

Dopaminergic and GABAergic amacrine cells are direct targets of melatonin: Immunocytochemical study of mt₁ melatonin receptor in guinea pig retina

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(RECEIVED April 4, 1999; ACCEPTED August 11, 1999)

Abstract

Distribution of the mt₁ melatonin receptor in the guinea pig retina was immunocytochemically investigated using peptide-specific anti-mt₁ receptor antibody. Western blots of the guinea pig retina showed a single band at approximately 37 kilodalton (kD) immunoreactive to the anti-mt₁ antibody. The most intense immunoreactivity for the mt₁ 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 mt₁-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 mt₁ receptor and tyrosine hydroxylase revealed that the majority of dopaminergic amacrine cells showed mt₁ immunoreactivity. Almost all the ICA type dopaminergic cells were mt₁ positive while the 2CA type cells less frequently exhibited mt₁ immunoreaction. By double immunolabeling for the mt₁ receptor and GABA, more than 50% of the mt₁-immunoreactive amacrine cells were shown to be GABAergic neurons. Approximately one-third of the GABAergic amacrine cells were immunolabeled for the mt₁ receptor. The present results demonstrate expression of the mt₁ 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 mt₁ receptor.

Keywords: Mt₁ 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 pig-

ment 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 D₁ 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-[¹²⁵I]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, mt₁ (Mel_{1a}) and MT₂ (Mel_{1b}) (Dubocovich et al., 1998), have been cloned in mammals (Reppert et al., 1994, 1995a), and mRNA of both subtypes with higher levels of the MT₂ subtype were detected in human retina by RT-PCR (Reppert et al., 1995a). 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., 1995b). Recently, by using immunocytochemistry and *in situ* hybridization, we have reported localization of the mt₁ receptor in the inner and outer plexiform layers of the rat retina and expression of the mt₁ 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 mt₁ 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 mt₁ 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-mt₁ receptor antibody directed against a peptide corresponding to the third intracellular loop of the human mt₁ receptor (residues 226–238; KPCLKPQDFRNfV) 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 MT₂ receptor was tested by preincubating the antibody with the peptide corresponding to the same region of the human MT₂ receptor (RLCLKPSDLRSFL). The MT₂ peptide did not block the specific reactions obtained both in Western blots and immunostaining, further showing the specificity of the antibody to the mt₁ 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-mt₁ 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 mt₁ 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-mt₁ 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-mt₁ 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 mt₁ 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-mt₁ 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 mt₁ 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 *mt₁* 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 *mt₁* 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-*mt₁* and anti-tyrosine hydroxylase (TH) antibodies demonstrated that the majority of TH-positive dopaminergic amacrine cells exhibit *mt₁* 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 *mt₁* receptor (Table 1). Although *mt₁* 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 *mt₁* positive (Figs. 3C and 3D), but 60% of this cell type was negative for the *mt₁* receptor (Table 1).

Double immunolabeling for the *mt₁* receptor and GABA revealed that more than half of the *mt₁*-immunoreactive amacrine cells are GABAergic (Figs. 4A–4F, Table 2). We estimated that approximately one-third of the entire GABA-positive cell population displayed *mt₁* immunoreactivity (Table 2). The *mt₁* immunoreactivity in this cell type was often restricted to the vitreal side of the cell cytoplasm (Figs. 4A–4F). Most of the *mt₁*-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



Fig. 1. Western blots of the *mt₁* receptor in the guinea pig retina showing a single 37-kilodalton (kD) band immunoreactive to the anti-*mt₁* receptor antibody (*mt₁*), which is blocked by preincubation with immunogen peptide (ppt).

Table 1. Degree of colocalization between the *mt₁* receptor and tyrosine hydroxylase in amacrine cells of the guinea pig retina^a

	Cell types			
	1CA		2CA	
	<i>mt₁</i> (+)	<i>mt₁</i> (-)	<i>mt₁</i> (+)	<i>mt₁</i> (-)
Cell number	37	0	14	21
%	100	0	40	60

^aCell numbers were counted from the whole areas of four sections.

GABAergic cells situated in the middle of the INL were negative for the *mt₁* receptor (Figs. 4A and 4B).

Discussion

The antibody we used in the present study was directed against a peptide corresponding to the third intracellular loop of the cloned human *mt₁* receptor (Song et al., 1997). This region of the *mt₁* receptor is highly conserved across mammalian species and shows 100% identity with the cloned *mt₁* 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 *mt₁* receptor (Reppert et al., 1994). It is thus anticipated that this antibody reacts with the *mt₁* receptor from a broad range of mammalian species. We have recently reported the native *mt₁* receptor in guinea pig kidney and intestine, human and rat brain, and rat retina as a 37-kD protein recognized by the anti-*mt₁* 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 *mt₁* receptor in this tissue.

The present immunocytochemical study has demonstrated distinct *mt₁* 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 *mt₁* receptor, as we observed the immunoreactivity in primary dendrites of some ganglion and amacrine cells. The immunoreactivity in the NFL indicates that the *mt₁* 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 *mt₁* receptor in the IPL and OPL of the rat retina, and expres-

Table 2. Degree of colocalization between the *mt₁* receptor and GABA in amacrine cells of the guinea pig retina^a

	GABA(+)		<i>mt₁</i> (+)	
	<i>mt₁</i> (+)	<i>mt₁</i> (-)	GABA(+)	GABA(-)
Cell number	97	186	97	75
%	34	66	56	44

^aCell numbers were counted from 33 microscopic fields on six sections.

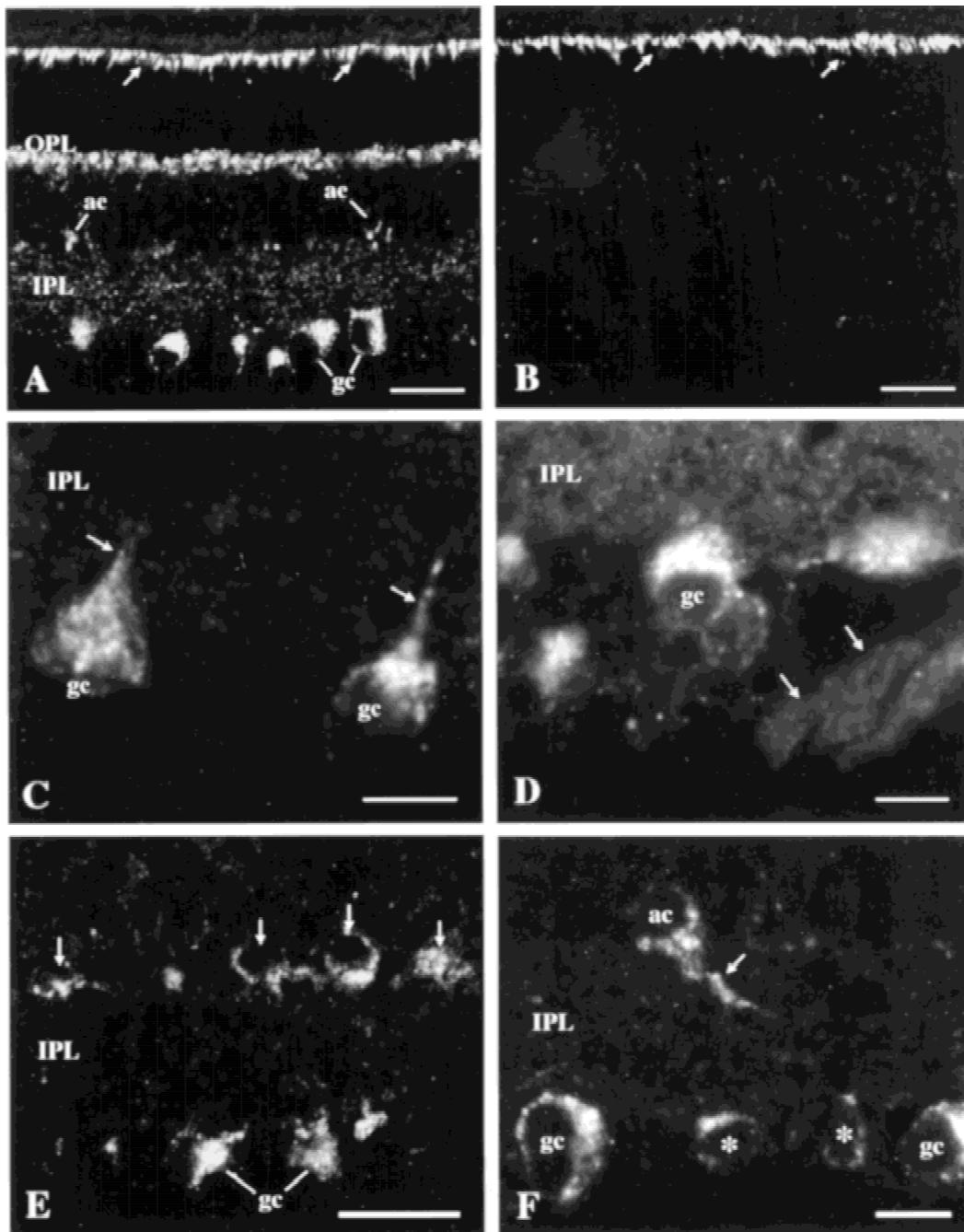


Fig. 2. Immunocytochemical staining of the mt_1 receptor in the guinea pig retina. A: Specific immunoreaction is detected in ganglion cells (gc), amacrine cells (ac), the inner plexiform layer (IPL), and the outer plexiform layer (OPL). Arrows indicate nonspecific labeling in presumptive Müller cell 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 μ m; in C, D, and F = 10 μ m.

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

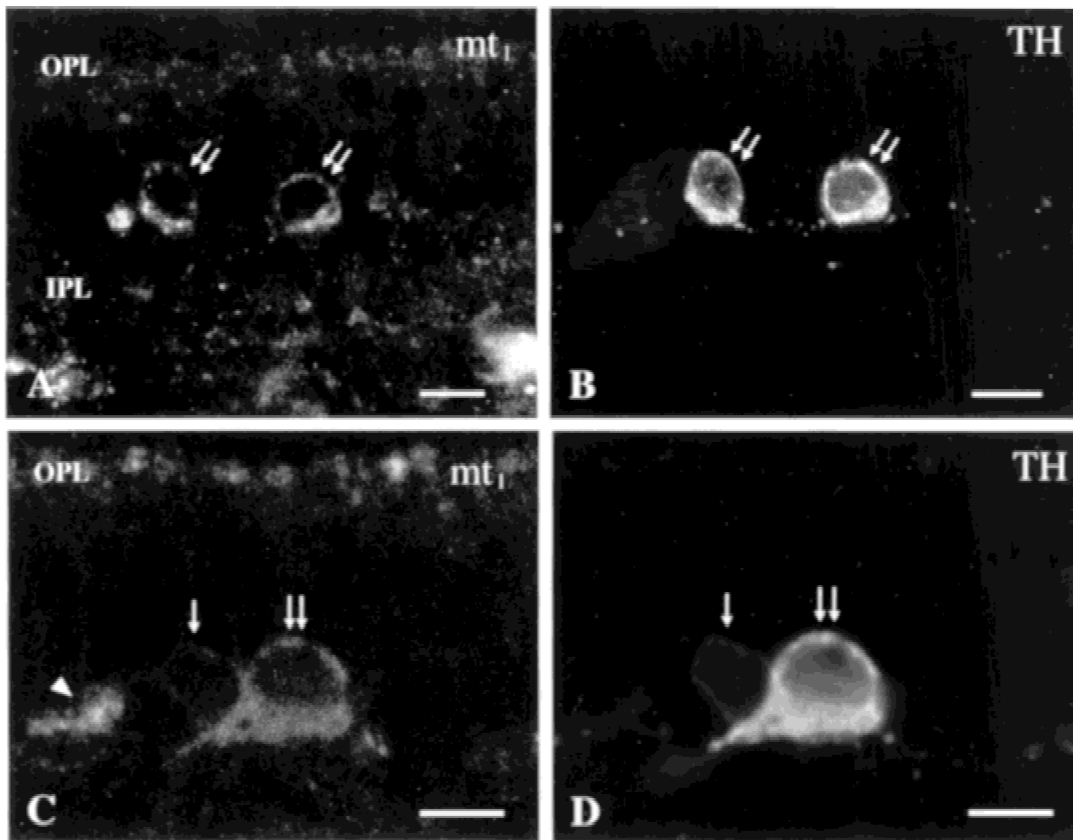


Fig. 3. Double immunolabeling for the mt_1 receptor (TRITC-labeled) and tyrosine hydroxylase (TH, FITC-labeled) in the guinea pig retina. A, B: Same section showing 1CA dopaminergic amacrine cells (double arrows) immunolabeled both for the mt_1 receptor (A) and TH (B). C, D: Same section. Arrow and double 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 μ m.

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-

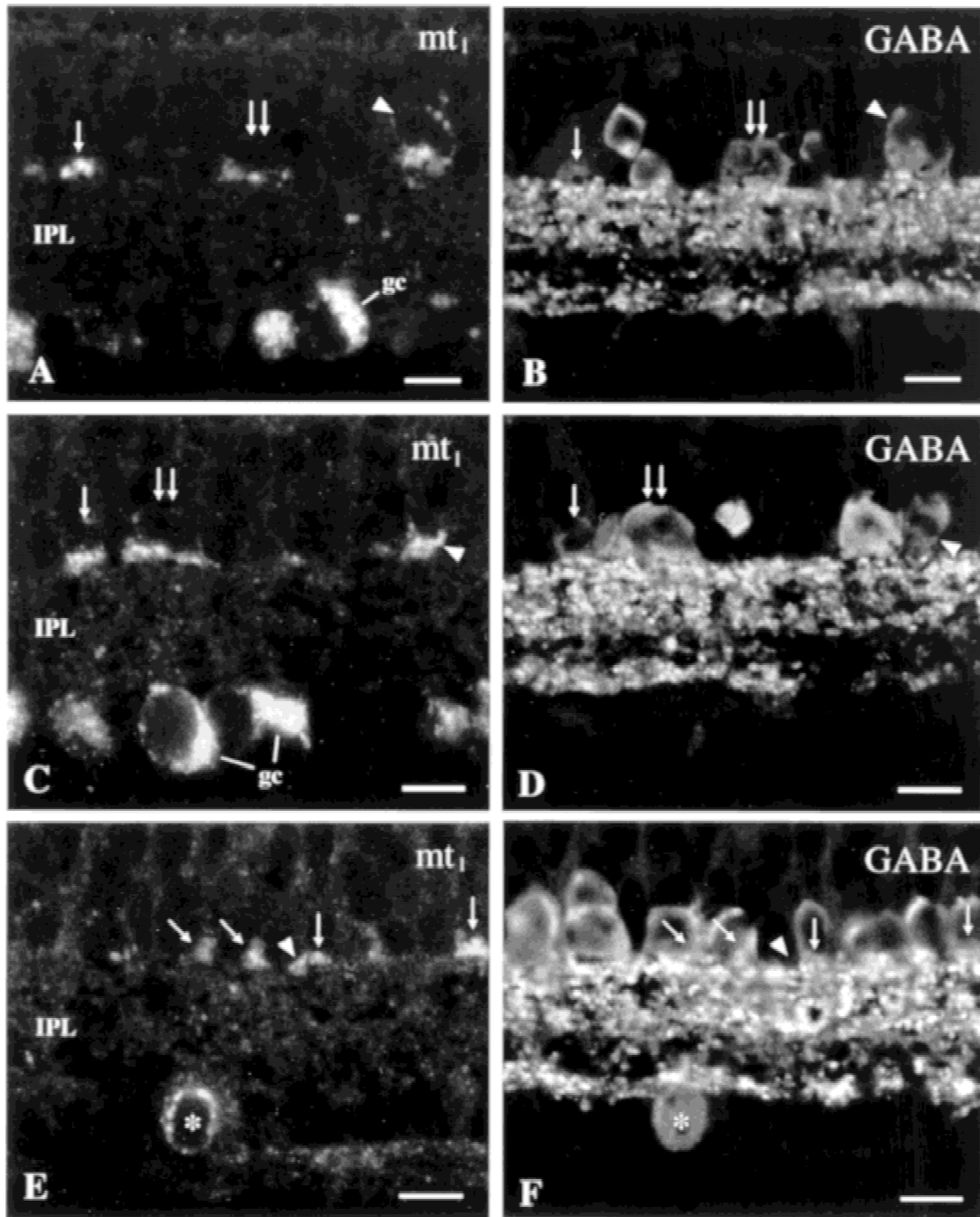


Fig. 4. Double immunolabeling for the mt_1 receptor (TRITC-labeled) and GABA (FITC-labeled) in the guinea pig retina. A, B: Same section showing colocalization of mt_1 receptor (A) and GABA (B) immunoreactivity in three amacrine cells (arrow, double arrow, and arrowhead). Some GABA-positive amacrine 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 μ m.

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 *mt₁* receptors (Wan et al., 1999), further supporting the possibility that the *mt₁* 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 MT₂ receptor, concluding that this melatonin effect in the rabbit retina is mediated through the MT₂ 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 *mt₁* 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 *mt₁*-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 *mt₁*-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 *mt₁* receptor may account for a part of the *mt₁*-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 *mt₁*-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 *mt₁* 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 *mt₁*-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 MT₂ as well as the *mt₁* subtype, will provide crucial knowledge for an understanding of the physiological roles of melatonin in the retina.

Acknowledgments

This work was supported by grants from the Clarke Foundation. We thank Erika Johansson for expert technical assistance and Lori Dixon for expertise in animal care.

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