

Research report

Binding characteristics and daily rhythms of melatonin receptors are distinct in the retina and the brain areas of the European sea bass retina (*Dicentrarchus labrax*)

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Abstract

Melatonin is synthesized, with a circadian rhythm, in the pineal organ of vertebrates, high levels being produced during the scotophase and low levels during the photophase. The retina also produces melatonin, although in the case of the European sea bass, its secretion pattern appears to be inverted. In the study described here, radioreceptor assay techniques were used to characterize the melatonin binding sites, their regional distribution and their daily variations. Brain and retina membrane preparations were used in all the binding assays and 2-[¹²⁵I]iodomelatonin ([¹²⁵I]Mel) as radioligand at 25°C. The specific binding of [¹²⁵I]Mel was seen to be saturable, reversible, specific and of high affinity. In all the tissues assayed, the power of the ligands to inhibit [¹²⁵I]Mel binding decreased in the following order: melatonin ≫ 4-P-PDOT > luzindole ≥ *N*-acetylserotonin, which points to the presence of Mel1-like receptors. The inhibition curves of 4-P-PDOT suggested the presence of two different binding sites in the brain areas, but only one type of site of low affinity in the neural retina. No daily variations in [¹²⁵I]Mel binding capacity (B_{\max}) or affinity (K_d) were detected in the brain areas, while a clear rhythm in K_d melatonin receptor affinity and B_{\max} binding capacity was observed in the retina. K_d and B_{\max} retinal rhythms were out of phase with the lowest K_d and the highest B_{\max} occurring at scotophase. This result suggests that retinal melatonin is a paracrine factor able to control receptor desensitization during photophase when ocular melatonin is higher in this species.

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Topic: Other neurotransmitters

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1. Introduction

The pineal organ of vertebrates synthesises melatonin, which is considered to be the time-keeping hormone, since

it is released during the dark period of the daily cycle. Melatonin synthesis is the result of the transduction of photoperiodic information, whose signal is conveyed to the brain and other peripheral organs, affecting the circadian rhythms of animals. However, this transduction process is not completely understood, although the development of radioreceptor assay techniques development using 2-[¹²⁵I]iodomelatonin ([¹²⁵I]Mel) as a radioligand has increased our knowledge of melatonin-binding sites in all species investigated.

Abbreviations: [¹²⁵I]Mel, 2-[¹²⁵I]iodomelatonin; 4-P-PDOT, 4-phenyl-2-propionamidotetralin; Mel, melatonin; NAS, *N*-acetylserotonin; Luz, luzindole; NSB, non-specific binding

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The main target of pineal melatonin action appears to be the brain [10]. The retina, other melatonin-producing organ in many vertebrate species, is another target for this hormone, regulating the adaptation to darkness [7]. In two species, including the European sea bass, the secretion of ocular melatonin follows a diurnal pattern [42,53], which contrasts with the pineal synthesis rhythm, which is nocturnal in all the species so far studied, such as catfish and pike [6,52]. This unusual fact led us to characterize the melatonin binding sites in the retina and to compare them with melatonin receptors in the brain of the European sea bass.

Based on their pharmacological characteristics, melatonin binding sites have been classified into two types, ML₁ and ML₂ [10]. The former is a subfamily of G-protein-coupled receptors, with three members, Mel 1a, Mel 1b and Mel 1c [41]. The Mel 1a receptor (currently known as MT₁) has a high affinity for [¹²⁵I]Mel and is expressed in the brain, retina and other peripheral tissues. The Mel 1b receptor (currently known as or MT₂) has a slightly lower affinity for [¹²⁵I]Mel than MT₁, and is mainly found in the brain and different peripheral tissues of some mammalian species [32]. In turn, the ML2 receptor, also known as MT₃, belongs to the family of the quinone reductases and has been recently purified [36], although its physiological role is still debated. Melatonin receptors have been cloned in several fish species, such as the rainbow trout and pike [16,34].

The sensitivity of the receptors fluctuates throughout the 24-h cycle, which may imply changes in melatonin receptor density, probably influenced by the lighting regime, time of day and developmental or endocrine status [44]. Nevertheless, contradictory results have been obtained regarding the influence of light on the density and affinity of melatonin binding sites. For example, some studies have reported a circadian variation in the binding capacity of brain receptors in avian and fish, while their affinity remained unchanged over 24 h [22,23,48,49,50]. On the other hand, circadian changes in both receptor density and affinity have been described in masu salmon and pike brain [2,15]. In the Siberian hamster, Gauer et al. [21] demonstrated that a light pulse given during nighttime failed to induce any variation in the melatonin receptor density of the suprachiasmatic nucleus (SCN). Thus, it appears that the photic input had no direct effect on melatonin receptor levels in the SCN of this species, a result that is in contrast with those obtained by the same authors in the rat [19,20]. When present, these variations in receptor density along the 24-h light/dark cycle have been described to occur out of phase with circulating melatonin levels [47].

In a previous study performed in the European sea bass, we described the existence of a single class of melatonin binding site in the brain of this species [5]. However, the distribution study suggested the existence of a remarkable degree of heterogeneity in the characteristics, and, possibly, in the functions of melatonin

receptors in discrete brain areas [5]. In order to clarify these aspects, we have attempted to characterize in detail the melatonin receptors in the neural retina and in the discrete brain areas, as well as its possible variations in binding capacity and affinity along a 24-h cycle in this species.

2. Materials and methods

2.1. Chemicals

The radioligand [¹²⁵I]Mel (specific activity: 2000 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). Melatonin and *N*-acetylserotonin (NAS) was from Sigma (St. Louis, MO). Luzindole (*N*-acetyl-2-benzyl-tryptamine) and 4-phenyl-2-propionamidotetralin (4-P-PDOT) were purchased from Tocris Cookson (Bristol, UK).

2.2. Animals and housing

Animals used in this study were 45 adult sea bass specimens, with mean body weights of 36.7±11.1 and 98.6±19.4 g and body lengths of 13.3±2.8 and 19.3±1.2 cm in Experiments 1 and 2, respectively. Fish were obtained from the “Laboratorio de Cultivos Marinos” (CASEM, University of Cádiz, Spain). Fish were maintained under natural photoperiod in circular tanks of 1000 l each provided with central drainage, in an open system continuously supplied with running borehole water of 39 ppt salinity at a temperature of 19.4±0.2 °C. Oxygen was supplied by aeration, the minimum level observed during trials being 5.6 mg/l or 77.8% saturation. Water renewal was set at four to five times total volume per day. Light was natural photoperiod conditions. Fish were fed with commercial extruded pellets (Trouw Aquaculture, Spain). Animals were treated in agreement with the European Union regulation concerning the protection of experimental animals.

2.3. Experimental designs

2.3.1. Experiment 1

This study was designed to characterize melatonin binding sites in sea bass. Fifteen fish were anaesthetized with 2-phenoxyethanol (0.3 ppm), weighed, measured and decapitated in the middle of the light period. Neural retina and the whole brain with the pituitary attached were dissected out and frozen in solid CO₂. Before freezing, the brain was divided into five regions: telencephalon, hypothalamus, mesencephalic tectum–tegmentum, cerebellum–vestibulolateral lobe and medulla oblongata–spinal cord, as previously described [5]. Samples were used to carry out saturation, kinetics and pharmacology competition studies.

2.3.2. Experiment 2

The aim of this experiment was to investigate the possible variation of the characteristics of melatonin binding sites in the sea bass brain and retina along a 24-h cycle. The experiment was performed near the summer solstice (sunrise at 0606 h, sunset at 2043 h) in Cádiz, Spain (latitude 36°32' N, longitude 6°19' W). Animals arranged in six experimental groups ($n=5$) were anaesthetized, weighed and measured at 16.00, 20.00, 00.00, 04.00, 08.00 and 12.00 h. Blood samples (1–2 ml) were taken by caudal puncture with heparinized syringes, and the plasma was separated by centrifugation before being frozen in solid CO₂ until melatonin determination. Fish were decapitated, and samples of brain areas and neural retina were taken and frozen in solid CO₂. Brain was divided into the same six regions as in Experiment 1. Samples were used to estimate the K_d and B_{max} by saturation studies.

2.4. Membrane preparation

Samples, which were pooled from 15 animals in Experiment 1, and treated individually in Experiment 2, were sonicated in Tris–HCl buffer (50 mM, pH 7.4), (6 pulses of 3 s at 30 W power) (Dr. Hielscher, Stuttgart, Germany). In order to reduce non-specific binding, homogenates were first centrifuged at 800× g for 5 min to eliminate melanin granules [26]. The resulting supernatants were centrifuged at 16000× g for 20 min. Pellets were resuspended in Tris–HCl buffer and centrifuged again. A final tissue concentration of approximately 20 mg protein/ml, determined by Lowry's method [30], was obtained from the crude membranes in Tris–HCl buffer. Membranes were manipulated at 4 °C during the process, and stored at –80 °C until assay.

2.5. Binding assays

All binding assays were performed in a total volume of 50 μ l containing 10–15 μ g of membrane, using [¹²⁵I]Mel as radioligand. Unlabeled melatonin (10 μ M) was used to quantify the non-specific binding. The binding of [¹²⁵I]Mel was measured in triplicate after incubation at 25 °C for 90 min, except in the kinetic assays, where incubation times varied. For the distribution study, saturation assays were performed in each brain region and retina separately, using [¹²⁵I]Mel concentrations from 3 to 250 pM, while in competition and kinetic studies, the radioligand concentration was 70 and 150 pM, respectively. The reaction was stopped by addition of 750 μ l of ice-cold Tris–HCl buffer and immediate vacuum filtration through 25-mm glass fiber filters (Millipore, APFC, Billerica, MA, USA) using a Millipore 1225 cell harvester. Filters were washed with 4 ml of ice-cold Tris–HCl buffer, and then radioactivity was quantified in a γ -counter (Perkin Elmer Life Science Wallac Wizard 1470, Boston, MA, USA) with 82%

efficiency. Non-specific binding was subtracted from total binding to obtain the specific binding of [¹²⁵I]Mel in each sample.

2.6. Melatonin analysis

Plasma melatonin concentrations were determined using a commercial kit of enzyme-linked immunosorbent assay (ELISA), by IBL (Hamburg, Germany). Briefly, plasma samples were purified after thawing, using C₁₈ reverse-phase extraction columns in the centrifuge. Samples were added to different wells of an ELISA plate (pre-coated with capture antibody), and incubated with the melatonin–biotin and antiserum solutions overnight. The wells were washed and the plate was incubated with the enzyme-labeled solution for 2 h. After washing again, the plate was incubated with the *p*-nitrophenyl phosphate solution for 30 min, a stop solution was added and absorbance was read at 405 nm.

2.7. Data analysis

In the saturation studies, equilibrium dissociation constants (K_d) and binding density (B_{max}) values were calculated by a non-linear regression of a four-parameter logistic model using the ALLFIT program [9]. To determine the existence of a single or multiple binding

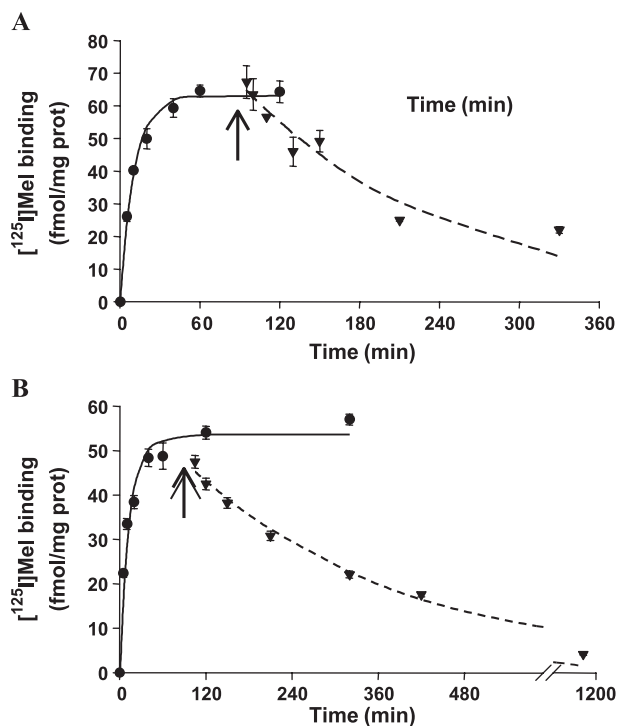


Fig. 1. Time courses of [¹²⁵I]Mel association (●) and dissociation (▼) in retina (A) and optic tectum (B) of European sea bass. The radioligand was used at a concentration of 155 and 107 pM, respectively. The arrows indicates the initiation of dissociation (addition of 10 μ M unlabeled melatonin). Each point represents mean \pm S.E.M. of triplicate determinations.

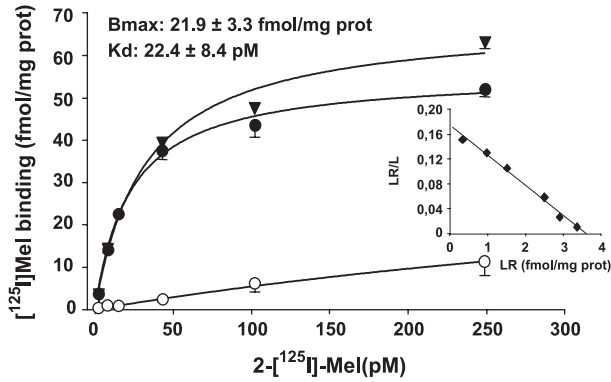


Fig. 2. Saturation curves of [¹²⁵I]Mel binding to membrane preparations from the neural retina of European sea bass. The radioligand concentration ranged from 2 to 250 pM. Each point represents the mean±S.E.M. of triplicate determinations Total binding (▼), specific binding (●) and non-specific binding (○). Inset graph depicts the Scatchard plot of the data. LR (concentration of bound ligand), LR/L (ratio of bound/free ligand).

sites in saturation assays, we used Scatchard plots. Linearity of the plot is considered significant when the *P*-value of the regression coefficient is lower than 0.05.

The association and dissociation rate constants (*K*₊₁ and *K*₋₁, respectively) from kinetic studies were obtained by non-linear regression [12], assuming a pseudo-first-order exponential rise to a maximum and a first-order exponential decay, respectively. The *K*_d from the kinetic studies was calculated as *K*_d=*K*₋₁/*K*₊₁.

For ligand competition studies, data were fitted to one- and two-site competition models. Curve fits were compared by *F*-test; a *P*-value of less than <0.05 indicates that the two-site model is a statistically better fit. *K*_i IC₅₀ values and Hill slope were estimated for each ligand by using the (Sigma Plot 8.0 program).

The melatonin results and the daily rhythms of *K*_d and *B*_{max} are expressed as mean±S.E.M. The statistical differences between the different time points were determined by one-way analysis of variance (ANOVA) followed by Duncan's test, with *P*<0.05 taken as the statistically significant threshold.

Table 1
Pharmacological profile of [¹²⁵I]Mel binding from several brain areas and neural retina of sea bass

<i>K</i> _i	Melatonin (pM)	Luzindole (nM)	NAS (nM)
Retina	945±128	706±83	1470±180
Optic tectum–thalamus	105±10	105±11	307±31
Hypothalamus	217±33	234±31	705±128
Cerebellum	222±49	124±16	286±52
Medulla oblongata	128±18	25.8±4.3	91.8±13.8
Telencephalon	71.5±10.8	28.7±5.1	106±15

Membranes have been incubated with [¹²⁵I]Mel and several concentrations of melatonin analogues. Data were fitted to one-site competition model. Inhibition constant (*K*_i±S.E.) of each ligand has been calculated from IC₅₀ values by application of Cheng and Prusoff equation: *K*_i=IC₅₀/(1+[L]/*K*_d).

3. Results

3.1. Experiment 1

Kinetic, saturation and competition assays were performed to characterize the melatonin binding sites in retina and discrete brain areas of sea bass.

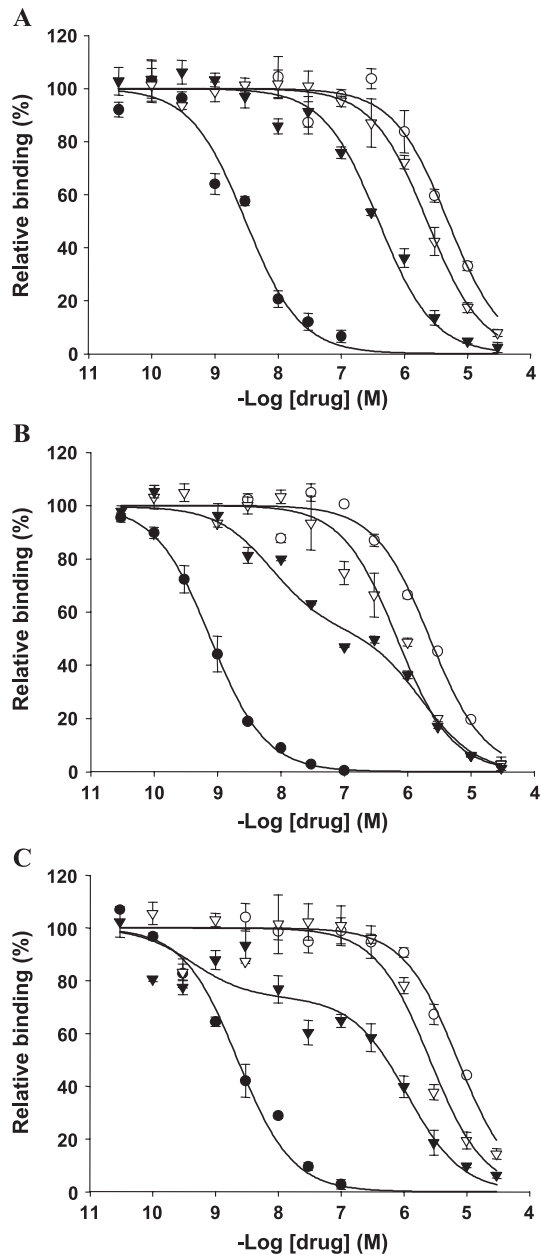


Fig. 3. Competition curves for inhibition of [¹²⁵I]Mel binding by melatonin analogues in membrane preparations from the retina (A), optic tectum (B) and hypothalamus (C) of European sea bass. The concentrations of radioligand were 50, 57 and 82 pM, respectively. Data are expressed as percentage of specific binding in the absence of competing drug (mean±S.E.M., *n*=3). Ligands were Mel (●), melatonin; NAS (○), *N*-acetylserotonin; 4-P-PDOT (▼), 4-phenyl-2-propionamidotetralin; and Luz (▽), luzindole.

3.1.1. Kinetic studies

The optic tectum–thalamus and neural retina membranes were incubated with the radioligand at different times until the steady state of [125 I]Mel binding was reached. The association reached equilibrium after 90 min for both, neural retina (Fig. 1A) and optic tectum–thalamus (Fig. 1B). The association rate constants (K_{+1}) were very similar, $(5.65 \pm 0.47) \times 10^{-4} \pm 0.47 \times 10^{-4} \text{ min}^{-1} \text{ pM}^{-1}$ for neural retina, and $(7.20 \pm 0.75) \times 10^{-4} \pm 0.75 \times 10^{-4} \text{ min}^{-1} \text{ pM}^{-1}$ for optic tectum–thalamus. Following the above association equilibrium, the addition of unlabeled melatonin (10 μ M) initiated the dissociation of [125 I]Mel, which occurred faster in the retina (around 7 h, Fig. 1A) than in the optic tectum–thalamus (18 h, Fig. 1B). The dissociation rate constants (K_{-1}) were $(6.3 \pm 1.0) \times 10^{-3} \pm 1.0 \times 10^{-3}$ and $(3.2 \pm 0.2) \times 10^{-3} \pm 0.2 \times 10^{-3} \text{ min}^{-1}$, respectively. These results showed the reversibility of specific [125 I]Mel binding in both tissues. The dissociation constants (K_d) derived from the kinetic constants (K_{-1}/K_{+1}) were 11.2 and 4.4 pM for retina and optic tectum–thalamus, respectively. This difference in affinity was mainly due to the lower value of the K_{-1} constant in optic tectum–thalamus.

3.1.2. Saturation studies

Increasing concentrations of [125 I]Mel from 2 to 250 pM were added to membrane preparations of neural retina. Specific binding increased with ligand concentration until saturation was reached above 100 pM (Fig. 2). The Scatchard plot was linear (inset of Fig. 2), indicating that [125 I]Mel binds to a single class of sites in the neural retina. The dissociation constant (K_d) was 22.1 ± 3.7 pM, and the B_{max} was 55.6 ± 3.3 fmol/mg protein.

3.1.3. Competition studies

In order to determine the pharmacological profiles of [125 I]Mel receptors in the discrete brain areas and retina of European sea bass, binding assays were performed with membranes of telencephalon, optic tectum–thalamus, hypothalamus, cerebellum, medulla oblongata and retina, using

different concentrations of melatonin, Luz, NAS and 4-P-PDOT as representative melatonin analogues. The pharmacological profiles obtained were similar for the different brain areas and retina, and showed the following order of inhibitory powers of the ligands: Mel \gg 4-P-PDOT > Luz > NAS. Table 1 shows the K_i values of Mel, Luz and NAS. The Hill coefficients of these drugs are close to 1 (data not shown), indicating a single class binding site. The K_i values for the retina were significantly higher than the respective K_i value in the brain areas. Thus, the low affinity of agonists and antagonists seems to be a general characteristic of the retinal receptor.

Pharmacology of 4-P-PDOT was more complex. In the optic tectum–thalamus (Fig. 3B), hypothalamus (Fig. 3C) and the rest of the brain areas (data not shown), inhibition curves for 4-P-PDOT were biphasic, revealing two distinct binding sites. In the same experimental conditions, only one class of site of low affinity was detected in the neural retina (Fig. 3A).

A clear difference in the Hill coefficients was found for 4-P-PDOT (Table 2). The coefficient is close to one in the retina, but was near 0.5 or even lower in the other tissues studied. This is the first indication that 4-P-PDOT binding may differentiate two different binding sites in the sea bass brain. The binding site with low affinity in the nanomolar range (site 2, Table 2) was present in all the areas studied, but the high affinity site in the picomolar range (or site 1) did not apparently exist in the retina. Site 1 rendered a significant fraction of the specific binding in the brain areas (from 35% in hypothalamus to 58% in medulla oblongata). These different levels of affinity between both 4-P-PDOT sensitive sites was very significant, as can be seen from the K_i values (100-fold difference in cerebellum and telencephalon, and 400–500-fold in optic tectum–thalamus, hypothalamus and medulla oblongata).

To characterize in detail the pharmacology of 4-P-PDOT, we compared the binding of optic tectum–thalamus membranes from sea bass at midday and midnight (Fig. 4; Table 2). Binding in the absence of competing ligand was

Table 2
Pharmacology of 4-P-PDOT from European sea bass retina and brain

4-P-PDOT	Hill coefficient	Site 1		Site 2	
		Binding (%)	K_i (pM)	Binding (%)	K_i (nM)
Retina	0.80 ± 0.07	0	–	100	119 ± 16
Optic tectum–thalamus	0.47 ± 0.03	46.2 ± 4.0	1150 ± 377	53.8 ± 4.0	274 ± 73
Hypothalamus	0.37 ± 0.03	35.0 ± 4.8	278 ± 180	65.0 ± 4.8	151 ± 50
Cerebellum	0.50 ± 0.06	42.5 ± 10.9	406 ± 295	57.5 ± 10.9	34.8 ± 19.0
Medulla oblongata	0.43 ± 0.04	57.4 ± 4.3	104 ± 33	42.6 ± 4.3	37.5 ± 15.6
Telencephalon	0.53 ± 0.06	53.5 ± 8.7	126 ± 63	46.5 ± 8.7	12.6 ± 7.3
Optic tectum–thalamus					
Midday	0.36 ± 0.03	45.5 ± 2.2	112 ± 24	54.5 ± 2.2	131 ± 25
Midnight	0.53 ± 0.03	26.4 ± 3.0	227 ± 105	73.6 ± 3.0	46.3 ± 6.6

Comparison of day and night pharmacology from optic tectum–thalamus.

Membranes have been incubated with [125 I]Mel and several concentrations of 4-P-PDOT. Data were fitted to two-site competition model. Inhibition constant ($K_i \pm \text{S.E.}$) has been calculated from IC_{50} values by application of Cheng and Prusoff equation: $K_i = \text{IC}_{50} / (1 + [L]/K_d)$. Hill coefficient values correspond to data fitted to one-site competition model.

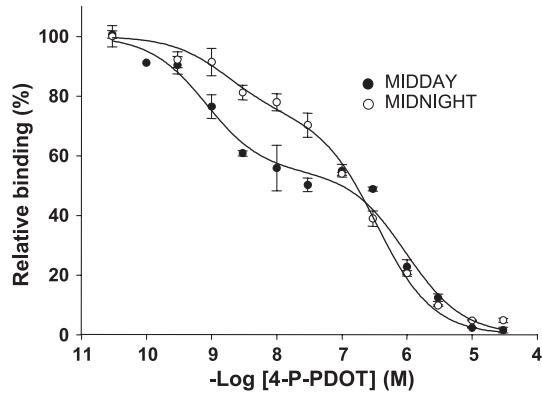


Fig. 4. Competition curves of 4-P-PDOT to [¹²⁵I]Mel binding in membranes from the optic tectum of European sea bass obtained at midday and midnight. Data are expressed as percentage of specific binding in the absence of 4-P-PDOT (mean±S.E.M., n=3). (●) Midday; (○) midnight.

not statistically different (midday: 36.2±3.0 fmol/mg protein, n=6; midnight: 39.0±1.8 fmol/mg protein, n=6).

The existence of two classes of binding sites is demonstrated by the biphasic competition curves obtained at both times of the daily cycle. In addition, the optic tectum–thalamus at midday had a larger population of high affinity sites (approximately 46% of the total binding sites, Table 2), meanwhile, at midnight the high affinity population of binding sites for 4-P-PDOT was significantly smaller (26%, *F*-test, *P*<0.05).

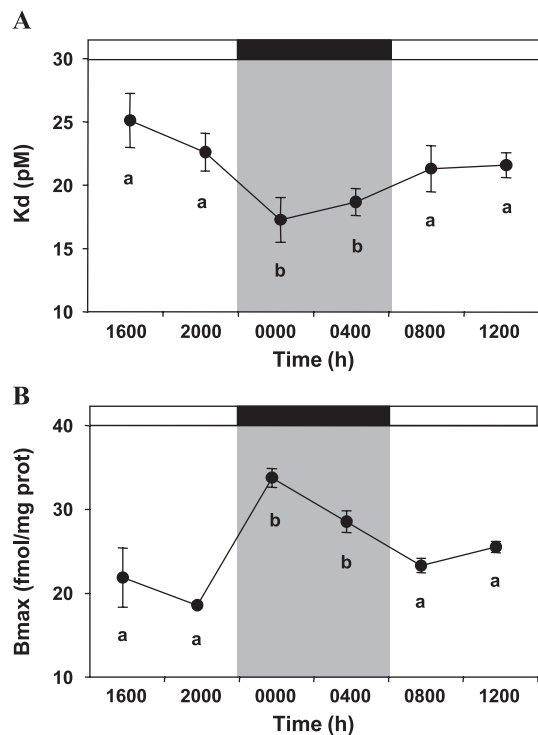


Fig. 5. Daily variations of *K_d* (A) and *B_{max}* (B) of [¹²⁵I]Mel binding in the neural retina membranes of European sea bass. Shaded area indicates night hours. Each point represents the mean±S.E.M. (n=4). Different letters indicate significant differences between mean values from different time groups (ANOVA, Duncan’s test, *P*<0.05).

3.2. Experiment 2

The saturation analysis of specific [¹²⁵I]Mel binding was carried out every 4 h throughout a 24-h cycle in neural retina and discrete brain areas. The *K_d* and *B_{max}* values were estimated individually for each fish and the results were analyzed by one-way ANOVA. In the case of neural retina (Fig. 5), significant differences (*P*<0.05) were found in both the receptor affinity (*K_d*) and binding capacity (*B_{max}*) during the 24-h cycle. The daily profiles showed the opposite pattern; *K_d* was lower during the night (00.00 and 04.00 h) than during daytime (16.00 h), while *B_{max}* at 0000 h was significantly higher than the daytime values. There were no

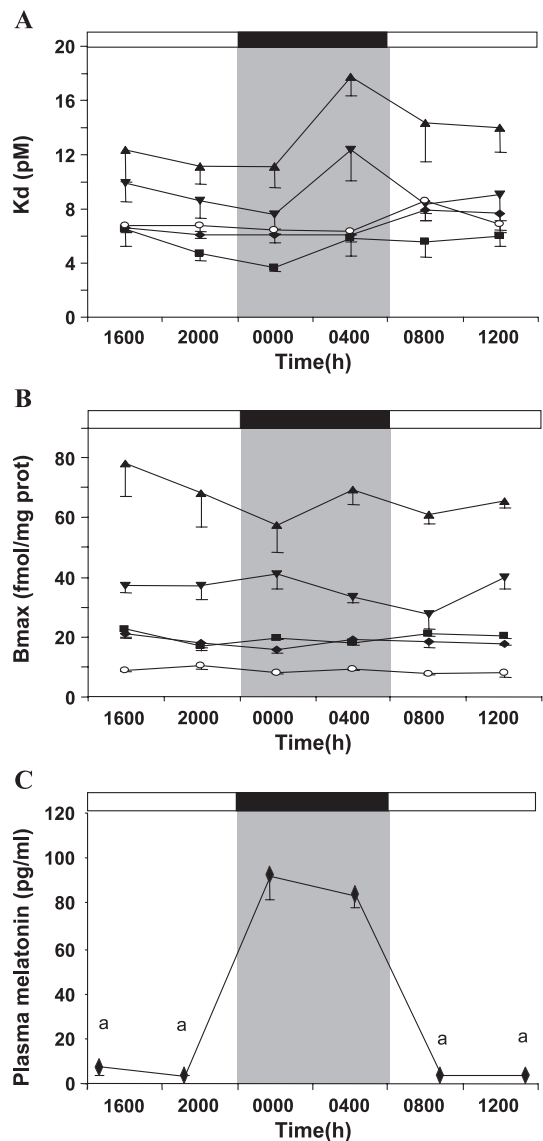


Fig. 6. Daily rhythms of *K_d* (A) and *B_{max}* (B) of [¹²⁵I]Mel binding in discrete brain areas and in circulating melatonin levels (C) of the European sea bass, in telencephalon (■), optic tectum–thalamus (▲), hypothalamus (▼), cerebellum (◆), and medulla oblongata (○). Shaded area indicates night hours. Each point represents the mean±S.E.M. (n=5). Statistical differences as in Fig. 5.

significant changes in K_d (Fig. 6A) or B_{max} (Fig. 6B) in the different brain areas analyzed.

A clear daily rhythm in plasma melatonin levels was observed in the same fish (ANOVA, $P < 0.05$). The profile exhibited very low levels during the day, and a significant rise during the night, with high melatonin concentrations at 00.00 and 04.00 h time points (Fig. 6C).

4. Discussion

The present study provides information about melatonin binding sites in nervous tissues of the European sea bass. The [125 I]Mel binding was seen to be specific, saturable and reversible in all the brain areas and the retina, characteristics that fulfill the criteria of a hormone receptor. The binding sites were of high affinity, with K_d values in the low picomolar range.

The association of [125 I]Mel to optic tectum–thalamus and retina binding sites reached the steady state within 90 min, while dissociation took more than 7 h in the retina and 20 h in the optic tectum–thalamus. Similar results have been obtained in the brain of other fish species, such as trout [8], sea bream [14], pike [15], goldfish [22,31], catfish [25] and masu salmon [1,2], and also in the retina of goldfish [24], where association occurred in less than 2 h, but dissociation proceeded slowly. In sea bream, results vary depending on age of fish [17]. However, despite our specimens were younger than fish used in the above-mentioned studies, association–dissociation curves were similar. The estimated rate constants (K_{+1} , K_{-1}) agree with the previously described values for whole brain homogenates in this species [5]. The dissociation constants (K_d) estimated from the kinetic studies for both optic tectum–thalamus and neural retina were quite similar to the K_d values obtained from the saturation studies. Both were in the low picomolar range (4.4 and 11.2, respectively), and comparable to those found in other fish brains [1,2,8,22,25,31,38]. In contrast, the K_d in the brain of other fish, such as the gilthead sea bream, is significantly higher, and well into the nanomolar range [14].

The binding capacity of neural retina ($B_{max} = 21.9 \pm 3.3$ fmol/mg prot) was intermediate in comparison with the different brain areas [5]. The highest B_{max} was present in the optic tectum–thalamus region and hypothalamus for this species. The retina showed a density similar to that of the telencephalon and cerebellum, and significantly higher than in medulla oblongata and olfactory bulbs.

In all of the tissues assayed, the power of ligands to inhibit [125 I]Mel binding was Melatonin > 4-P-PDOT > Luz > NAS, which reflects the pharmacological profile of a Mel₁-like receptor. The Mel K_i value for melatonin obtained from the brain binding sites (0.1–0.2 nM) led us to conclude that this affinity for the natural ligand is sufficient to transduce the nocturnal increase of circulating melatonin in this species (≈ 0.4 nM, Fig. 6C). In contrast, the affinity of

melatonin to retinal binding sites was significantly lower ($K_i = 0.95$ nM), as was the affinity of other ligands (NAS, Luz; Table 1). The distinctive low affinity of Mel receptors in the retina during the photophase may be easily justified as an adaptation mechanism to the active Mel synthesis in the eye during this period [4].

As regards the inhibition curves obtained for the melatonin antagonist, 4-P-PDOT, it appears that the European sea bass brain contains two distinct populations of melatonin binding sites. Biphasic displacement curves have also been found in other teleost fish brain, the gilthead sea bream and pike [14,15]. However, most pharmacological studies in non-mammalian vertebrates have described the existence of monophasic curves, e.g. in frog [28], masu salmon [1,2], skate [45], dark-dwelling deep-sea fish [43], trout [8], goldfish [22,24] and catfish [25].

The co-expression of various receptor subtypes in the same tissue has also been described in several species [40,46], but few selective ligands are available for characterizing native melatonin receptor subtypes in native tissue samples. The 4-P-PDOT has been proposed as a selective antagonist for mammalian MT₂ receptor [11,37]. In the case of European sea bass brain, the biphasic 4-P-PDOT inhibition curve allowed two K_i values to be estimated, one in the low nanomolar range, corresponding to a high affinity site, and the other one in the high nanomolar range for a low affinity site. These affinity ranges are consistent with mammalian MT₂ (≈ 1 nM) and MT₁ (220–446 nM) receptor affinities for 4-P-PDOT [11,37].

Another possible explanation could be the existence of one sole receptor with two affinity states, such as has been described for several G-protein-coupled receptors, including human MT₁ [35]. However, this possibility seems improbable in the European sea bass, because the two affinity states are only revealed by an antagonist, such as 4-P-PDOT, but not by agonists. Furthermore, the inhibition curve for 4-P-PDOT in the neural retina is monophasic and only exhibits the low affinity site ($K_i = 120$ nM, Table 1). Until definitive molecular identification would be possible, the most probable explanation is the co-expression of two melatonin receptor subtypes in the European sea bass brain with MT₁- and MT₂-like pharmacology.

In the present study, the daily rhythms in melatonin receptor affinity and binding capacity were analyzed in European sea bass, and substantial differences were found between the brain and retina. As occurred in whole brain homogenates [5], no significant variations in receptor affinity or density appeared along the 24-h cycle in any of the brain areas assayed [5]. The brain of several vertebrates, including fish, has been the focus of melatonin binding sites studies, and significant daily variations have been observed in receptor density alone [22,27,29,39,51], or in both receptor affinity and binding capacity [1,2,14,15].

Even though the K_d and B_{max} did not show any daily variation, the European sea bass brain might still exhibit a rhythmic receptor expression. In the optic tectum–thalamus,

we detected a clear effect of daytime on subtype proportions: at midday both MT₂-like (site 1, Table 2) and MT₁-like (site 2) receptors were present in a similar rate, whereas at midnight the MT₁-like binding site was the more abundant subtype (75%).

In contrast to the results obtained in the brain, both the K_d and B_{max} exhibited significant daily rhythms in the European sea bass retina. Interestingly, these rhythms appeared out of phase, with the minimal K_d and the maximal B_{max} occurring at the beginning of the dark period (00.00 h). The rhythm was more patent for B_{max} , where the maximum/minimum ratio was close to 2, while it was only 1.5 for the K_d rhythm. The very few studies that have analyzed daily variations of melatonin binding sites in retina have showed these rhythms to be in phase [13]. When both rhythms are found in brain, they usually appear also in phase [2,14,27]. The affinity and density of melatonin receptors have been reported to fluctuate throughout the light/dark cycle due to variations in their sensitivity status [47].

The mechanisms controlling melatonin receptor sensitization or desensitization remain unknown, but it has been suggested that photoperiod, estrogen [47] or melatonin itself [18,22,23,27,33] may be involved. The inverse relationship between the circulating melatonin rhythm and receptor affinity and density supports the key role played by endogenous hormone. In almost all the species so far studied, melatonin receptors appear to be desensitized (lower affinity and density) coincident with high plasma melatonin levels during darkness. Thus, circulating melatonin rhythm is out of phase with receptor affinity and binding capacity rhythms [14,27,47]. In contrast, in the European sea bass retina, melatonin is synthesized during the light period [3,17,26,42]. This uncommon characteristic of the European sea bass ocular melatonin rhythm may be closely related to the mechanisms that regulate the daily oscillation of melatonin binding oscillations in the retina. In this sense, the low density and affinity of melatonin binding sites during the daytime coincide with the peak of ocular melatonin, suggesting that melatonin may act as a direct desensitizing factor. Moreover, the presence of circadian B_{max} and K_d rhythmic patterns in fish brain points out to the existence of a clock-driven rhythm of melatonin receptor gene expression. This is also the case with *Xenopus laevis* retina, which expresses a daily rhythm of melatonin receptor mRNA [46].

In summary, in the present study, we have characterized [¹²⁵I]Mel binding sites in the retina of the European sea bass, demonstrating their distinct properties from those of brain receptors, although both receptors are probably members of the same Mel1 subfamily. The pharmacological studies using 4-P-PDOT suggested the existence of a single class of melatonin binding site in the retina and the presence of two distinct binding sites in the brain of this species. However, we cannot rule out the existence of other melatonin receptor subtypes at very low density in the

retina. Finally, our results also reveal that daily regulation of the binding capacity and K_d is tissue-dependent, both rhythms being inverted in the retina.

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These experiments comply with the *Principles of Animal Care* of the Institute of Health (1985), and also with the laws of the country in which the experiments were conducted.

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