed illumination in experiments similar to those described above for the reversed illumination experiment. This time the perifusion columns were also wrapped into stained cellophane filters. The light absorbency of the filters were compensated in each case by moving the light source until 1200 lux light intensity was resulted on the surface of the columns behind the filters.

In all groups, a complete reversal of the daily melatonin rhythm was obtained in three days (Fig. 3). However, the speed of the modifications was apparently different. Behind the red filter, during the first light

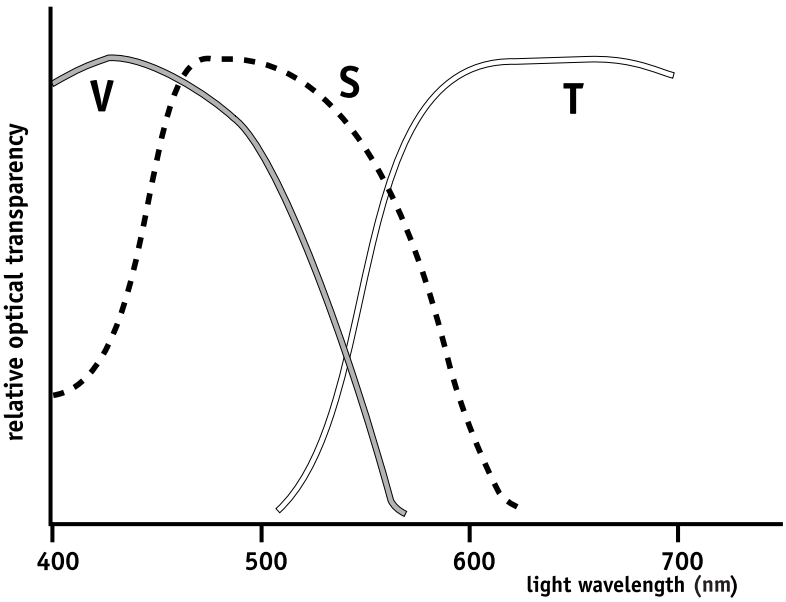

Fig. 2. Light transparency spectra of the filters used in the experiments. Relative optical transparencies of the cellophane filters stained with Victoria blue (V), Smaragdgrün (S) and Trypanrot (T) are plotted.

period (8 P.M. to 8 A.M.), a large melatonin peak was detected (Fig. 3 R). This peak was apparently larger than that obtained in the similar phase of the normal, natural reversed illumination. A similar peak was just detectable behind the green filter (Fig. 3 G) and completely missing behind the blue filter (Fig. 3 B). During the next dark phase (between 8 A.M. and 8 P.M. on the second day) a melatonin peak was detected. This peak was small under red and much larger under green and blue filters. On the third day in the middle of the dark phase, a large melatonin peak was measured in each case.

Discussion

Unlike mammalian, avian pineals show circadian melatonin secretion patterns also *in vitro* [19, 23]. This rhythmic melatonin secretion is primarily controlled by a circadian "biological clock" [24]. The complete biological clock resides inside the avian pineal, thus it is fully functional *in vitro*. For its genetically determined development no circadian changes in the environment are required [20]. While the frequency of the circadian clock in the explanted chicken pineal can be altered only within very narrow limits, its actual phase can be shifted easily with alteration of the *in vitro* illumination pattern [18, 20]. Reversed *in vitro* illumination has the most striking effect on the phase shift of the circadian pacemaker of the explanted pineal. The perifusion method is suitable to follow subtle changes in the melatonin secretion from explanted avian pineals for several days [22, 19]. This is why perifusion experiments on chicken pineals, exposed to reversed illumination, were applied in this

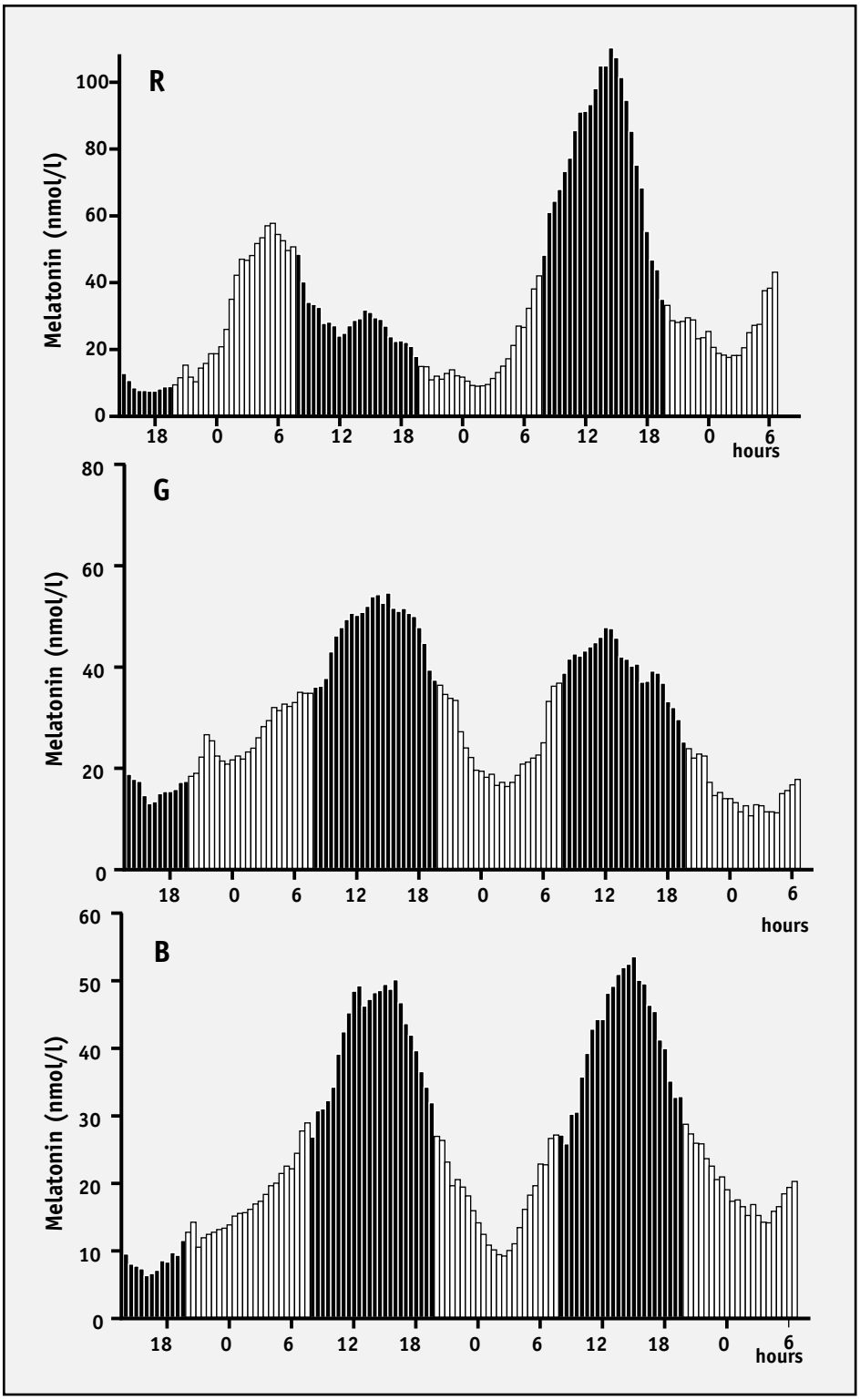

Fig. 3. Melatonin release from explanted chicken pineals exposed to reversed illumination (18 W flourescent light on between 8 P.M. and 8 A.M.) through red (R, Trypanrot), green (G, Smaragdgrün) or blue (B, Victoria blue) filters. The light intensity on the surface of the columns was set to 1200 lux. The columns on the plots represent melatonin concentration of consecutive 30 minute samples. On the horizontal axis, the real time-of the-day is indicated. Black columns show the time of the dark periods.

study to investigate the effect of the wavelength of the light on the circadian pacemaker in the avian pineal.

Reversed *in vitro* illumination resulted in a complete, 180 degree phase shift in the melatonin rhythm of the explanted chicken pineals in two days (Fig. 1 R). Filtered light with different dominant light spectrum (Fig. 2) also resulted in a similar reversal of the melatonin rhythm, but the rate of the phase shift was apparently different (Fig. 3). Compared to changes induced by the normal, unfiltered light, the red light (Trypanrot filter) induced more limited changes on the second day; the night-time peak (during the first red-illuminated period) was higher than the peak on the following dark phase (Fig. 3 A). Green and especially blue light advanced the phase shift significantly. The night-time (illuminated phase) peak was completely missing under

blue, and just marginally present under green light, and there was a significant peak on the following dark period similar in size to the normal, dark peak.

These data suggest that the shorter the wavelength of the light the stronger the effect of the environmental light is on the circadian pacemaker of the avian pineals. Our results are in agreement with the findings of Okano et al. [16, 17] who identified the photosensor in the chicken pineal gland, the pinopsin, as a blue, light-sensitive photoreceptive molecule with an absorption maximum of 470 nm. The blue light sensitivity of the chicken pineal gland is also supported by the observation that the chicken pinopsin showed considerable cross-reactivity with the green-blue, light-sensitive photoreceptors of the reptiles using immunohistochemistry at light- and electron-microscopic levels [25, 26]. Data of our experiments reveal that this blue sensitive pigment is not only present but it is fully functional in the chicken pineal gland acting on the circadian biological clock controlling the melatonin secretion from avian pineal glands.

Acknowledgments

The authors are grateful to Beatrix Bruman and Gabriella Végi for their excellent technical assistance. The work was supported by grants from the Hungarian Science Foundation (OTKA T-017451 and T-029553).

REFERENCES

1 Cardinali DP, Larin F, Wurtman RJ. Control of the rat pineal gland by light spectra. Proc Natl Acad Sci USA 1972; **69**:2003–2005.
2 Cardinali DP, Larin F, Wurtman RJ. Action spectra for effects of light on hydroxyindole-O-methyl transferases in rat pineal, retina and Harderian gland. Endocrinology 1972; **91**:877–886.
3 Brainard GC, Barker FM. Regulation of pineal-reproductive axis by near ultraviolet radiation (UV.A) in three rodent species. In: Trentini G, DeGaetani C, Pevet P, editors. New York: Raven Press; 1987. p. 207–210.
4 Reiter RJ. The role of light and age in determining melatonin production in the pineal gland. In: Axelrod J, Fraschini F, Velo P, editors. New York: Plenum Press; 1983. p. 227–242.
5 Reiter RJ. Visible and non-visible electromagnetic field exposure: induced changes in pineal indoleamine metabolism. In: Foldes A, Reiter RJ, editors. London: John Libbely and Co. Ltd.; 1991. p. 111–121.
6 Brainard GC, Leny A, Menaker M, Frederickson R, Miller S, Webber R, et al. Effect of light wavelength on the suppression of nocturnal plasma melatonin in human volunteers. Ann NY Acad Sci 1985; **453**:316–378.
7 Schapiro S, Salas M. Effects of age, light and sympathetic innervation on electrical activity of the rat pineal gland. Brain Res 1971; **28**:47–55.
8 Reuss S. Electrical activity of the mammalian pineal gland. In: Reiter RJ, editor. New York: Alan R. Liss Inc.; 1987. p. 153–189.
9 Nelson RJ, Zucker I. Absence of extraocular photoreception in diurnal and nocturnal rodents exposed to direct sunlight. Comp Biochem Physiol A 1981; **69**:145–148.
10 Groos GA, van der Kooy D. Functional absence of brain photoreceptors mediating entrainment of circadian rhythms in the adult rat. Experientia 1981; **37**:71–72.
11 Dodt E. The parietal eye (pineal and parapineal organs) of lower vertebrates. In: Jung R, editor. Berlin-Heidelberg-New York: Springer; 1973. p. 113–140.
12 Ueck M, Ohnishi R, Wake K. Photoreceptor-like outer segments in the pineal organ of the lovebird, Urolоncha domestica (Aves: passeriformes). A scanning electron microscopic study. Cell Tissue Res 1977; **182**:139–143.
13 Vigh B, Vigh-Teichmann I. Light- and electron-microscopic demonstration of immunoreactive opsin in the pinealocytes of various vertebrates. Cell Tissue Res 1981; **221**:451–463.
14 Deguchi T Rhodopsin-like photosensitivity of isolated chicken pineal gland. Nature 1981; **290**:706–707.
15 Sun JH, Reiter RJ, Mata NL, Tsin AT. Identification of 11-cis-retinal and demonstration of its light- induced isomerization in the chicken pineal gland. Neurosci Lett 1991; **133**:97–99.
16 Okano T, Yoshizawa T, Fukada Y. Pinopsin is a chicken pineal photoreceptive molecule. Nature 1994; **372**:94–97.
17 Okano T, Takanaka Y, Nakamura A, Hirunagi K, Adachi A, Ebihara S, et al. Immunocytochemical identification of pinopsin in pineal glands of chicken and pigeon. Brain Res Rev 1997; **50**:190–196.
18 Ghosh M, Csernus V, Mess B. In vitro melatonin secretion pattern of the avian pineal gland with special reference to light reactivity. Neuroendocrinol Lett 1994; **3**:195–200.
19 Mess B, Rekasi Z, Ghosh M, Csernus V. Regulation of pineal melatonin secretion: comparison between mammals and birds. Acta Biol Hung 1996; **47**:313–322.
20 Csernus V, Ghosh M, Mess B. Development and control of the circadian pacemaker for melatonin release in the chicken pineal gland. Gen Comp Endocrinol 1998; **110**:19–28.
21 Csernus VJ, Schally AV. The dispersed cell superfusion system. In: Greenstein BD, editor. London: Harwood Academic Publishers; 1991. p. 71–109.
22 Rekasi Z, Csernus V, Horvath J, Vigh S, Mess B. Long-term dynamic in vitro system for investigating rat pineal melatonin secretion. J Neuroendocrinol 1991; **3**:563–568.
23 Ghosh M, Csernus V, Rekasi Z, Mess B. Comparative studies on the regulation of pineal melatonin secretion in mammals and birds. In: Haldar C, editor. New Delhi: Hindustan Publishing Corporation; 1996. p. 5–15.
24 Binkley SA, Riebman JB, Reilly KB. The pineal gland. A biological clock in vitro. Science 1978; **202**:1198–1200.
25 Vigh B, Rohlich P, Gorcs T, Manzano, Szel A, Fejer Z, et al. The pineal organ as a folded retina: immunocytochemical localization of opsins. Biol Cell 1998; **90**:653–659.
26 Fejer Z, Szel A, Rohlich P, Gorcs T, Manzano, Vigh B. Immunoreactive pinopsin in pineal and retinal photoreceptors of various vertebrates. Acta Biol Hung 1997; **48**:463–471.