Annu. Rev. Pharmacol. Toxicol. 1991. 31:549–68

MELATONIN RECEPTORS

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KEY WORDS: N-acetyl-5-methoxytryptamine, 2-[^{125}I]-iodomelatonin binding, ML-1 melatonin receptor, melanophores, pineal hormone

INTRODUCTION

The hormone melatonin (N-acetyl-5-methoxytryptamine) is secreted primarily from the pineal gland and the retina during the hours of darkness in all vertebrates studied, i.e. fish, amphibians, reptiles, birds, and mammals (see 1–6). Production of the hormone is controlled by endogenous circadian oscillators, referred to as body clocks. Light dramatically inhibits melatonin synthesis, and thus the secretion of melatonin is influenced by the daily and seasonal changes in the environmental light-dark cycle. Melatonin is of considerable interest for its regulatory influence on a variety of physiological processes including biological rhythms and neuroendocrine function. The many biologic effects of melatonin and the complex regulation of its synthesis from the precursors serotonin and N-acetylserotonin have been well reviewed (e.g. 1–9).

During much of the thirty years that melatonin has been known and studied, however, the search for its physiological receptor sites has been elusive. The difficulties reflect technical problems and the lack of necessary pharmacological tools such as quantitative bioassays, high-affinity ligands with high specific activity and selective pharmacological agents. Without a conclusive

0362-1642/91/0415-0549$02.00

definition of melatonin receptors or reliable means with which to obtain this information, it is not surprising that the area has received relatively little attention from pharmacologists. This picture, however, has been changing rapidly over the past several years; and for the first time, a consistent description of high-affinity, functionally related melatonin receptors is emerging. The problems encountered in earlier attempts to define mammalian melatonin receptor sites (see 10) are now understandable given that it appears melatonin receptors of picomolar affinity are very discretely localized within the brain, retina, and pituitary. Certain aspects of these and other putative melatonin receptor sites have been summarized (6, 10, 11–13). The focus of the present review is to bring together what is currently known regarding the pharmacological characteristics of specific receptor sites that show high affinity for melatonin and can be correlated to a functional response.

IN VITRO FUNCTIONAL RECEPTOR BIOASSAYS

A key step in defining a specific hormone receptor is the ability to measure a specific physiological response triggered by receptor activation. Not only do such studies provide insight into the physiological role of the hormone but they allow the generation of pharmacological criteria for the functional receptor. This information is particularly useful for validating that specific binding sites are related to functional receptors (see below). In addition, a sensitive and specific assay of biological activity is necessary for defining the agonist or antagonist nature of compounds with affinity for the receptor site. Three specific in vitro bioassays that have been described to date for melatonin are based on the hormone's ability to (*a*) condense pigment granules in skin melanophores, (*b*) inhibit dopamine release in the retina, and (*c*) affect production of second messengers, particularly adenosine 3',5'-monophosphate (cyclic AMP). The following describes each of these bioassays and the known structure-activity relationships for the functional receptors involved. The structures of melatonin, its synthetic precursors serotonin and N-acetylserotonin, and key melatonin agonists and antagonists are shown in Figure 1.

Melanophore Pigment Condensation

The first bioassay used to measure an effect of melatonin was the frog skin lightening test in which melatonin produces an aggregation of pigment granules (melanosomes) within the dermal melanophores, thus causing the skin to become translucent. The effect was first seen in 1917 by McCord & Allen (14) who looked at effects of bovine pineal extracts on *Rana pipiens* tadpoles. This sensitive assay was next used to measure melatonin during the original isolation of the hormone in 1959 by Lerner and coworkers (15), and the name

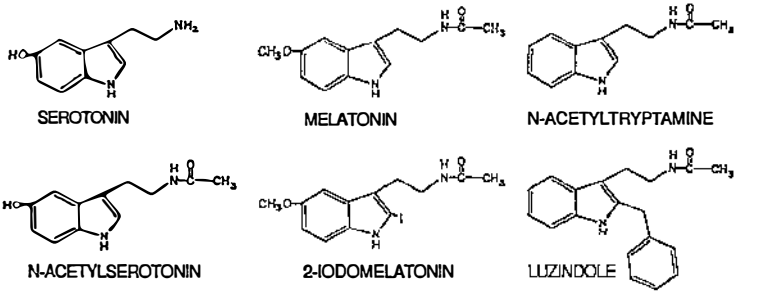

Figure 1 The chemical structures of melatonin (N-acetyl-5-methoxytryptamine), its synthetic precursors serotonin (5-hydroxytryptamine) and N-acetylserotonin (N-acetyl-5-hydroxytryptamine) and the melatonin receptor agonists and antagonists 2-iodomelatonin, N-acetyltryptamine and luzindole (2-benzyl-N-acetyltryptamine; N-0774).

"melatonin" was derived from the hormone's first defined biological activity. Subsequently, the ability of melatonin to condense dermal pigment granules has been assayed in amphibians or fish in vivo (16, 17) and in vitro (18, 19). More recently, isolated melanophores from *Xenopus laevis*, studied in culture, have been found to be exquisitely sensitive to melatonin through a mechanism that is dependent on calcium influx but not mediated through adrenergic or serotonergic receptors (20, 21). In this preparation the effect of melatonin is blocked by pertussis toxin, suggesting the involvement of a toxin-sensitive G protein in the response (22).

STRUCTURE-ACTIVITY RELATIONSHIPS The earliest structure-activity analysis of melatonin receptors using frog skin bioassays was semiquantitative. By determining the minimum concentration that was effective in vivo, the following order of potency was obtained: melatonin (0.1 pg/ml)<< 5-methoxytryptamine<<serotonin (1 μg/ml) (18, 23). It was realized early on that two key features of the melatonin molecule, i.e. the 5-methoxy and N-acetyl groups, are important for optimal biological activity (19, 23). Melatonin also is unique among the bioactive amines in that the nitrogen atom of the amine group is acetylated and therefore not charged (see Figure 1).

Structure-activity data are shown in Table 1 from the in vitro studies of the skin-lightening activity of melatonin and related indoleamines conducted on the frog *Rana pipiens* by Heward & Hadley (19). N-acetyltryptamine, which lacks a 5-methoxy group, had no intrinsic activity but was found to antagonize the skin-lightening effect of melatonin. These authors concluded that the intrinsic activity of indolic compounds on the melatonin receptor is determined primarily by the moiety substituted on the 5th carbon atom, whereas the affinity for the receptor binding site is determined primarily by the moiety

Table 1 Pharmacology of functional melatonin receptors

	Inhibition of DA release		Pigment condensation	
	Rabbit retina (11, 28, 29, 31)	Chicken retina (30, 27)	Frog skin (19)	Isolated frog melan (20, 21[a])
AGONISTS	IC_{50} (nM)	IC_{50} (nM)	MEC[b] (nM)	IC_{50} (nM)
2-Chloromelatonin	—	—	—	0.0003[a]
2-Iodomelatonin	0.005	0.01	—	0.0006[a]
2-Methyl-6,7-dichloro-melatonin	0.01	—	—	—
6-Chloromelatonin	0.04	0.5	—	—
Melatonin	0.04	1.0	0.05	0.003[a] 0.000009
5-Methoxyluzindole	1.3[c]	—	—	—
6-Methoxymelatonin	1.6	100		—
6-Hydroxymelatonin	2.0	30	0.1	43
N-acetyltryptamine	5.0[c]	>1000[d]	N.E.[d,	—
N-acetyl-5-methoxy-kynurenamine	10.0	—	—	860
N-acetylserotonin	54.0	300	50,000[c]	860
5-Methoxytryptamine	63.0	200	700	86
5-Methoxytryptophol	4000	>10,000	—	430
Serotonin	>1000	>10,000	N.E.	860
Luzindole	>10,000[d]	—	—	—
ANTAGONISTS	K_B (nM)	K_B (nM)		
5-Methoxyluzindole	0.063	—	—	—
N-acetyltryptamine	9.0	33	+[f]	—
Luzindole	20.0	—	—	—
N-acetylserotonin	N.E.[e]	—	+	N.E.

[a] Indicates data from ref. 21. All other data in this column were calculated from ref. 20.
[b] Minimal effective concentrations were determined in this study.
[c] Partial agonist.
[d] Antagonist.
[e] Not effective.
[f] Antagonized the effect of melatonin but K_B not determined.

substituted on the 3rd carbon atom of the indole nucleus. More recently, melanophores isolated from *Xenopus laevis* have been used to quantitate responses to melatonin and related indole agonists (20, 21; see Table 1). In the cultured melanophores, these compounds exhibit very high potency, however the rank order of potencies is in general agreement with what was found in more intact preparations (19).

A study of melatonin's effects on the melanocytes of hair follicles from Syrian hamsters (24) provides an interesting mammalian correlate to the frog skin studies. Melanogenesis in hair follicles in short-term culture was found to

be inhibited by melatonin (10^{-10}–10^{-6} M) in a concentration-dependent manner. The pharmacological characteristics of the melatonin receptors mediating the melanocyte response of the hamster hair follicle (24) appear similar to those found for melatonin receptors in frog skin melanocytes (19). Through its actions on the hair follicle, melatonin may mediate the striking seasonal changes observed in the fur color of Syrian hamsters, i.e. dark brown in the summer and almost pure white in the winter (25).

The amphibian skin receptors for melatonin have not been characterized much beyond the early studies, and for many years it was not clear how these receptors might relate to putative central mammalian receptors for melatonin. It now appears that the melanophore melatonin receptors are pharmacologically similar to the high-affinity melatonin receptors described recently for mammals as well as birds and reptiles (see Tables 1 and 3). This bioassay thus appears to be a useful test system for melatonin receptors.

Inhibition of Retinal Dopamine Release

In 1983 Dubocovich reported that melatonin at picomolar concentrations inhibits electrically evoked release of dopamine from the rabbit retina in vitro (26). This discovery led to the first in-depth quantitative analysis of functional melatonin receptors in neural tissue of mammalian as well as avian origin (11, 27–31; Table 1). In both the rabbit and chicken retina, melatonin selectively inhibits the calcium-dependent release of [^{3}H]dopamine, elicited by either electrical stimulation or potassium-induced depolarization, through activation of a site possessing the pharmacological and functional characteristics of a specific melatonin receptor (11, 26–31). Melatonin's effects are concentration-dependent, and an IC_{50} value of 40 pM was reported for the rabbit retina (28). Serotonin antagonists such as spiperone, methysergide, or methiothepin did not alter the response to melatonin, suggesting that the site activated by melatonin in the retina is pharmacologically distinct from a serotonin receptor (26). The inhibitory effect of melatonin was also unaffected by specific inhibitors of alpha-adrenergic, dopamine, and opiate receptors (26), ruling out a possible effect of melatonin at other presynaptic receptors known to regulate dopamine release in the retina (32). In addition, the site of melatonin's actions appears to be distinct from monoamine uptake sites (26).

The potency of melatonin to inhibit [^{3}H]-dopamine release from slices of rabbit retina depends on the external concentration of calcium in the superfusion medium (33). In general, it appears that conditions that decrease the total amount of transmitter released, i.e. by reducing either the external calcium concentration (33), the duration of field stimulation (34), or the concentration of potassium that is used as a depolarizing stimulus (34), significantly enhance the ability of melatonin to inhibit the release process. These observa-

Table 2 2-[^{125}I]Iodomelatonin binding sites of picomolar affinity

Tissue	K_d (pM) Assay Temp. (°C) 0	22–28	37	GTPγS[a] or NaCl[b]	Reference (M/A)[c]
Retina					
Rabbit	353	172[d]	—	—	11; M
Chicken	434	91	38	—	30, 83; M
Pars Tuberalis[e]					
Rat	-	21	—	—	61; M
	- -	43	—	—	62; A
Sheep	--	--	32		70; M[f]
Hamster	-	59	—	—	71; M
	- -	45	—	—	63; M
	-	28	—	318[a]	48; A
SCN					
Rat	—	53	—	843[a]	72; A
		761[g]		364[b]	
Area Postrema					
Rat	-	46	—	200[a]	74; A
				545[b]	
Optic Tectum					
Chicken	—	35	—	—	77; A
Whole Brain					
Chicken	344	99	37	—	82; M
	-	47	—	—	79; M
Lizard	- -	18	—	--	80; M
		220[h]			

[a,b] Binding in the presence of either 10–100 μM GTPγS[a] or 50–150 mM NaCl[b].

[c] M: Membrane binding assay terminated by filtration (or centrifugation[f]) A: Receptor autoradiography using tissue slices.

[d] M. L. Dubocovich, unpublished observations.

[e] Identified as either the median eminence or medial basal hypothalamus in some studies.

[g] Two brief (1 min) washes were used to separate free from bound ligand in contrast to the 5–30 min wash step used in other autoradiographic studies.

[h] Two sites detected by nonlinear regression analysis.

tions suggest melatonin may act to decrease the availability of calcium for the secretory process, perhaps by affecting the entry of calcium ions into the presynaptic nerve terminal (32–34).

Recently, the inhibitory effect of picomolar concentrations of melatonin on [^{3}H]dopamine release from the rabbit retina was confirmed (35) and a similar effect reported for amphibian retina (36). In the latter study, melatonin suppressed the light-evoked release of endogenous DA from *Xenopus* eye cups in vitro with an IC_{50} of 5 nM, a similar potency to that reported for melatonin in the chicken retina (30; Table 1).

Table 3 Comparison of affinity constants of melatonin analogs for 2-[^{125}I]iodomelatonin binding sites and potencies at presynaptic melatonin heteroreceptors in rabbit retina

Compound	Inhibition of DA release[a] -log IC_{50}	2-[^{125}I]Iodomelatonin Binding (-log K_i)					
		Ovine pars tuberalis (70)	Rat[b] SCN (72)	Lizard brain (80)	Chick brain (79)	Chicken brain[c] (68)	Chicken retina[c] (30)
2-Iodomelatonin	11.3	11.0		10.5	9.7	8.0	8.6
6-Chloromelatonin	10.4			9.5	8.1	8.0	8.3
Melatonin	10.4	9.9	10.3	9.6	8.4	7.7	8.2
6-Hydroxymelatonin	8.7		9.4	9.0	7.0	6.8	7.1
N-Acetylserotonin	7.3	6.7	7.6	6.2	6.1	6.4	5.5
5-Methoxytryptamine	7.2	4.5	5.7			5.7	5.3
5-Methoxytryptophol	5.4	5.9				5.0	4.3
Serotonin	<6	5.5	<4	<4	<4	5.6	<4

[a] Data from refs. 28, 29; DA: dopamine.
[b] -Log IC_{50} values reported in this study.
[c] Binding assayed at 0°C. All other binding and release studies shown here were run at 22–37°C.

Presynaptic melatonin receptors show regional specificity in that dopamine release by slices of rabbit striatum or olfactory tubercle was unaffected by 0.1 μM melatonin (28). Micromolar concentrations of melatonin have been reported to inhibit dopamine release in hypothalamic slices and other regions of rat brain in vitro (37–40). However, this effect does not correlate with the mammalian retinal melatonin receptor in terms of pharmacological profile or potency for melatonin (37, 39).

AGONISTS The most extensive quantitative structure-activity analysis of a functional melatonin receptor has been conducted for the presynaptic heteroreceptors mediating the inhibition of dopamine release in the retina (26–31; Table 1). The rank order of potencies for a series of melatonin analogs tested as agonists in the chicken retina correlate well with that determined for the rabbit retina, although the absolute potencies measured under these conditions were about tenfold greater in the mammalian tissue (11, 30).

The agonists that were most effective in mimicking the inhibitory effect of melatonin on dopamine release were compounds possessing a 5-methoxy group on carbon 5 of the indole nucleus and an N-acetyl group in the same position as melatonin (28; see Table 1). This confirms what was reported earlier for melatonin receptors in amphibian melanophores (19, 20). Thus, the most potent agonists tested in both the rabbit and chicken retina (28–30) were 2-iodomelatonin and 2-methyl-6,7-dichloromelatonin, a compound designed to be more stable metabolically than melatonin in vivo (41). 6-Chloromelatonin was equipotent with melatonin in inhibiting dopamine release. In contrast, inhibitory potencies were about 1000-fold less for N-acetylserotonin, the synthetic precursor for melatonin that lacks the 5-methoxy group, and 5-methoxytryptamine, a 5-methoxyindole that lacks the N-acetyl group (26, 28).

Melatonin analogs with non-halogen substitutions on position 6, i.e. 6-hydroxymelatonin and 6-methoxymelatonin, are about 50 times less potent than melatonin to activate the melatonin receptors of the rabbit retina (28). The former compound is the primary liver metabolite of melatonin (42) and has utility as a urine marker for melatonin secretion in humans (43). Another melatonin metabolite, N-acetyl-5-methoxykynurenamine, which is formed in the brain (44), also exhibits some activity in the rabbit retina; however, it is several orders of magnitude less potent than melatonin (11, 28). Only very weak agonist responses were seen in the retinal release assay (28) with two other putative pineal products with biologic activity, i.e. 5-methoxytryptophol, which is also formed from serotonin (2, 9), and 6-methoxyharmalan, which is a related β-carboline compound in which a third ring is incorporated into the melatonin structure (2). The above structure-activity data for the retinal presynaptic melatonin receptor have been used recently to generate a model of the melatonin binding site of the receptor (44a).

ANTAGONISTS Further evidence for the presence of specific melatonin receptors in the retina comes from studies showing blockade by putative melatonin receptor antagonists N-acetyltryptamine (chicken; 27) and luzindole (rabbit; 29). In previous structure-activity studies in melanocytes, it was shown that N-acetyltryptamines lacking the 5-methoxy group are potential melatonin receptor antagonists (19, 24; Table 1). N-acetyltryptamine, an indole with no substitution on the indole nucleus (Figure 1), antagonized the melatonin-induced lightening of frog skin (19). In chicken retina, this compound also competitively antagonized the melatonin-induced inhibition of [^{3}H]dopamine release, but had no direct effect on release when added alone (27). N-acetylserotonin, which has a hydroxy group on carbon 5 (Figure 1), also appears to antagonize the effect of melatonin in some tissues, i.e. the hamster hair follicle (24) and amphibian skin melanophores (19). However, this compound behaves as a relatively weak agonist in both chicken and rabbit retina (28, 30) and in isolated *Xenopus* melanophores in culture (20). N-acetylserotonin thus appears to act as a partial agonist that shows different intrinsic activities in different tissues. Taken together, the above studies confirm the suggestion that the 5-methoxy group on the indole ring of the melatonin molecule is important for agonist activation of melatonin receptors (19, 27, 28).

In the rabbit retina, N-acetyltryptamine acts as a partial agonist in that it directly inhibited the calcium-dependent release of dopamine (IC_{50} = 5 nM) but also antagonized the effects of melatonin in a competitive manner (10–100 nM; 28). Adding a 2-benzyl substitution to N-acetyltryptamine (luzindole, N-0774; see Figure 1) created a compound that antagonized melatonin's effects in the rabbit retina but by itself had no direct effect on [^{3}H]dopamine release (29; Table 1). Schild plot analysis of luzindole's effects gave a straight line with a slope of 0.91 indicating a competitive interaction between melatonin and luzindole at the melatonin receptor site with a pA2 value of 7.7 for luzindole (29). The corresponding dissociation constant of 20 nM was not statistically different from that determined when luzindole was tested against 6-chloromelatonin (40 nM) and 2-methyl-6,7-dichloromelatonin (16 nM), suggesting that these agonists all activate the same presynaptic site to mediate inhibition of dopamine release in the rabbit retina (29). The action of luzindole appears specific; it does not affect the inhibition of dopamine release elicited through the activation of either D-2 dopamine autoreceptors by apomorphine or alpha-2 adrenoceptors by clonidine (29). Luzindole also shows little or no effect on the binding of specific radioligands to monoamine, muscarinic, adenosine, or benzodiazepine receptors (11; D. N. Krause, unpublished observations). Luzindole is thus the first competitive and selective antagonist to be characterized for a mammalian melatonin receptor. Recently, luzindole has been reported to antagonize the effects of melatonin on dopamine release from *Xenopus* eye cups in vitro (36).

The addition of a 5-methoxy group to luzindole increases both the potency and the intrinsic activity of the compound at the melatonin receptor of the rabbit retina (31). 5-methoxyluzindole competitively antagonizes the ability of melatonin to inhibit [^{3}H]dopamine release with a K_B of 63 pM as calculated from the Schild plot (slope=0.93). However, this compound by itself also inhibits dopamine release (IC_{50}=1.4 nM) and therefore, it appears to act as a partial agonist at the retinal melatonin receptor (31).

Several other compounds have been suggested as putative melatonin antagonists because of their effects in other in vitro and in vivo studies, e.g. 6-methoxy-2-benzoxazoline (45), N-(3,5-dinitrophenyl)-5-methoxytryptamine (ML-23; 39) and 5-methoxy-indole-N-methyl-3-propionamide, the reversed amide of melatonin (46). However, none of these compounds was able to antagonize melatonin's inhibition of dopamine release in the rabbit retina (29).

Second Messenger Responses

An important signal tranduction mechanism for at least some melatonin receptors appears to involve the inhibition of cyclic AMP formation. The involvement of cyclic AMP in a biologic effect of melatonin was first suggested about twenty years ago in a study of amphibian skin (47). Melatonin (4.3 pM–4.3 nM) inhibited not only skin darkening in response to αMSH (melanophore stimulating hormone) but also inhibited the concomitant rise in cyclic AMP. In 1989, three laboratories reported that melatonin was also a potent inhibitor of cyclic AMP production by the pars tuberalis region of the pituitary in mammals, i.e. Djungarian hamster (48), sheep (49), and neonatal rat (50). A similar effect has recently been reported for this same region in the white-footed mouse (51).

In primary cultures of ovine pars tuberalis cells (49) and explants of the pars tuberalis/median eminence region from hamster (48) and neonatal rat (50), melatonin caused a dose-dependent inhibition of the cyclic AMP production stimulated by forskolin (1–10 μM). The IC_{50} values for melatonin in the three studies were 6, about 10, and 250–500 pM respectively. The cyclic AMP response of the pars tuberalis culture appears very specific to melatonin in that almost complete inhibition of the forskolin stimulation was observed (49). This is in contrast to the explant preparations where the maximal effect of melatonin was about a 50% reduction in cyclic AMP levels. This probably reflects a greater heterogeneity of the latter preparation with cells that produce cyclic AMP but are not responsive to melatonin. In all of the tissues, melatonin had no effect on basal levels of cyclic AMP. Melatonin did not directly affect phosphodiesterase, suggesting that decreases in cyclic AMP may be due to inhibition of adenylyl cyclase (48). No effect of melatonin was

detected, however, on cyclic AMP production in broken cell homogenates of pars tuberalis tissue, a preparation in which forskolin also was less effective (49). Melatonin has recently been reported to decrease the activity and levels of calmodulin in MDCK and N1E-115 cells (51a). These results, taken together with the lack of a direct effect of melatonin on cyclase activity in cell homogenates, suggest the possibility that the effect of melatonin on cyclic AMP levels may be indirect.

The anterior pituitary of the rat also appears to be sensitive to melatonin during the neonatal period (50). Using explants of the more distal portion of the anterior pituitary (hemipituitaries) from 10-day old rats, it was shown that melatonin decreased both the basal levels of cyclic AMP as well as the increased levels of cyclic AMP stimulated by either luteinizing hormone-releasing hormone (LH-RH; 10 nM) or forskolin (50). The studies were performed in the presence of 1mM isobutylmethylxanthine to inhibit phosphodiesterase. In this tissue, melatonin also inhibited cyclic GMP production stimulated by LH-RH, and similar potencies (IC_{50} about 250 pM) were seen for melatonin's effects on the two cyclic nucleotides (50). Recently melatonin was reported to modulate diacylglycerol metabolism in the neonatal rat pituitary (52); this finding raises questions as to which is the primary transduction mechanism for melatonin receptors in this tissue. It was suggested that, in neonatal rats at least, melatonin mediates a decrease in the release of luteinizing hormone that leads to reproductive regression (50). This effect may only be relevant to the neonatal rat because melatonin receptors are not found in this region in older animals (53). Melatonin had no effect on forskolin-stimulated cyclic AMP accumulation in hamster pituitary preparations nor did it affect cyclic AMP in the hamster pineal (48).

The structure-activity analysis of melatonin's inhibitory effect on cyclic AMP has not been extensive; however, the initial pharmacological profile in hamster (48) is consistent with those of the retinal and melanophore melatonin receptors. Melatonin and 6-chloromelatonin were equipotent agonists (IC_{50} about 10^{-11}M) whereas serotonin had no effect at concentrations up to 10^{-6}M (48). The effects of melatonin were blocked by pretreatment of the tissue with pertussis toxin (1μg/ml, 6 hr) suggesting involvement of a G protein in melatonin receptor transduction (48). In the neonatal rat hemipituitary, N-acetylserotonin and 5-methoxytrypamine were about 1000-fold less potent than melatonin in inhibiting cyclic AMP (50). Similar potencies were reported earlier for these agents in inhibiting the release of luteinizing hormone stimulated by LH-RH (54). The potencies of the above compounds in the functional cyclic AMP assay correlate well with their binding potencies in these same tissues (see below). This bioassay appears to be a useful test system in which to develop additional structure-activity data on G protein-coupled melatonin receptors affecting second messenger systems.

PICOMOLAR AFFINITY 2-IODOMELATONIN BINDING SITES

The initial attempts, over 10 years ago, to identify receptors using radioligand binding techniques involved the use of [^{3}H]melatonin as ligand (55–57). Although specific binding to membrane and cytosolic preparations from a variety of organs was described (see 10, 13), a reproducible and detailed characterization of melatonin binding sites was hampered by the low specific activity and instability of the [^{3}H]melatonin available (10). In addition, there was no direct correlation of the melatonin binding sites with any known functional responses.

The recent development of 2-[^{125}I]iodomelatonin as a selective, high-affinity ligand of high specific activity has provided a long-awaited tool for probing melatonin receptors and has stimulated a flurry of studies in the past several years. The radioiodinated compound was initially synthesized for use as a tracer in melatonin radioimmunoassays by Vakkuri and coworkers in 1984 (58). ^{1}H NMR analysis showed that iodine was incorporated at the C-2 position of the indole moiety. The biological activity of 2-iodomelatonin was demonstrated three years later when the compound was shown to mimic melatonin as a potent inhibitor of evoked dopamine release in the chicken retina (IC_{50} = 100 pM; 30). 2-Iodomelatonin is also a very effective melatonin agonist in the rabbit retina (IC_{50} = 5 pM; 29) and in *Xenopus* melanophores in culture (EC_{50} = 3.4 pM; 21). When administered in vivo, 2-iodomelatonin mimicks the actions of melatonin in Djungarian hamsters by affecting the reproductive development of fetuses (59) and inhibiting testes growth and reducing body weight gain in adult animals (60). 2-Iodomelatonin has low affinity for serotonin receptor binding sites (D. N. Krause & M. L. Dubocovich, unpublished observations), and the selectivity of this compound is further confirmed by the very discrete localization of 2-[^{125}I]iodomelatonin binding sites seen in mammalian brain with autoradiographic techniques (e.g. 61–67).

2-[^{125}I]Iodomelatonin was first shown in 1987 to be a useful radioligand for membrane homogenate (chicken retina; 30) as well as autoradiographic (rat hypothalamus; 61) binding studies of melatonin receptors. In both studies, the picomolar affinity of the binding sites correlated with the known functional data from the rabbit retina (26, 28). Biological and binding activities should ideally be measured in the same tissue under in vitro conditions where molar concentrations can be precisely specified before a receptor is conclusively defined. The chicken retina provided such a tissue for extensive pharmacological analysis. Examination of over 15 melatonin analogs indicated that the rank order of potency in inhibiting 2-[^{125}I]iodomelatonin binding was well correlated with that for inhibiting retinal dopamine release (11, 30).

Picomolar affinity binding sites for 2-[^{125}I]iodomelatonin have now been characterized pharmacologically in a variety of mammalian, avian, and reptilian tissues, including the retina of chicken (11, 30, 68, 69) and rabbit (11, 69); the pars tuberalis/median eminence region of sheep (70), rat (61, 62), and hamster (48, 63, 71); the hypothalamic suprachiasmatic nuclei (SCN) of rat (72) and human (73) brain; the area postrema of rat brain (74); the thalamic paraventricular nucleus of mouse brain (75); cerebral and caudal arteries of the rat (76); the optic tectum (77, 78), nucleus rotundus, neostriatum, and visual suprachiasmatic nuclei of chicken brain (77), as well as whole brain homogenates from chicken (68, 79) and lizard (80). As discussed below, the overall characteristics of these various sites are generally in good agreement with each other (Tables 2 and 3) and with the pharmacological characteristics described above for functional melatonin receptors in the retina, pars tuberalis, and skin (28, 31, 48, 19). The high affinities of these 2-[^{125}I]iodomelatonin binding sites also appear physiologically relevant to the range of melatonin levels that are normally found in the blood and/or cerebrospinal fluid of the various species studied (see 2, 70, 79).

Affinity and Density of 2-Iodomelatonin Binding Sites

Specific binding sites for 2-[^{125}I]iodomelatonin have been characterized by saturation analysis using either membrane homogenate or autoradiographic radioligand binding techniques (see Table 2). The binding is reversible and saturable, with affinities for 2-[^{125}I]iodomelatonin in the picomolar range (see Table 2). Kinetic analysis of association/dissociation data give K_d values similar to that obtained from saturation studies (e.g. 30, 68, 70, 74, 77). Generally, the data have been consistent with a single class of sites; however, recent studies (48, 72, 74, 80–82) suggest that melatonin receptors exist in two affinity states. The low Hill coefficient (79) and multiple off rates (62, 68) observed in several other binding studies also suggest the presence of multiple sites. A review of picomolar K_d values reported to date indicates they are all consistent with one of two affinity states: a high affinity form exhibiting an average K_d of 45 pM and a second state with about tenfold lower affinity (average K_d of 400 pM) (Table 2, see p. 554).

The affinity state(s) and K_d values measured for 2-[^{125}I]iodomelatonin binding sites appear to be affected by the assay conditions employed, e.g. temperature, washing procedures, and the presence of specific cations and/or guanine nucleotides (see Table 2). In homogenate binding assays of retina (11, 30, 83; M. L. Dubocovich, unpublished observations) and chicken brain (68, 82), an increase in the incubation temperature from 0°C to 25°C and 37°C caused a progressive increase in affinity (e.g. K_d values from 434 pM to 38 pM; 30, 83; see Table 2). Detection of the lower affinity state thus appears more likely when using lower assay temperatures (0°C) or with very quick

washing procedures (72; Table 2). In most autoradiographic studies to date, only the high-affinity site has been observed because the lower affinity binding of 2-[^{125}I]iodomelatonin appears to have been washed off under the conditions used (62, 72). Sodium and lithium ions also were found to decrease specific binding of 2-[^{125}I]iodomelatonin, apparently by altering the affinity for the ligand (72), in both chicken retina (30) and rat brain (72, 74; Table 2). Calcium promotes high affinity binding in the SCN (72) but not in the area postrema of the rat brain (74). The physiological implications of these ion effects, however, are not yet clear.

G Protein Linkage

Micromolar concentrations of guanine nucleotides dose-dependently and specifically inhibit 2-[^{125}I]iodomelatonin binding in a number of tissues including lizard (80) and chicken brain homogenates (82; M. L. Dubocovich, unpublished observations), mammalian pars tuberalis (48, 84), and the SCN (72) and area postrema of the rat (74). The nonhydrolyzable GTP analog guanosine 5'-[γ-thio]triphosphate (GTPγS) was found to be the most potent analog tested (48, 72, 84). GTP itself inhibited binding at higher concentrations and was equipotent with GDP, while ATP and its analog ATPγS had no effect (48, 72, 74, 84). GTPγS was also shown to either shift the affinity of the 2-[^{125}I]iodomelatonin binding sites by tenfold (48, 72, 74; see Table 2) or decrease the number of high-affinity sites with no change in K_d (80, 84). Any shift to the lower affinity state may have been undetectable under the conditions of the latter studies.

The above observations suggest the high-affinity melatonin receptor belongs to the superfamily of G protein-coupled receptors (85). In a study of 2-[^{125}I]iodomelatonin binding complexes solubilized from lizard brain, treatment with GTPγS eliminated the complex of higher molecular size consistent with an uncoupling of a G protein from the receptor (80). A variety of G proteins regulate a number of intracellular events, e.g. cyclic nucleotides, calcium, phosphoinositides, prostaglandins and membrane potential (85), but the nature of the G proteins coupled to melatonin receptors is not yet known. The order of potency for the guanine nucleotides described above (48, 72, 74, 84) is consistent, however, with the involvement of a Gi-like rather than Gs protein. In explants of hamster pars tuberalis, specific 2-[^{125}I]iodomelatonin binding was inhibited by pertussis toxin, a treatment that appeared to affect both Gi and Go proteins in this tissue (48). Other receptor types that inhibit calcium channels through the involvement of Go and/or Gi can also inhibit adenylyl cyclase and hyperpolarize cells by stimulating potassium channels via Gi (85). Such receptors may be a model for melatonin receptors that lower cyclic AMP levels in the pars tuberalis (48, 49), inhibit retinal transmitter release possibly by lowering calcium (28, 31, 33), and may inhibit electrical

activity of cells in the SCN (86). The latter in vitro effect, however, was obtained with micromolar concentrations of melatonin but could be blocked by N-acetyltryptamine (86).

Structure-Activity Relationships

HIGH-AFFINITY (ML-1) BINDING SITES A number of the specific sites shown to bind 2-[^{125}I]iodomelatonin with picomolar affinity (Table 2) have been analyzed with competition experiments using a series of compounds structurally related to melatonin. In general, the rank order of binding potencies for these compounds correlates well with that obtained for the presynaptic melatonin receptor of the rabbit retina, a model for a physiologically relevant receptor (Table 3, see p. 555). When comparing the ability to inhibit 2-[^{125}I]iodomelatonin binding to a variety of tissues (30, 68, 70, 72, 79, 80) with effectiveness at inhibiting dopamine release (28, 29, 31), 2-iodomelatonin is the most potent compound, followed by melatonin and 6-chloromelatonin, which are roughly equipotent (see Table 3). 6-Hydroxymelatonin is somewhat less potent than melatonin while the potencies of N-acetylserotonin and 5-methoxytryptamine are three or more orders of magnitude lower. 5-Methoxytryptophol and serotonin show little or no affinity for either receptor binding or the functional response in the rabbit retina. Overall the characteristics of the specific 2-[^{125}I]iodomelatonin binding sites described above are consistent with a G protein-coupled melatonin receptor that can exist in two states of differing picomolar affinity that have specific pharmacological characteristics consistent with functional melatonin receptors in the retina and frog skin (Table 1). It has been suggested that receptors exhibiting these pharmacological properties be called ML-1 receptors (11) to distinguish them from other potential melatonin receptor binding sites that have been described (31, 87–91).

LOWER AFFINITY (ML-2) BINDING SITES The pharmacological profile for specific 2-[^{125}I]iodomelatonin binding sites in hamster brain homogenates (31, 87–90) was found to be different from that described above, even though the assay conditions were identical to those used to detect ML-1 receptors in retina and chicken brain (30, 68, 69). The binding in hamster homogenates exhibits nanomolar affinity for [^{125}I]iodomelatonin, different kinetics, and different structure-activity relationships, most notably a relatively high potency of N-acetylserotonin, 6-hydroxymelatonin, 6-methoxymelatonin and 5-methoxytryptophol (31, 88, 90). Prazosin is potent in inhibiting binding at this site, but the effect of this compound appears unrelated to adrenergic receptors (90). Because the binding sites observed in the hamster brain homogenates are of relatively high affinity and the pharmacology is unique

and selective for melatonin analogs, they appear to be relevant melatonin receptors. It has been proposed that 2-[^{125}I]iodomelatonin binding sites exhibiting the characteristics seen in hamster brain homogenates be referred to as ML-2 sites (11). No functional correlate, however, has yet been established for these sites.

SUMMARY AND FUTURE DIRECTIONS

Specific functional receptors for the hormone melatonin mediate the presynaptic inhibition of dopamine release in the retina, inhibition of cyclic nucleotide production and condensation of pigment granules in melanophores. The receptors all exhibit a similar high potency for melatonin and consistent structure-activity profiles. Using the recently available, high-affinity radioligand 2-[^{125}I]iodomelatonin, discrete, picomolar affinity binding sites have been found in the brain, retina, and pituitary that have the same pharmacological characteristics as the functional responses, thus validating the use of this ligand to label physiologically relevant melatonin receptors. These melatonin receptors, designated ML-1, appear coupled to G proteins and can exist in two affinity states, both in the picomolar range.

With the knowledge and tools necessary to identify and characterize high-affinity melatonin receptors, it is now possible to study the physiological roles of these receptors and how they might be involved in various clinical states (92). Receptor autoradiographic studies are providing insight into the location of melatonin target sites that, in many cases, are in regions consistent with the known physiological and behavioral effects of melatonin; e.g. the hypothalamic SCN, site of the mammalian circadian clock, is likely to mediate melatonin's ability to synchronize biological rhythms (43, 73, 93), while the pars tuberalis region, shown to be particularly sensitive to seasonal changes (94), is suggested to mediate melatonin's involvement in neuroendocrine regulation (95). Now it is also possible to monitor specific melatonin receptor populations, i.e. by measuring their number, affinity and/or effector coupling, under different physiological or pathological conditions. Initial studies have begun to address the interesting questions of how melatonin receptors may be altered during times of dramatic changes in melatonin secretion, i.e. during circadian (53, 81) and seasonal (71, 96) cycles as well as during development (53, 59, 73, 97). It should be possible to determine the primary structure of the ML-1 receptor through purification and cloning techniques. Initial progress in this direction has been made using lizard (80) or chicken brain (98), two tissues relatively rich in melatonin receptors. A better understanding of possible melatonin receptor subtypes and further development of selective melatonin receptor agonists and antagonists will provide the means to elucidate the physiological roles of melatonin and its receptors and

allow the development of a novel class of therapeutic agents that act specifically through melatonin receptors.

ACKNOWLEDGMENTS

The authors thank E. L. Nelson and K. L. Krause for helpful discussion and support. Recent work was supported in part by USPHS grants MH42922 and DK38607 to M.L.D.

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