Stimulation of Melatonin Receptors Decreases Calcium Levels in Xenopus Tectal Cells by Activating GABA<sub>C</sub> Receptors

Claudia Prada, Susan B. Udin, Allan F. Wiechmann, and Irina V. Zhdanova. Stimulation of melatonin receptors decreases calcium levels in Xenopus tectal cells by activating GABA<sub>C</sub> receptors. J Neurophysiol 94: 968–978, 2005. First published April 7, 2005; doi:10.1152/jn.01286.2004. To investigate the physiological effects of melatonin receptors in the Xenopus tectum, we have used the fluorescent indicator Fluo-4 AM to monitor calcium dynamics of cells in tectal slices. Bath application of KCl elicited fluorescence increases that were reduced by melatonin. This effect was stronger at the end of the light period than at the end of the dark period. Melatonin increased γ-aminobutyric acid-C (GABA<sub>C</sub>)–receptor activity, as demonstrated by the ability of the GABA<sub>C</sub>–receptor antagonists, picrotoxin and TPMPA, to abolish the effects of melatonin. In contrast, neither the GABA<sub>A</sub>–receptor antagonist bicuculline nor the GABA<sub>B</sub>–receptor antagonist CGP 35348 diminished the effects of melatonin. RT-PCR analyses revealed expression of the 3 known melatonin receptors, MT1 (Mel1a), MT2 (Mel1b), and Mel1c. Because the effect of melatonin on tectal calcium increases was antagonized by an MT2-selective antagonist, 4-P-PDOT, we performed Western blot analyses with an antibody to the MT2 receptor; the data indicate that the MT2 receptor is expressed primarily as a dimeric complex and is glycosylated. The receptor is present in higher amounts at the end of the light period than at the end of the dark period, in a pattern complementary to the changes in melatonin levels, which are higher during the night than during the day. These results imply that melatonin, acting by MT2 receptors, modulates GABA<sub>C</sub> receptor activity in the optic tectum and that this effect is influenced by the light–dark cycle.

INTRODUCTION

Although the physiological effects of many neurotransmitters and neuromodulators in the tectum have been studied, little is known about the role of the hormone melatonin in the tectum. In cold-blooded vertebrates, melatonin receptors are widely distributed but are particularly dense in areas associated with visual processing, such as the retina, tectum, and nucleus isthmi (Mazzucchelli et al. 1996; Wiechmann and Wirsig-Wiechmann 1993). In the tectum, melatonin binding has been reported both presynaptically (Brooks and Cassone 1992; Wiechmann et al. 1999) and postsynaptically (Krause et al. 1994). In Xenopus laevis, 3 G-protein–coupled melatonin receptors, denoted MT1 (Mel1a), MT2 (Mel1b), and Mel1c, have been identified and cloned (Ebisawa et al. 1994; Reppert et al. 1995b). Binding and molecular studies indicate that melatonin receptors undergo a daily rhythm of expression, increasing late during the light period and decreasing late during the dark period (Brooks and Cassone 1992; Wiechmann et al. 1999). Melatonin is synthesized at night by retinal photoreceptors where it is involved in photoreceptor outer disc shedding and phagocytosis (White and Fisher 1989), photomechanical movements (Pierce and Besharse 1987), increases in sensitivity of horizontal cells (Wiechmann et al. 1988), and changes in photoreceptor conductance (Brzezinski 1997). Melatonin also is synthesized at night in the pineal gland (Borjigin et al. 1999; Reiter 1991), from which it is released into the cerebrospinal fluid and blood; it modulates functions such as circadian rhythms, sleep, seasonal reproduction, neuroimmunological activities, and avian seasonal neuroplasticity (Arendt 2000; Brzezinski 1997; Guerrero and Reiter 2002).

Melatonin receptors mediate inhibitory effects of melatonin on increases of intracellular calcium induced by depolarizing influences (Zisapel and Laudon 1983). Using electrophysiological and fluorescence calcium-imaging approaches, this effect has been demonstrated in rat pituitary cells (Vanecek and Cardinali 1990; Rosenstei et al. 1989) and in rat brain synaptosomes (Vacas et al. 1984). Melatonin also decreases the spontaneous discharge rate in cells of the striate cortex of the cat (Reuss and Kiefer 1989). In addition, several studies indicate that melatonin exerts an enhancing influence on GABAergic activity, increasing γ-aminobutyric acid (GABA) binding, turnover, and GABA-induced chloride ion uptake (Boatright et al. 1994; Rosenstein and Cardinali 1990; Rosenstei et al. 1989; Wan et al. 1999b). These observations raised the possibility that melatonin might influence tectal GABA receptors, which include ionotropic GABA<sub>A</sub> (Xiao et al. 1999) and GABA<sub>C</sub> receptors (Sivilotti and Nistri 1989) and metabotropic GABA<sub>B</sub> Receptors (Sivilotti and Nistri 1988).

The prominent expression of melatonin receptors in the optic tectum and the evidence of melatonin’s modulatory effects in other parts of the nervous system thus led us to test melatonin’s effects by measuring calcium levels in tectal cells. For this purpose, we administered melatonin to tectal slices and compared the depolarization-induced calcium increase in slices pretreated with melatonin to nonpretreated slices. We also evaluated whether the effect of melatonin in the tectum is influenced by the light–dark cycle by assessing the effect of melatonin on tectal slices from animals killed at 2 different times in a 12 h:12 h light–dark cycle. Finally, we tested the possible involvement of GABA receptors as mediators of melatonin’s effects. Our results support a role of melatonin in

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the tectal physiology of *Xenopus* through modulation of GABA<sub>C</sub> receptors.

**METHODS**

*Xenopus laevis* were bred in our laboratory and were used between 6 and 12 mo postmetamorphosis, except for tadpoles and juveniles 0–3 mo postmetamorphosis that were also used for RT-PCR studies. Animals were housed in tanks maintained at room temperature on a 12 h:12 h light–dark cycle. To limit the differences between the 2 groups to their light–dark cycle, animals from the 2 different groups were killed at the same time of the experimenter’s day. For this purpose, animals that would be killed at ZT (Zeitgeber) 23 were housed under conditions that were time-shifted by 12 h for ≥4 wk before experiments were performed. Slice preparation and incubation times were equal for both groups of animals. All procedures were conducted with the approval of the State University of New York at Buffalo Animal Care Committee and in accordance with their regulations.

**Solutions and drugs**

The ionic composition of the sucrose solution used for dissection and first hour recovery was (in mM): 3.3 KCl, 0.8 MgCl<sub>2</sub>, 0.1 NaH<sub>2</sub>PO<sub>4</sub>, 10 dextrose, 223 sucrose, 1.8 CaCl<sub>2</sub>, and 26 NaHCO<sub>3</sub>. 1 pyruvic acid, 0.05 glutathione, 1 kynurenic acid, pH 7.4, 295–300 mOsm equilibrated with a gas mixture of 95% O<sub>2</sub>-5% CO<sub>2</sub>. The composition of the amphibian Ringer solution used for overnight incubation of the slices was (in mM): 111 NaCl, 3.3 KCl, 0.8 MgCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 1.8 CaCl<sub>2</sub>, 10 dextrose, and 0.1 NaH<sub>2</sub>PO<sub>4</sub> bubbled with a gas mixture of 95% O<sub>2</sub>-5% CO<sub>2</sub>. The ionic composition of the recording solution was (in mM): 134.5 NaCl, 3.3 KCl, 0.8 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 dextrose, 5 HEPES, bubbled with O<sub>2</sub>, pH 7.4, 295–300 mOsm. The recording solution containing 22 mM KCl was prepared by substituting equimolar amounts of NaCl and KCl. Except as noted below, bicuculline (40 μM), 1 mM kynurenic acid, and 0.5 μM tetrodotoxin (TTX; Calbiochem, La Jolla, CA) were added to the recording solutions to block GABA<sub>A</sub> receptors, ionotropic glutamate receptors, and voltage-sensitive sodium channels, respectively. Melatonin stocks were dissolved in ethanol and then further diluted in the perfusion solution. The maximum concentration of ethanol applied to the brain slice was 0.0002%; tests of ethanol vehicle showed that this level of ethanol did not influence the responses of the slices. Stock solutions (1 mM) of the AM-ester form of Fluo-4 (a fluorescent calcium indicator) were made in absolute dimethylsulfoxide (DMSO). Aliquots were diluted with Ringer solution to give a final concentration of 1 μM. The nonionic detergent Pluronic F-127 (final concentration of 0.01%) was added to increase solubility.

Chloromelatonin, 4-phenyl-2-propionamidotetralin (4-P-DOT), and picrotoxin were purchased from Tocris (Ellisville, MO). Fluo-4 AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). All the other chemicals were purchased from Sigma (St. Louis, MO).

**Slice preparation**

Animals were anesthetized by subcutaneous injection of 3-amino-benzoic acid ethyl ester (MS-222) and were then decapitated. Animals were killed at Zeitgeber time (ZT) 10 h except as noted. The brains were rapidly removed and placed in a chamber filled with chilled sucrose solution. Brains were then embedded in 4% low-melting-point agarose for slicing. The tissue was cut at 300 μM with a Vibratome (Vibratome, St. Louis, MO) while bathed in the same sucrose solution. Slices were then incubated in this solution for 1 h, after which they were transferred to chilled amphibian Ringer solution. Slices were incubated in this solution overnight. We compared slices prepared the same day with slices incubated overnight and found that overnight incubation produces a far higher yield of responsive tissue.

**Labeling of tectal cells**

Brain slices were labeled by soaking in a solution of Fluo-4 AM as described by Feller et al. (1996) (modifying the incubation time from 1 h to 7 min). After staining, slices were washed for 30 min with perfusion saline before use.

**Calcium imaging**

All experiments were performed at room temperature (20–24°C). Imaging experiments were performed with an Olympus BX51WI microscope equipped for epifluorescence. An Olympus 40 × (N.A. 0.8) water-immersion lens was used. Slices were placed in a perfusion chamber (Warner Instruments, Hamden, CT) with a 230-μL working bath volume and perfused at a rate of about 2 ml/min. Excitation light was provided by a 103 W/2 mercury short arc lamp (Olympus) and was attenuated by using neutral density filters to avoid photobleaching. Excitation and emission wavelengths were obtained by using a fluorescein-isothiocyanate (FITC) filter (excitation wavelength 490, emission 520). Images were collected with a Cooke Sensicam QE (Cooke, Auburn Hills, MI). Exposure times were between 100 and 300 ms, and frames were taken every 10 s. Acquisitions and analyses were performed with SLIDEBOOK software (Intelligent Imaging Innovations, Denver, CO).

Fluorescence was measured from regions of interest containing multiple cell bodies in layer 6, where cells that receive inputs from the retina and nucleus isthmi reside. Only slices that showed a similar increase in fluorescence to 3 initial depolarization stimuli were studied; about 60% of the slices met this criterion. Responses are presented as normalized ΔF/F, where F is the resting fluorescence (before stimulation) and ΔF is the peak change in fluorescence from resting levels. Normalization was accomplished by computing the ratio of the ΔF/F response to melatonin to the ΔF/F of a prior response to KCl. This normalization allowed us to standardize values across slices. Only responses to melatonin in slices that showed recovery, evidenced by subsequent response to KCl of nearly 95% of the initial response, were included in computations.

**Pertussis toxin (PTX) treatment**

Slices were incubated for 4 h in a 5% CO<sub>2</sub>, 95% O<sub>2</sub> bubbled, static bath with 2 μg/ml PTX in Ringer solution. Control slices were incubated for equal amounts of time in Ringer solution.

**Antagonist pretreatment**

Slices were incubated for 1 h in a static bath, bubbled with 5% CO<sub>2</sub>, 95% O<sub>2</sub>, with 1 μM 4-P-PDOT in Ringer solution before perfusion was resumed and either melatonin or vehicle was applied. 4-P-PDOT was dissolved to 10 mM in 95% ethanol and diluted to 1 mM in 50% ethanol. Further serial dilutions were performed in Ringer solution.

**RT/PCR**

Total RNA was isolated from pooled samples of optic tectum, using a RNeasy total RNA isolation kit (Qiagen, Santa Clarita, CA), according to the manufacturer’s instructions. Total RNA (500 ng/ sample) was reverse-transcribed (RT) using components from an RNA PCR Kit (Perkin–Elmer, Norwalk, CT) in a volume of 10 μl. The cDNA was then amplified by the polymerase chain reaction (PCR), using oligonucleotide primers specific for the melatonin MT1, MT2, or Mel<sub>C</sub> receptors, in a final volume of 50 μl. PCR primers were based on the *Xenopus laevis* melatonin receptor cDNA sequences (Ebisawa et al. 1994; Reppert et al. 1995b). For the MT1
receptor, the 5′ primer sequence was [5′ATT CCG CTA TTC TCT AAT TCC GGC3′] and the 3′ primer sequence was [5′AGA ATC TCC AAG GGG TGG ATA GAT3′], which corresponds to positions 16–39 and 426–403, respectively (GenBank accession number U31826), to generate a PCR product of 459 bp. For the MT2 receptor, the 5′ primer sequence was [5′GAA AAG CTG TTC AGC CTG TGG3′] and the 3′ primer sequence was [5′TTT GGG TGC TAC TTC TGT AAG3′], which corresponds to positions 16–36 and 423–403, respectively (GenBank accession number U31827), to generate a PCR product of 388 bp. For the Mel1α receptor, the 5′ primer sequence was [5′ATG ATG GAG GTG AAT AGC AC3′] and the 3′ primer sequence was [5′ACA CAG CAA CAA CCA GAT CG3′], which corresponds to positions 1–20 and 250–231 of the coding region, respectively (GenBank accession number U09561), to generate a PCR product of 251 bp. PCR reactions were performed with 1 cycle of 95°C for 5 min, then 40 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, followed by 1 cycle of 72°C for 7 min; then 15 μl of the amplified cDNA was electrophoresed on an agarose gel and stained with ethidium bromide.

Melatonin assay

To extract melatonin, brain samples were sonicated in methanol and the supernatant was removed after centrifugation and diluted with water to contain 10% methanol. Melatonin was then extracted using C18 columns, and samples were dried (Savant Speed Vac) and reconstituted with assay buffer. The melatonin assay was conducted using a Buehlemann Lab radioimmunoassay kit (ALPCO Diagnostics, Windham, NH), according to protocol. This is a sensitive method with a detection limit of 1 pg/ml, according to the manufacturer’s protocol (Supersignal West Pico Kit, Pierce). Immunoreactive bands were analyzed using a Bio-Rad GS-700 imaging densitometer and Multi-Analyist software (Version 1.1).

Western blot

After incubation, slices were homogenized directly in 1 × Laemmli sample buffer. The lysates were denatured at 95°C for 10 min. Insoluble material was removed by centrifugation (14,000g for 10 min), and the protein concentration for each sample was measured by the method of Bradford (1976). Stringent denaturing conditions were achieved by separating denatured proteins by electrophoresis on a 6 M urea and 10% SDS–PAGE gel with prestained protein standards (Bio-Rad, Palo Alto, CA) and the proteins were transferred to nitrocellulose membranes. The membrane was blocked in tris-buffered saline (TBS) plus 5% nonfat dry milk (Carnation, Solon, OH) for 1 h at room temperature. Blots were incubated overnight at 4°C with antibodies generated against *Xenopus* MT2 receptors diluted 1:500 with blocking solution. After rinsing, blots were incubated with horseradish peroxidase–conjugated anti-rabbit antibodies (Pierce, Rockford, IL) diluted 1:20,000 for 1 h at room temperature. After washing, chemiluminescence detection was performed using the manufacturer’s protocol (Supersignal West Pico Kit, Pierce). Immunoreactive bands were analyzed using a Bio-Rad GS-700 imaging densitometer and Multi-Analyist software (Version 1.1).

MT2 antibody

A polyclonal antibody directed against a 13 amino acid peptide (VKSEFKPRMQSDF), corresponding to a region of an intracellular loop of the *Xenopus laevis* MT2 receptor (Reppert et al. 1995b), was generated in rabbits (Invitrogen, Carlsbad, CA).

Statistical analysis

All data were analyzed by a 2-tailed, unpaired Student’s t-test or by a one-way ANOVA followed by the Bonferroni post hoc test (SPSS Software, SPSS, Chicago, IL). Significance was defined as *P < 0.05.*

**RESULTS**

**Depolarization conditions**

To determine changes in tectal calcium levels, slices were incubated with the membrane-permeant form of Fluo-4, rinsed, and placed in a perfusion chamber (Fig. 1A). A rapid increase in Fluo-4 fluorescence was observed in response to depolarization (Fig. 1B). We tested increasing concentrations of KCl (15, 22, 26, and 30 mM) to establish a concentration of KCl that would permit us to observe either increases or decreases in calcium resulting from melatonin coapplication. Brain slices were stimulated at least 3 times with the same KCl concentration for 90 s. A 5-min interval was allowed between stimuli for

**FIG. 1.** Response of tectal cells to depolarization. A: fluorescence image of a tectal slice incubated with the membrane-permeant tetracacetoxymethyl ester (AM) of Fluo-4 (a fluorescent calcium indicator). Slices were perfused with amphibian Ringer solution and depolarized with 22 mM KCl (10× magnification). Box indicates the area where the images in B were obtained. B: Fluo-4 fluorescence of tectal cells. Black indicates all pixels with brightness above arbitrary threshold. Images shown at 20-s intervals after start of 90-s perfusion with 22 mM KCl (40× water immersion lens). C: example of changes in fluorescence in a single tectal slice caused by depolarization with KCl with and without melatonin. Sliced was perfused with 500 nM melatonin for 4 min before being challenged by 22 mM KCl plus 500 nM melatonin for 90 s. Values represent percentage of ΔF/F.
washing. Each healthy slice was stimulated 8–10 times over a period of 1.5–2 h; responses to KCl were consistent over that period. The data were fitted to a Hill plot (not shown), and 22 mM KCl, which elicited a response of 80% of saturation levels, was chosen for subsequent experiments. In addition, for each graph presented in the results, every tectal slice contributing to the data was exposed to all the experimental conditions tested; the relatively high values of n for exposure to KCl reflect the repeated testing with KCl before and after each drug trial. Calcium transients were measured as ΔF/F values.

Effect of melatonin on depolarization-induced calcium increase

To investigate whether melatonin exerts an effect on depolarization-evoked calcium increases, tectal slices were pretreated for 4 min with melatonin followed by perfusion with 22 mM KCl plus melatonin. Pretreatment with melatonin inhibited the increase in calcium in tectal cells (Fig. 1C). This effect was concentration dependent, with a significant inhibition observed even at the lowest concentration tested, 50 nM (Fig. 2A). At the maximum melatonin concentration tested (5 μM), the peak Fluo-4 fluorescence averaged 58 ± 6% (SE) of control (n = 3, P < 0.05). The response to depolarization recovered to control levels after a 5-min washout period. In addition, we assessed whether melatonin had a similar inhibitory influence on increases of calcium elicited by a lower KCl concentration (15 mM); 0.05 μM melatonin elicited peak Fluo-4 fluorescence values that were 78% of control levels, essentially the same as those obtained with 22 mM KCl (data not shown).

The physiological relevance of the melatonin concentrations used for these experiments was assessed by comparing them with the values estimated based on radioimmunoassay of melatonin in brain tissue of animals killed during the dark period, which indicated a content of about 0.9 ng melatonin/mg protein (Fig. 2B). Using the method of Bradford (1976), we measured the tectal protein content to be about 630 μg and, based on these values, the amount of tectal melatonin was estimated as 0.57 ng. Because tectal volume is about 10 cm³, the concentration of melatonin in the tectum was estimated as 245 nM, the molecular weight of melatonin is 232.28 g/M, the concentration of melatonin in the tectum was estimated as 245 nM, close to the value used in most of our experiments.

The specificity of the effect of melatonin was tested using chloromelatonin (0.5 μM), a melatonin analog. Pretreatment of tectal slices for 4 min with chloromelatonin followed by perfusion with 22 mM KCl plus chloromelatonin mimicked the effect of melatonin by decreasing the peak Fluo-4 fluorescence to 73% of control (n = 5, P < 0.05) (Fig. 2C). This effect is reversible.

Role of GABA receptors in melatonin-induced inhibition of depolarization-mediated calcium increase

Several studies indicate that melatonin potentiates GABA-induced increases of chloride ion influx by regulating GABA-receptor activity (Rosenstein et al. 1989; Wan et al. 1999a; Wu et al. 1999). Because GABA-immunoreactive structures are found throughout the frog’s optic tectum (Antal 1991; Li and Fite 2001; Rybicka and Üdin 1994), we next studied whether the effect of melatonin on depolarization-evoked calcium increases in tectal slices is mediated by GABA-receptor activation. For this purpose, we examined the response of tectal cells to depolarization stimuli in conditions where GABA_A-, GABA_B-, or GABA_C-receptor antagonists were added in combination with melatonin during depolarization (Fig. 3). Exogenous GABA was not added because depolarization was expected to cause release of GABA from tectal interneurons. In addition, to specifically test the effect of each GABA antagonist, bicuculline was omitted from the amphibian Ringer solution in these experiments. Bath application of the GABA-receptor antagonists alone had no significant effect on fluorescence increases elicited by 22 mM KCl compared with control levels (Fig. 3, black bars). Bicuculline (40 μM) and CPG 35348 (100 μM), GABA_A- and GABA_B-receptor antagonists,
respectively, did not alter the effects of melatonin (Fig. 3, A and B). In contrast, picrotoxin (100 μM), a GABA<sub>A</sub>-receptor antagonist, counteracted the effect of melatonin (Fig. 3C). Furthermore, 2,5,6-tetrahydropyridine-4-yl methylphosphonic acid (TPMPA, 100 μM), a highly selective GABA<sub>C</sub>-receptor antagonist, significantly diminished the melatonin effect (Fig. 3D); changes in fluorescence resulting from 22 mM KCl/melatonin/TPMPA were not significantly different from the changes caused by 22 mM KCl alone (Fig. 3D). Because TPMPA, a purely GABA<sub>C</sub>-receptor antagonist, did not appear to be as effective an antagonist as picrotoxin, a GABA<sub>A,C</sub>-receptor antagonist, we also tested the 2 drugs in combination to evaluate further whether GABA<sub>A</sub> receptors might be playing a role. As Fig. 3D indicates, the effects of the antagonists were not additive. These results are consistent with the hypothesis that the effect of melatonin on tectal cells is mediated by GABA<sub>C</sub> receptors.

Detection of melatonin receptors in optic tectum of Xenopus

We used RT-PCR to determine which of the 3 known Xenopus melatonin receptors are expressed in the tectum. PCR primers specific for all 3 melatonin receptors (MT1, MT2, and Mel1c) were reverse-transcribed and PCR-amplified (RT-PCR)
RT-PCR of Mel receptors

FIG. 4. Evidence for melatonin receptors in Xenopus tectum. Agarose gel electrophoresis shows RT/PCR products of tectal RNA, amplified with primers complementary to the MT1 (Mel1a), MT2 (Mel1b), and Mel1c DNA sequences. MT1 tissue in this example was harvested from 2-wk postmetamorphosis froglets, whereas the MT2 and Mel1c tissues were obtained from adults. PCR bands of the predicted size appear in the receptor MT1 (459 bp) and MT2 (388 bp) and Mel1c lanes (251 bp).

from RNA isolated from Xenopus tectum. Agarose gel electrophoresis demonstrated PCR products of the expected size in tectum samples of late tadpoles, juveniles, and adult animals amplified with the MT2 and the Mel1c primers, but MT1 product was observed only in tadpoles and juveniles ≈ 2 wk postmetamorphosis (Fig. 4). These results indicate that the Xenopus tectum expresses the 3 known melatonin receptors and suggest that their expression is influenced by age.

Effect of melatonin on depolarization-induced calcium increase is receptor mediated

EFFECT OF PERTUSSIS TOXIN (PTX). To determine whether the effects of melatonin are mediated by melatonin receptors, which are G-protein coupled, we performed experiments in which tectal slices were preincubated with PTX, a blocker of inhibitory (Gi) and other (Gq) G-proteins (Hildebrandt et al. 1983). Pretreatment of slices with PTX did not affect increases in Fluo-4 fluorescence resulting from KCl alone. Also, control slices preincubated in a static bath for 3–5 h in the absence of PTX showed the expected inhibitory effect of melatonin (66% of the response to KCl alone). In contrast, PTX pretreatment resulted in a significant inhibition of the melatonin effect on depolarization-induced calcium increases (Fig. 5); values in PTX-treated slices were 90% of control. These observations suggest that melatonin acts through a pertussis toxin–sensitive G-protein to decrease elevations of intracellular calcium induced by depolarization stimuli.

EFFECT OF MT2 RECEPTOR ANTAGONIST. Because functional studies indicate that the MT2 receptor mediates melatonin effects on retinal physiology (Dubocovich et al. 1997), we sought to determine whether the MT2 receptor is also involved in the modulation of retinal input to the tectum by evaluating the effect of melatonin in tectal slices. For this purpose, we used 4-P-PDOT, a melatonin receptor antagonist with a 300-fold higher selectivity for the MT2 receptor over the MT1 receptor (Dubocovich et al. 1997). 4-P-PDOT (1 μM) counteracted the inhibitory effect of 50 nM melatonin in tectal cells (Fig. 6). Preincubation of slices with 4-P-PDOT had no effect on increases of fluorescence in response to 22 mM KCl alone. These results suggest that in tectal cells, the effect of melatonin on stimulated calcium increases is mediated by the MT2 receptor.

Detection of MT2 receptor protein in Xenopus tectum

Because the effect of melatonin on calcium increases in tectal slices was antagonized by 4-P-PDOT, an antagonist with
a high selectivity for the MT2 receptor, we decided to test whether the protein for this receptor is present in the *Xenopus* tectum. Using sequence data for the MT2 receptor, we raised an antibody to a peptide corresponding to an intracellular loop of the receptor. With this antibody, we performed Western blot analysis using nonreducing SDS–PAGE, which revealed an immunoreactive band of about 85 kD (data not shown). Because molecular characterization of this receptor predicts a molecular mass of about 40 kD (Reppert et al. 1995a), we considered the possibility that the receptor is assembled as a dimer (Ayoub et al. 2002), and we therefore tested the effect of more stringent reducing conditions (as described in Methods). This modification resulted in the detection of a lower band, with apparent molecular mass roughly corresponding to the expected monomeric species of the receptor (about 45 kD) (Fig. 7, left lane). In addition, isolated membranes of tectal tissue were treated with endoglycosidase H (Endo H), a recombinant glycosidase that cleaves oligosaccharides from N-linked glycoproteins (Maley et al. 1989). This treatment resulted in a decrease of the intensity of the higher and lower molecular weight immunoreactive bands and the subsequent detection of a new, nearly 38-kD band (Fig. 7, right lane). This effect is consistent with reports of sites for asparagine-linked glycosylation in the amino terminal of the MT2 receptor (Reppert et al. 1995a).

**Effect of melatonin on depolarization-induced calcium increase is influenced by the light–dark cycle**

To determine whether the inhibitory influence of melatonin on depolarization-induced calcium increase is influenced by the light–dark cycle, the effects of melatonin on tectal tissue obtained from animals killed at 2 different ZT were compared. We found that the effect of melatonin was more pronounced in animals killed at ZT 10 (ZT 0 = lights on) than in animals killed at ZT 23 (Fig. 8A). The decrease of ΔF/F at ZT 10 was significantly different from the values obtained at ZT 23 (n = 15, P < 0.05).

**Western blot of MT2 receptors**

![Western blot of MT2 receptors](image)

**FIG. 7.** Immunodetection of MT2 receptor in isolated membranes with MT2 receptor antibody. Membrane preparations were separated by SDS–PAGE–urea gel, blotted to nitrocellulose, and labeled with an antibody against the *Xenopus* MT2 receptor. Two immunoreactive bands appear at about 85 and 45 kD. Right lane: treatment with endoglycosidase H (Endo H) results in a lower immunoreactive band at about 38 kD and in a decrease of the intensity of the higher MW bands. Molecular weights were determined using Kaleidoscope prestained standards (Bio-Rad).

**DISCUSSION**

Our results show that melatonin inhibits the calcium increases induced by KCl in tectal slices and that this effect is mediated by melatonin receptors, through a mechanism involving activation of GABA<sub>a</sub> receptors. We also present evidence that the MT2 receptor is expressed in the *Xenopus* tectum, in both monomeric and dimeric states. In addition, the effect of...
melatonin is influenced by the light–dark cycle, with a stronger effect in tissue obtained from animals killed at the end of the light period (ZT 10) than near the end of the dark period (ZT 23), consistent with our data showing a higher MT2 receptor density at ZT 10 than that at ZT 23.

Melatonin modulation of GABA<sub>C</sub>-receptor function and possible impact on tectal activity

A link between melatonin and GABAergic pathways was previously reviewed (Rosenstein and Cardinali 1990), and several groups provided evidence of such links in Xenopus retina, where melatonin receptor immunoreactivity is colocalized with GABAergic amacrine cells (Wiechmann and Wirsig-Wiechmann 2001b), and the GABA-receptor blockers, picrotoxin and bicuculline, block melatonin-induced suppression of dopamine release (Boatright et al. 1994).

We also demonstrated that suppression of depolarization-induced calcium accumulation by melatonin involves GABA<sub>C</sub> receptor activation. Picrotoxin and TPMPA, nonselective and selective GABA<sub>C</sub> antagonists, respectively, reversed the melatonin-induced inhibition of calcium increase (Fig. 3).

Pharmacological experiments have indicated that GABA<sub>C</sub> receptors are present in the frog tectum (Sivilotti and Nistri 1989). In addition, studies in the mammalian superior colliculus, the mammalian counterpart of the frog tectum, have suggested a postsynaptic role of GABA<sub>C</sub> receptors in GABAergic transmission (Kirischuk et al. 2003). These reports reveal that GABA<sub>C</sub> receptors mediate an excitatory action of GABA on the activity of projection neurons induced by optic nerve stimulation (Boller and Schmidt 2003; Pasternack et al. 1999; Platt and Wittington 1998; Sivilotti and Nistri 1989). These results appear to contradict our observations that GABA<sub>C</sub> receptors mediate the inhibitory effect of melatonin. However, these authors attributed the excitatory action of GABA<sub>C</sub> receptors to disinhibition, a multisympathetic effect. Because we used TTX, bicuculline, and kynurenic acid, we observed only direct effects. In contrast, retinotectal activity initiates a chain of events, in which disinhibition may be crucial for triggering the firing of tectal projection neurons that mediate prey catching or predator avoidance. If so, the presence of melatonin would alter such cascades of activity.

Because GABA<sub>C</sub> receptors are down-modulated by a cAMP-dependent protein kinase (PKA) (Dong and Werblin 1994; Wellis and Werblin 1995), and melatonin decreases cAMP (Wiechmann and Wirsig-Wiechmann 1993), melatonin could initiate an intracellular signaling pathway involving inhibition of adenylyl cyclase, a decrease of cAMP, and relief of cAMP-dependent down-modulation of GABA<sub>C</sub> receptors (Fig. 9). The resulting increase in chloride influx by the GABA<sub>C</sub> receptor would then oppose the depolarizing effect of KCl and thereby reduce the opening of voltage-sensitive calcium channels. This model is supported by reports that melatonin-induced hyperpolarization of plasma membrane is an upstream event to its influence on calcium influx through voltage-dependent channels (Vanecek 1999; Vanecek and Klein 1992). The impact of melatonin on tectal functioning is thus mediated in part by GABA<sub>C</sub> receptors, and the circadian periods when melatonin levels are high are predicted to be periods of high GABA<sub>C</sub>-mediated disinhibition within the tectum. However, melatonin also affects other components of the visual system that influence tectal function; as noted above, melatonin receptors directly affect retinal functioning (Brzezinski 1997; Wiechmann et al. 1988), and our work in progress also shows that melatonin influences the responses of retinotectal axons (Prada and Udin, unpublished observations). Moreover, melatonin also affects calcium levels in the nucleus isthmi, a midbrain structure with reciprocal connections to the tectum (Gruberg and Udin 1978; R. Lima and S. Udin, unpublished observations). The net effect of these multiple sites of melatonin’s influences has yet to be established.

MT2 receptor expression

We propose that the effect of melatonin in tectal slices is mediated at least in part by the MT2 receptor, for several reasons: 1) PCR and Western blotting show expression of this receptor in the tectum; 2) diurnal variations in responses to melatonin correlate well with changes in MT2 receptor expression; and 3) the MT2 receptor antagonist 4-P-PDOT (Dubocovich et al. 1997) counteracts the effect of melatonin in tectal cells. In the case of interpretation of the 4-P-PDOT data, however, some caution is required because the selectivity of the drug has not been established in Xenopus.

In Xenopus, evidence that MT2 receptors are also expressed in the retina (Wiechmann and Wirsig-Wiechmann 2001a) also supports the idea that this receptor contributes to the effect of melatonin on visual function.

In the optic tectum of Xenopus, evidence of melatonin receptors has been indicated by autoradiographic receptor
binding (Mazzucchelli et al. 1996). Our data imply that a substantial amount of binding is likely to involve the MT2 receptor. In addition, our Western blot analyses performed under different denaturing conditions suggest that MT2 is expressed primarily as a dimeric complex. This result is in line with a report that demonstrated ligand-independent dimerization of melatonin receptors (Ayyob et al. 2002). Our Western blot analyses revealed 2 immunoreactive bands of about 45 and 85 kDa in the membrane fraction of the optic tectum. The predicted molecular weight of the MT2 receptor is about 40 kDa, not including posttranslational modifications (Reppert et al. 1995a). We hypothesize that the higher band observed in our immunoblots represents the glycosylated dimeric form of the receptor and that the lower band represents the glycosylated monomer form, as indicated by the appearance of a new, nearly 38-kDa band after treatment of membrane fractions with Endo H. Glycosylation of this receptor is supported by analyses of its sequence, which contains a consensus N-terminal glycosylation site (Reppert et al. 1995a). It is interesting that Endo H treatment did not result in a lower band of the dimeric form. This observation suggests that glycosylation plays an important role in stabilization of the dimeric complexes.

The effect of melatonin was prevented by pretreatment of the slices with PTX (Fig. 5). Because PTX blocks the effects of agents that act through Gs/Gt proteins (Katada and Ui 1982), we postulate that melatonin’s effect involves a G-protein-mediated mechanism. Altogether, these data indicate that in tectal slices, melatonin decreases depolarization-induced calcium increase through a receptor-mediated mechanism that probably involves the MT2 receptor. The present data, however, do not preclude an involvement of the Mel1c receptor in the effect of melatonin on tectal cells.

**Tectal melatonin content**

We have calculated that the concentration of melatonin in the *Xenopus* tectum is about 250 nM. This concentration is much higher than the circulating concentration of the hormone reported in amphibians (0.5–1 nM) (d’Istria et al. 1994; Wright et al. 2003). However, several studies have reported that the brain concentration of melatonin greatly exceeds its plasma concentration (Cardinali et al. 1997; Hedlund et al. 1977), a result that probably stems from the ability of brain tissue to accumulate melatonin (Ferreira et al. 1996).

Most of our experiments were conducted using 500 nM melatonin, which is about twice the dark-period value that we have estimated for tectal melatonin. Because we used bath perfusion and slices of 300 μm thickness, the effective concentration of melatonin reaching the center of the slice, where the healthiest cells are most likely to be located, should be close to the physiological level.

**Influence of the light–dark cycle on melatonin’s effect and on receptor density**

Not surprisingly, the effect of melatonin on tectal slices was influenced by the light–dark cycle. Melatonin’s effect was less pronounced in tissue removed late during the dark period (ZT 23) than late in the light period (ZT 10). This finding is consistent with a decrease in MT2 receptor protein in tecta of animals killed at ZT 23 as compared with ZT 10 (Fig. 8). These results support previous studies indicating that melatonin receptors undergo a diurnal rhythm of expression (Guerrero et al. 2000; Masana et al. 2000; Wiechmann and Smith 2001). Furthermore, melatonin levels in the *Xenopus* brain were high during the dark period and low during the light period (Fig. 2B), consistent with the possibility that high levels of melatonin downregulate melatonin receptor expression. Our data in *Xenopus* are in general agreement with observations in other species (Aste et al. 2001; Brooks and Cassone 1992) and indicate that the light–dark cycle influences the levels of melatonin and of its receptors. The net effect of the changes in melatonin and melatonin receptors is likely to be dominated by the large magnitude of the circadian fluctuations in melatonin levels rather than by the relatively small changes in melatonin receptor numbers, although our data do not directly address this point.

**Melatonin’s effect on calcium levels**

The present results are consistent with previous work indicating an inhibitory effect of melatonin on calcium accumulation resulting from excitatory inputs (Ayar et al. 2001; Dubocovich 1983; Vacas et al. 1984; Vanecek and Klein 1992; Zisapel and Laudon 1983). Several studies indicate that the effects of melatonin on calcium accumulation include a downstream influence on calcium influx through calcium channels (Ayar et al. 2001; Mei et al. 2001; Vanecek and Klein 1995). Our results suggest that in tectal cells, melatonin regulates calcium increases by an action involving an inhibitory G-protein and GABA receptors. We hypothesize that melatonin may in part regulate calcium levels by enhancing GABA-mediated hyperpolarization, which would then decrease calcium influx through voltage-sensitive calcium channels.

In conclusion, we have demonstrated that the optic tectum of *Xenopus* has functional melatonin receptors. In tectal slices, exogenous melatonin inhibits increases in cytosolic calcium evoked by depolarization stimuli with 22 mM KCl. Our data indicate that this effect is receptor mediated and involves activation of GABA  receptors. In addition, the light–dark cycle influences melatonin levels in the *Xenopus* brain, the effect of melatonin in tectal slices, and the density of expression of the MT2 receptor.

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