Direct Modulation of Rod Photoreceptor Responsiveness through a Mel$_{1c}$ Melatonin Receptor in Transgenic Xenopus laevis Retina

Allan F. Wiechmann,1,2 Melissa J. Vrieze,1 Radhika Dighe,1 and Ying Hu1

PURPOSE. Retinal circadian signals may have a role in maintaining the normal function and health of photoreceptors. Melatonin is an output of the retinal circadian oscillator and provides nocturnal signaling that is mediated through specific G-protein–coupled receptors. Melatonin receptors are expressed in retinal photoreceptor cells, and this study was undertaken to test the hypothesis that melatonin directly increases photoreceptor responses through melatonin receptors.

METHODS. Transgenic Xenopus laevis frogs were generated using a DNA construct containing a Xenopus opsin promoter driving expression of a melatonin Mel$_{1c}$ receptor-green fluorescent protein (GFP) fusion protein (XOP-MEL$_{1c}$-GFP). Electrotetinogram (ERG) analysis on transgenic and normal tadpole eyes was performed in response to melatonin treatment, and the eyes were subsequently examined by confocal microscopy and GFP immunocytochemistry.

RESULTS. XOP-MEL$_{1c}$-GFP transgenic frogs demonstrated GFP immunoreactivity in rod photoreceptor inner segments throughout the retina, indicating the rod-specific expression of the Mel$_{1c}$-GFP fusion protein. ERG analysis of transgenic tadpole eyes showed that 1 to 100 nM melatonin increased the a- and b-wave amplitudes. Control transgenic (XOP-GFP) and normal frogs exhibited modest ERG responses to 100-nM melatonin treatment. The effect of melatonin on a- and b-wave amplitudes in XOP-MEL$_{1c}$-GFP transgenic frogs was dose dependent, with ERG responses occurring at physiological concentrations.

CONCLUSIONS. The results suggest that melatonin, acting through Mel$_{1c}$ receptors on rod photoreceptor membranes, directly stimulates the responsiveness of rod photoreceptors to light. This supports the hypothesis that melatonin acts both as an intracrine and paracrine circadian signal of darkness, and binds to specific receptors in photoreceptors and other retinal cells to increase visual sensitivity. (Invest Ophthalmol Vis Sci. 2003;44:4522–4531) DOI:10.1167/iovs.03-0529

Copyright © Association for Research in Vision and Ophthalmology

From the Departments of 1Cell Biology and 2Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma. Supported by National Eye Institute Grant EY13686 (AFW) and Core Grant EY12191; Grant R002-135R from the Oklahoma Center for Science and Technology; the Presbyterian Health Foundation; and Publication Grant P20 RR017703 from the COBRE Program of the National Center for Research Resources.

Submitted for publication March 31, 2003; revised June 13, 2003; accepted June 20, 2003.

Disclosure: A.F. Wiechmann, None; M.J. Vrieze, None; R. Dighe, None; Y. Hu, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Allan F. Wiechmann, Department of Cell Biology, University of Oklahoma Health Sciences Center, PO Box 26901, Oklahoma City, OK 73190; allen-wiechmann@ouhsc.edu.

Circadian rhythms are thought to influence many cellular processes that occur in the retina and other tissues. The cyclic rhythm of photoreceptor outer segment disc shedding is considered to be driven by the circadian signaling molecule melatonin in the retina of the African clawed frog Xenopus laevis, but the mechanism of how this occurs is unknown. Melatonin is synthesized by the retinal photoreceptors of all species studied so far, including Xenopus.1,2 As in the pineal gland, retinally synthesized melatonin is produced at a relatively higher rate during the dark period than during the light period.4–6 There are many reports documenting the likely paracrine role of melatonin signaling in the inner retina, in which melatonin diffuses throughout the retina and binds to melatonin receptors on target cells of the inner retina to modulate the release of dopamine and GABA.7,8 Melatonin may inhibit dopamine release from the inner retina by binding directly to receptors on dopaminergic amacrine cells, and/or indirectly by GABA released from amacrine cells that are stimulated by melatonin receptor binding.9,10 Dopamine suppresses the synthesis of melatonin in the photoreceptor cells.9 Melatonin and dopamine are considered to be chemical signals of darkness and light, respectively, and appear to exert their effects by a mutual antagonism. The modulation of dopamine release from amacrine cells may be at least partially responsible for cyclic changes in visual sensitivity.10

Our recent discovery that Mel$_{1c}$ melatonin receptors are expressed in the rod and cone photoreceptors of the Xenopus retina suggests that the photoreceptors themselves may be direct targets of melatonin action.11,12 The localization of MT1 (Mel$_{1a}$) melatonin receptors in photoreceptor cells has been observed in the human and rodent retina13–15 and in chicken retina (Mel$_{1c}$),16 thus confirming our reports of melatonin receptor expression in photoreceptors. Two other melatonin receptors Mel$_{1a}$ and Mel$_{1b}$ are also expressed in the Xenopus retina.12,17 Based on the results of these new studies, we propose that photoreceptor cells are direct targets of melatonin signaling, and that melatonin drives the circadian rhythm of retinal sensitivity to light.

We tested our novel hypothesis that photoreceptors are directly responsive to melatonin and that their cyclic sensitivity to light is driven by melatonin which is produced at night by the photoreceptors. Many cell types in the retina (pigment epithelium, photoreceptor, amacrine, horizontal, and ganglion cells) express melatonin receptors,11,17 and three melatonin receptor types (Mel$_{1a}$, Mel$_{1b}$, and Mel$_{1c}$) are expressed in cells of the Xenopus retina.12,17 The many possible combinations of cells and receptors that are affected by melatonin at various times of the circadian cycle result in a complex system in which it is difficult to determine the mechanisms of melatonin action on specific receptors in specific retinal cells.

To meet the inherent challenges in studying this complex circadian signaling system, we generated a transgenic Xenopus that overexpresses functional Mel$_{1c}$ receptor-green fluorescent protein (GFP) fusion proteins in rod photoreceptors. The overexpressed Mel$_{1c}$-GFP fusion proteins in rods is driven by an opsin promoter.18,19 This approach is dependent on the as-
sumption that the overexpression of melatonin receptor-GFP fusion proteins produces an enhanced response to melatonin in the specific transgenic cells. Physiological analysis of retinas that overexpress a specific melatonin receptor in a specific population of cells would then render this complex circadian signaling system into its simpler component parts that can be effectively studied. This strategy has the potential to identify the functions of specific melatonin receptors in specific retinal cell types. In this study, we report that melatonin directly increases rod responsiveness to light in transgenic frogs that overexpress the Mel₁<sub>r</sub> receptor in rod photoreceptors.

**MATERIALS AND METHODS**

**Generation of DNA Constructs**

A plasmid containing a 541-bp upstream promoter sequence (−503/+41) from the 5’ region of the *Xenopus laevis* rhodopsin gene (XOP), which drives rod-specific expression in *Xenopus*, was kindly provided by Barry Knox (Upstate Medical Center, Syracuse, NY).<sup>15</sup> In this plasmid, cDNA encoding enhanced green fluorescent protein (GFP) was located directly downstream of the XOP promoter sequence in a pGL2 expression vector (Promega, Madison, WI) and is referred to in this report as the XOP-GFP construct. The XOP-GFP plasmid was used to construct a plasmid encoding a *Xenopus laevis* melatonin Mel<sub>1c</sub> receptor-GFP fusion protein (XOP-Mel<sub>1c</sub>-GFP). A full-length Mel<sub>1c</sub> cDNA clone in a pcdNA I mammalian expression vector was kindly provided by Steven Reppert (University of Massachusetts Medical School, Worcester, MA)<sup>20</sup> and we used this plasmid to prepare an XOP-Mel<sub>1c</sub>-GFP construct.

The XOP-GFP construct was digested with the restriction enzyme AgeI (Promega) which separated the opsin promoter DNA from the GFP DNA sequence. A full-length melatonin receptor cDNA was prepared using polymerase chain reaction (PCR) with primers complementary to the 5’ (5’-CCA AGG AGA GAA ATG ATG CAG GTG-3’) and the 3’ (5’-ATA GTG CAA CCG GTG TGA CCT TTG GGA-3’) ends of the coding regions, using the Mel<sub>1c</sub> plasmid as the template. Specific restriction sites for AgeI were added to the 3’ end of the primer and to the 5’ end of the 5’ primer, to aid in the subsequent cloning, and also contained one additional nucleotide (the italic thymidine inserted between GTC and TGA on the 3’ primer) to create an open reading frame between the melatonin receptor sequence and the GFP sequence. The Mel<sub>1c</sub> melatonin receptor cDNA PCR product was digested with AgeI, which removed the noncomplementary ends of the Mel<sub>1c</sub> receptor cDNA, leaving sticky ends that could be ligated into the AgeI-cut opsin promoter-GFP plasmid. The digested melatonin receptor cDNA was ligated into the AgeI-cut opsin promoter-GFP plasmid, which inserted the melatonin receptor cDNA downstream of the promoter sequence, and upstream of the GFP sequence, maintaining an open reading frame between the melatonin receptor sequence and the GFP sequence. The ligated plasmid was cloned into bacteria (XL1 Blue *Escherichia coli*; Stratagene, La Jolla, CA), and the plasmids were purified with a kit (QiAprep spin miniprep kit; Qiagen, Valencia, CA). Plasmids of several clones were analyzed and identified by VspI restriction profiling and DNA sequencing. The DNA sequences of the clones containing the melatonin receptor sequence demonstrated 100% identity with the known Mel<sub>1c</sub> receptor sequence<sup>21</sup> plus an open reading frame between the Mel<sub>1c</sub> receptor sequence and the GFP sequence. For use in transgenic frogs, both the XOP-GFP and XOP-Mel<sub>1c</sub>-GFP constructs were digested with EcoRI and Hpal to separate the XOP-GFP fragment and the XOP-Mel<sub>1c</sub>-GFP fragment from the vectors.

**Generation of Transgenic Xenopus laevis Embryos**

Adult *Xenopus laevis* frogs were obtained from *Xenopus* 1 (Dexter, MI) and maintained in aquaria for at least 2 weeks in a 12-hour light–dark cycle at a temperature of 20°C. A modification of the protocol of Kroll and Amaya<sup>22</sup> was used for generating transgenic *Xenopus laevis* frogs. This research adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Declaration of Helsinki and Principles of Laboratory Animal Care of the National Institute of Health.

*Xenopus* sperm nuclei were prepared by first macerating testes removed from adult male *Xenopus laevis* that were killed by immersion in 2% tricine methylsulfoxide (MS 222; Sigma-Aldrich, St. Louis, MO) for 20 minutes. Nuclei were isolated from sperm, as described previously<sup>21</sup> and cryoprotected and stored at −80°C until time of use.

The oocyte high-speed cytoplasmic extract was prepared according to previously published methods.<sup>21,22</sup> Briefly, ovulation was induced in mature female *Xenopus* frogs by injection of 50 IU of pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich) followed 48 hours later by injection of 500 IU of human chorionic gonadotropin (HCG; Chorulon, Millsboro, DE). After 18 hours, oocytes were retrieved from the 1× Marc’s modified Ringer’s (MMR: 100 mM NaCl, 2 mM KCl, 1 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, and 5 mM HEPES [pH 7.5]) in the tanks in which the frogs were housed. Oocytes were dejellied with 2% cysteine (pH 8.2; Sigma-Aldrich), packed by low-speed centrifugation, and centrifuged at 15,000g for 10 minutes at 2°C. After incubation with an adenosine triphosphate (ATP)-regenerating system and 0.04 mM CaCl<sub>2</sub> cystosol was obtained from the cytoplasmic fraction by two centrifugations at 200,000g. The first centrifugation was for 1.5 hours at 4°C and the second for 20 minutes, and then aliquots were stored at −80°C until time of use.

To prepare oocytes for microinjections, mature female *Xenopus laevis* frogs were injected in the dorsal lymphatic cavities with 50 IU of PMSG to induce maturation of the oocytes. Then, 3 to 5 days later, the injected females were injected with 500 IU of HCG to induce ovulation. Oocytes were retrieved from the frogs at 18 hours after the HCG injections and were dejellied with 2% cysteine, rinsed in 1× MMR and placed in 0.4× MMR and 6% Ficol (Sigma-Aldrich) for immediate use in transgenesis.

EcoRI/Hpal (Promega) fragments containing the XOP-GFP construct or the XOP-Mel<sub>1c</sub>-GFP construct were purified by phenol-chloroform extractions and spin columns (Qiagen). Each fragment (200 ng) was incubated with approximately 2 × 10<sup>5</sup> sperm nuclei for 5 minutes at room temperature (RT) in sperm dilution buffer (SDB; 250 mM sucrose, 75 mM KCl, 0.5 mM spermidene, and 0.2 mM spermine [pH 7.5]), and then 5 μL oocyte cytosolic extract, MgCl<sub>2</sub> to a final concentration of 10 mM, and 0.5 U each of EcoRI and Hpal were added to the sample in a final volume of 32 μL SDB for 3 minutes at RT. The mixture was diluted to 120 nucleic/μL and approximately 10 nL was injected into each oocyte. Normally cleaving embryos were selected at the four- to eight-cell stage and incubated in 0.4× MMR and 6% Ficol (Sigma-Aldrich) for 48 hours at 18°C. When gastrulation was reached, the embryos were cultured in 0.1× MMR with 50 μg/mL gentamicin at 18°C. Tadpoles were transferred to 22°C at approximately stage 20. At approximately stage 45, tadpoles were maintained in a solution of 0.2 M NaH<sub>2</sub>P<sub>4</sub> and 50 g/L of a salt mixture (Instant Ocean; Aquarium Systems, Mentor, OH) at 22°C. Staging of the *Xenopus* embryos was performed according Nieuwkoop and Faber.<sup>25</sup>

**Genomic DNA Analysis**

The heads of anesthetized XOP-Mel<sub>1c</sub>-receptor-GFP transgenic tadpoles used for ERG analyses were removed from the bodies and fixed for GFP analysis and immunocytochemistry. The tadpole bodies without the heads were quick frozen in liquid nitrogen, and genomic DNA was isolated from them with a genomic DNA isolation kit (Qiagen). The genomic DNA was amplified by PCR, using oligonucleotide primers specific for the GFP, in a final volume of 50 μL. PCR primers were based on the GFP sequences. The 5’ primer sequence was 5’ CCG ATC TTG AAG TAC TTC CTT ATG 3’ and the 3’ primer sequence was 5’ CAA GAT CCT GAC CCT GAA GTT CAT CAT GTC 3’, which corresponds to positions 506-882 and 146-123, respectively of the GFP DNA sequence.<sup>18,19</sup> to generate a PCR product of 385 bp. PCR reactions were
performed with 1 cycle of 95°C for 5 minutes, then 35 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute, followed by 1 cycle of 72°C for 10 minutes, and then 15 μL of the amplified cDNA was electrophoresed on a 1% agarose gel and stained with ethidium bromide.

The PCR products were ligated into a PCR cloning vector (pCR II: Invitrogen, San Diego, CA). Competent XLI Blue E. coli (Stratagene) were transformed with the ligated plasmid and selected by ampicillin resistance and blue-white color expression, followed by restriction mapping of the purified plasmids. The cDNA inserts in the plasmid from some positive clones were sequenced and revealed a 99% identity with the known GFP sequence.

**Tissue Preparation and Immunocytochemistry**

Whole-head preparations of transgenic and normal tadpoles were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and transferred to 30% sucrose in phosphate buffer for 16 to 20 hours at 4°C. Sagittal 30-μm sections were cut on a cryostat microtome and collected on glass slides. To analyze the tissue distribution of GFP expression, sections were labeled with 0.0005% 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Sections were rinsed in phosphate-buffered saline (pH 7.4; PBS), and coverslips were mounted onto the slides with mounting medium (Cytoseal 60; Stephens Scientific, Kalamazoo, MI).

For immunocytochemical localization of the Mel1a and Mel1c receptors in the retina, postmetamorphic Xenopus frogs were anesthetized with MS-222, and whole eyes were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Cryostat sections were rinsed in PBS, and then incubated in incubation buffer (1% normal goat serum [Sigma-Aldrich], 0.2% Triton X-100, and 0.004% sodium azide in PBS) for 30 minutes at RT. In control experiments, the melatonin receptor antibodies were incubated overnight at 4°C with 1 μM of their corresponding antigen peptides. Sections were then incubated with 2 μg/mL of the Mel1a melatonin receptor antibody in incubation buffer for 3 days at 4°C. Polyclonal antibodies directed against peptides corresponding to regions of the third cytoplasmic loop of the Xenopus laevis Mel1a receptor (residues 231-243; KQKLTQTDLRNFL),20 and the homologous region of the Mel1c receptor (HHQTVPNYHGFI)24 were generated in rabbits and chickens, respectively (Research Genetics, Huntsville, AL). The 13-amino acid peptides were conjugated to keyhole limpet hemocyanin (KLH), and used to immunize the rabbits and chickens. Pooled antisera against each receptor peptide were affinity purified against the appropriate antigen peptide conjugated to a solid support matrix. The peptide synthesis, conjugation, immunizations, and affinity purification were all performed by Research Genetics, Inc. Characterization and use of these antibodies have been described previously.5,12,25

Sections were rinsed in PBS and incubated in 5 μg/mL of Alexa Fluor 488 (green) conjugated to goat anti-rabbit (Molecular Probes, Eugene, OR) for 30 minutes at RT. Sections were rinsed in PBS and incubated with 2 μg/mL of Mel1a receptor antibody in PBS for 3 days at 4°C. Sections were rinsed in PBS, and incubated in 5 μg/mL of Alexa Fluor 568 (red) conjugated to anti-chicken IgG (Molecular Probes) for 30 minutes at RT. Sections were rinsed in PBS and incubated in 0.0005% DAPI nuclear stain for 10 seconds at RT. Sections were rinsed in PBS, and coverslips were mounted onto the slides with mounting matrix (Cytoseal 60; Stephens Scientific). GFP-labeled sections were viewed under a fluorescence microscope (AX70; Olympus, Melville, NY) equipped with a color digital camera (Spot; Diagnostic Instruments, Sterling Heights, MI). For confocal microscopy, sections were viewed under a laser-scanning confocal microscope (LSM 510; Carl Zeiss Meditec, Jena, Germany). In the specimen shown in this report, the section thickness analyzed was 23.8 μm, with 32 sections in the Z-series.

For GFP immunocytochemistry, tissues were prepared as described for melatonin receptor immunocytochemistry. Sections were rinsed in PBS, incubated in 2% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 20 minutes at RT, and incubated for 30 minutes at RT in mouse anti-GFP antiserum (BD Biosciences Clontech, Palo Alto, CA) diluted 1:1000 in PBS. Sections were rinsed in PBS and incubated for 20 minutes in 2% normal goat serum (Sigma-Aldrich) and 2% BSA in PBS. Sections were rinsed in PBS, incubated for 30 minutes in Alexa Fluor 568-conjugated goat anti-mouse antibody (Molecular Probes) diluted 1:500 in PBS, rinsed in PBS, incubated in 0.0005% DAPI for 10 seconds at RT, rinsed in PBS, and coverslipped. Sections were viewed by standard fluorescence microscopy and by confocal microscopy. The confocal image in Figure 3B is a projection image of a section with a thickness of 9.4 μm, with 16 sections in the Z-series. In the single-slice confocal image in Figure 3D, the section thickness is 4.4 μm, with 10 sections in the Z-series.

**Electroretinogram Analysis of Transgenic Tadpoles**

The retinas of premetamorphic normal and transgenic frogs (late-stage tadpoles; approximate stages 46–52), were analyzed by electroretinogram (ERG) recording. Tadpoles were entrained to a 12-hour light-dark cycle and placed into constant darkness for 24 hours before ERG analysis. They were anesthetized with 0.01% MS-222 and placed on a sponge soaked with the MS-222 solution. One drop of 1% atropine sulfate (Butler Co., Columbus, OH) was applied directly to the tadpole eyes to dilate the pupils. ERGs were recorded in vivo using silver wire corneal electrodes. A ground electrode was placed on the tadpole’s tail, another electrode was placed on the head near the eye as a reference electrode, and another electrode was placed onto the corneal surface as the recording electrode. Tadpole eyes were allowed to recover for 1 minute between flashes. Animals were maintained in a salt solution (described earlier) containing 0.01% MS-222 throughout the experiments. The ERG components consisted of a photic stimulator (model PS33-Plus) a preamplifier (model PS11 AC), and a data acquisition and analysis system (PolymVIEW; Grass Telefactor). ERGs were elicited with a 10-μs LED flash (470 nm).

In experiments to analyze the ERG response to applied melatonin, tadpoles were placed in a salt solution, and ERG recordings were made at relative flash intensities of 1, 2, 4, 8, and 16 (flash intensity 1 = 140 lux). The solution was replaced with various concentrations of melatonin (1–10.000 nM) for 30 minutes before each ERG recording. Small lipophilic molecules, such as melatonin, are readily absorbed through Xenopus skin.26 After the ERG analysis, the head and trunk were preserved for later analysis of fusion protein expression. After the tadpole was killed by immersion in 1% MS-222, the head was fixed for immunocytochemistry, and the trunk was stored at ~80°C and used later for genomic DNA analysis.

**Results**

**Melatonin Receptor Localization**

Immunocytochemical labeling of sections of Xenopus retina with the Mel1a and Mel1c antibodies revealed both differential distribution and colocalization in many cells of the frog retina (Fig. 1), as has been reported.14 A high-magnification confocal image of the Mel1a and Mel1c double-labeling has not been reported and is included in this study to illustrate the immunolabeling of the photoreceptors by the Mel1a antibody, but not by the Mel1c antibody. The observation of Mel1c immunolabeling of rod photoreceptors formed the basis of the present study to use a Xenopus rod opsin promoter to drive overexpression of the Mel1c receptor in the rod photoreceptors of transgenic frogs.

The green fluorescence of the Mel1a immunolabeling was observed in the photoreceptor inner segments, but not the outer segments (Fig. 1). There appeared to be a punctate or mottled appearance in the pattern of Mel1c labeling of the inner segments. Mel1c immunolabeling was also prevalent in the inner retina, as indicated by green and yellow fluorescence.
Mel1c-GFP Fusion Protein Expression in Transgenic Xenopus Photoreceptors

Analysis of the XOP-GFP control transgenic frogs showed green fluorescent labeling of rod photoreceptors in the retinas, as expected (Fig. 2). In these animals, the opsin promoter was driving the expression of the soluble GFP in the cytoplasm of the rods. Essentially all rods throughout the retina displayed GFP fluorescence, although many specimens displayed instead a mosaic pattern of expression (Fig. 3). Immunocytochemical labeling of the XOP-GFP transgenic retinas with a GFP antibody labeled with a red fluorescent dye-secondary antibody conjugate revealed that essentially all of the rod photoreceptor cell inner segments that displayed the green GFP fluorescence also displayed the red GFP immunoreactivity (Fig. 2D). In many specimens, only approximately 10% to 30% of the rod photoreceptors demonstrated the green fluorescence (Fig. 5). Immunocytochemical labeling of XOP-GFP transgenic retinas with just the clusters of green fluorescent rods showed that the green-labeled rods were immunoreactive for GFP, as were essentially all rods throughout the retina (Fig. 3). Other areas of the rods, such as the synaptic terminal regions and cell soma, also appeared to express GFP immunoreactivity in the Mel1c receptor transgenic frog retinas. Red GFP immunoreactivity also occurred within the entire cell soma, including the rod outer segments, of the green fluorescent cells because of the reaction of the red-labeled GFP antibody with the GFP expressed in the inner and outer segments by the opsin promoter in a subset of rods.

Because the Mel1c-GFP fusion protein transgenic tadpoles did not demonstrate obvious green fluorescence in the photoreceptors, we performed immunocytochemistry on these tissues, using a commercial antibody against GFP, with the expectation that it would provide a higher level of sensitivity for the localization of GFP expression. The Mel1c-GFP fusion protein transgenic retinas demonstrated GFP immunoreactivity in the inner segments of essentially all rod photoreceptors (Figs. 2, 5). This was precisely the location where the Mel1c-GFP fusion protein was predicted to be expressed, based on the localization of Mel1c immunoreactivity in the rod inner segments (Fig. 1). Retinas of normal tadpoles did not exhibit any

The yellow fluorescence was the result of the combined red and green images from the Mel1a and Mel1c immunolabeling, respectively. Mel1a immunolabeling was present in the inner retina, as indicated by the red (differential distribution) and yellow (colocalized with Mel1c) fluorescence, but no Mel1a immunolabeling could be detected in the photoreceptor cells.

Figure 1. Confocal image of the differential distribution and colocalization of Mel1a and Mel1c receptor immunoreactivity in the Xenopus laevis retina. The merged image of Mel1a (green) and Mel1c (red) immunolabeling demonstrates the differential expression and colocalization of the two receptor types. The two receptors appeared to have some colocalization in the OPL and IPL (black arrowheads), as indicated by the yellow fluorescence. Mel1c red immunolabeling appeared in the outer and inner plexiform layers (OPL, IPL: white arrowheads), but was absent from the photoreceptor inner (IS) or outer (OS) segments. Mel1c green immunolabeling appeared primarily in the photoreceptor IS and in the OPL and IPL. The yellow fluorescence representing colocalization of Mel1a and Mel1c immunoreactivity was observed in the OPL, but the two receptor types are also observed in separate populations of cells in the INL. Also, Mel1a and Mel1c immunoreactivity were both located in ganglion cell soma in the ganglion cell layer (GCL), but were in different populations of cells in that layer. Scale bar, 50 μm.

Figure 2. Distribution of GFP fluorescence and GFP immunoreactivity in XOP-GFP transgenic frogs. (A) Retina section stained with blue nuclear dye (DAPI). (B) Same section as in (A) showing the green labeling of the opsin-driven GFP expression. The rod inner segments (RIS) were intensely labeled (arrowheads), and the rod outer segments (ROS) displayed a much lower intensity of GFP fluorescence. (C) Same section as in (A) and (B), incubated with GFP antibody followed by incubation in secondary antibody conjugated to a red fluorescent dye. GFP immunoreactivity is intense in the RIS (arrowheads) and is less intense in the ROS. Neither the green GFP fluorescence in (A) or the red GFP immunoreactivity in (B) was located in any other cell layer except the photoreceptor layer (arrowheads). (D) Merged images of (A), (B), and (C) showing colocalization of the green GFP fluorescence and the red GFP immunoreactivity. The merged images show the yellow labeling (arrowheads) resulting from the colocalization of the green GFP fluorescence and the red GFP antibody labeling in the rod photoreceptors. Some red GFP immunolabeling also occurred in the inner segment layer. Remaining abbreviations as in Figure 1. Scale bar, 50 μm.
specific green fluorescence or specific GFP immunoreactivity (Fig. 3E).

PCR cloning and DNA sequencing of genomic DNA isolated from the XOP-Mel1c receptor-GFP transgenic tadpoles demonstrated that DNA encoding GFP (99% homology) was expressed in these animals (Fig. 4). This confirmed that the XOP-Mel1c-GFP transgene encoding the Mel1c-GFP fusion protein was incorporated into the genome of the putative Mel1c receptor transgenic animals.

Electroretinogram Analysis of Transgenic Xenopus Eyes

Eyes of normal, XOP-GFP transgenic control, and Mel1c-GFP fusion protein transgenic tadpoles (stages 46–52) were analyzed in vivo by ERG recording in response to applied melatonin. The ERG analysis was performed to determine whether opsin promoter–driven overexpression of the Mel1c-GFP fusion protein in rod photoreceptors alters the ERG response to applied melatonin. ERG recordings of untreated normal, XOP-GFP transgenic, and Mel1c-GFP fusion protein transgenic tadpoles demonstrated typical ERG responses in the a-, b-, and c-waves (Fig. 5). After the application of 100 nM melatonin for 30 minutes, little or no change in the ERG responses was observed in the normal and XOP-GFP transgenic eyes (Fig. 5). However, application of 100 nM melatonin caused two- to threefold increases in the a- and b-wave amplitudes of the Mel1c-GFP fusion protein transgenic tadpoles (Fig. 5). Because the 100 nM melatonin dose was dissolved in a final concentration of 0.0001% ethanol, we tested for the effect of ethanol and normal salt solution and 100 nM melatonin dissolved in 0.0001% ethanol in normal salt solution on ERG responses at flash intensities of 1.40 to 2.240 lux in normal and Mel1c-GFP transgenic tadpoles. In normal tadpoles, no obvious differences in a- or b-wave amplitudes were observed among the three treatment groups (Fig. 6), indicating that the ethanol carrier had no significant effect on the ERG responses and 100 nM melatonin had only a small effect. In the Mel1c-GFP transgenic tadpoles, however, 100 nM melatonin in 0.0001% ethanol resulted in a- and b-wave amplitudes that were two to three times higher than in the ethanol control or normal salt solution.
groups (Fig. 6). This is consistent with the ERG recordings described in Figure 5. The increases in a- and b-wave amplitudes of the Mel1c-GFP transgenic tadpoles in response to 100 nM melatonin were two to three times higher at all flash intensities tested, with the higher responses occurring at the higher flash intensities.

ERG responses to various concentrations of melatonin were performed to determine the optimal physiological concentration of melatonin that elicits increases in a- and b-wave amplitudes in the Mel1c-GFP transgenic tadpoles. Dose–response analyses were also performed on XOP-GFP control transgenic and normal tadpoles. When treated with 1 to 10,000 nM melatonin, dosages of 100, 1,000, and 10,000 nM melatonin resulted in very modest increases (approximately 3%–10%) in a-wave amplitudes in normal tadpoles (Fig. 7). The b-wave amplitudes demonstrated even lower increases (approximately 1%–3%) in response to 100, 1,000, and 10,000 nM melatonin in normal tadpoles (Fig. 7). Similar results were observed in the XOP-GFP control transgenic frogs, but the amplitude increases in response to almost all melatonin concentrations (1–10,000 nM) were somewhat higher (approximately 2%–25%) than in the normal frogs (Fig. 7). However, the amplitude changes did not show a consistent relationship with the different concentrations of melatonin administered. In the Mel1c-GFP transgenic tadpoles, there was a direct correlation between amplitude increases and melatonin concentration at the lower concentrations of melatonin. Amplitude increases of the a-wave directly corresponded to higher melatonin concentrations (1–1,000 nM), except for the highest dose (10,000 nM), which elicited a response comparable to that elicited by the 1,000-nM concentration. Similarly, the b-wave amplitudes increased as a direct correlation of increasing melatonin concentrations at the lower concentrations (1, 10, and 100 nM; Fig. 7), but the higher concentrations (1,000 and 10,000 nM) of melatonin showed a lower response than the response to 100 nM, suggesting that, of the concentrations analyzed, 100 nM melatonin was the optimal concentration to elicit increases in the b-wave amplitude.

**DISCUSSION**

This study suggests that by binding to Mel1c receptors on rod photoreceptor inner segments, melatonin increases the sensitivity of rods to light. Over-expression of a Mel1c receptor-GFP fusion protein driven by an opsin promoter significantly increases the expression of Mel1c receptors in rods, thus enabling electrophysiological measurements of rod responses to applied melatonin. Mel1c receptor overexpression in rods thus provides the ability to determine the action of a specific melanin receptor in a specific cell type. This is a noteworthy feature of this experimental approach, because three melanin receptor types are expressed in the *Xenopus* retina, and multiple retinal cell types express different combinations of these receptors. This transgenic approach enables us to render a complex system into its simpler component parts that can be more readily studied. The receptor overexpression is required to be able to differentiate between the activation of the recombinant fusion protein receptor and the endogenous activity of the other melanin receptor types expressed in the various cells of the retina.

We have noted that GFP immunoreactivity (red) is present throughout the retinas of all transgenic animals (XOP-GFP and
XOP-Mel1c-GFP), although the GFP fluorescence driven by the opsin promoter in the XOP-GFP frogs is sometimes visible in only a subset of rods and not at all in the XOP-Mel1c-GFP frog retinas. These observations, together with the supporting evidence reported here suggest that the visible expression of GFP fluorescence may not necessarily indicate the entire cellular distribution of the reporter gene. Perhaps most or all of the rod photoreceptors express the transgene, but sometimes only a subset express it in high enough levels to be detected by fluorescence alone, and GFP immunocytochemistry may be necessary to obtain a more accurate determination of the cellular distribution of transgene expression. The low levels of visibly detectable GFP fluorescence in the rods of XOP-Mel1c-GFP retinas is not surprising, because unlike the soluble GFP, the melatonin receptor is located only on the cell membrane, and so its level of expression would be subject to the spatial constraints of the inner segment membrane.

We have reported that the retinal photoreceptors of *Xenopus laevis* express Mel1c melanin receptors. This discovery has been confirmed in humans and chickens, and is further illustrated in this study. We have therefore proposed that melatonin produced by retinal photoreceptors at night acts both as a paracrine signal of darkness for neurons of the inner retina (amacrine, horizontal, and ganglion cells), and as an intracrine (or autocrine) signal for the photoreceptors. It has been well-documented that stimulation of melatonin receptors on specific inner retinal neurons, such as amacrine cells, exerts an indirect influence on photoreceptor function, such as the synthesis of melatonin. The expression of melatonin receptors on photoreceptors suggests that melatonin may also exert a direct effect on photoreceptor function, as documented in the present study.

There is some evidence from other laboratories that supports the concept of a direct action of melatonin on retinal photoreceptors. It has been reported that melatonin induces membrane conductance changes in isolated frog rod photoreceptors. Also, melatonin appears to have a role in photoreceptor outer segment disc shedding in *Xenopus*, although it could be the result of indirect signals from the inner retina, it could also be due to melatonin's acting directly on the photoreceptors. It has been reported recently that melatonin delays the onset of photoreceptor degeneration in a mouse model of retinal degeneration, and we have reported that melatonin increases the degree of light-induced photoreceptor cell death in rat retina. Both of these phenomena could be mediated by photoreceptor melatonin receptors. In addition, the enhancement of light-induced photoreceptor cell death by melatonin was shown to be mediated by a retinal melanin receptor. These reports, combined with the new evidence in

---

**Figure 6.** Analysis of ERG recordings of normal and melatonin receptor transgenic frogs treated with 100 nM melatonin. ERG a- and b-wave amplitudes are plotted as a function of flash intensity. ERGs were first recorded in normal salt solution (control); and then in salt solution containing 0.0001% ethanol (EtOH), which is the final concentration used in the melatonin-treated groups; and then with 100 nM melatonin (Mel+EtOH). All solutions contained 0.01% of the MS-222 anesthetic. (A) The a-wave amplitude was more than two times higher in response to melatonin treatment in Mel1c receptor-GFP-overexpressing transgenic frogs. (B) There was no measurable effect of melatonin on the a-wave amplitude in normal frogs. (C) The b-wave amplitude was higher by approximately threefold in response to melatonin in the Mel1c receptor-GFP-overexpressing transgenic frogs. (D) There was no measurable effect of melatonin on the b-wave amplitude in normal frogs. The ethanol carrier had no significant effect on the ERG responses. All data points represent the average of measurements from three different animals and are normalized to control values.
the present report, offer compelling support for a direct action of melatonin on photoreceptor function.

The observation that melatonin causes a stimulation of the amplitude of the a-wave (rod photoreceptors) and the b-wave (inner retinal cells responding to the photoreceptor input) in transgenic frogs overexpressing the Mel1c receptor-GFP fusion protein supports our hypothesis that melatonin increases retinal sensitivity to light as part of a dark-adaptation mechanism. It has been shown that melatonin enhances the sensitivity of the central visual system to light, and we have shown that melatonin increases the sensitivity of horizontal cells to light in the salamander retina. Melatonin may therefore bind to receptors in the retina and brain at night to increase the sensitivity of the visual system to facilitate dark adaptation. In the retina, one mechanism by which melatonin may increase dark adaptation is to increase horizontal cell-coupling through inhibition of dopamine release. However, in addition to the events induced by melatonin in the inner retina, evidence in the present study suggests that melatonin acts directly on the rod photoreceptors to increase dark adaptation. The role of melatonin in dark adaptation suggests a potential mechanism by which melatonin increases the degree of light-induced photoreceptor cell death. Because one function of melatonin may be to increase the sensitivity of the retina to light as part of a dark-adaptation mechanism, an undesirable consequence of this may be an increased sensitivity to the damaging effects of light. Although signals from the inner retina undoubtedly play a major role in the circadian activities of retinal photoreceptors, intracrine melatonin signaling in photoreceptors probably contributes substantially to circadian regulation in the retina.

The dissociation constant of the Xenopus Mel1c receptors is reported to be 630 nM. This value is consistent with our observation on the concentrations of melatonin that elicit ERG responses in the Mel1c transgenic frogs. Melatonin concentrations in the range of 1 to 100 nM exerted dose-dependent effects on the rod ERG, whereas the higher dosages (1,000 and 10,000 nM) were generally less effective or only slightly more effective.

Previous studies on the effect of melatonin on ERG responses have shown that melatonin reduces the circadian rhythm of the ERG b-wave amplitude in iguanas. Also, the b-wave, but not the a-wave shows a peak in the daytime, whereas both the a- and b-waves show a circadian rhythm in implicit time. Reduction in circulating levels of melatonin abolishes the ERG circadian rhythm, suggesting that melatonin modulates retina responses. In chickens, ERG b-wave, but not the a-wave, amplitude has a circadian rhythm with peak amplitude in the daytime. The a- and b-wave implicit times are higher during the day than during the night in chickens. Melatonin abolishes the rhythm of b-wave amplitude and of a- and b-wave implicit times in continuous darkness. These

**Figure 7.** Dose response of the effect of melatonin treatment on ERG recordings of transgenic and normal Xenopus tadpoles. ERG a- and b-wave amplitudes are plotted as a function of flash intensity versus the percent change from the baseline (0 nM melatonin) measurements from the same animal, at increasing concentrations of melatonin (1-10,000 nM). (A, B, C) ERG a-wave amplitudes in response to increasing concentrations of melatonin in Mel1c transgenic, XOP-GFP control transgenic, and normal tadpoles. (A) The a-wave amplitude increased as the melatonin concentration and flash intensity increased in transgenic tadpoles overexpressing the Mel1c receptor-GFP expression protein. There were modest increases in the a-wave amplitudes in response to melatonin in the control XOP-GFP transgenic frogs (B) and in normal frogs (C). (D, E, F) ERG b-wave amplitudes in response to increasing concentrations of melatonin in Mel1c transgenic, XOP-GFP control transgenic, and normal tadpoles. (D) The b-wave amplitude increased as the melatonin concentration and flash intensity increased in transgenic tadpoles overexpressing the Mel1c receptor-GFP expression protein. There were some modest increases in the b-wave amplitudes in response to melatonin in the control XOP-GFP transgenic (E) and normal (F) frogs. All data points represent the average of measurements from three different animals, and are normalized to control values.
studies suggest that the circadian system regulates visual function in the retina at least partially through melatonin.

There is a strong correlation between cone ERG recordings and levels of salivary melatonin in humans, in which there are lower ERG amplitudes and retinal sensitivity in the early daytime. This suggests a direct effect of melatonin on the physiology of cones or of the circadian phase. In human subjects, the b-wave implicit time exhibits a diurnal variation, with greatest times occurring during the daytime. Melatonin decreases the b-wave amplitude in the light and dark in human retina. It should be noted that the ERG recordings in these earlier studies were performed on normal subjects, whereas the present report has analyzed the effect of melatonin essentially only on rod photoreceptors, rather than on the whole normal retina. Further study with this transgenic model is needed to correlate the effects of melatonin on specific transgenic melatonin receptor overexpression in specific retinal cells, with the effects of melatonin on whole normal retinas.

The discovery that Mel1c receptors that are expressed in rod photoreceptors stimulate rod sensitivity to light flashes supports our hypothesis that melatonin acts directly on photoreceptors to modulate their functions, such as responsiveness to light, and perhaps on other cyclic events in the retina, such as photoreceptor outer segment disc shedding and phagocytosis. The further use of transgenic frogs to investigate the mechanism of melatonin action on each melatonin receptor type expressed in the many different retinal cell types offers a potentially effective approach to elucidate the role of melatonin in the overall health and diseases of the retina.

Acknowledgments

The authors thank the many individuals who have generously contributed their ideas, advice, and reagents to aid in this study: Gustav Engbrecht (University of Syracuse) for generous contribution of time and ideas and for the transfer of the Xenopus transgenic technology; Barry Knox (Upstate Medical Center, Syracuse) for the XOP-GFP construct and for the use of his laboratory for learning the frog transgenic technology; Steven Reppert (University of Massachusetts Medical School, Worcester, MA) for providing the Xenopus Mel1c receptor clone; Carla Green, Eduardo Solessio, John Ash, Wei Cao, and May Nour for providing stimulating ideas and technical advice that were of great help to the study; and Rajendra Dhote and Courtney McClearn for providing excellent technical assistance with the transgenic procedures.

References


