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Dopaminergic and GABAergic amacrine cells are direct targets of melatonin: Immunocytochemical study of mt_1 melatonin receptor in guinea pig retina

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Abstract

Distribution of the mt_1 melatonin receptor in the guinea pig retina was immunocytochemically investigated using peptide-specific anti-mt_1 receptor antibody. Western blots of the guinea pig retina showed a single band at approximately 37 kilodalton (kD) immunoreactive to the anti-mt_1 antibody. The most intense immunoreactivity for the mt_1 receptor was detected in the cell bodies of ganglion cells. Their dendrites and axons were also immunolabeled. Subpopulations of amacrine cells, the inner plexiform layer, and the outer plexiform layer also exhibited moderate to weak immunolabeling. The mt_1-positive amacrine cells were located either at the vitreal border of the inner nuclear layer or displaced in the ganglion cell layer. Double immunolabeling using antibodies to the mt_1 receptor and tyrosine hydroxylase revealed that the majority of dopaminergic amacrine cells showed mt_1 immunoreactivity. Almost all the 1CA type dopaminergic cells were mt_1 positive while the 2CA type cells less frequently exhibited mt_1 immunoreaction. By double immunolabeling for the mt_1 receptor and GABA, more than 50% of the mt_1-immunoreactive amacrine cells were shown to be GABAergic neurons. Approximately one-third of the GABAergic amacrine cells were immunolabeled for the mt_1 receptor. The present results demonstrate expression of the mt_1 receptor in diverse neuronal cell types in the guinea pig retina and provide the first evidence for the direct effect of melatonin on dopaminergic and GABAergic amacrine cells *via* the mt_1 receptor.

Keywords: Mt_1 Melatonin receptor, Immunocytochemistry, Tyrosine hydroxylase, GABA, Retina

Introduction

Melatonin is a putative neuromodulator in the retina (Besharse, 1982; Pang & Allen, 1986). It is synthesized by retinal photoreceptor cells (Bubenik et al., 1976; Vivien-Roels et al., 1981; Wiechmann & Craft, 1993; Bernard et al., 1997) in a marked diurnal rhythm with increased levels during the dark period (Pang et al., 1980; Yu et al., 1981). The rhythm of melatonin synthesis is controlled by a circadian pacemaker located in the retina itself (Besharse & Iuvone, 1983; Cahill & Besharse, 1993; Tosini & Menaker, 1996, 1998). Melatonin has been implicated in several regulatory processes in retinal physiology, such as melanosome aggregation in the pigment epithelium (Pang & Yew, 1979), inhibition of pigment cell phagocytic activity (Ogino et al., 1983), dark-adaptive cone elongation (Pierce & Besharse, 1985), activation of rod disc shedding (Besharse & Dunis, 1983; White & Fisher, 1989), and enhancement of horizontal cell sensitivity to light (Wiechmann et al., 1988). There is considerable evidence for functional interactions of melatonin and dopamine in the retina. Melatonin inhibits dopamine release and synthesis elicited by electrical or light stimulation and high potassium (Dubocovich, 1983; Dubocovich & Takahashi, 1987; Nowak et al., 1992). In the *Xenopus* retina, suppression of light-evoked dopamine release by melatonin is blocked by $GABA_A$ receptor antagonists, suggesting that melatonin enhances GABAergic inhibition of dopamine release (Boatright et al., 1994). Melatonin also inhibits D_1 dopamine receptor-mediated cAMP accumulation in cultured chick retinal neurons (Iuvone & Gan, 1995). Dopamine, on the other hand, inhibits melatonin biosynthesis and release (Iuvone & Besharse, 1986; Zawilska & Iuvone, 1989). Thus, melatonin and dopamine appear to interact as mutually inhibitory neuromodulators in the retina (Besharse et al., 1988).

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To understand the functional role of melatonin in the retina, it is essential to define precise cell targets of melatonin in this tissue. By *in vitro* autoradiography using the melatonin analog 2-[^{125}I]iodomelatonin, melatonin binding sites have been localized to the inner plexiform layer in various vertebrate species (Laitinen & Saavedra, 1990; Blazynski & Dubocovich, 1991; Wiechmann & Wirsig-Wiechmann, 1991). Two melatonin receptor subtypes, mt_1 (Mel_{1a}) and MT_2 (Mel_{1b}) (Dubocovich et al., 1998), have been cloned in mammals (Reppert et al., 1994, 1995*a*), and mRNA of both subtypes with higher levels of the MT_2 subtype were detected in human retina by RT-PCR (Reppert et al., 1995*a*). In the chick retina, mRNA of the Mel_{1a} and Mel_{1c} subtypes has been localized to the ganglion cell layer and the inner nuclear layer by *in situ* hybridization (Reppert et al., 1995*b*). Recently, by using immunocytochemistry and *in situ* hybridization, we have reported localization of the mt_1 receptor in the inner and outer plexiform layers of the rat retina and expression of the mt_1 mRNA by ganglion cells, amacrine cells, and horizontal cells, suggesting diverse physiological functions of melatonin in the rat retina (Fujieda et al., 1999). Since retinal neurons, especially amacrine cells, are noted for their neurochemical heterogeneity (Vaney, 1990), further characterization of the cells expressing melatonin receptors with respect to their neurochemical contents is essential for an understanding of the nature of the melatonin–neurotransmitter interaction in the retina. As melatonin has a modulatory effect on the retinal dopaminergic system (Dubocovich, 1983; Dubocovich & Takahashi, 1987; Nowak et al., 1992) and this effect may be mediated by GABAergic neurotransmission (Boatright et al., 1994), it is of special interest to determine whether dopaminergic or GABAergic amacrine cells express melatonin receptors.

Here, we report immunocytochemical localization of the mt_1 receptor in the guinea pig retina, with special focus on its localization in amacrine cells. We show that the majority of dopaminergic amacrine cells and a significant proportion of GABAergic amacrine cells express the mt_1 receptor, providing the first evidence for the direct effect of melatonin on these cell types.

Methods

Animals and tissue preparation

Male Hartley guinea pigs (250 g body weight), kept under an artificial 12-h light/12-h dark (LD 12:12) photoperiod with lights on at 08:00, were sacrificed during the photophase (12:00–14:00). Six retinas were used for Western blotting and eight retinas were examined for immunocytochemistry. The animals used for Western blots were decapitated after euthanasia with carbon dioxide. Retinas were dissected, frozen on dry ice, and stored at -70°C until use. The animals for immunocytochemistry were anesthetized with sodium pentobarbital and transcardially perfused either with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) or with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS. Whole eyeballs were dissected, rinsed in 30% sucrose in PBS, frozen with dry ice/acetone, sliced into 10-μm-thick sections on a cryostat, and collected on gelatin-coated glass slides.

Antibodies

Polyclonal anti-mt_1 receptor antibody directed against a peptide corresponding to the third intracellular loop of the human mt_1 receptor (residues 226–238; KPKLKPQDFRNFV) was used in the present study. Specificity of the antibody was previously described (Song et al., 1997; Fujieda et al., 1999). Possible cross-reactivity of the antibody with the MT_2 receptor was tested by preincubating the antibody with the peptide corresponding to the same region of the human MT_2 receptor (RLCLKPSDLRSFL). The MT_2 peptide did not block the specific reactions obtained both in Western blots and immunostaining, further showing the specificity of the antibody to the mt_1 receptor (data not shown). Affinity-purified antibody was used for immunocytochemical studies (Fujieda et al., 1999).

Monoclonal antibodies to tyrosine hydroxylase (TH) and γ-aminobutyric acid (GABA) were purchased from Sigma (St. Louis, MO) and used to label dopaminergic and GABAergic neurons, respectively.

Gel electrophoresis and Western blots

Gel electrophoresis and Western blots were performed as previously described (Fujieda et al., 1999). In brief, membrane fractions of retinal tissues solubilized with 1% Triton X-100 were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The blots were sequentially incubated in anti-mt_1 antibody and HRP-conjugated donkey anti-rabbit IgG (Amersham, Buckinghamshire, England) and processed for ECL detection (Amersham). Control experiments were performed by preincubating the primary antibody with immunogen peptide.

Immunocytochemistry

The mt_1 immunostaining was carried out by the streptavidin-biotin method (Fujieda et al., 1997, 1999) and by subsequent signal amplification using TSA-Direct Kit (NEN Life Science, Boston, MA). Sections were treated with 0.3% hydrogen peroxide in methanol for 20 min and sequentially incubated with Blocking Reagent (NEN) for 30 min, affinity-purified anti-mt_1 antibody overnight, biotinylated swine anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) for 30 min, peroxidase-conjugated streptavidin (DAKO) for 30 min, and finally tetramethylrhodamine (TRITC)-conjugated tyramide (NEN) for 10 min. Immunocytochemical controls were performed by using primary antibody preabsorbed with immunogen peptide.

Double immunolabeling was performed using a monoclonal antibody to either TH or GABA in combination with anti-mt_1 antibody. TH and GABA were labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson, West Grove, PA) as a secondary antibody whereas the mt_1 receptor was visualized with TRITC as described above. Sections were sequentially incubated in a mixture of primary antibodies overnight, a mixture of FITC-conjugated goat anti-mouse IgG and biotinylated swine anti-rabbit immunoglobulins for 30 min, peroxidase-conjugated streptavidin for 30 min, and finally TRITC-tyramide for 10 min. Cross-reactivity of secondary antibodies was tested by omitting one of the primary antibodies. The signals of TRITC were completely eliminated by the omission of anti-mt_1 antibody and FITC signals disappeared by omitting either anti-TH or anti-GABA antibody (data not shown).

Fluorescence signals were observed by confocal laser scanning microscope (Zeiss).

Results

Western blotting of the mt_1 melatonin receptor in the guinea pig retina showed a single immunoreactive band of approximately

37 kD, which was completely blocked by preincubation of the antibody with immunogen peptide (Fig. 1).

Immunocytochemical staining of the mt_1 receptor revealed intense immunolabeling in ganglion cells and moderate to weak staining in amacrine cells, the inner plexiform layer (IPL), and the outer plexiform layer (OPL) (Fig. 2A). The immunoreaction was completely blocked with immunogen peptide on the control sections, except for nonspecific labeling in the structures adjacent to the outer limiting membrane, which seemed to be Müller cell terminals (Fig. 2B). The majority of ganglion cells exhibited strong immunoreaction in their cell bodies. Some were seen to project their immunoreactive primary dendrites into the IPL (Fig. 2C). Faint immunolabeling was also associated with the nerve fiber layer, indicating the presence of mt_1 immunoreactivity in the ganglion cell axons (Fig. 2D). Most immunoreactive amacrine cells were located in the inner nuclear layer (INL) immediately adjacent to the IPL (Fig. 2E), occasionally showing their immunolabeled primary dendrites in the IPL (Fig. 2F). Small immunoreactive cells which seemed to be displaced amacrine cells were often encountered in the ganglion cell layer (GCL) (Fig. 2F).

Double immunolabeling using anti-mt_1 and anti-tyrosine hydroxylase (TH) antibodies demonstrated that the majority of TH-positive dopaminergic amacrine cells exhibit mt_1 immunoreactivity (Figs. 3A–3D, Table 1). The type 1CA cells, characterized by a large cell body and intense TH immunoreactivity (Nguyen-Legros et al., 1997), were always immunoreactive for the mt_1 receptor (Table 1). Although mt_1 immunoreactivity in this cell type was weak, it was diffusely distributed throughout the cell body as typically shown in Fig. 3C. The type 2CA cells, characterized by a small cell body and weak TH immunoreactivity (Nguyen-Legros et al., 1997), were occasionally found to be mt_1 positive (Figs. 3C and 3D), but 60% of this cell type was negative for the mt_1 receptor (Table 1).

Double immunolabeling for the mt_1 receptor and GABA revealed that more than half of the mt_1-immunoreactive amacrine cells are GABAergic (Figs. 4A–4F, Table 2). We estimated that approximately one-third of the entire GABA-positive cell population displayed mt_1 immunoreactivity (Table 2). The mt_1 immunoreactivity in this cell type was often restricted to the vitreal side of the cell cytoplasm (Figs. 4A–4F). Most of the mt_1-positive GABAergic neurons were located in the vitreal margin of the INL or in the GCL as displaced amacrine cells (Figs. 4E and 4F), and GABAergic cells situated in the middle of the INL were negative for the mt_1 receptor (Figs. 4A and 4B).

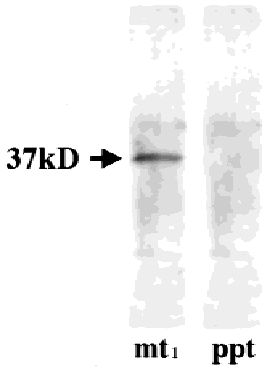

Fig. 1. Western blots of the mt_1 receptor in the guinea pig retina showing a single 37-kilodalton (kD) band immunoreactive to the anti-mt_1 receptor antibody (mt_1), which is blocked by preincubation with immunogen peptide (ppt).

Table 1. *Degree of colocalization between the mt_1 receptor and tyrosine hydroxylase in amacrine cells of the guinea pig retina*[a]

	Cell types			
	1CA		2CA	
	$mt_1(+)$	$mt_1(-)$	$mt_1(+)$	$mt_1(-)$
Cell number	37	0	14	21
%	100	0	40	60

[a]Cell numbers were counted from the whole areas of four sections.

Discussion

The antibody we used in the present study was directed against a peptide corresponding to the third intracellular loop of the cloned human mt_1 receptor (Song et al., 1997). This region of the mt_1 receptor is highly conserved across mammalian species and shows 100% identity with the cloned mt_1 receptor of sheep, cow, rat, and mouse (Reppert et al., 1994; Roca et al., 1996; Messer et al., 1997) and only one amino acid variation with the hamster mt_1 receptor (Reppert et al., 1994). It is thus anticipated that this antibody reacts with the mt_1 receptor from a broad range of mammalian species. We have recently reported the native mt_1 receptor in guinea pig kidney and intestine, human and rat brain, and rat retina as a 37-kD protein recognized by the anti-mt_1 antibody (Song et al., 1997, Fujieda et al., 1999). In the present study, we detected an immunoreactive protein of the same molecular size in the guinea pig retina, again showing the specificity of the antibody as well as expression of the mt_1 receptor in this tissue.

The present immunocytochemical study has demonstrated distinct mt_1 immunoreactivity in ganglion cells and amacrine cells in the guinea pig retina. The immunostaining was also observed in the nerve fiber layer (NFL), inner plexiform layer (IPL), and outer plexiform layer (OPL). The staining in the IPL is probably due to the dendrites of both ganglion and amacrine cells expressing the mt_1 receptor, as we observed the immunoreactivity in primary dendrites of some ganglion and amacrine cells. The immunoreactivity in the NFL indicates that the mt_1 receptor is present not only in the dendrites of ganglion cells but also in their axons. The cell types which contribute to the immunoreaction in the OPL were not clear. We have recently reported localization of the immunoreactive mt_1 receptor in the IPL and OPL of the rat retina, and expres-

Table 2. *Degree of colocalization between the mt_1 receptor and GABA in amacrine cells of the guinea pig retina*[a]

	GABA(+)		$mt_1(+)$	
	$mt_1(+)$	$mt_1(-)$	GABA(+)	GABA(−)
Cell number	97	186	97	75
%	34	66	56	44

[a]Cell numbers were counted from 33 microscopic fields on six sections.

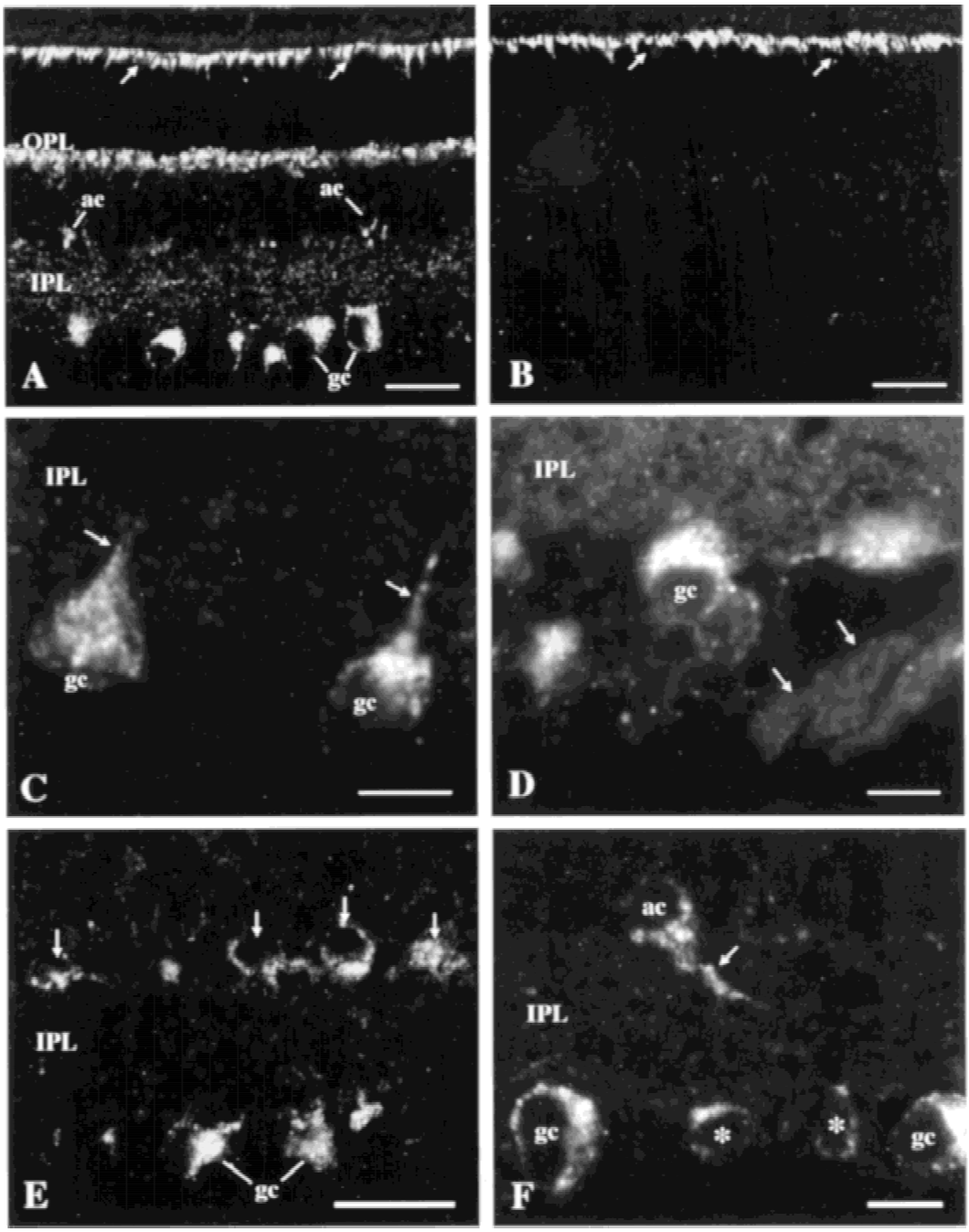

Fig. 2. Immunocytochemical staining of the mt_1 receptor in the guinea pig retina. A: Specific immunoreaction is detected in ganglion cells (gc), amacrine cells (ac), the inner plexiform layer (IPL), and the outer plexiform layer (OPL). Arrows indicate nonspecific labeling in presumptive Müller cell processes. B: Control section reacted with the mt_1 antibody preabsorbed with immunogen peptide shows complete loss of specific immunolabeling except a nonspecific reaction indicated by arrows. C: High-power view of mt_1-immunoreactive ganglion cells (gc) showing punctate immunolabeling in their cell soma and primary dendrites (arrows). D: Shows faintly labeled ganglion cell axons in the nerve fiber layer (arrows). E: High-power view of mt_1-immunoreactive amacrine cells (arrows) located immediately adjacent to the inner plexiform layer (IPL). F: Shows an immunoreactive amacrine cell (ac) projecting its labeled primary dendrite (arrow) into the inner plexiform layer (IPL). Note two presumptive displaced amacrine cells immunolabeled for the mt_1 receptor (asterisks). Scale bars in A, B, and E = 25 μm; in C, D, and F = 10 μm.

sion of the mt_1 mRNA in ganglion cells, amacrine cells, and horizontal cells (Fujieda et al., 1999). We thus concluded that, in the rat retina, the immunoreactivity found in the IPL is localized in the dendritic processes of ganglion cells and amacrine cells and that the staining in the OPL is localized in horizontal cell processes. Localization of mt_1 immunoreactivity in ganglion and amacrine cells of the guinea pig retina is consistent with the findings in the rat retina, providing further evidence for expression of this

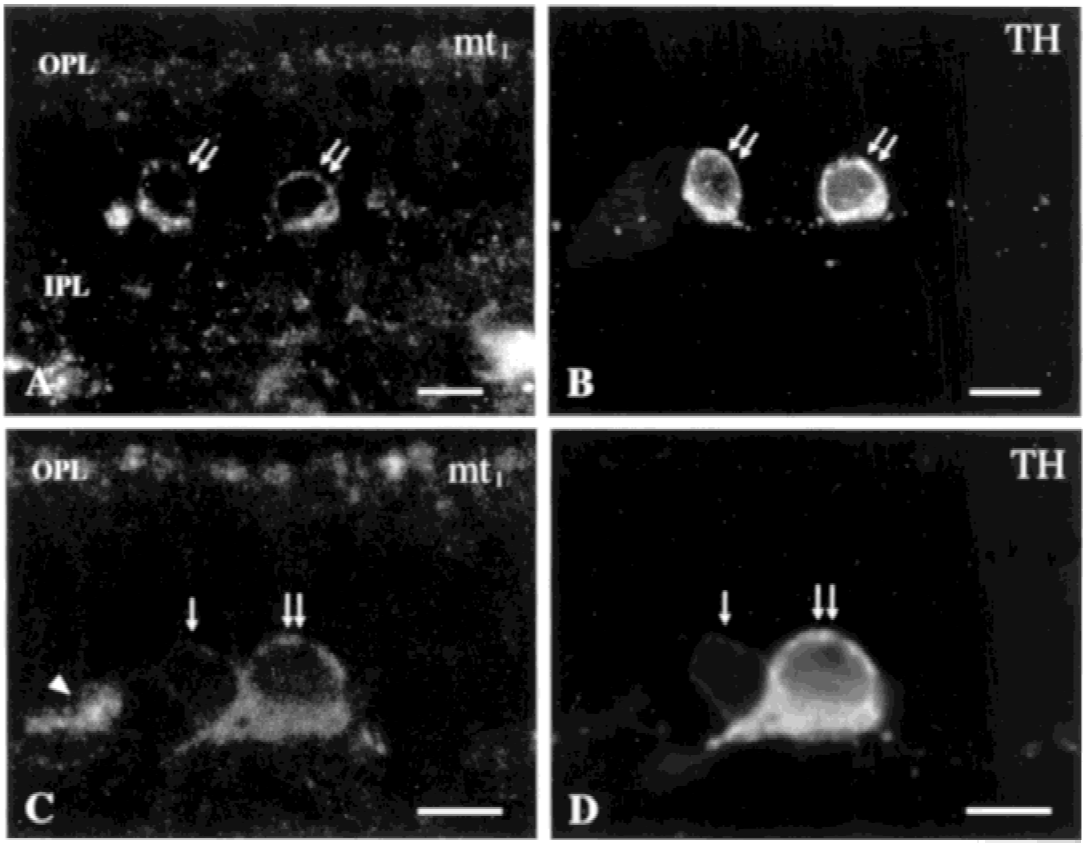

Fig. 3. Double immunolabeling for the mt_1 receptor (TRITC-labeled) and tyrosine hydroxylase (TH, FITC-labeled) in the guinea pig retina. A, B: Same section showing 1CA dopaminergic amacrine cells (double arrows) immunolabeled both for the mt_1 receptor (A) and TH (B). C, D: Same section. Arrow and double arrow respectively indicate the 2CA and 1CA dopaminergic cells, both showing mt_1 (C) and TH immunoreactivity (D). Arrowhead in C indicates an mt_1 labeled but TH-negative amacrine cell. IPL: inner plexiform layer; and OPL: outer plexiform layer. Scale bars in A–D = 10 μm.

melatonin receptor subtype in these cell types. Considering the similar staining pattern of the rat and guinea pig retina, we assume that horizontal cell processes may be the site of immunoreaction in the OPL also in the guinea pig retina. The characteristic feature of the mt_1 immunoreactivity in the guinea pig retina compared to that in the rat retina is the strong reactivity of the neuronal somata of ganglion cells and amacrine cells. It may indicate higher expression or slower metabolism of the receptor in these cell types in the guinea pig retina. It is also possible that the intracellular transport mechanism of melatonin receptors from the neuronal cell soma to the processes is different between the rat and guinea pig retina.

Considerable evidence suggests melatonin influences the function of photoreceptor cells and the retinal pigment epithelium (RPE). In the *Xenopus* retina, melatonin has been implicated in the regulation of cone retinomotor movements (Pierce & Besharse, 1985) and activation of rod disc shedding (Besharse & Dunis, 1983). In rat, melatonin administration by a subcutaneous implant increases photoreceptor disk shedding (White & Fisher, 1989). Melatonin promotes melanosome aggregation in the guinea pig RPE (Pang & Yew, 1979), inhibits phagocytosis by chick RPE cells in culture (Ogino et al., 1983), and affects electrical activity of the RPE of chick (Nao-I et al., 1989) and rabbit (Textorius & Nilsson, 1987). In the present study, we did not find detectable mt_1 immunoreactivity in photoreceptors and the RPE in the guinea pig retina. We also failed to detect mt_1 immunoreactivity and mt_1 mRNA in these cell types in the rat retina (Fujieda et al., 1999). The absence of the mt_1 receptor in photoreceptor cells and the RPE supports the previous suggestions that melatonin may indirectly affect the function of these cell types through its effect on dopamine release (Pierce & Besharse, 1985; Besharse et al., 1988). Alternatively, another subtype of melatonin receptor, such as the MT_2 receptor, might be involved in these melatonin effects.

There is substantial evidence that melatonin inhibits the stimulation-evoked release of dopamine in the retina *via* a specific, receptor-mediated mechanism (Dubocovich, 1983; Dubocovich & Takahashi, 1987; Nowak et al., 1992; Boatright et al., 1994). It was not known, however, whether melatonin directly acts on dopaminergic neurons or the effect is mediated by other neurotransmitters. The present finding showing the mt_1 immunoreactivity in the majority of dopaminergic amacrine cells in the guinea pig retina strongly suggests that melatonin modulates dopaminergic function by directly influencing dopaminergic amacrine cells through the mt_1 receptor. The so-called type 1CA cells, which display a large cell body and intense tyrosine hydroxylase (TH) immunoreactivity (Nguyen-Legros et al., 1997), were 100% positive for the mt_1 receptor. This indicates an intimate functional relationship between melatonin and this type of dopaminergic cells. The second type of dopaminergic neurons, called the type 2CA cells, which show a small cell body and weak TH immunoreactivity (Nguyen-Legros et al., 1997), were less frequently positive (40%) for the mt_1 re-

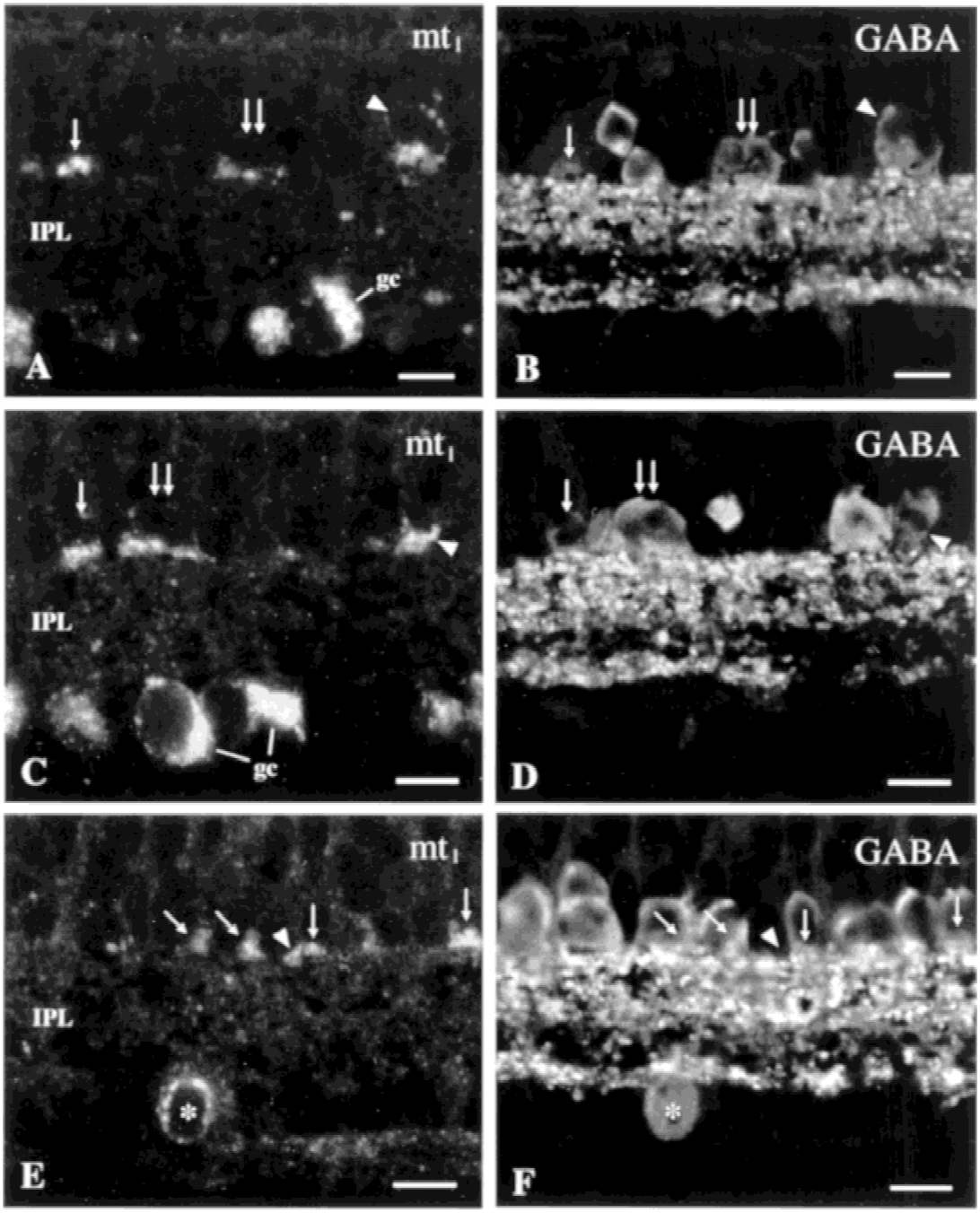

Fig. 4. Double immunolabeling for the mt_1 receptor (TRITC-labeled) and GABA (FITC-labeled) in the guinea pig retina. A, B: Same section showing colocalization of mt_1 receptor (A) and GABA (B) immunoreactivity in three amacrine cells (arrow, double arrow, and arrowhead). Some GABA-positive amacrine cells are mt_1 negative. C, D: Same section showing three amacrine cells (arrow, double arrow, and arrowhead) immunoreactive both for the mt_1 receptor (C) and GABA (D). E, F: Arrows indicate colocalization of the mt_1 receptor (E) and GABA (F) immunoreactivity in amacrine cells. Note an mt_1-positive but GABA-negative amacrine cell (arrowhead). Asterisk: a displaced amacrine cell immunoreactive both for the mt_1 receptor and GABA; gc: ganglion cells; and IPL: inner plexiform layer. Scale bars in A–F = 10 μm.

ceptor. The functional difference between these two subtypes of dopaminergic neurons is not well known, but different branching patterns of their dendritic processes suggest possible different functional roles. The mechanism by which the mt_1 receptor activation influences dopaminergic neurons is a matter of interest. Suppression of light-evoked dopamine release by melatonin is blocked by $GABA_A$ receptor antagonists in the *Xenopus* retina (Boatright et al., 1994). Dopaminergic amacrine cells express $GABA_A$ receptors (Greferath et al., 1995) and GABA is a potent inhibitor of retinal dopaminergic release (Morgan & Kamp, 1980; Marshburn & Iuvone, 1981; Kamp & Morgan, 1981). It is thus likely that melatonin inhibits dopamine release by enhancing the $GABA_A$ receptor

activity in the dopaminergic neurons. We have recently reported that melatonin potentiates $GABA_A$ receptor-mediated currents in HEK293 cells transiently co-transfected with the $GABA_A$ and mt_1 receptors (Wan et al., 1999), further supporting the possibility that the mt_1 receptor enhances $GABA_A$ receptor function in retinal dopaminergic neurons. Dubocovich et al. (1997) have recently reported that high-affinity melatonin receptors of the rabbit retina mediating inhibition of calcium-dependent dopamine release show a pharmacological profile similar to that of the human recombinant MT_2 receptor, concluding that this melatonin effect in the rabbit retina is mediated through the MT_2 subtype. Although no information is available regarding the cellular localization of melatonin receptor subtypes in the rabbit retina, there may be interspecies variation in the distribution and functional significance of the two subtypes of melatonin receptor in the retina.

Another interesting finding of the present study is localization of mt_1 immunoreactivity in a subset of GABAergic amacrine cells. Approximately 30 to 40% of the amacrine cell population in mammalian retina are known to be GABAergic and another 40 to 50% are glycinergic amacrine cells (Vaney, 1990). We showed that a large proportion (56%) of the mt_1-immunoreactive amacrine cells were GABA positive, suggesting a significant physiological role of melatonin in the regulation of this inhibitory neurotransmitter. As melatonin has been reported to increase brain GABA concentration (Rosenstein & Cardinali, 1986; Xu et al., 1995), melatonin may also influence retinal GABA metabolism, modulating GABA synthesis and release from amacrine cells. The proportion of the mt_1-positive GABAergic neurons was estimated to be 34% of the entire GABAergic cell population. Since most dopaminergic neurons are known to also contain GABA (Kosaka et al., 1987; Wässle & Chun, 1988), dopaminergic cells expressing the mt_1 receptor may account for a part of the mt_1-positive GABAergic cell population. As GABAergic neurons are known to comprise different subpopulations containing other neuroactive substances such as acetylcholine, substance P, somatostatin, vasoactive intestinal polypeptide (VIP), as well as dopamine (Vaney, 1990), further characterization of mt_1-positive GABAergic cells with respect to their additional neurochemical contents may illuminate the modulatory effect of melatonin on these cells.

The functional effect of melatonin on retinal ganglion cells is also a matter of interest. Our present and previous studies (Fujieda et al., 1999) suggest that almost all ganglion cells both in the guinea pig and rat retina express the mt_1 receptor. The present finding that both dendrites and axons of these cells contain the melatonin receptor raises the interesting possibility that the receptors located in ganglion cell dendrites are mainly activated by retinal melatonin whereas the receptors located in retinal terminals in brain are stimulated by pineal melatonin. Thus, retinal and pineal melatonin, both of which are produced at night, seem to cooperatively regulate the function of retinal ganglion cells. It is known that the circadian rhythms of the suprachiasmatic nucleus (SCN) are entrained by the environmental lighting, which is mediated by retinal ganglion cells projecting directly to the SCN *via* the retinohypothalamic tract (Moore & Lenn, 1972). Evidence suggests that glutamate is a neurotransmitter of retinal terminals in the SCN (Liou et al., 1986; Castel et al., 1993), which mediates the phase-shifting effect of light (Meijer et al., 1988; Shirakawa & Moore 1994; Ebling, 1996). It is thus tempting to speculate that the melatonin-induced entrainment of SCN rhythms (Cassone et al., 1986) might be mediated not only by its direct effect on SCN neurons (McArthur et al., 1991) but also by its effect on the glutamate release by retinal ganglion cells.

In the present study, we have provided evidence for the direct effect of melatonin on dopaminergic and GABAergic amacrine cells. However, we also found mt_1-positive amacrine cells which were neither labeled for tyrosine hydroxylase nor for GABA, suggesting that melatonin may also regulate the functions of other types of amacrine cells, such as glycinergic cells. Further systematic analyses of the cell types expressing melatonin receptors, including the MT_2 as well as the mt_1 subtype, will provide crucial knowledge for an understanding of the physiological roles of melatonin in the retina.

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References

Bernard, M., Iuvone, P.M., Cassone, V.M., Roseboom, P.H., Coon, S.L. & Klein, D.C. (1997). Avian melatonin synthesis: Photic and circadian regulation of serotonin N-acetyltransferase mRNA in the chicken pineal gland and retina. *Journal of Neurochemistry* **68**, 213–224.

Besharse, J.C. (1982). The daily light–dark cycle and rhythmic metabolism in the photoreceptor-pigment epithelial complex. In *Progress in Retinal Research, Vol. 1*, ed. Osborne, N.N. & Chader, G.J., pp. 81–124. Oxford: Pergamon Press.

Besharse, J.C. & Dunis, D.A. (1983). Methoxyindoles and photoreceptor metabolism: Activation of rod shedding. *Science* **219**, 1341–1343.

Besharse, J.C. & Iuvone, P.M. (1983). Circadian clock in *Xenopus* eye controlling retinal serotonin N-acetyltransferase. *Nature* **305**, 133–135.

Besharse, J.C., Iuvone, P.M. & Pierce, M.E. (1988). Regulation of rhythmic photoreceptor metabolism: A role for post-receptoral neurons. In *Progress in Retinal Research, Vol. 7*, ed. Osborne, N.N. & Chader, G.J., pp. 21–61. Oxford: Pergamon Press.

Blazynski, C. & Dubocovich, M.L. (1991). Localization of 2-[^{125}I]Iodomelatonin binding sites in mammalian retina. *Journal of Neurochemistry* **56**, 1873–1880.

Boatright, J.H., Rubim, N.M. & Iuvone, P.M. (1994). Regulation of endogenous dopamine release in amphibian retina by melatonin: The role of GABA. *Visual Neuroscience* **11**, 1013–1018.

Bubenik, G.A., Brown, G.M. & Grota, L.G. (1976). Differential localization of N-acetylated indolealkylamines in CNS and the harderian gland using immunohistology. *Brain Research* **118**, 417–427.

Cahill, G.M. & Besharse, J.C. (1993). Circadian clock functions localized in *Xenopus* retinal photoreceptors. *Neuron* **10**, 573–577.

Cassone, V.M., Chesworth, M.J. & Armstrong, S.M. (1986). Entrainment of rat circadian rhythms by daily injection of melatonin depends upon the hypothalamic suprachiasmatic nuclei. *Physiology and Behavior* **36**, 1111–1121.

Castel, M., Belenky, M., Cohen, S., Ottersen, O.P., Storm-Mathisen, J. (1993). Glutamate-like immunoreactivity in retinal terminals of the mouse suprachiasmatic nucleus. *European Journal of Neuroscience* **5**, 368–381.

Dubocovich, M.L. (1983). Melatonin is a potent modulator of dopamine release in the retina. *Nature* **306**, 782–784.

Dubocovich, M.L. & Takahashi, J.S. (1987). Use of 2-[^{125}I]Iodomelatonin to characterize melatonin binding sites in chicken retina. *Proceedings of the National Academy of Sciences of the U.S.A.* **84**, 3916–3920.

Dubocovich, M.L., Masana, M.I., Iacob, S. & Sauri, D.M. (1997). Melatonin receptor antagonists that differentiate between the human Mel1a and Mel1b recombinant subtypes are used to assess the pharmacological profile of the rabbit retina ML1 presynaptic heteroreceptor. *Naunyn-Schmiedebergs Archives of Pharmacology* **355**, 365–375.

Dubocovich, M.L., Cardinali, D.P., Guardiola-Lemaitre, B., Hagan, R.M., Krause, D.N., Sugden, D., Vanhoutte, P.M. & Yocca, F.D. (1998). Melatonin receptors. In *The IUPHAR Compendium of Receptor Characterization and Classification*, pp. 187–193. London: IUPHAR Media.

Ebling, F.J. (1996). The role of glutamate in the photic regulation of the suprachiasmatic nucleus. *Progress in Neurobiology* **50**, 109–132

Fujieda, H., Sato, T., Shi, J. & Wake, K. (1997). Remodeling of pineal epithelium in the fetal rat as delineated by immunohistochemistry of laminin and cadherin. *Cell and Tissue Research* **287**, 263–274.

Fujieda, H., Hamadanizadeh, S.A., Wankiewicz, E., Pang, S.F. & Brown, G.M. (1999). Expression of mt_1 melatonin receptor in rat retina: Evidence for multiple cell targets for melatonin. *Neuroscience* **93**, 793–799.

Greferath, U., Grünert, U., Fritschy, J.M., Stephenson, A., Möhler, H. & Wässle, H. (1995). $GABA_A$ receptor subunits have differential distributions in the rat retina: *In situ* hybridization and immunohistochemistry. *Journal of Comparative Neurology* **353**, 553–571.

Iuvone, P.M. & Besharse, J.C. (1986). Dopamine receptor-mediated inhibition of serotonin N-acetyltransferase activity in retina. *Brain Research* **369**, 168–176.

Iuvone, P.M. & Gan, J. (1995). Functional interaction of melatonin receptors and D1 dopamine receptors in cultured chick retinal neurons. *Journal of Neuroscience* **15**, 2179–2185.

Kamp, C.W. & Morgan, W.W. (1981). GABA antagonists enhance dopamine turnover in the rat retina *in vivo*. *European Journal of Pharmacology* **69**, 273–279.

Kosaka, T., Kosaka, K., Hataguchi, Y., Nagatsu, I., Wu, J.Y., Ottersen, O.P., Storm-Mathisen, J. & Hama, K. (1987). Catecholaminergic neurons containing GABA-like and/or glutamic acid decarboxylase-like immunoreactivities in various brain regions of the rat. *Experimental Brain Research* **66**, 191–210.

Laitinen, J.T. & Saavedra, J.M. (1990). The chick retinal melatonin receptor revisited: Localization and modulation of agonist binding with guanine nucleotides. *Brain Research* **528**, 349–352.

Liou, S.Y., Shibata, S., Iwasaki, K. & Ueki, S. (1986). Optic nerve stimulation-induced increase of release of ^{3}H-glutamate and ^{3}H-aspartate but not ^{3}H-GABA from the suprachiasmatic nucleus in slices of rat hypothalamus. *Brain Research Bulletin* **16**, 527–531.

Marshburn, P.B. & Iuvone, P.M. (1981). The role of GABA in the regulation of the dopamine/tyrosine hydroxylase-containing neurons of the rat retina. *Brain Research* **214**, 335–347.

McArthur, A.J., Gillette, M.U. & Prosser, R.A. (1991). Melatonin directly resets the rat suprachiasmatic circadian clock *in vitro*. *Brain Research* **565**, 158–161.

Meijer, J.H., van der Zee, E.A. & Dietz, M. (1988). Glutamate phase shifts circadian activity rhythms in hamsters. *Neuroscience Letters* **86**, 177–183.

Messer, L.A., Wang, L., Tuggle, C.K., Yerle, M., Chardon, P., Pomp, D., Womack, J.E., Barendse, W., Crawford, A.M., Notter, D.R. & Rothschild, M.F. (1997). Mapping of the melatonin receptor 1a (MTNR1A) gene in pigs, sheep, and cattle. *Mammalian Genome* **8**, 368–370.

Moore, R.Y. & Lenn, N.J. (1972). A retinohypothalamic projection in the rat. *Journal of Comparative Neurology* **146**, 1–14.

Morgan, W.W. & Kamp, C.W. (1980). A GABAergic influence on the light-induced increase in dopamine turnover in the dark-adapted rat retina *in vivo*. *Journal of Neurochemistry* **34**, 1082–1086.

Nao-I, N., Nilsson, S.E., Gallemore, R.P. & Steinberg, R.H. (1989). Effects of melatonin on the chick retinal pigment epithelium: Membrane potentials and light-evoked responses. *Experimental Eye Research* **49**, 573–589.

Nguyen-Legros, J., Versaux-Botteri, C. & Savy, C. (1997). Dopaminergic and GABAergic retinal cell populations in mammals. *Microscopy Research and Technique* **36**, 26–42.

Nowak, J.Z., Kazula, A. & Golembiowska, K. (1992). Melatonin increases serotonin N-acetyltransferase activity and decreases dopamine synthesis in light-exposed chick retina: *In vivo* evidence supporting melatonin–dopamine interaction in retina. *Journal of Neurochemistry* **59**, 1499–1505.

Ogino, N., Matsumura, M., Shirakawa, H. & Tsukahara, I. (1983). Phagocytic activity of cultured retinal pigment epithelial cells from chick embryo: Inhibition by melatonin and cyclic AMP, and its reversal by taurine and cyclic GMP. *Ophthalmic Research* **15**, 72–89.

Pang, S.F. & Allen, A.E. (1986). Extra-pineal melatonin in the retina: Its regulation and physiological function. *Pineal Research Reviews* **4**, 55–95.

Pang, S.F. & Yew, D.T. (1979). Pigment aggregation by melatonin in the retinal pigment epithelium and choroid of guinea-pigs, *Cavia porcellus*. *Experientia* **35**, 231–233.

Pang, S.F., Yu, H.S., Suen, H.C. & Brown, G.M. (1980). Melatonin in the retina of rats: A diurnal rhythm. *Journal of Endocrinology* **87**, 89–93.

Pierce, M.E. & Besharse, J.C. (1985). Circadian regulation of retinomotor movements. I. Interaction of melatonin and dopamine in the control of cone length. *Journal of General Physiology* **86**, 671–689.

Reppert, S.M., Weaver, D.R. & Ebisawa, T. (1994). Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron* **13**, 1177–1185.

Reppert, S.M., Godson, C., Mahle, C.D., Weaver, D.R., Slaugenhaupt, S.A. & Gusella, J.F. (1995*a*). Molecular characterization of a second melatonin receptor expressed in human retina and brain: The Mel_{1b} melatonin receptor. *Proceedings of the National Academy of Sciences of the U.S.A.* **92**, 8734–8738.

Reppert, S.M., Weaver, D.R., Cassone, V.M., Godson, C., & Kolakowski, L.F., Jr. (1995*b*). Melatonin receptors are for the birds: Molecular analysis of two receptor subtypes differentially expressed in chick brain. *Neuron* **15**, 1003–1015.

Roca, A.L., Godson, C., Weaver, D.R. & Reppert, S.M. (1996). Structure, characterization, and expression of the gene encoding the mouse Mel_{1a} melatonin receptor. *Endocrinology* **137**, 3469–3477.

Rosenstein, R.E. & Cardinali, D.P. (1986). Melatonin increases *in vivo* GABA accumulation in rat hypothalamus, cerebellum, cerebral cortex and pineal gland. *Brain Research* **398**, 403–406.

Shirakawa, T. & Moore, R.Y. (1994). Glutamate shifts the phase of the circadian neuronal firing rhythm in the rat suprachiasmatic nucleus *in vitro*. *Neuroscience Letters* **178**, 47–50.

Song, Y., Chan, C.W.Y., Brown, G.M., Pang, S.F. & Silverman, M. (1997). Studies of the renal action of melatonin: Evidence that the effects are mediated by 37kDa receptors of the Mel_{1a} subtype localized primarily to the basolateral membrane of the proximal tubule. *FASEB Journal* **11**, 93–100.

Textorius, O. & Nilsson, S.E. (1987). Effects of intraocular irrigation with melatonin on the c-wave of the direct current electroretinogram and on the standing potential of the eye in albino rabbits. *Documenta Ophtalmologica* **65**, 97–111.

Tosini, G. & Menaker, M. (1996). Circadian rhythms in cultured mammalian retina. *Science* **272**, 419–421.

Tosini, G. & Menaker, M. (1998). The clock in the mouse retina: Melatonin synthesis and photoreceptor degeneration. *Brain Research* **789**, 221–228.

Vaney, D.I. (1990). The mosaic of amacrine cells in the mammalian retina. In *Progress in Retinal Research, Vol. 9*, ed. Osborne, N.N. & Chader, G.J., pp. 49–100. Oxford: Pergamon Press.

Vivien-Roels, B., Pévet, P., Dubois, M.P., Arendt, J. & Brown, G.M. (1981). Immunohistochemical evidence for the presence of melatonin in the pineal gland, the retina and the harderian gland. *Cell and Tissue Research* **217**, 105–115.

Wan, Q., Man, H.Y., Liu, F., Braunton, J., Niznik, H.B., Pang, S.F., Brown, G.M. & Wang, Y.T. (1999). Differential modulation of $GABA_A$ receptor function by MEL_{1a} and MEL_{1b} receptors. *Nature Neuroscience* **2**, 401–403.

Wässle, H. & Chun, M.H. (1988). Dopaminergic and indoleamine-accumulating amacrine cells express GABA-like immunoreactivity in the cat retina. *Journal of Neuroscience* **8**, 3383–3394.

White, M.P. & Fisher, L.J. (1989). Effects of exogenous melatonin on circadian disc shedding in the albino rat retina. *Vision Research* **29**, 167–179.

Wiechmann, A.F. & Craft, C.M. (1993). Localization of mRNA encoding the indolamine synthesizing enzyme, hydroxyindole-*O*-methyltransferase, in chicken pineal gland and retina by in situ hybridization. *Neuroscience Letters* **150**, 207–211.

Wiechmann, A.F. & Wirsig-Wiechmann, C.R. (1991). Localization and quantification of high-affinity melatonin binding sites in *Rana pipiens* retina. *Journal of Pineal Research* **10**, 174–179.

Wiechmann, A.F., Yang, X.L., Wu, S.M. & Hollyfield, J.G. (1988). Melatonin enhances horizontal cell sensitivity in salamander retina. *Brain Research* **453**, 377–380.

Xu, F., Li, J.C., Ma, K.C. & Wang, M. (1995). Effects of melatonin on hypothalamic gamma-aminobutyric acid, aspartic acid, glutamic acid, beta-endorphin and serotonin levels in male mice. *Biological Signals* **4**, 225–231.

Yu, H.S., Pang, S.F. & Tang, P.L. (1981). Increase in the level of retinal melatonin and persistence of its diurnal rhythm in rats after pinealectomy. *Journal of Endocrinology* **91**, 477–481.

Zawilska, J. & Iuvone, P.M. (1989). Catecholamine receptors regulating serotonin N-acetyltransferase activity and melatonin content of chicken retina and pineal gland: D2-dopamine receptors in retina and alpha-2 adrenergic receptors in pineal gland. *Journal of Pharmacology and Experimental Therapeutics* **250**, 86–92.