Melatonin-Binding in the Frog Retina: Autoradiographic and Biochemical Analysis

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Binding of melatonin was examined in the retina of Rana pipiens. When intact frog retinas were incubated with 3H-melatonin and processed for autoradiography, most of the radioactivity was localized to the melanosomes of the retinal pigment epithelium-choroid (RPE-choroid) and to the outer plexiform layer of the retina. Melanosome-enriched fractions of the RPE-choroid and membrane-enriched fractions of the neural retina demonstrated saturable melatonin binding when incubated with increasing melatonin concentration. Thin-layer chromatography showed that >98% of the bound radioactivity was authentic melatonin. Scatchard analysis revealed a single population of binding sites with apparent Kd values of 6 × 10⁻⁷ M for both the RPE-choroid and neural retina. When various indole analogs were tested for their ability to inhibit ³H-melatonin binding to the neural retina, both 5-methoxytryptophol and 6-chloromelatonin demonstrated complete displacement of melatonin binding. Endogenous retinal melatonin levels were measured by radioimmunoassay. A twofold increase in melatonin levels was observed during the dark period with peak levels at 384.5 ± 28.8 pgms melatonin/pair retinas. Melatonin levels persisted in constant darkness, but were suppressed in constant light. Our data suggest that in the frog, the sites of action of retinal melatonin are the melanosomes of the RPE-choroid and the outer plexiform layer of the neural retina. Invest Ophthalmol Vis Sci 27:153-163, 1986

The indolamine hormone, melatonin, is synthesized in the vertebrate retina on a diurnal rhythm, with peak levels during the dark period.¹⁻³ Since the melatonin-synthesizing enzyme, hydroxyindole-O-methyltransferase (HIOMT) has been immunocytochemically localized to the photoreceptors,⁴ and melatonin immunofluorescence has been detected in the outer nuclear layer,⁵,⁶ the photoreceptors are considered to be the site of retinal melatonin synthesis. Several investigations have suggested that melatonin may be involved in photoreceptor outer segment disc shedding,⁷ retinal pigment epithelium (RPE) phagocytosis,⁸ photomechanical movements,⁹⁻¹¹ and modulation of neurotransmitter release.¹² The sites of action of melatonin in the retina, however, are not known. Specific melatonin binding has been detected in the cytosol of the rodent eye,¹³ and in the membrane-cytosol fraction of the teleost RPE-choroid.¹⁴ In this paper, we report the diurnal variation in melatonin content, and the localization of melatonin binding sites in the retina of Rana pipiens.

Materials and Methods

Melatonin Radioimmunoassay (RIA)

Adult Rana pipiens (West Jersey Biological; Wenonah, NJ) utilized for diurnal, constant light, and constant dark studies were entrained to a 12 h light:12 h dark cycle for at least 1 month, with a light intensity of 65 foot-candles during the photophase. For the diurnal study, groups of five frogs were killed by decapitation at 3-hr intervals over a 24-hr period. For the constant light and dark studies, groups of four frogs were killed at 4-hr intervals over a period of 3 days under continuous illumination or darkness. The animals were maintained at a constant temperature of 20°C. The eyes were enucleated, and RPE-choroids and neural retinas were separated and frozen immediately on dry ice. Tissues obtained during the dark period were dissected under a sodium vapor lamp. These investigations adhered to the ARVO Resolution on the Use of Animals in Research.

Individual neural retinas or RPE-choroids were placed in 0.2 mls of 0.01 M phosphate-buffered saline, 0.1% gelatin pH 7.4 (PBS-Gel), and sonicated at 50 watts for 5 sec with a Braunsonic 1510 sonicator (B. Braun Instruments; South San Fransisco, CA). Two, 0.1-ml aliquots of each tissue homogenate were assayed for melatonin according to the method of Rollag and Niswender.¹⁵ Briefly, melatonin was extracted into 2.0 mls chloroform, the aqueous phase was removed by
aspiration, then the volume of the organic phase adjusted to 1.0 ml by aspiration. The remaining chloroform was evaporated to dryness, and the residue resuspended with 0.5 ml PBS-Gel, and washed with 2.0 ml petroleum ether. The petroleum ether was removed by aspiration. To each sample was added 0.1 ml 125I-melatonin tracer (approximately 40,000 cpm; Meloy Laboratories; Springfield, VA), and 0.2 ml melatonin antibody (1:256,000 final dilution). The latter was generously provided by Drs. Mark Rollag and Gordon Niswender. The mixture was incubated for 48 hr at 4°C, then 3.0 ml cold 95% ethanol was added to precipitate the antigen–antibody complex. The samples were centrifuged at 2000 g for 30 min at 4°C, the supernatant decanted, and the gamma-.emissions of the precipitate quantified. Melatonin values were obtained from a standard curve.

3H-melatonin Autoradiography

3H-melatonin (2-aminoethyl-2-3H-N-acetyl-5-methoxy tryptamine; 26.4 Ci/m mole) was purchased from New England Nuclear (Boston, MA). Its radio-purity was checked by thin layer chromatography prior to use. Light- or dark-adapted adult Rana pipiens were killed by decapitation, and the eyes enucleated. The anterior segment and sclera were removed, and the retinas placed in modified Ringer’s solution consisting of 70 mM NaCl, 2 mM KCl, 2 mM CaCl2, 2 mM MgSO4, 1.25 mM KH2PO4, 35 mM NaHCO3, 10 mM glucose, and 10% fetal bovine serum (M. A. Bioproducts; Walkersville, MD). The choroid-retinas were then incubated in 3 ml modified Ringer’s solution containing 400 nM 3H-melatonin (10 μCi/ml) with or without 400 μM unlabeled melatonin (Sigma; St. Louis, MO) oxygenated with 95% O2 and 5% CO2 at 0°C for 1 hr in room light or darkness. After incubation, the tissue was placed in fresh, nonradioactive incubation medium, and the RPE-choroid was separated from the neural retina. The tissues were cut into 2 X 2 mm rectangles with a razor blade, placed onto perforated aluminum foil spatalas, and plunged into liquid nitrogen-cooled Freon 22 slush (Matheson; Cucamonga, CA). The frozen tissues were freeze-dried for 5 days at –50°C, exposed to osmium vapors under vacuum for 2 hr at room temperature, and vacuum-embedded in Spurr resin (Polysciences; Warrington, PA). Sections were cut at 0.5 μm thickness, collected on glass slides, and prepared for autoradiography by coating with Kodak NTB-2 emulsion (Rochester, NY) diluted 1:1 with distilled water at 40°C. The slides were stored at 4°C in light-proof boxes for several months. Autoradiograms were developed at 17°C in Kodak Dektol developer, and the neural retina sections stained with 1% Toluidine Blue, and RPE-choroid sections stained with 1% Basic Fuchsin. Quantification of melatonin binding was performed on a series of photomicrographs taken of the entire length of a retina section, extending from the ora serrata to the posterior fundus, and of micrographs of six separate sections from the same retina. This method of analysis provided comparison of melatonin binding in the various retinal layers of several areas of the same retina, and of similar areas of different retina sections. The retinas were subdivided into six layers, and each layer of each photomicrograph was individually quantified. The autoradiograms were quantified using a Microplan II Image Analysis System (Nikon; Garden City, NY).

Saturation Binding Assays

Melatonin binding assays were performed by a combination of methods used by Cardinali et al16 and Cohen et al13 with some modifications. Adult Rana pipiens were dark-adapted for one hr to facilitate separation of retinal layers. The frogs were killed, and neural retina and RPE-choroid isolated as described above. The tissues were sonicated at 50 watts for 30 sec in 10 volumes cold 50 mM Tris-HCl, 6 mM CaCl2, 0.1% ascorbic acid pH 7.4, then centrifuged at 3000 g for 5 min 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 27,000 g for 15 min at 4°C, then the pellet resuspended in assay buffer to yield a crude membrane fraction. Protein content was determined by the method of Lowry et al17 using bovine serum albumin (Sigma) as the standard.

Aliquots (4.0 ml) of tissue pellet suspension were incubated in triplicate for 5 hr at 0°C in darkness with 10–1000 nM 3H-melatonin with or without 100 μM unlabeled melatonin. The 3000 g tissue pellet incubations were terminated by centrifugation at 3000 g for 10 min at 4°C. The supernatant was aspirated, and the pellet resuspended in 0.8 ml 30% hydrogen peroxide, to bleach the pigment. After bleaching, 10 ml Aqueous Counting Scintillant (Amersham; Arlington Heights, IL) was added, and the radioactivity was measured by liquid scintillation spectrometry.

Tissue supernatant suspensions were incubated as described above for the tissue pellet suspensions. As a control, a parallel series of tubes containing buffer instead of tissue homogenate (blanks) were assayed. At the end of the tissue supernatant incubations, 0.8 ml of dextran-coated charcoal (5% activated charcoal, 2.5%
pellet were analyzed by electron microscopy. Tetroxide, and embedded in araldite 502 (Polysciences; Warrington, PA). Sections of the entire width of the membrane-enriched fraction was performed according to a modification of the method of Cotman and Flansburg. Retinal homogenate was centrifuged at 3000 g for 5 min, and the pellet was discarded. The supernatant was centrifuged at 27,000 g for 15 min, and the pellet was discarded. The supernatant was fixed at 27,000 g for 15 min, and the pellet was discarded. The supernatant was fixed in 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, then post-fixed in 1% osmium tetroxide, and embedded in araldite 502 (Polysciences; Warrington, PA). Sections of the entire width of the pellet were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, then post-fixed in 1% osmium tetroxide, and embedded in araldite 502 (Polysciences; Warrington, PA). Sections of the entire width of the pellet were analyzed by electron microscopy.

Electron Microscopic Analysis of Membrane Fraction

Electron microscopic analysis of the neural retina membrane-enriched fraction was performed according to a modification of the method of Cotman and Flansburg.19 Retinal homogenate was centrifuged at 3000 g for 5 min, and the pellet was discarded. The supernatant was centrifuged at 27,000 g for 15 min, and the pellet was fixed in 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, then post-fixed in 1% osmium tetroxide, and embedded in araldite 502 (Polysciences; Warrington, PA). Sections of the entire width of the pellet were analyzed by electron microscopy.

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 melatonin was extracted into chloroform and chromatographed on a Kodak silica gel TLC plate with fluorescent indicator in a chloroform-methanol (9:1, vol:vol) solvent system.

Competitive Binding Assays

Displacement studies similar to the method of Gern et al24 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 with or without 5 μM or 500 μM ouabain (Sigma) for 30 min at 20°C. After the preincubation with ouabain, the $^3$H-melatonin (46.9 Ci/m mole, New England Nuclear) and nonradioactive melatonin (for measurement of non-specific binding) was added, to result in a final concentration of 50 nM and 25 μM, respectively. The mixtures were incubated at 0°C or 20°C for 5 hr. Bound $^3$H-melatonin was separated from unbound melatonin as described above, then the radioactivity was measured by liquid scintillation spectrometry.

Results

Measurement of Endogenous Retinal Melatonin

Endogenous melatonin levels were measured by radioimmunoassay in the RPE-choroid and neural retina of Rana pipiens under a normal 12 h light:12 h dark lighting schedule. The diurnal variation in retinal melatonin content is shown in Figure 1. We interpret the degree of melatonin immunoreactivity obtained from the melatonin radioimmunoassay to represent endogenous levels of melatonin. The melatonin levels were low during the light period, but increased twofold during the dark period to a peak level of 384.5 ± 28.8 pgms/pair retinas at 18.00 hr. The levels of retinal melatonin at 15.00, 18.00, 21.00 and 24.00 hr were significantly higher ($P < 0.005$) than those at 03.00, 06.00, 09.00 and 12.00 hr. Melatonin levels appeared to rise before the onset of darkness (12.00 h), and continued to rise until mid-scotophase. Melatonin levels then decreased during the latter part of the dark period and continued to decrease until the latter part of the light period (09.00 h). The retinal melatonin content was higher at 12.00 h than at 09.00 h, but the difference was not statistically significant ($P > 0.05$). The level at 24.00 h was significantly lower ($P < 0.05$) than the level at 21.00 h, suggesting that the decrease in melatonin levels during the light period is not due solely to light inhibition. Melatonin levels in the RPE-choroid were below the range of detectability of this assay (<1.0 picogram/retina).

To determine the influence of environmental illumination on retinal melatonin levels, melatonin was measured in the neural retina of frogs maintained under constant illumination or darkness for 3 days. The re-
Fig. 1. Diurnal rhythm of melatonin in the retina of Rana pipiens. Each point is the mean of values from five animals (10 retinas) ± standard error of the mean. Animals killed in the light are indicated by the open circles, and the animals killed in the dark are indicated by closed circles. The period of darkness is indicated by the solid bar.

Fig. 3. Effects of 3 days constant light and constant darkness on frog retinal melatonin levels. Each point is the mean of values from four animals (8 retinas). Animals killed in the light are indicated by the open circles, and the animals killed in the dark are indicated by closed circles.

Melatonin levels in the retinas of the constant dark-treated animals (Fig. 3) showed several fluctuations during the 3-day sampling period. Statistically significant differences were observed only at 44.00, 48.00, and 60.00 hr, however, when compared to the 56.00-hr time point ($P < 0.05$). These observations suggest that, although melatonin levels fluctuate in constant darkness, a light cue is necessary to maintain a normal diurnal rhythm.

The retinal melatonin levels in the constant light-treated frogs showed very little fluctuation during the 3-day sampling period (Fig. 3), and no statistically significant differences between any time points were observed. These observations indicate that light has an inhibitory effect on retinal melatonin levels.

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. Any bound radioactivity occurring in the tissue incubated with the excess unlabeled melatonin was considered to represent non-specific binding of the ligand to the tissue. After processing the tissue for autoradiography, radioactivity was found associated with the melanosomes of the RPE-choroid (Fig. 4), and was concentrated in the outer plexiform layer of the neural retina (Fig. 5). The melanosomes were dispersed throughout the cells in the light-adapted pigment epithelium in Figure 4. The 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.
Although silver grains were present in all retinal layers, the radioactive labeling was concentrated in the outer plexiform layer of the neural retina (Fig. 5b). This pattern of radioactivity was present across the entire length of the retina. The same distribution of radioactivity was observed in both light-adapted and dark-adapted retinas. Quantitative measurements of silver grain concentration in the various retinal layers are graphically demonstrated in Figure 6. 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.

**Kinetics of $^{3}$H-Melatonin Binding**

Saturation of melatonin binding in the neural retina and RPE-choroid was determined by incubating tissue
Fig. 6. Grain count distributions in retinas incubated for 1 hr 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 measurements 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.

homogenates with increasing concentrations of \(^3\)H-melatonin. Non-specific binding of melatonin to the tissue was represented by \(^3\)H-melatonin binding in the presence of excess nonradioactive melatonin. Specific melatonin binding to the tissue was determined as the difference between the total binding and the non-specific binding. Specific melatonin binding was almost completely saturated at 1000 nM in both the RPE-choroid (Fig. 7), and in the neural retina (Fig. 8). 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. Specific binding in the neural retina was 95% of total binding, while specific binding in the RPE-choroid was 40% of total binding. Scatchard analysis of the specific binding curves revealed linear plots (Figs. 9, 10), suggesting the presence of a single class of bind-
No 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 (27,000 g pellet), but not in the cytosol fraction. Electron microscope analysis of the neural retina membrane fraction pellet showed that the fraction was composed of synaptosomes and membrane fragments (Fig. 11). The synaptosomes appeared to be damaged, since they probably were not in an optimal osmotic environment. These observations suggest that melatonin binding occurs on membrane-associated components of the neural retina. The $^3$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

![Fig. 10. Scatchard analysis of specific melatonin binding in the membrane-enriched fraction of frog neural retina.](image)

![Fig. 11. Electron micrograph of membrane-enriched neural retina fraction. Synaptosomes are indicated by the arrows. Magnification bar = 1.0 μm.](image)
Table 1. Percent displacement of a 1000-fold excess of unlabeled indole competitor with the melatonin binding component of the melanosome-enriched fraction of RPE-choroid, and the membrane-enriched fraction of neural retina

<table>
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<th>Competitor</th>
<th>Percentage of displacement</th>
<th>Competitor</th>
<th>Percentage of displacement</th>
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<td>Neural retina</td>
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<td>RPE-choroid</td>
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<td>melatonin</td>
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<td>melatonin</td>
<td>100</td>
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The results of this study are presented on Table 1. In the neural retina, both 6-chloromelatonin and 5-methoxytryptophol demonstrated complete displacement of melatonin binding. Colchicine did not displace melatonin binding, suggesting that the melatonin binding we have observed does not involve microtubules. The order of degree of melatonin displacement by indole analogs was quite different for the RPE-choroid and neural retina, suggesting that the binding components of these two tissues are different. There appeared to be significant displacement by several indole analogs.

To determine if the observed melatonin accumulation in the retina was the result of binding or active uptake, the effect of ouabain and temperature on melatonin binding was measured. Ouabain is an inhibitor of Na+/K+ ATPase, and has been shown to inhibit uptake of dopamine in the goldfish retina, taurine uptake in rat RPE, and serotonin uptake in bovine retina synaptosomes. In the presence of ouabain, a decrease in melatonin accumulation would be expected if it were the result of active uptake. As can be seen in Figure 12, 500 μM ouabain did not significantly suppress melatonin binding in the neural retina or RPE-choroid (P > 0.90). Identical results were obtained with 5 μM ouabain (data not shown).

Temperature elevation caused a small increase in melatonin binding (Fig. 12) in both the neural retina (P < 0.01) and the RPE-choroid (P < 0.001). The temperature coefficient (Q10) of the neural retina and RPE-choroid was 0.21 and 0.08, respectively. This is an order of magnitude lower than expected for active uptake (Q10 = 2). Although the possibility of active uptake of melatonin in the retina should not be discounted, this study favors the conclusion that melatonin accumulation in the retina is due to binding, rather than active uptake.

Discussion

The results of this study have confirmed the existence of melatonin in Rana pipiens retina and have demonstrated that melatonin binding occurs in specific layers of that tissue. The diurnal cycle of melatonin levels in the frog retina is similar to that observed in other species. Diurnal melatonin variation in the retina has been measured by radioimmunooassay in rat and chick, and has been corroborated by high pressure liquid chromatography in chick retina. In all species studied, peak melatonin levels occurred during the dark period. This same pattern of melatonin rhythmicity...
has been observed in the pineal gland and plasma of several species. Melatonin also has been detected in the human retina by radioimmunoassay and gas chromatography mass spectrometry.

N-Acetyltransferase (NAT), the rate-limiting enzyme in melatonin synthesis, converts serotonin to N-acetylserotonin on a terminal enzyme in melatonin synthesis, has been identified in the pineal gland and melatonin levels do not show a diurnal rhythm of activity in the frog. Melatonin-like immunoreactivity has been detected in the outer nuclear layer of the vertebrate retina and HIOMT immunoreactivity has been observed in the retina of several species, including *Rana pipiens* and does not show a diurnal rhythm of activity in the frog. Melatonin-like immunoreactivity has been detected in the outer nuclear layer of the vertebrate retina and HIOMT immunoreactivity has been observed in the retina of several species. These lines of evidence strongly suggest that the retinal photoreceptors synthesize melatonin on a diurnal rhythm.

It is interesting to note the close parallels between retinal melatonin levels and the frequency of photoreceptor outer segment disc shedding under similar lighting regimes. Retinal melatonin levels (presumably due to retinal melatonin synthesis) in *Rana pipiens* persist in constant darkness, although they are somewhat reduced in comparison to the dark peak of the normal diurnal cycle. Although there appear to be some fluctuations, melatonin levels do not undergo a regular oscillation in constant darkness. Basinger et al and Hollyfield et al have observed a persistent but sporadic frequency of disc shedding in constant light-treated *Rana pipiens*. This may somehow be related to the sporadic fluctuations in melatonin levels observed in the current study. Suppression of disc shedding occurs in constant light-treated *Rana pipiens*, which is similar to the suppression of melatonin synthesis we have observed. Disc shedding in *Rana pipiens* does not follow a free-running circadian rhythm.

In contrast, NAT activity (and presumably, melatonin levels) in *Xenopus laevis* retinas exhibit a free-running circadian rhythm in constant darkness. This is very similar to the disc shedding pattern observed in retinas of constant dark-treated *Xenopus laevis*. Both NAT activity and disc shedding are suppressed in constant light in *Xenopus laevis* retinas. Besharse and Dunis have demonstrated that exogenous melatonin and its analogs activate disc shedding in *Xenopus* eyecups, suggesting that melatonin may be involved in the regulation of disc shedding. Although the cyclic nature of disc shedding appears to differ between *Xenopus* and *Rana*, the similar patterns of melatonin synthesis and disc shedding may reflect a similar regulatory role of melatonin in the retinas of these two species.

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 and in the membrane fraction of trout RPE-choroid. Ehinger and Florén, however, did not detect melatonin uptake in rabbit retina. This lack of binding may be due to species difference, or different incubation conditions. It should also be noted that prolonged exposure of the autoradiograms was necessary in order to achieve the result reported here.

Although melatonin binding to melanosomes has not been demonstrated previously, the action of melatonin on melanosome aggregation of dermal melanophores and RPE cells has been demonstrated. Melatonin administration induces melanosome aggregation in dermal melanophores of *Xenopus* and *Rana*, and intracellular administration of melatonin induces melanosome aggregation in the RPE of frogs, *trout*, and guinea pigs. Since retinal melatonin levels and degree of RPE melanosome aggregation are highest during the dark period, 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 also is apparent. Many investigators have reported that ring compounds, such as chloroquine, prochlorperazine, and kanamycin, accumulate in melanin granules of the RPE and choroid. This drug affinity for melanin granules indicates that these organelles 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 have shown saturable binding of melatonin to bovine hypothalamic membranes, and Niles et al have shown melatonin binding to the cytosol of several brain regions of the rat. Binding of melatonin also occurs to membrane preparations of bovine pineal gland. Melatonin has an inhibitory effect on neurotransmitter uptake in synaptosome-rich homogenates of rat hypothalamus, and on evoked dopamine release of rat hypothalamic slices. Based on these observations, it was suggested that melatonin modulates neurotransmission in the brain. Melatonin has recently been shown to inhibit the calcium-dependent release of dopamine from isolated rabbit retina. Therefore, melatonin may therefore modulate neurotransmission in the retina, as well as in the brain.

The dissociation constants (Kd) obtained on this study suggest that melatonin binds with high affinity in both the RPE-choroid and neural retina (Kd = 6 × 10⁻⁷ M). This observation is similar to those of other...
studies on melatonin binding in the eye (Kd = 5.5 × 10^{-7} M),14 pineal (Kd = 7.0 × 10^{-7} M),31 and midbrain (Kd = 3 × 10^{-7} M).50 Lower dissociation constants for melatonin binding also have been reported,13,14,16 suggesting that there may be variation among different species and tissues in the kinetics of melatonin binding. The experiments with ouabain show that the accumulation of melatonin in the retina is a process which is not dependent on metabolic energy. Accumulation of melatonin also does not appear to be strongly dependent on temperature. Although some active uptake of melatonin may occur in the retina, the melatonin accumulation observed in this study is most likely due to binding. Whether this represents receptor binding remains to be determined. The neural retina 3000 g pellet, which contains nuclei and photoreceptor synaptosomes,25 displayed no melatonin binding at the incubation temperatures of 0°C. Since this fraction was not assayed for melatonin binding at higher temperatures, the possibility that active uptake of melatonin occurs in photoreceptor terminals should be considered.

The competition experiments indicate that several indole analogs bind to the melatonin binding component in the RPE-choroid and neural retina. This is similar to the results of Gern et al14 in which several indole analogs cross reacted with the melatonin-specific binding component in the 15,000 g supernatant of trout RPE-choroid. These results suggest that the methoxy group on the indole nucleus is important for binding in the neural retina. Since lowest displacement of melatonin binding in neural retina occurs with indole compounds carrying a terminal carboxyl group on the side chain, the N-acetyl group may also be important for binding in the neural retina. Reasons for the differences between the neural retina and RPE-choroid in degree of displacement of melatonin binding of the various indole analogs are presently unknown.

Since colchicine does not appear to bind to the melatonin binding component, the action of colchicine on disc shedding reported by Besharse and Dunis54 may be mediated through a mechanism different from the methoxyindole-induced disc shedding.7 It is interesting to note that both 6-chloromelatonin and 5-methoxytryptophol demonstrate complete displacement of melatonin binding in the neural retina, since Besharse and Dunis5 have reported that melatonin, 6-chloromelatonin and 5-methoxytryptophol all activate photoreceptor outer segment disc shedding in Xenopus eyecups.

Heward and Hadley55 suggested that if melatonin receptors can be identified in a particular tissue, then the hypothesis that the tissue is a target for melatonin is strongly supported. The present study has demonstrated the presence of saturable, low capacity hormone binding. Other investigators7,12 have shown that melatonin exerts a physiological effect on the retina. Together, these studies support the hypothesis that the retina is a target tissue for melatonin.

Reppert and Sagar25 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 previously shown that photoreceptors are the site of retinal melatonin synthesis.4 Melatonin (and some indole analogs) have been shown to affect outer segment disc shedding,7 RPE phagocytosis,8 RPE-choroid melanosome migration,9-11 cone retinomotor movements,56,57 and retinal neurotransmitter release.12 The present study suggests 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.

Key words: melatonin, receptor, retina, autoradiography

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References


