Melatonin Binding Sites in the Brain of European Sea Bass (Dicentrarchus labrax)

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Melatonin Binding Sites in the Brain of European Sea Bass (*Dicentrarchus labrax*)

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ABSTRACT—Characteristics, day-night changes, guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) modulation, and localization of melatonin binding sites in the brain of a marine teleost, European sea bass *Dicentrarchus labrax*, were studied by radioreceptor assay using 2-[^{125}I]iodomelatonin as a radioligand. The specific binding to the sea bass brain membranes was rapid, stable, saturable and reversible. The radioligand binds to a single class of receptor site with the affinity (Kd) of 9.3±0.6 pM and total binding capacity (Bmax) of 39.08±0.86 fmol/mg protein (mean±SEM, n=4) at mid-light under light-dark (LD) cycles of 12:12. Day-night changes were observed neither in the Kd nor in the Bmax under LD 12:12. Treatment with GTPγS significantly increased the Kd and decreased the Bmax both at mid-light and mid-dark. The binding sites were highly specific for 2-phenylmelatonin, 2-iodomelatonin, melatonin, and 6-chloromelatonin. Distribution of melatonin binding sites in the sea bass brain was uneven: The Bmax was determined to be highest in mesencephalic optic tectum-tegmentum and hypothalamus, intermediate in telencephalon, cerebellum-vestibulolateral lobe and medulla oblongata-spinal cord, and lowest in olfactory bulbs with the Kd in the low picomolar range. These results indicate that melatonin released from the pineal organ and/or retina plays neuromodulatory roles in the sea bass brain via G protein-coupled melatonin receptors.

Key words: sea bass *Dicentrarchus labrax*, melatonin receptor, daily rhythm, brain

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) is an indoleamide synthesized in the pineal organ and retina of vertebrates. Generally, melatonin production exhibits a clear daily rhythm with high levels observed during the dark phase under light-dark (LD) cycles. The duration of nocturnal increase in melatonin production is influenced by day length with longer duration under short photoperiod than under long photoperiod. These changes in melatonin regulate seasonal rhythms such as reproduction and moulting (Binkley, 1987; Yu and Reiter, 1991).

In addtion to the regulation by light, melatonin production is regulated by circadian clock. In the mammalian pineal gland, the circadian clock that regulates melatonin production in the pineal locates in the suprachiasmatic nucleus (SCN) of the hypothalamus, the site of primary circadian pacemaker. The photic information is received in the retina and sent to the SCN, which in turn regulates the pineal via sympathetic nervous system (Binkley, 1987; Yu and Reiter, 1991). On the contrary, melatonin production in the pineal of

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non-mammalian vertebrates and in the retina of vertebrates is regulated by the circadian clocks located in these photo-receptive structures (Iigo *et al.*, 1994a; Cahill *et al.*, 1991; Falcón, 1999). Thus, the pineal organ, retina and melatonin could be important components of the vertebrate circadian system.

The European sea bass (*Dicentrarchus labrax*) provides an interesting opportunity to analyze the roles of melatonin in the teleostean circadian system. Recent studies demonstrated the presence of daily and circadian feeding rhythms with a dual phasing capacity: some individual fed during the light phase (i.e. diurnal) but the other fed during the dark phase under LD (i.e. nocturnal) (Sánchez-Vázquez *et al.*, 1995a,b, 1998). The information on such feeding habits provides some insights into the development of feeding regimes in aquaculture farms because this species is of great commercial values in Mediterranean regions. In addition, the presence of reversed melatonin profiles in the retina (with high ocular contents during the light phase under LD) has been demonstrated (Sánchez-Vázquez *et al.*, 1997; Iigo *et al.*, 1997d). The melatonin rhythms in the plasma and eyes were also influenced by environmental temperature, day length and light conditions (Iigo *et al.*, 1997d; Garcia-Allegue *et al.*, 2001; Bayarri *et al.*, 2002, 2003). Thus, this species is an interesting model to analyze the circadian system in marine fish.

In this study, in order to make the roles of melatonin in the sea bass circadian system clear, we characterized melatonin binding sites in the brain by radioreceptor assay using 2-[^{125}I]iodomelatonin as the radioligand. Here we report characteristics, day-night changes, guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) modulation, and distribution of melatonin binding sites in the sea bass brain.

MATERIALS AND METHODS

Materials

2-[^{125}I]Iodomelatonin (81.4 TBq/mmol) were obtained from Dai-ichi Pure Chemicals (Tokyo, Japan). Unlabeled 2-iodomelatonin was obtained from Research Biochemicals Inc. (Natick, MA). Other indole derivatives, bovine serum albumin (BSA), polyethylenimine, dopamine, noradrenaline and GTPγS (tetra lithium salt) were obtained from Sigma (St. Louis, MO). Acetylcholine chloride was obtained from Nakarai Tesque, Inc. (Kyoto, Japan). Ordinary chemicals were obtained from commercial sources.

Membrane preparations and binding assays

Membrane preparations and binding assays were performed according to the procedure as previously described (Iigo *et al.*, 1994b, 1997c) unless otherwise stated. In the present study, 50 mM Tris-HCl buffer containing 4 mM $CaCl_2$ (pH 7.4) was used as the assay buffer. The binding of 2-[^{125}I]iodomelatonin was routinely measured in duplicate after incubation at 25°C for 2 hr with the exception of kinetic studies where incubation duration varied. Protein contents in the incubation mixture were determined by the method of Bradford (1976) with bovine γ-globulin as a standard. Nonspecific binding was defined as the binding in the presence of 10 μM melatonin. Specific binding was calculated by subtracting nonspecific binding from total binding and expressed as fmol/mg

protein. A preliminary study demonstrated the specific binding increased linearly at least up to 0.2 mg protein/tube.

Characterization, day-night changes and GTPγS modulation of melatonin binding sites in the sea bass whole brain

Sea bass (approx. 170 g in body weight) reared in net cages were supplied by a local fish farm (Culmarex, S.A., Aguillas, Murcia, Spain). Fish were reared in 350 l experimental tanks filled with artificial seawater (hw-Marinemix, Wiegandt GmgH, Germany) of 2.3% salinity at 24°C. Illumination (200 lx at the water surface) was supplied by 40 W daylight blue bulbs for the light phase of LD cycles (LD 12:12; light on 0800–2000 hr). During this period, sea bass were fed using demand feeding devices (Sánchez-Vázquez *et al.*, 1997), displaying mainly nocturnal feeding behavior (data not shown).

Fish were anesthetized with 2-phenoxyethanol (0.6 ml/l) around mid-light (1400 hr) or mid-dark (0200 hr). Blood samples were taken from caudal vasculature with a heparinized syringe with a 23-G needle and collected in test tubes on ice. Plasma was later separated by centrifugation and melatonin contents were determined by radioimmunoassay as previously described (Iigo *et al.*, 1997e; Sánchez-Vázquez *et al.*, 1997). Fish were then decapitated and whole brains were dissected out, frozen on dry ice and later used for kinetic, saturation and competition studies. Saturation studies with or without GTPγS (10^{-4} M were performed using a range of 2-[^{125}I]iodomelatonin concentration from 6.1 to 189.6 pM. Kinetic and competition studies were performed using a 2-[^{125}I]iodomelatonin concentration of 49.9 and 48.0 pM, respectively.

Localization of melatonin binding sites in discrete brain areas

Sea bass (approx. 540 g in body weight) were obtained from the Laboratorio de Cultivos Marinos (CASEM, University of Cádiz, Spain). Fish were reared in 1000 l experimental tanks continuously supplied with running borehole water of 3.9% salinity at a temperature of 19.4°C under natural photoperiod. Fish were fed with commercial extruded pellets (Trouw Aquaculture, Spain).

On June 14, 2001, fish were decapitated during the light phase (around 1700 hr). Brain regions (olfactory bulbs, telencephalon, hypothalamus, mesencephalic optic tectum-tegmentum, cerebellum-vestibulolateral lobe, and medulla oblongata-spinal cord; see Fig. 1) were immediately dissected out, rapidly frozen on dry ice and later used for saturation analysis using 2-[^{125}I]iodomelatonin concentration from 6.0 to 200.0 pM.
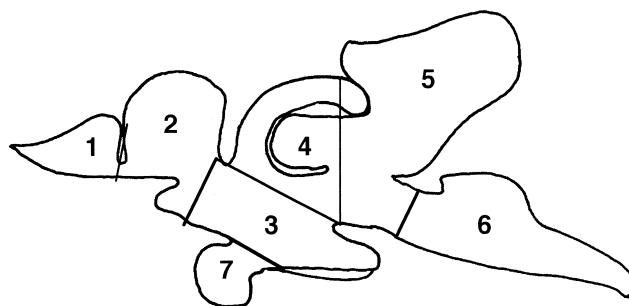
Fig. 1. Schematic representation of the sea bass brain (sagittal view) that depicts dissections used in the present study to localize melatonin binding sites in the sea bass brain. 1: olfactory bulbs; 2: telencephalon; 3: hypothalamus; 4: mesencephalic optic tectum-tegmentum; 5: cerebellum-vestibulolateral lobe; 6: medulla oblongata-spinal cord; 7: pituitary gland.

Data analysis

Data obtained from the kinetic, saturation and competition experiments were analyzed as previously described to calculate association rate constants (k_1), dissociation rate constants (k_{-1}),

affinity (Kd), total binding capacity (Bmax), Hill coefficients (n_H), and Ki values (Iigo et al., 1994b). Day-night changes in plasma concentrations of melatonin and in the Kd and Bmax of melatonin binding sites in the brain were analyzed by t-test. The Kd and Bmax in the GTPγS-treated group were compared with respective control values by paired t-test. Analysis was performed using the GraphPad Prism program (GraphPad Software, San Diego, CA).
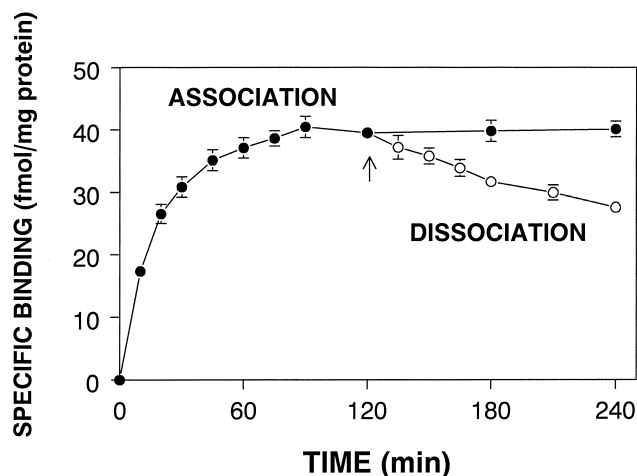
RESULTS

Kinetic Studies

The time course of the association of 2-[^{125}I]iodomelatonin (48.6 pM) to sea bass brain membranes at 25°C is shown in Fig. 2. The specific binding of 2-[^{125}I]iodomelatonin was rapid, stable and reversible. Association of 2-[^{125}I]iodomelatonin to sea bass brain membranes proceeded rapidly and reached a steady state at 2 hr. The k_1 determined from the pseudo-first-order plot was $8.84 \times 10^8 \pm 0.57 \times 10^8$ $M^{-1}min^{-1}$ (n=5). The binding of 2-[^{125}I]iodomelatonin was stable during 2–4 hr of incubation. After 2 h of incubation with 2-[^{125}I]iodomelatonin, dissociation was initiated by the addition of 8 μM melatonin to several of the tubes. The k_{-1} calculated from the first-order regression analysis was $2.83 \times 10^{-3} \pm 0.34 \times 10^{-3}$ min^{-1} (n=5). The kinetic dissociation constant calculated from the ratio k_{-1}/k_1 was 3.45 ± 0.60 pM.
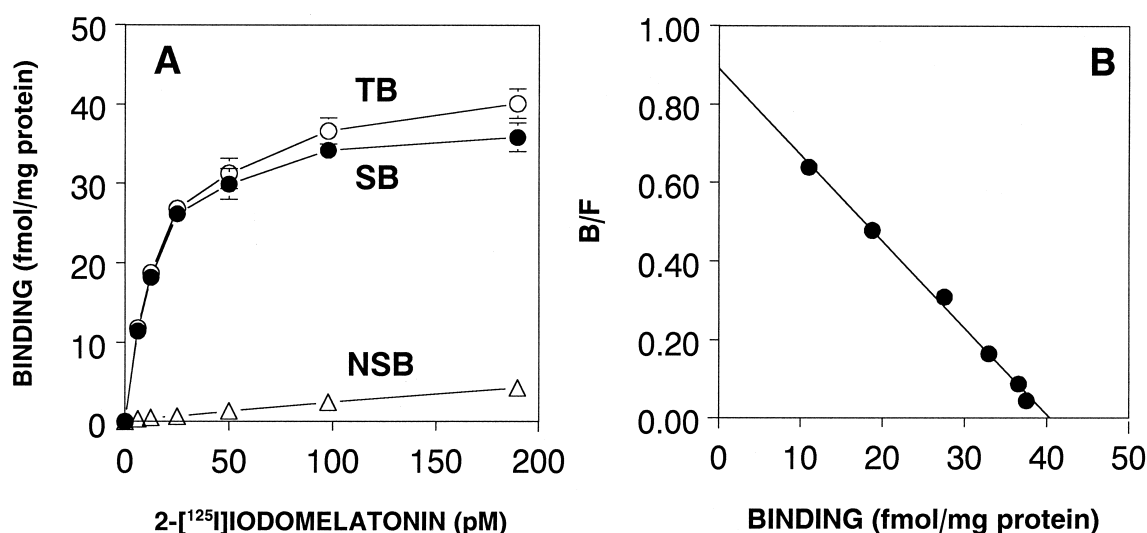
Saturation studies

Membranes prepared from the sea bass brain collected both at mid-light and mid-dark were used for saturation studies using a range of radioligand concentrations from 6.1 to 189.6 pM with or without GTPγS. Saturation curves, Scatchard plots, and calculated Kd, Bmax and n_H values were shown in Figs. 3 and 4 and Table 1. The specific binding reached a plateau at approximately 100 pM (Fig 3A). A linear Scatchard plot (Fig. 3B) and the n_H close to unity


Fig. 2. The time course of association and dissociation of 2-[^{125}I]iodomelatonin (48.6 pM) to sea bass brain membranes at 25°C. Values shown are the means±SEM (n=5). The SEM is not visible when it is smaller than the symbol. The arrow indicates the addition of melatonin (8 μM) for the initiation of dissociation. The data were analyzed by pseudo-first-order plotting to estimate the association rate constant ($k_1 = 8.84 \times 10^8 \pm 0.57 \times 10^8$ M^{-1} min^{-1}), and by first-order plotting to calculate the dissociation rate constant ($k_{-1} = 2.83 \times 10^{-3} \pm 0.34 \times 10^{-3}$ min^{-1}) after the addition of melatonin.

(1.05±0.02) revealed that 2-[^{125}I]iodomelatonin binds to a single class of sites with the Kd of 9.3±0.6 pM and the Bmax of 39.08±0.86 fmol/mg protein (n=4) at mid-light.

Day-night changes in the Kd, Bmax and n_H and modulation by GTPγS of melatonin binding sites in the sea bass brain are exhibited in Fig. 4 and Table 1. Although plasma concentrations of melatonin exhibited significant day-night changes with higher levels at mid-dark than at mid-light (P<0.05), neither the Kd nor the Bmax exhibited day-night changes (Table 1). Treatment with GTPγS induced signifi-

Fig. 3. Results of a saturation study using whole brain membranes of the sea bass collected at mid-light. (A) A saturation curve of melatonin binding sites in the sea bass brain collected at mid-light. Values shown are the means±SEM (n=5). The SEM is not visible when it is smaller than the symbol. Total binding (TB): open circles; specific binding (SB): closed circles; nonspecific binding (NSB): open triangles. (B) A representative Scatchard plot.
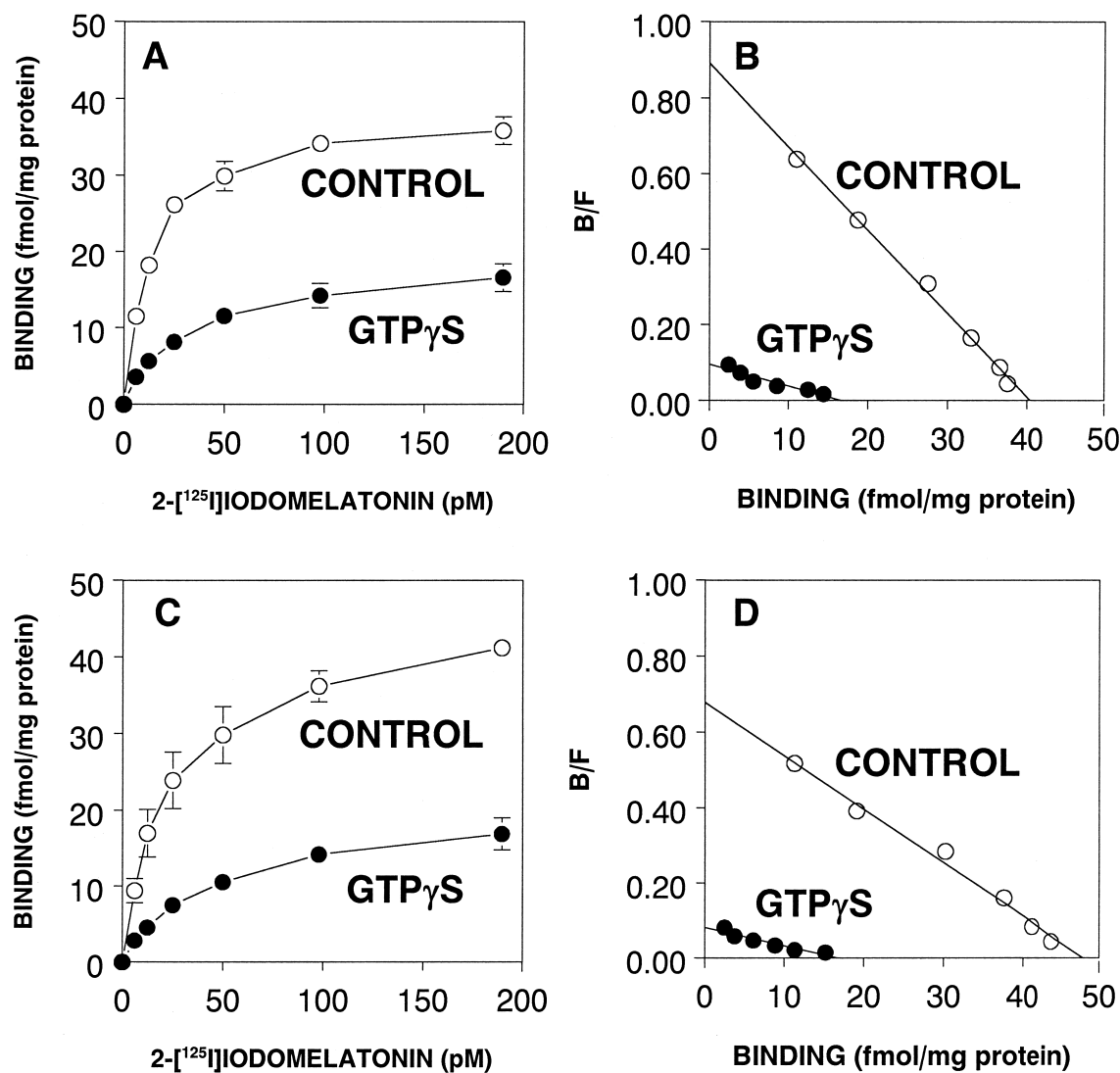
Fig. 4. Day-night changes of melatonin binding sites in the sea bass whole brain and their modulation by GTPγS. Saturation curves of melatonin binding sites in the sea bass brain collected at mid-light (A) and mid-dark (C). Values shown are the means±SEM (n=5). The SEM is not visible when it is smaller than the symbol. Representative Scatchard plots at mid-light (B) and mid-dark (D). The data for the mid-light control is the same as shown in Fig. 3. Open circles, the control group; solid circles, the GTPγS-treated group. The Kd, Bmax and n_H values calculated from these data are shown in Table 1.

Table 1. Day-night changes of the Kd and Bmax and n_H of melatonin binding sites in the brain and plasma melatonin concentrations of the sea bass under LD 12:12 and modulation of melatonin binding sites by GTPγS.

Time	Group	Kd (pM)	Bmax (fmol/mg protein)	n_H	Melatonin (pg/ml)
Mid-light	Control	9.3±0.6	39.08±0.86	1.05±0.02	18.3±1.8
	GTPγS	26.9±3.5**	18.29±2.44***	1.04±0.04	–
Mid-dark	Control	10.9±1.0	43.45±2.35	1.04±0.06	167.7±51.5[a]
	GTPγS	31.8±5.6*	19.22±3.75**	1.05±0.06	–

The Kd, Bmax and nH values were calculated from the data obtained from saturation experiments (shown in Fig. 4) using 2-[125I]iodomelatonin concentrations from 6.1 to 189.6 pM. Melatonin concentrations in the plasma were determined by radioimmunoassay. The data shown are the means±SEM (n=4 for mid-light and n=3 for mid-dark). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. respective control values. [a] $P<0.05$ vs. the mid-light value.
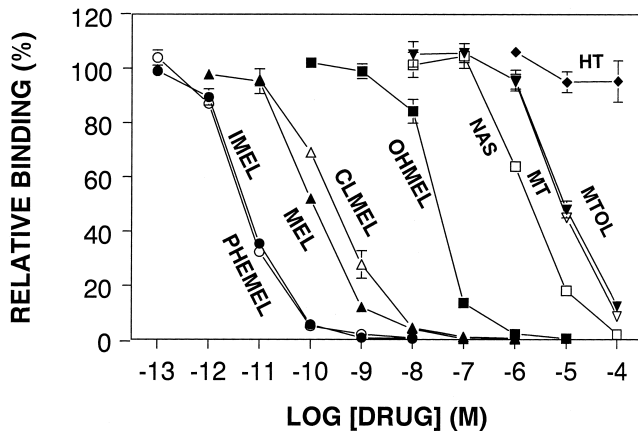
Fig. 5. Competition curves for inhibition of 2-[^{125}I]iodomelatonin binding by various indole derivatives in sea bass brain membranes. Values shown are means±SEM (n=3). The SEM is not visible when it is smaller than the symbol.The membrane preparations were incubated with 2-[^{125}I]iodomelatonin (48.0 pM) and various concentrations of melatonin analogues and neurotransmitters. The K_i values were calculated from the IC_{50} values of the inhibition curves: 2-phenylmelatonin (PHEMEL; Ki=0.00067±0.00008 nM); 2-iodomelatonin (IMEL; Ki=0.00087±0.0004 nM), melatonin (MEL; Ki=0.0155±0.0016 nM), 6-chloromelatonin (CLMEL; Ki=0.0526±0.0112 nM), 6-hydroxymelatonin (OHMEL; Ki=4.48±0.31 nM), N-acetylserotonin (NAS; Ki=261±45 nM), 5-methoxytryptophol (MTOL; Ki=1,212±244 nM) 5-methoxytryptamine (MT; Ki=1,524±54 nM) and 5-hydroxytryptamine (HT; Ki>970,000 nM). Acetylcholine, dopamine and noradrenaline exhibited no inhibition at 10-4 M (Ki>970,000 nM; data not shown).

cant changes both in the Kd and Bmax with no change in the n_H both at mid-light and mid-dark (Table 1).
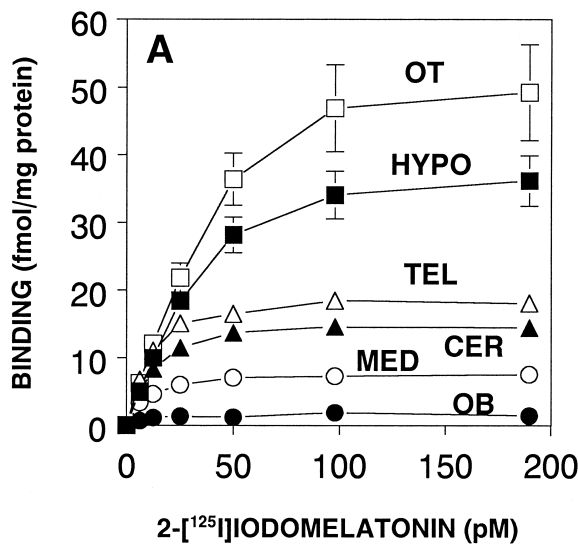
Specificity

Specificity of melatonin binding sites in the sea bass brain is shown in Fig. 5. Competition experiments carried out using 2-[^{125}I]iodomelatonin (48.0 pM) with several indole compounds and neurotransmitters demonstrated the following order of potency to inhibit specific binding: 2-phenylmelatonin = 2-iodomelatonin > melatonin > 6-chloromelatonin > 6-hydroxymelatonin > N-acetylserotonin> 5-methoxytryptophol = 5-methoxytryptamine. 5-hydroxytryptamine. Acetylcholine, dopamine and noradrenaline exhibited no inhibition even at a concentration of 10^{-4} M (data not shown).

Localization of melatonin binding sites in discrete brain areas

Saturable 2-[^{125}I]iodomelatonin binding was detected in all brain areas with the Kd values in the low picomolar range (Fig. 6 and Table 2). However, melatonin binding sites in the sea bass brain exhibited uneven distribution: The Bmax values were highest in mesencephalic optic tectum-tegmentum and hypothalamus, intermediate in telencephalon, cerebellum-vestibulolateral lobe and medulla oblongata-spinal cord, and lowest in olfactory bulbs.

DISCUSSION

European sea bass has been used as an experimental model to analyze the circadian system in marine fish (Sánchez-Vázquez et al., 1995a, b, 1997, 1998; Iigo et al., 1997d; Garcia-Allegue et al., 2001; Bayarri et al., 2002, 2003). In the present study, in order to make the sites of melatonin action in the sea bass circadian system clear, we have examined characteristics of melatonin binding sites in the brain of the European sea bass.
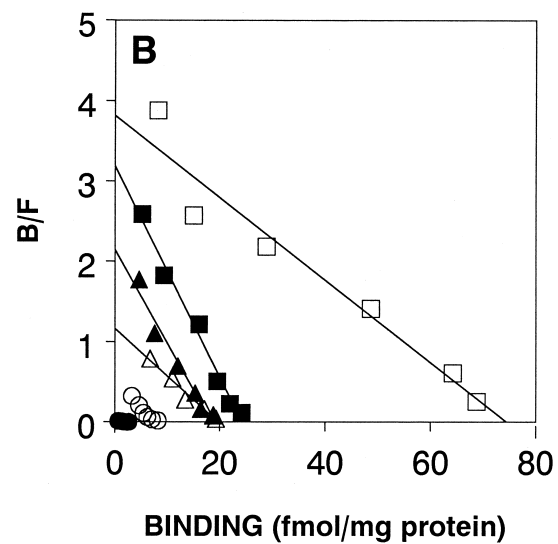
Fig. 6. Localization of melatonin binding sites in discrete brain areas of the sea bass. (A) Saturation curves. Values shown are the means±SEM (n=5 except for the olfactory bulbs where n=3). The SEM is not visible when it is smaller than the symbol. (B) Representative Scatchard plots. OB: olfactory bulbs (solid circles); TEL: telencephalon (open triangles); HYPO: hypothalamus (solid squares); OT: mesencephalic optic tectum-tegmentum (open squares); CER: cerebellum-vestibulolateral lobe (solid triangles); MED: medulla oblongata-spinal cord (open circles). The Kd, Bmax and n_H in each brain areas calculated from these data are shown in Table 2.

Table 2. Localization of melatonin binding sites in the sea bass brain.

Brain area	Kd (pM)	Bmax (fmol/mg protein)	n_H
Olfactory bulbs	15.9±3.0	2.27±0.23	1.00±0.12
Telencephalon	6.6±1.3	19.56±0.57	0.97±0.10
Hypothalamus	9.9±1.4	41.12±5.18	1.22±0.04
Optic tectum- tegmentum	12.4±2.4	57.30±8.95	1.21±0.02
Cerebellum-vestibulolateral lobe	6.6±0.4	15.77±1.06	0.97±0.11
Medulla oblongata-spinal cord	6.8±0.4	7.88±0.37	0.98±0.08

The Kd, Bmax and nH values were calculated from the data obtained from saturation experiments (shown in Fig. 6) using 2-[^{125}I]iodomelatonin concentrations from 6.0 to 200.0 pM in six brain regions as depicted in Fig. 1. Results are represented as the mean±SEM (n=5 except for the olfactory bulbs where n=3).

The specific binding of a radioiodinated melatonin agonist 2-[^{125}I]iodomelatonin to sea bass brain membranes fulfills almost all the criteria for binding to a functional receptor site. First, kinetic studies demonstrated that the specific binding was rapid, stable and reversible. Second, saturation studies showed that the binding is of low capacity and high affinity and that the radioligand appeared to label a single class of binding sites as is evident from linear Scatchard plots and the n_H close to unity. Finally, competition studies demonstrated that the binding sites are highly specific to melatonin and its related analogues. The Kd values obtained from the kinetic and saturation studies are consistent with picomolar affinity. The Kd values in discrete brain areas of the sea bass (6.6–15.9 pM) are also well within the physiological range of plasma melatonin concentrations described above (~100 pM at night) (Iigo *et al.*, 1997d; Sánchez-Vázquez *et al.*, 1997; Garcia-Allegue *et al.*, 2001; Bayarri *et al.*, 2002, 2003).

Melatonin binding sites in the sea bass brain are highly specific for melatonin and its related compounds that possess both 5-methoxy- and *N*-acetyl-substituents. Especially, 2-phenylmelatonin, 2-iodomelatonin, melatonin and 6-chloromelatonin exhibited a high affinity. Melatonin precursors such as *N*-acetylserotonin or metabolites such as 6-hydroxymelatonin were less effective as compared to the compounds described above. Furthermore, compounds known to act at serotonergic, dopaminergic, cholinergic and adrenergic sites were ineffective. These results indicate that the sites labeled by 2-[^{125}I]iodomelatonin are specific binding sites for melatonin. The pharmacological order of potency of melatonin analogs to inhibit specific binding in the sea bass brain was almost identical to that of high-affinity melatonin receptors (ML-1 melatonin receptors) in vertebrates (Dubocovich, 1988, 1995; Reppert, 1997). Although recent molecular studies demonstrated the presence of at least three subtypes of melatonin receptors among the ML-1 subtype, MT_1 (Mel_{1a}), MT_2 (Mel_{1b}) and Mel_{1c} subtypes (Reppert, 1997), the subtype(s) expressed in the sea bass brain remains to be determined. Further molecular biological and pharmacological studies are required to elucidate this subject in future.

In the present study, a non-hydrolyzable GTP analog (GTPγS) was used to examine whether the melatonin binding sites in the sea bass brain are coupled to G protein pharmacologically. Treatment of sea bass brain membranes with GTPγS affected both the Kd and Bmax of melatonin binding sites both at mid-light and mid-dark, indicating that they are coupled to G protein as in other vertebrate species (Reppert, 1997; Davies *et al.*, 1994; Iigo *et al.*, 1997a, b; Gaildrat *et al.*, 1998; Amano *et al.*, 2003). Further identification of the type(s) of G protein and the effector system should be required to elucidate the signal transduction pathway of melatonin in this species.

In several teleost species such as the goldfish, catfish, gilthead sea bream, pike, and masu salmon, the Bmax and/or the Kd of melatonin binding sites in the brain exhibit daily variations under LD (Iigo *et al.*, 1994b, 1995, 1997c, 2003; Falcón *et al.*, 1996; Gaildrat *et al.*, 1998: Amano *et al.*, 2003b). However, in the present study, neither the Kd nor Bmax of melatonin binding sites in the sea bass brain showed day-night changes, whereas plasma concentrations of melatonin exhibited significant day-night changes. Similar results were obtained in other teleost species such as rainbow trout, Atlantic salmon and Arctic charr (Davies *et al.*, 1994; Pang *et al.*, 1994). These results indicate that the regulatory mechanism of melatonin binding sites differ among species. Alternatively, melatonin binding sites in the brain do exhibit daily variation but the use of whole brain homogenates for binding assays and/or the sampling at only two time points a day may have obscured variations in restricted brain areas. This possibility should be verified in future.

Several studies demonstrated widespread distribution of melatonin binding sites in the teleostean brain (Martinoli *et al.*, 1991; Ekström and Vanecek, 1992; Davies *et al.*, 1994; Iigo *et al.*, 1994b, 1997c; Vernadakis *et al.*, 1998; Mazurais *et al.*, 1999). The present study on the sea bass brain also demonstrated a similar uneven distribution: Localization of melatonin binding sites in discrete brain areas was determined to be highest in the mesencephalic optic tectum-tegmentum and hypothalamus, intermediate in the telencephalon, cerebellum-vestibulolateral lobe and medulla oblongata-spinal cord, and lowest in the olfactory bulbs. The present results are consistent with the previous reports on the goldfish, catfish, Atlantic salmon and rainbow trout.

(Martinoli *et al.*, 1991; Ekström and Vanecek, 1992; Davies *et al.*, 1994; Iigo *et al.*, 1994b, 1997c; Vernadakis *et al.*, 1998; Mazurais *et al.*, 1999).

The highest density in the optic tectum, a primary retinorecipient area, is a conserved feature among nonmammalian vertebrates, indicating importance of melatonin in the visual signal transduction (Rivkees *et al.*, 1989; Wiechmann and Wirsig-Wiechmann, 1993, 1994). High levels of melatonin binding in the hypothalamus were also notable among vertebrates. Melatonin binding sites in this area may modulate neuroendocrine functions. Furthermore, the SCN of the hypothalamus, the site of primary circadian pacemaker in mammals, is a target of melatonin in mammals and melatonin is involved in the resetting of the circadian clock (Yu and Reiter, 1992). It is of great interest to examine whether the SCN homologue of the sea bass expresses melatonin receptors. Furthermore, localization of melatonin binding sites in the other brain areas (olfactory bulbs, telencephalon, cerebellum-vestibulolateral lobe and medulla oblongata-spinal cord) indicates a variety of roles of melatonin in the central nervous system in fish. Further studies using *in vitro* autoradiography should be required to demonstrate precise localization of melatonin binding sites in the sea bass brain.

The present study also demonstrated the heterogeneity in the affinity of melatonin binding sites in discrete brain area of the sea bass, i.e., the Kd values were not uniform among brain areas. Relatively high Kd values (9.9–15.9 pM) were seen in the olfactory bulbs, optic tectum and hypothalamus while in the other brain areas the Kd values were in the range of 6.6–6.8 pM. These suggest site-specific expression of different subtypes of melatonin receptors in the sea bass brain. Recently, subtype selective melatonin receptor ligands such as 4-phenyl-2-propionamidotetralin (4P-PDOT) were developed and used to differentiate melatonin receptor subtypes expressed not only in cells transfected melatonin receptor subtype cDNAs but also in native tissues (Dubocovich *et al.*, 1997, 1998). Further pharmacological studies using these selective ligands will be required to examine the subtype of melatonin receptors expressed in each brain area of the sea bass. In addition, molecular cloning of melatonin receptors and *in situ* hybridization histochemistry will be required to elucidate this subject in more details.

In several vertebrate species, melatonin binding sites are localized not only in the brain but also in the retina (Dubocovich and Takahashi JS, 1987; Lu *et al.*, 1991; Wiechmann and Wirsig-Wiechmann, 1994; Iigo *et al.*, 1997b). Since the melatonin profile in the retina of the European sea bass is reversed (with high values during the light phase under LD cycles), it is of great interest to examine whether melatonin binding sites are localized in the sea bass retina. If so, to examine characteristics and daily variations of the melatonin binding sites in the retina may help to elucidate distinct roles of melatonin produced in the pineal organ and retina in the sea bass circadian system that regulates locomotor and feeding rhythms.

In conclusion, the present study has characterized melatonin binding sites in the brain of European sea bass. The results suggest that melatonin produced in the pineal organ act via melatonin binding sites (putative melatonin receptors) characterized in this study. Further studies using the sea bass will help to elucidate the roles of melatonin in the teleostean circadian system.

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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 countries in which the experiments were conducted.

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