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Melatonin Binding Sites in the Goldfish Retina

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ABSTRACT—Melatonin binding sites in the goldfish retina were characterized by radioreceptor assay using 2-[^{125}I]iodomelatonin as the radioligand. The specific binding to goldfish retinal membranes is rapid, stable, saturable and reversible. Saturation studies demonstrated that 2-[^{125}I]iodomelatonin binds to a single class of sites with an affinity constant (K_d) of 61.9 ± 5.7 pM, a total binding capacity (B_{max}) of 6.52 ± 0.79 fmol/mg protein and Hill coefficients (n_H) of 1.07 ± 0.03 (mean ± SEM, n=6). Competition experiments with various indoles and neurotransmitters revealed the following order of affinities: 2-iodomelatonin > melatonin > 6-hydroxymelatonin > 5-methoxytryptamine = *N*-acetylserotonin > 5-methoxytryptophol. The other indoles and neurotransmitters tested were much less effective. The order resembles with those reported for the goldfish brain and the ML-1 subtype melatonin receptors in vertebrates. Co-incubation of retinal membranes with a non-hydrolyzable GTP analog, guanosine 5'-*O*-(3-thiotriphosphate), significantly reduced the specific binding. These results suggest that in the goldfish, ocular melatonin plays neuromodulatory roles in the retina via G protein-coupled melatonin receptors with picomolar affinity.

INTRODUCTION

Melatonin is synthesized in the pineal organ and the retina of vertebrates in a rhythmic fashion with peak production during the dark phase of light-dark (LD) cycles. Melatonin produced in the pineal organ is secreted into the blood and/or the cerebro-spinal fluid and acts as a time keeping hormone that regulates circadian and seasonal rhythms (for review, see Underwood and Goldman, 1987). Melatonin produced in the retina, on the other hand, is considered to act within the retina itself. Melatonin is known to affect several aspects of retinal physiological processes such as dopamine release, retinomotor movements, disc shedding of photoreceptor outer segments, aggregation of the retinal pigment epithelium and sensitivity of horizontal cells (for review, see Pang and Allen, 1986; Cahill *et al.*, 1991).

The actions of melatonin are thought to be mediated through its specific receptors. Although several attempts have been made to characterize them using [^{3}H]melatonin as a radioligand, only the binding sites with low (nanomolar) affinity were labeled (Cohen *et al.*, 1978; Cardinali *et al.*, 1979; Niles *et al.*, 1979; Gern *et al.*, 1980; Wiechmann *et al.*, 1986). The physiological functions of these sites still remain unclear. However, introduction of a melatonin agonist, 2-[^{125}I]-iodomelatonin with high specific radioactivity (Vakkuri *et al.*, 1984), enables us to characterize melatonin binding sites with picomolar affinity by radioreceptor assay and *in vitro* autoradiography. These sites are considered as functional melatonin receptors in various tissues including the central nervous system and peripheral tissues (for review, see Dubocovich, 1988,1995; Pang *et al.*, 1993; Morgan *et al.*, 1994). In the vertebrate retina, melatonin binding sites with picomolar affinity were identified in mammals, birds, reptiles and amphibians (Dubocovich and Takahashi, 1987; Vaněček, 1988; Laitinen and Saavedra, 1990; Blazynski and Dubocovich, 1991; Chong and Sugden, 1991; Lu *et al.*, 1991; Wiechmann and Wirsig-Wiechmann, 1991, 1994; Skene *et al.*, 1993; Failace *et al.*, 1995; James *et al.*, 1995).

In teleosts, melatonin is produced in the retina (Quay, 1965; Gern and Ralph, 1979; Wiechmann and Hollyfield, 1988; Nowak *et al.*, 1989; Falcón and Collin, 1991; Grace *et al.*, 1991; Cahill, 1996; Iigo *et al.*, 1997a) and known to affect retinal rhythmic processes (Chèze and Ali, 1976; Mangel and Wong, 1996) as well as in other vertebrate classes. Thus, it is probable that melatonin receptors exist in the teleostean retina. However, to our knowledge, melatonin binding sites with picomolar affinity have not yet identified in the retina of fishes.

In order to obtain more insights on the physiological functions of melatonin in the fish retina, in the present study, melatonin binding sites in the retina of the goldfish (*Carassius auratus*) were characterized by radioreceptor assay using 2-

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[^{125}I]iodomelatonin as the radioligand. This fish was selected as our experimental model because we have studied the regulation of melatonin production in the pineal organ and eye, and characteristics and regulation of melatonin binding sites in the brain in this species (Kezuka *et al.*, 1992; Iigo *et al.*, 1994a,b, 1995, 1997a,b).

MATERIALS AND METHODS

Chemicals

2-[^{125}I]Iodomelatonin (specific radioactivity, 2,000-2,200 Ci/mmol) was obtained from Amersham (Buckinghamshire, England) or New England Nuclear (Boston, MA). Unlabeled 2-iodomelatonin was obtained from Research Biochemicals Inc. (Natick, MA). The other indole derivatives, norepinephrine, polyethlenimine, and guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS; tetralithium salt) were obtained from Sigma (St. Louis, MO). Acetylcholine was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Ordinary chemicals were obtained from commercial sources.

Goldfish

The goldfish (61–164 g in body weight) purchased from a local dealer were maintained in experimental aquaria under LD cycles of 12 hr:12 hr (lights on 06.00–18.00) with controlled temperature at 24–25°C for at least two weeks. Illumination (300–500 lx at the water surface) was supplied by a white fluorescent bulb (10 W) for the light phase. Fish were fed commercial trout pellets.

The goldfish were anesthetized with 2-phenoxyethanol (0.6 ml/l) and decapitated during the light phase (11.00–16.00) when ocular melatonin contents are low (Iigo *et al.*, 1994a, 1997a). The retina (neural retina with the pigment epithelium) was dissected out, immediately frozen on dry ice, and stored at –80°C until used.

Membrane preparation and binding assay

Membrane preparation and binding assay were performed according to the procedure as previously described (Iigo *et al.*, 1994b) unless otherwise stated. Briefly, the retina was homogenized in 50 mM Tris-HCl buffer (pH 7.5), centrifuged (40,000 × g for 20 min at 4°C). The pellet was resuspended in the buffer and centrifuged for the second time. The pellet was used for binding assay. Incubation was performed in a total volume of 200 µl and the binding of 2-[^{125}I]iodomelatonin was routinely measured in duplicate after incubation at 25°C for 1.5 hr with the exception of kinetic studies where incubation duration and temperature 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.

Identification of bound radioligand

Stability of 2-[^{125}I]iodomelatonin during the incubation period was examined since melatonin is known to be metabolized in the eye of nonmammalian vertebrates including the goldfish (Grace *et al.*, 1991). After filtration of a binding assay, filters were removed and extracted with methanol (2 ml). The methanol extract was evaporated under reduced pressure to a volume of 100 µl. An aliquot of this extract (50 µl) was applied to a silica gel plate for thin layer chromatography (TLC) (Whatman LK6F, Whatman International Ltd., Maidstone, England). Melatonin (10 µg) and 2-iodomelatonin (10 µg) were also applied as tracers. Chromatograms were developed with ethyl acetate for 10 cm. The position of melatonin and 2-iodomelatonin on the TLC plate was determined by fluorescence during exposure to UV lights and the R_f values were calculated. Radioactivity of sections of silica gel (1 cm) were counted in a γ-counter. The peak radioactivity in the extracts of the bound radioligand was compared with the position of 2-iodomelatonin.

Characterization and GTPγS modulation of melatonin binding sites in the goldfish retina

Saturation studies were performed using a range of 2-[^{125}I]iodomelatonin concentration from 4.4 to 269.9 pM. Kinetic and competition studies were performed using a 2-[^{125}I]iodomelatonin concentration of 49.9 and 49.6 pM, respectively. To study the effects of GTPγS on melatonin binding sites in the goldfish retina, the specific binding of 2-[^{125}I]iodomelatonin (68.9 pM) was determined in the presence or absence of GTPγS (10^{-4} M).

Data analysis

Data obtained from kinetic, saturation and competition experiments were analyzed as previously described (Iigo *et al.*, 1994b) to calculate association rate constants (k_1), dissociation rate constants (k_{-1}), K_d, B_{max}, n_H and K_i values. Correlation between the specific binding and the protein concentration in the incubation mixture, and between log K_i values of some indoles obtained in the present study and those reported for the goldfish brain (Iigo *et al.*, 1994b) was analyzed by linear-regression analysis. The specific binding in the GTPγS-treated group was compared with the vehicle-treated control by paired *t*-test.

RESULTS

Binding conditions

At first, the time course of the association of 2-[^{125}I]-iodomelatonin (50.0 pM) to goldfish retinal membranes at 0, 25 and 37°C was compared (data not shown). The specific binding at equilibrium was similar at 0 and 25°C but the equilibrium was reached faster at 25°C (1 hr) than at 0°C (12 hr). At 37°C, the specific binding did not show a steady state. Therefore, further experiments were performed at 25°C.

In a preliminary study, retinal homogenates were prepared in 50 mM Tris-HCl buffer (pH 7.5) containing 0.32 M sucrose and fractionated by differential centrifugation at 4°C to yield a crude nuclear pellet (P_1, 1,000 × g for 10 min), a crude mitochondrial pellet (P_2, 40,000 × g for 10 min), and a crude microsomal pellet (P_3, 100,000 × g for 60 min) to study the intracellular distribution of melatonin binding sites. The specific binding of 2-[^{125}I]iodomelatonin (50.0 pM) in these membrane fractions was found to be greatest in the P_2 fraction (7.35 fmol/mg protein, 42% of the specific binding in the total retinal membranes) as compared to the P_1 fraction (2.01 fmol/mg protein, 28%) and the P_3 fraction (4.40 fmol/mg protein, 30%). These results indicate that the 40,000 × g pellet used in the present study contained 70% of the specific binding in the total retinal membranes.

Linear regression analysis revealed that there was a significant relationship between the specific binding of 2-[^{125}I]iodomelatonin (50.0 pM) and the protein concentration in the incubation mixture at least up to 0.35 mg protein/tube (r=0.999, P<0.001, n=6; Fig. 1).

Identification of bound radioligand

Radioactivity of the bound radioligand, extracted from filters after a binding assay with methanol, comigrated with synthetic 2-iodomelatonin. The R_f values for synthetic melatonin

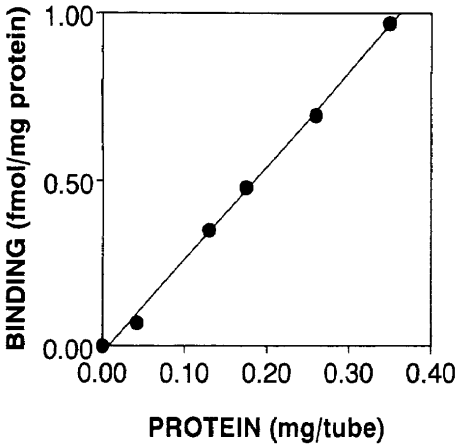

Fig. 1. The relationship between the specific binding of 2-[^{125}I]iodomelatonin (50.0 pM) to goldfish retinal membranes and the protein concentration in the incubation mixture. Values shown are means from a experiment performed in duplicate. A significant relationship was obtained between the two at least up to 0.35 mg protein/tube (r=0.999, P<0.001, n=6).

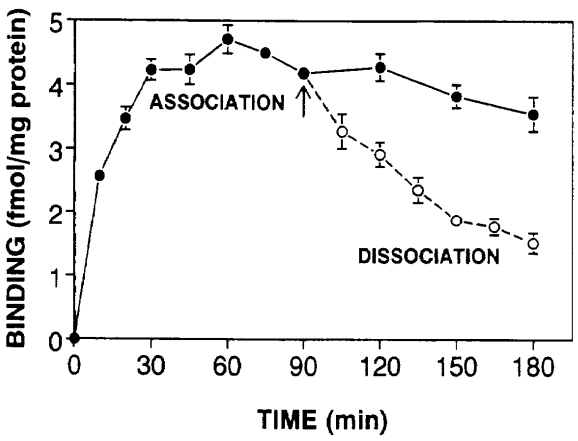

Fig. 2. The time course of the association (closed circles) and dissociation (open circles) of 2-[^{125}I]iodomelatonin (49.9 pM) to goldfish retinal membranes at 25°C. Values represent the means ± SEM (n=6). A steady state was reached at 1 hr and stable during 1–3 hr. The specific binding was reversible upon the addition of 8 μM melatonin (arrow).

and 2-iodomelatonin was 0.32 and 0.55, respectively. Peak radioactivity (>96% of total) recovered between R_f values of 0.5 and 0.6.

Kinetic study

At 25°C, the specific binding was rapid, stable and reversible (Fig. 2). The k_1 determined from the pseudo-first-order plot was $4.69 \times 10^8 \pm 0.74 \times 10^8$ M^{-1} min^{-1} (n=6). The specific binding was stable from 1–3 hr of incubation. After 1.5 hr of incubation with 2-[^{125}I]iodomelatonin, dissociation was initiated by addition of melatonin (8 μM) to some tubes. The k_{-1} calculated from the first-order regression analysis was $1.05 \times 10^{-2} \pm 0.08 \times 10^{-2}$ min^{-1} (n=6). The kinetic dissociation constant calculated from the ratio k_{-1}/k_1 was 24.2 ± 3.0 pM.

Saturation study

Saturation experiments using a range of 2-[^{125}I]-iodomelatonin concentrations from 4.4 to 269.9 pM demonstrated that the specific binding reached a plateau at approximately 130 pM (Fig. 3A). Scatchard analysis of the data (Fig. 3B) revealed that 2-[^{125}I]iodomelatonin binds to a single class of sites with the K_d of 61.9 ± 5.7 pM, the B_{max} of 6.52 ± 0.79 fmol/mg protein, the n_H of 1.07 ± 0.03 (n=6).

Specificity

Specificity of melatonin binding sites in the goldfish retina is shown in Fig. 4 and Table 1. Competition experiments carried out using a concentration of 2-[^{125}I]iodomelatonin (49.6 pM) with several indole compounds and neurotransmitters revealed the following order of potency to inhibit the specific binding: 2-iodomelatonin > melatonin > 6-hydroxymelatonin > 5-methoxytryptamine = *N*-acetylserotonin > 5-methoxytryptophol. Even at a concentration of 10^{-4} M, 5-methoxyindole-3-acetic acid and serotonin exhibited less than 50% inhibition, and 5-hydroxytryptophol, 5-hydroxyindole-3-acetic acid, norepinephrine and acetylcholine showed no inhibition (data not shown).

Linear regression analysis demonstrated a significant correlation between the log K_i values obtained in the present

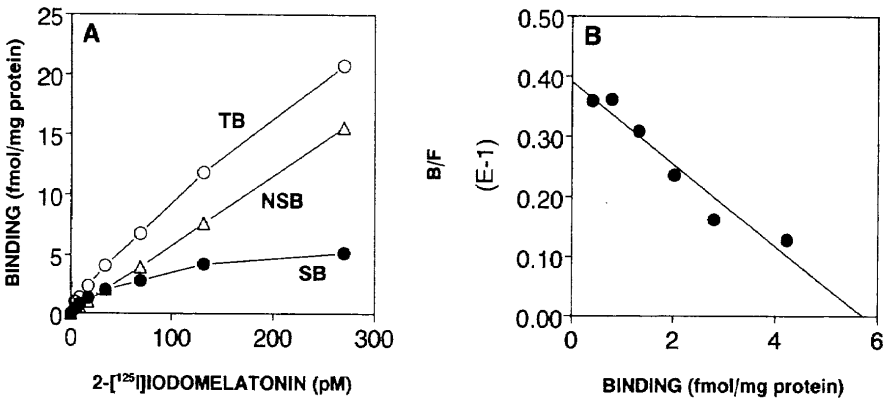

Fig. 3. (**A**) A saturation curve of 2-[^{125}I]iodomelatonin (4.4-269.9 pM) binding to goldfish retinal membranes. Each point represents the mean of duplicate determinations from a representative experiment of six such studies. Nonspecific binding (NSB) was measured in the presence of 10 μM melatonin. Specific binding (SB) is defined as total binding (TB) minus nonspecific binding. (**B**) A Scatchard plot of the data shown in (**A**). In this case, the K_d, B_{max} and n_H values were 53.1 pM, 5.71 fmol/mg protein and 1.03, respectively.

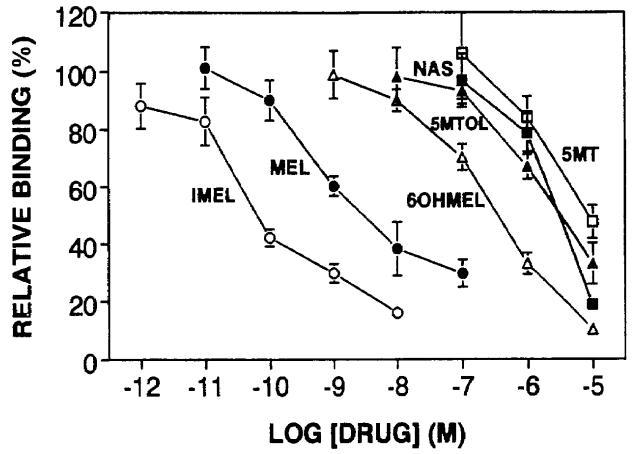

Fig. 4. Competition curves for inhibition of 2-[^{125}I]iodomelatonin (49.6 pM) binding to goldfish retinal membranes by various indole derivatives. Values represent the means ± SEM (n=3). The membrane preparations were incubated with 2-[^{125}I]iodomelatonin (49.6 pM) and various concentrations of 2-iodomelatonin (IMEL), melatonin (MEL), 6-hydroxymelatonin (6OHMEL), 5-methoxytryptophol (5MTOL), *N*-acetylserotonin (NAS), and 5-methoxytryptamine (5MT). The K_i values were calculated from these data are shown in Table 1.

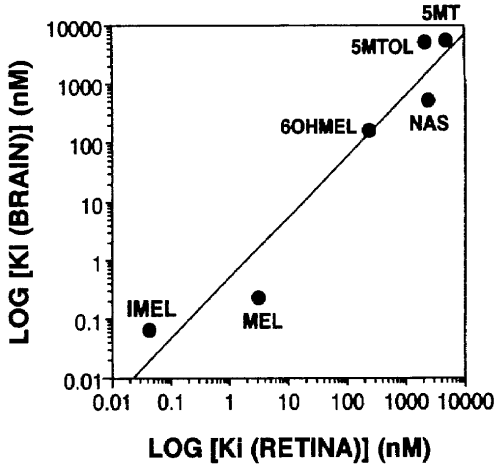

Fig. 5. Correlation between the affinities of melatonin analogs for melatonin binding sites in the retina and brain of the goldfish. The K_i values for the goldfish brain are taken from Iigo *et al.* (1994b). Linear regression analysis after logarithmic transformation of the data revealed a significant correlation (slope=1.038, r=0.965, P<0.01, n=6). Abbreviations of the compounds are the same as in Fig. 4.

Table 1. Specificity of melatonin binding sites in the goldfish retina

Drug	[a]K_i (nM)	[b]Affinity ratio to melatonin
2-Iodomelatonin	0.043 ± 0.014	0.014
Melatonin	3.12 ± 1.23	1
6-Hydroxymelatonin	234 ± 17	75
5-Methoxytryptamine	2140 ± 320	690
N-Acetylserotonin	2370 ± 700	760
5-Methoxytryptophol	4830 ± 100	1550
5-Methoxyindole-3-acetic acid	>79000	>141000
Serotonin	>79000	>141000
5-Hydroxytryptophol	>79000	>141000
5-Hydroxyindole-3-acetic acid	>79000	>141000
Acetylcholine	>79000	>141000
Norepinephrine	>79000	>141000

[a]Relative inhibition for the binding of 2-[^{125}I]iodomelatonin (49.6 pM) to retinal membranes was determined for 3-5 concentrations of competing drugs. The K_i values were calculated from the IC_{50} values obtained from the competition studies as previously described (Iigo *et al.*, 1994b). Results are the mean±SEM (n=3).
[b]The ratio of the K_i value of competing drug versus the K_i value of melatonin.

study and those reported for the goldfish brain (slope=1.038, r=0.965, P<0.01, n=6; Fig. 5).

Effects of GTPγS

Effects of GTPγS (10^{-4} M) on the specific binding of 2-[^{125}I]iodomelatonin (68.9 pM) to goldfish retinal membranes are exhibited in Fig. 6. The specific binding of the GTPγS-treated group was significantly reduced as compared with the vehicle-treated control (P<0.01).

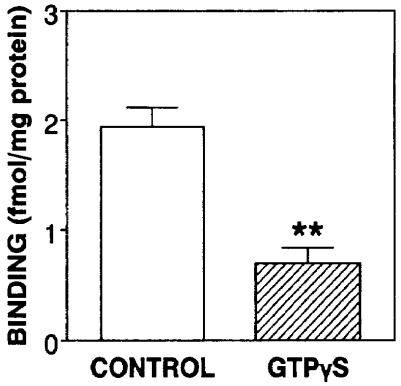

Fig. 6. Effects of GTPγS (10^{-4} M) on the specific binding of 2-[^{125}I]iodomelatonin (68.9 pM) to goldfish retinal membranes. Values represent the means ± SEM (n=5). The control group was treated with vehicle (distilled water). Significance: **, P<0.01 compared with the control values (paired *t*-test).

DISCUSSION

Presence of melatonin and its synthesizing enzymes have been demonstrated in the eye of several teleost species (Quay, 1965; Gern and Ralph, 1979; Wiechmann and Hollyfield, 1988; Nowak *et al.*, 1989; Falcón and Collin, 1991; Grace *et al.*, 1991; Cahill, 1996; Iigo *et al.*, 1997a,c,d; Sánchez-Vázquez *et al.*, 1997). To investigate the sites of melatonin action in the fish retina, in the present study, existence of melatonin binding sites in the goldfish retina was investigated by radioreceptor assay using 2-[^{125}I]iodomelatonin as the radioligand.

We successfully detected the specific binding of 2-[^{125}I]iodomelatonin to the goldfish retinal membranes as in the goldfish brain membranes (Iigo *et al.*, 1994b). The nonspecific binding was relatively high as compared with those in the brain. This might be caused by the binding of the radioligand

to the melanosomes in the retinal pigmented epithelium (James *et al.*, 1995).

The specific binding of 2-[^{125}I]iodomelatonin to goldfish retinal membranes fulfills all the criteria for binding to a receptor site: the binding was rapid, stable, saturable, reversible, and of high specificity, low capacity, and high affinity. The radioligand was stable during the incubation period and appeared to label a single class of binding sites as is evident from linear Scatchard plots, monophasic inhibition curves and the n_H close to unity. The K_d values obtained from the kinetic and saturation experiments and the K_i values obtained from the competition study were consistent with picomolar affinity as those in the goldfish brain (Martinoli *et al.*, 1991; Iigo *et al.*, 1994b, 1995). These values are also well within the physiological range of ocular melatonin contents in the goldfish (0.1–2.5 ng/eye) (Iigo *et al.*, 1994a, 1997a), suggesting that the sites labeled with 2-[^{125}I]iodomelatonin are physiological melatonin receptors as in the retina of chicken and rabbit (Dubocovich and Takahashi, 1987; Blazynski and Dubocovich, 1991).

Pharmacological specificity of melatonin binding sites in the goldfish retina was determined using several indoles and neurotransmitters. The results indicate that the binding sites are highly specific for 2-iodomelatonin and melatonin. Precursors and metabolites of melatonin were much less effective. Furthermore, compounds known to act at serotonergic, adrenergic or cholinergic sites were ineffective. The specificity of melatonin binding sites in the retina and brain of the goldfish are highly correlated, and resembles with those of the ML-1 but not with the ML-2 melatonin receptor subtype (Dubocovich, 1988, 1995). These results indicate that melatonin binding sites in the goldfish retina and brain belong to the ML-1 subtype.

During the course of this study, cDNAs encoding melatonin receptors with picomolar affinity were cloned. The results indicate that there are at least three subtypes of melatonin receptors among the ML-1 subtype, namely Mel_{1a}, Mel_{1b} and Mel_{1c} subtypes (for review, see Reppert and Weaver, 1995). However, no melatonin analogue is available to distinguish differences in the pharmacological properties among these three subtypes (Pickering *et al.*, 1996). Thus, molecular biological studies are necessary to characterize the subtype(s) of melatonin receptors expressed in a given tissue. As far as teleost fish are concerned, 5 different DNA fragments of putative melatonin receptors (2 clones belong to the Mel_{1a}, 2 to the Mel_{1b} and 1 to the Mel_{1c} subtype) were isolated from genomic DNA of the zebrafish (Reppert *et al.*, 1995, 1996). However, it is still controversial whether these are functional melatonin receptors and in which tissues they are expressed. Thus, further molecular biological approach will be required to determine which subtype is expressed in the goldfish retina.

In the present study, the nature of melatonin binding sites-G protein coupling was examined in the goldfish retinal membranes by the use of a non-hydrolyzable GTP analog, GTPγS, because several authors have failed to detect the effects of GTP and its analogues on 2-[^{125}I]iodomelatonin binding to retinal membranes (Dubocovich and Takahashi, 1987; Chong and Sugden, 1991). The treatment of GTPγS significantly reduced the specific binding of 2-[^{125}I]iodomelatonin to the goldfish retinal membranes, indicating that melatonin binding sites in the goldfish retina are coupled to G protein as those in the goldfish brain (Iigo *et al.*, 1997b) and in the retina, brain and pars tuberalis of other vertebrate species (Skene *et al.*, 1993; Morgan *et al.*, 1994; James *et al.*, 1995). These results are also consistent with the recent results of cDNA cloning: melatonin receptors are members of the G protein-coupled receptor superfamily that contain seven hydrophobic segments (Reppert and Weaver, 1995). Further studies to identify the type(s) of G protein and the effector enzyme associated with melatonin receptor-G protein complex will help to elucidate the function of melatonin in the goldfish retina.

In the retina of rabbit, chicken, lizard and frog, melatonin binding sites were localized mainly in the inner plexiform layer as revealed by *in vitro* autoradiography (Laitinen and Saavedra, 1990; Blazynski and Dubocovich, 1991; Wiechmann and Wirsig-Wiechmann, 1991, 1994). Furthermore, localization of functional melatonin receptors are reported in the iris-ciliary processes of rabbit and in the retinal pigmented epithelium of human and rat (Osborne and Chidlow, 1994; Nash and Osborne, 1995). These structures may be possible sites of melatonin action in the goldfish retina. By the use of an *in vitro* autoradiographic technique, we have localized the specific binding in the neural retina of the goldfish (our unpublished results). However, precise localization was not apparent because of low resolution caused by high non-specific binding in the retinal pigmented epithelium. Further studies should be required to elucidate this subject.

In the goldfish, melatonin produced in the retina does not contribute to its blood levels and is metabolized in the eye (Quay, 1965; Grace *et al.*, 1991; Kezuka *et al.*, 1992; Iigo *et al.*, 1997a), suggesting that melatonin may have local neuromodulatory roles in the retina. Visual sensitivity and inputs from photoreceptor to horizontal cells have been reported to exhibit circadian rhythms in the goldfish (Bassi and Powers, 1987; Wang and Mangel, 1996) and melatonin is considered to be involved in the regulation of these retinal events (Iigo *et al.*, 1997a). Recently, melatonin is proved to increase rod input but decrease cone input to the L-type cone horizontal cells in the goldfish (Mangel and Wong, 1996). These processes may be mediated by high-affinity melatonin binding sites characterized in the present study.

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