Direct Modulation of Rod Photoreceptor Responsiveness through a Mel1c Melatonin Receptor in Transgenic Xenopus laevis Retina

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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 melanotin Mel1c receptor-green fluorescent protein (GFP) fusion protein (XOP-MEL1c-GFP). Electrotetrogram (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-MEL1c-GFP transgenic frogs demonstrated GFP immunoreactivity in rod photoreceptor inner segments throughout the retina, indicating the rod-specific expression of the Mel1c-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 only modest ERG responses to 100-nM melatonin treatment. The effect of melatonin on a- and b-wave amplitudes in XOP-MEL1c-GFP transgenic frogs was dose dependent, with ERG responses occurring at physiological concentrations.

Conclusions. The results suggest that melatonin, acting through Mel1c 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

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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.2,3 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.7–9 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.7,8,10,11 Dopamine suppresses the synthesis of melatonin in the photoreceptor cells.12 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 Mel1c 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.3,11,12 The localization of MT1 (Mel1a) melatonin receptors in photoreceptor cells has been observed in the human and rodent retina4–5 and in chicken retina (Mel1b),6,7 thus confirming our reports of melatonin receptor expression in photoreceptors. Two other melatonin receptors Mel1b and Mel1a are also expressed in the Xenopus retina.12,13 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 (Mel1a, Mel1b, and Mel1c) are expressed in cells of the Xenopus retina.12,13 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 Mel1c receptor-green fluorescent protein (GFP) fusion proteins in rod photoreceptors. The overexpressed Mel1c-GFP fusion proteins in rods is driven by an opsin promoter.14,15 This approach is dependent on the a-
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 Mel1c receptor in rod photoreceptors.

**Materials and Methods**

**Generation of DNA Constructs**

A plasmid containing a 544-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).19 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 Mel1c receptor-GFP fusion protein (XOP-Mel1c-GFP). A full-length Mel1c cdNA clone in a pcdNA I mammalian expression vector was kindly provided by Steven Reppert (University of Massachusetts Medical School, Worcester, MA),20 and we used this plasmid to prepare an XOP-Mel1c-GFP construct.

The XOP-GFP construct was digested with the restriction enzyme AgeI (Promega) which separated the opsins 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 CTG 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 Mel1c plasmid as the template. Specific restriction sites for AgeI were added to the 5′ end of the primers 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 Mel1c melatonin receptor cdNA PCR product was digested with AgeI, which removed the noncomplementary ends of the Mel1c 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 Mel1c receptor sequence28 plus an open reading frame between the Mel1c receptor sequence and the GFP sequence. For use in transgenic frogs, both the XOP-GFP and XOP-Mel1c-GFP constructs were digested with EcoRI and Hpal to separate the XOP-GFP fragment and the XOP-Mel1c-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 Amaya27 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% tricaine methanesulfonate (MS 222; Sigma-Aldrich, St. Louis, MO) for 20 minutes. Nuclei were isolated from sperm, as described previously,21 and then cryoprotected and stored at −80°C until time of use.

The oocyte high-speed cytoplasmic extract was prepared according to previously published methods.21,22 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, Millisde, DE). After 18 hours, oocytes were retrieved from the 1× Marc’s modified Ringer’s (MMR: 100 mM NaCl, 2 mM KCl, 1 mM MgSO4, 2 mM CaCl2, and 5 mM HEPES [pH 7.5]) in the tanks in which the frogs were housed. Oocytes were dejellied with 2% cysteine, rinsed in 1× MMR, and placed in 0.4× MMR and 6% Ficoll (Sigma-Aldrich) for immediate use in transgenesis.

*EcoRI/Hpal* (Promega) fragments containing the XOP-GFP construct or the XOP-Mel1c-GFP construct were purified by phenol-chloroform extractions and spin columns (Qiagen). Each fragment (200 ng) was incubated with approximately 2 × 10⁵ sperm nuclei for 5 minutes at room temperature (RT) in sperm dilution buffer (SDB: 250 mM sucrose, 75 mM KCl, 0.5 mM spermine, and 0.2 mM spermidine [pH 7.5]), and then 5 μL oocyte cytosolic extract, MgCl₂ 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 5 minutes at RT. The mixture was diluted to 120 nuclei/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/6% Ficoll with 50 μg/ml gentamicin (Sigma-Aldrich) 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 NaH₂PO₄ 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.25

**Genomic DNA Analysis**

The heads of anesthetized XOP-Mel1c-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′ CGG ATC TTG AAG TAC TCC TTG ATG 3′ and the 3′ primer sequence was 5′ CAA GGC GCC CCT GAA GTT CAT CTG 3′, which corresponds to positions 506-482 and 146-123, respectively of the GFP DNA sequence.25

*Melatonin Receptors in Transgenic Xenopus* 4523

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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 XL1 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 on the slides with mounting matrix (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). Crystall 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 corresponding to regions of the third cytoplasmic loop of the Mel1c melatonin receptor (residues 231-243; KQKLTQTDLRNFL),20 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.5,11,25 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 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 Clontec, Palo Alto, CA) diluted 1:10000 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-labeled 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 eye 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 (PolyVIEW; 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 = 40 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.11 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.40 For 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 Mel1a 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. 3). 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, 3). 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 Mel1c 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 arrowbeads), as indicated by the yellow fluorescence. Mel1a red immunolabeling appeared in the outer and inner plexiform layers (OPL, IPL; white arrowbeads), 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 (arrowbeads), 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 (arrowbeads) 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 (arrowbeads). (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 (arrowbeads) 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.

**Figure 3.** Distribution of GFP fluorescence and GFP immunoreactivity in transgenic frogs. (A, B) Opsin promoter-driven expression of GFP in rod photoreceptors. Standard fluorescent (A) and confocal (B) images show the yellow labeling (arrows) resulting from the colocalization of the green GFP fluorescence and the red GFP antibody labeling in a subset of rods. The red GFP immunolabeling occurs in rod inner segments (RIS; arrowheads) throughout the retina. A subset of rod photoreceptors display the green fluorescence caused by GFP expression in those cells driven by the opsin promoter, in which the entire rod cell somas (RIS and outer segments [ROS]) are filled with the green fluorescence. The red GFP immunoreactivity also occurs within the entire cell soma of the green fluorescent cells because of the reaction of the red-labeled GFP antibody with the GFP expressed by the opsin promoter in a subset of rods. Most ROS and RIS are negative for GFP fluorescence. (C, D) Opsin promoter-driven expression of the Mel1c receptor-GFP fusion protein. Standard fluorescent (C) and confocal (D) images show the red GFP antibody labeling in the RIS throughout the retina (arrowheads). ROS do not express any specific GFP immunolabeling. Note the presence of GFP immunoreactivity in what appear to be the axoneme of the ROS (✱). (E) Normal tadpole retina incubated with the GFP antibody demonstrates no specific GFP fluorescence or specific GFP immunolabeling of the photoreceptors (arrowheads). Abbreviations as in Figure 1. Scale bars: (A, C, E) 100 μm; (B) 20 μm; (D) 50 μm.

**Figure 4.** Green fluorescent protein (GFP) expression in transgenic frogs. Genomic DNA isolated from two Mel1c receptor-GFP fusion protein (XOP-Mel1c-GFP) transgenic tadpoles was isolated and PCR cloned with oligonucleotide probes complementary to GFP DNA sequences. The presence of GFP PCR fragments (385 bp) in an agarose gel of the PCR-amplified genomic DNA from the Mel1c receptor-GFP transgenic frogs indicates that the transgene is expressed in these 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 140 to 2240 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 a specific cell type. This is a noteworthy feature of this experimental approach, because three melatonin 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 melatonin 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 melatonin receptors.2,11,12 This discovery has been confirmed in humans and chickens15,16 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.9 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.27 Also, melatonin appears to have a role in photoreceptor outer segment disc shedding in Xenopus,1 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,28 and we have reported29 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 melatonin receptor.30 These reports, combined with the new evidence in

![Figure 6](https://example.com/fig6.png)

**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 reti-
nal 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,31,32 and we have shown that
melatonin increases the sensitivity of horizontal cells to light in
the salamander retina.33 This is supported by the report that
horizontal cells in the rat retina appear to express melatonin
receptor RNA and protein.13 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.10,34,35 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
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 photore-
ceptors,7–9,36,37 intracrine melatonin signaling in photorecep-
tors probably contributes substantially to circadian regulation
in the retina.

The dissociation constant of the\textit{Xenopus} Mel1c receptors is
reported to be 630 nM.20 This value is consistent with our
observation on the concentrations of melatonin that elicit ERG
responses in the Mel 1c transgenic frogs. Melatonin con-
centrations in the range of 1 to 100 nM exerted dose-depen-
dent 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 re-
sponses have shown that melatonin reduces the circadian
rhythm of the ERG b-wave amplitude in iguanas.38 Also, the
b-wave, but not the a-wave shows a peak in the daytime,39
whereas both the a- and b-waves show a circadian rhythm in
implicit time. Reduction in circulating levels of melatonin abol-
ishes 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 am-
plitude in the daytime.40 The a- and b-wave implicit times are
higher during the day than during the night in chickens.41
Melatonin abolishes the rhythm of b-wave amplitude and of a-
and b-wave implicit times in continuous darkness.41 These
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,42 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.43 Melatonin decreases the b-wave amplitude in the light and dark in human subjects, suggesting that it transduces the dark signal in the retina.44 It should be noted that the ERG recordings in these subjects, suggesting that it transduces the dark signal in the retina. Further study with this transgenic model is needed to correlate the effects of melatonin on specific transgenic melanin receptor overexpression in specific retinal cells, with the effects of melanin 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 melanin action on each melanin receptor type expressed in the many different retinal cell types offers a potentially effective approach to elucidate the role of melanin in the overall health and diseases of the retina.

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