

International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, Classification, and Pharmacology of G Protein-Coupled Melatonin Receptors

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Abstract—The hormone melatonin (5-methoxy-*N*-acetyltryptamine) is synthesized primarily in the pineal gland and retina, and in several peripheral tissues and organs. In the circulation, the concentration of melatonin follows a circadian rhythm, with high levels at night providing timing cues to target tissues endowed with melatonin receptors. Melatonin receptors receive and translate melatonin's message to influence daily and seasonal rhythms of physiology and behavior. The melatonin message is translated through activation of two G protein-coupled receptors, MT₁ and MT₂, that are potential therapeutic

targets in disorders ranging from insomnia and circadian sleep disorders to depression, cardiovascular diseases, and cancer. This review summarizes the steps taken since melatonin's discovery by Aaron Lerner in 1958 to functionally characterize, clone, and localize receptors in mammalian tissues. The pharmacological and molecular properties of the receptors are described as well as current efforts to discover and develop ligands for treatment of a number of illnesses, including sleep disorders, depression, and cancer.

I. Introduction

Melatonin (5-methoxy-*N*-acetyltryptamine) is an important hormonal output of the circadian system, and it circulates nightly to provide timing cues to any tissue that can read the message. Melatonin receptors, of course, are the entities that receive and translate this message to influence daily and seasonal rhythms of physiology and behavior. Our evolving understanding of melatonin receptors is providing new insights into where and how this hormone exerts its physiological effects in the body as well as how these receptors may be useful therapeutic targets in disorders ranging from insomnia and jet lag to depression, cancer, and cardiovascular disease.

The field of mammalian melatonin receptors got off to a slow start; the first pharmacological characterization of a functional mammalian melatonin receptor (Dubocovich, 1983) and the cloning of the first human melatonin receptor (Reppert et al., 1994) came 25 and 36 years, respectively, after melatonin itself was discovered (Lerner et al., 1959). Now, however, the field is moving quickly, and there are sufficient data upon which to organize and classify the known mammalian melatonin receptors. That topic is the focus of this review.

A. Melatonin Physiology and Function

In mammals, melatonin is secreted primarily by the pineal gland during the dark period of the daily light/dark cycle (for reviews, see Cardinali, 1981; Reiter, 1991; Borjigin et al., 1999; Klein, 1999; Olcese, 1999).

The circadian rhythm of pineal melatonin synthesis and release is driven by circadian pacemaker cells (the “master clock”) located in the suprachiasmatic nucleus (SCN¹) of the hypothalamus that project to the pineal via a multisynaptic pathway (Fig. 1). Melatonin is also synthesized in the retina and a clock mechanism within the retina itself seems to drive the melatonin rhythm in this tissue (Tosini and Menaker, 1998; Tosini et al., 2007). In both cases, the clock rhythm is entrained to a 24-h period by environmental light (the photoperiod) that is sensed by a subset of retinal ganglion cells containing the photopigment melanopsin, which conveys photic stimuli to the SCN via the retinohypothalamic tract (Berson et al., 2002).

The melatonin rhythm is a consequence of the regulation of the hormone's synthetic enzymes, which are highly active at night (see Fig. 2). Melatonin is synthesized from serotonin through two enzymatic steps. First,

¹ Abbreviations: 4P-ADOT, 4-phenyl-2-acetamidotetraline; 4P-PDOT, 4-phenyl-2-propionamidotetraline; 5-HEAT, 5-hydroxyethoxy-*N*-acetyltryptamine; AA-NAT, arylalkylamine *N*-acetyltransferase; AFMK, *N*¹-acetyl-*N*²-formyl-5-methoxykynuramine; AMK, *N*¹-acetyl-5-methoxykynuramine; AMMTC, *N*-acetyl-4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole; BK_{Ca}, calcium-activated potassium channel; CHO, Chinese hamster ovary; CREB, cAMP responsive element binding protein; CT, circadian time; GLP-1, glucagon-like peptide; GPCR, G protein-coupled receptor; H-89, 5-isoquinolinesulfonamide; HIOMT, hydroxyindole-*O*-methyltransferase; KO, knockout; PKC, protein kinase C; PTX, pertussis toxin; RT-PCR, reverse transcription-polymerase chain reaction; SCN, suprachiasmatic nucleus; TM, transmembrane; WT, wild type.

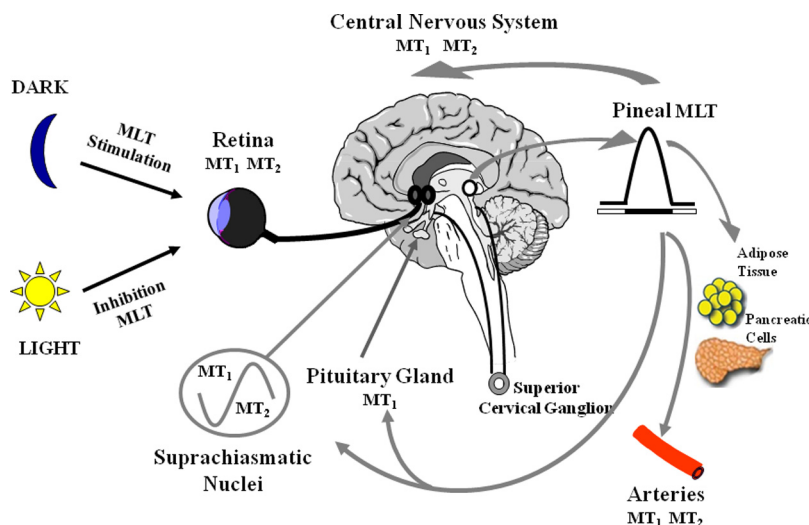


FIG. 1. Regulation of melatonin production and receptor function. Melatonin is synthesized in the pineal gland and in the retina. In the pineal gland, melatonin (MLT) synthesis follows a rhythm driven by the suprachiasmatic nucleus, the master biological clock. Neural signals from the SCN follow a multisynaptic pathway to the superior cervical ganglia. Norepinephrine released from postganglionic fibers activates α_1 - and β_1 -adrenoceptors in the pinealocyte, leading to increases in second messengers (i.e., cAMP and inositol trisphosphate) and the activity of AA-NAT, the rate-limiting step in melatonin synthesis. The system is dramatically inhibited by light, the external cue that allows entrainment to the environmental light/dark cycle. The photic signal received by the retina is transmitted to the SCN via the retinohypothalamic tract, which originates in a subset of retinal ganglion cells. Pineal melatonin thus serves as the internal signal that relays day length, allowing regulation of neuronal activity (MT_1) and circadian rhythms (MT_1 , MT_2) in the SCN (Dubocovich, 2007), of neurochemical function in brain through the MT_1 and MT_2 receptors (Dubocovich, 2006), of vascular tone through activation of MT_1 (constriction) and MT_2 receptors (dilation) in arterial beds (Masana et al., 2002), and seasonal changes in reproductive physiology and behavior through activation of MT_1 receptors in the pars tuberalis (Duncan, 2007). The pars tuberalis of the pituitary gland interprets this rhythmic melatonin signal and generates a precise cycle of expression of circadian genes through activation of MT_1 receptors. Melatonin synthesis in the photoreceptors of the retina follows a similar circadian rhythm generated by local oscillators (Tosini et al., 2007). Activation of MT_1 and MT_2 melatonin receptors regulate retina function and hence transmission of photic information to the brain (Dubocovich et al., 1997). [Adapted from Dubocovich ML and Masana M (2003) Melatonin receptor signaling, in *Encyclopedia of Hormones and Related Cell Regulators* (Henry H and Norman A eds), pp 638–644, Academic Press, San Diego, CA. Copyright © 2003 Academic Press. Used with permission.]

serotonin *N*-acetyltransferase [arylalkylamine *N*-acetyltransferase (AA-NAT)] acetylates serotonin to yield *N*-acetylserotonin. The second step involves transfer of a methyl group from (*S*)-adenosylmethionine to the 5-hydroxy group of *N*-acetylserotonin via the enzyme hydroxyindole-*O*-methyltransferase (HIOMT). The regulation of these enzymes is fascinating and much studied (Wurtman and Axelrod, 1968; Cardinali, 1981; Klein, 1999, 2007; Olcese, 1999). Most recently, molecular approaches are revealing the transcriptional and post-transcriptional mechanisms responsible for daily fluctuations in AA-NAT (Borjigin et al., 1995, 1999; Roseboom et al., 1996; Gastel et al., 1998; Klein, 1999; Fukuhara et al., 2001) and regulation of HIOMT (Gauer and Craft, 1996). Use of sensitive RT-PCR techniques suggests that low levels of AA-NAT and HIOMT, and therefore local melatonin synthesis, may also occur in other tissues, such as gut, testis, spinal cord, raphe nucleus, and striatum (Stefulj et al., 2001). In mammals, most of the nocturnal rhythm in circulating melatonin is abolished by pinealectomy (Simonneaux and Ribelayga, 2003). However, sporadic reports suggested that as much as 20% of circulating melatonin is derived from tissues other than the pineal gland (Ozaki and Lynch, 1976).

The lipophilicity of the melatonin molecule allows it to diffuse passively across cell membranes as well as cell layers, and thus it can diffuse from the pinealocytes as soon as it is synthesized. Early studies by

Cardinali et al. (1972) showed melatonin binding to human plasma albumin. This observation was later confirmed by Pardridge and Mietus (1980). These authors also reported that albumin-bound melatonin crosses the blood-brain barrier. Melatonin binding to plasma albumin was confirmed by Morin et al. (1997) and provided evidence of high-affinity melatonin binding to α_1 -acid glycoprotein (Cardinali et al., 1972; Morin et al., 1997). An excellent correlation was demonstrated between saliva melatonin in humans and levels of unbound serum melatonin, suggesting for the first time that melatonin binding to plasma proteins may affect the levels of free melatonin and hence physiological responses (Kennaway and Voultsios, 1998). Because the levels of albumin and α_1 -acid glycoprotein may vary with age and disease, particularly during inflammatory processes, the level of free melatonin and drugs in human plasma cannot be predicted and has to be considered on a case-by-case basis (Viani et al., 1992; Morin et al., 1997; Waldhauser et al., 1988).

Melatonin rapidly disappears from the blood, with a half-life of approximately 30 min, depending on the species examined (Cardinali et al., 1972; Waldhauser et al., 1984). In humans, most melatonin in the general circulation is converted to 6-hydroxymelatonin by the liver, which clears 92 to 97% of circulating melatonin in a single pass (Tetsuo et al., 1980; Young et al., 1985). Then 6-hydroxymelatonin

MELATONIN SYNTHESIS

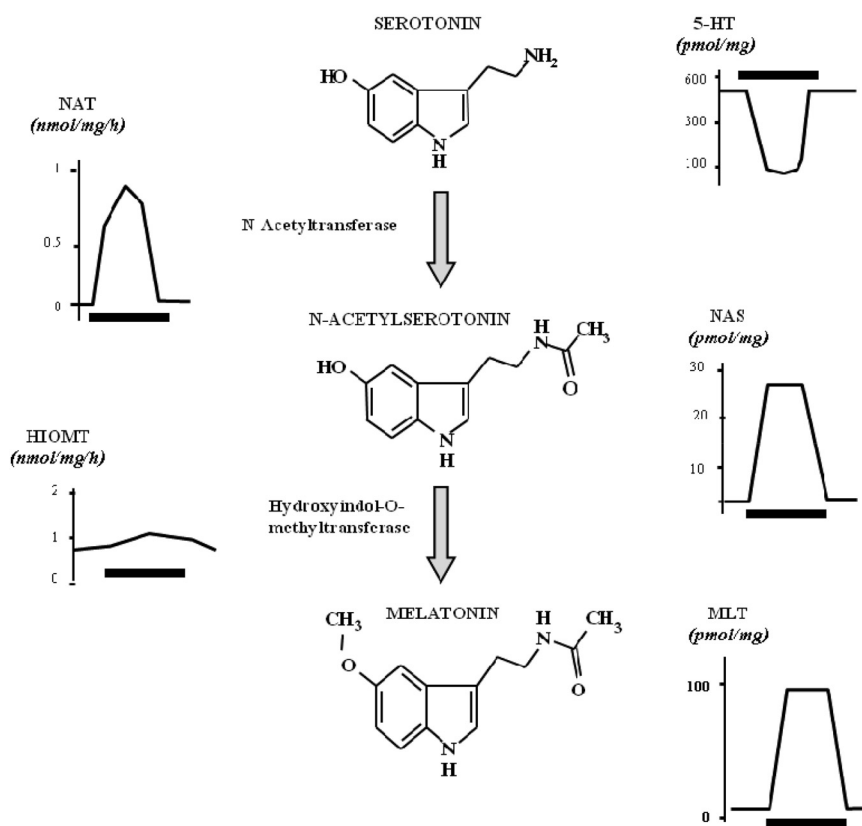


FIG. 2. Melatonin synthesis. Melatonin (MLT) is synthesized from serotonin through two enzymatic steps. First, serotonin is acetylated by NAT to yield *N*-acetylserotonin (NAS). The second step involves transfer of a methyl group from (*S*)-adenosylmethionine to the 5-hydroxyl group of *N*-acetylserotonin via the enzyme HIOMT. The rhythms of melatonin and serotonin have opposite phase during subjective night and day (Klein, 1999).

is conjugated and excreted into the urine, approximately 50 to 80% as the sulfate derivative and 5 to 30% as the glucuronide (Ma et al., 2008). It is important to point out that the most abundant metabolite in mouse urine is 6-hydroxymelatonin excreted as the glucuronide conjugate (75–88%) (Kennaway et al., 2002; Ma et al., 2008). Melatonin metabolism in the brain, however, may involve oxidative pyrrole-ring cleavage. No 6-hydroxymelatonin is detected after melatonin injection into the cisterna magna (Hirata et al., 1974). This pathway may be particularly important because melatonin is also released via the pineal recess into the cerebrospinal fluid as well as into the circulation (Tricoire et al., 2002). The primary cleavage product is *N*¹-acetyl-*N*²-formyl-5-methoxykynuramine (AFMK), which is deformylated, either by arylamine formamidase or hemoperoxidases, to *N*¹-acetyl-5-methoxykynuramine (AMK). Surprisingly, numerous enzymatic (indoleamine 2,3-dioxygenase, myeloperoxidase), pseudoenzymatic (oxoferryl hemoglobin, hemin), photocatalytic, or free-radical reactions lead to the same product: AFMK (Hardeland 2005). Some estimates indicate that pyrrole ring cleavage contributes to approximately one third of the total catabolism of melatonin (Ferry et al., 2005), but the percentage may be even higher in certain tissues. Other oxidative

catabolites are cyclic 3-hydroxymelatonin, which can also be metabolized to AFMK, and a 2-hydroxylated analog that does not cyclize but turns into an indolinone (Hardeland 2005). Additional hydroxylated or nitrosated metabolites have been detected, but they seem to be present in minor quantities only. AFMK and AMK also form metabolites by interactions with reactive oxygen and nitrogen species (Tan et al., 2007). Antioxidative protection, safeguarding of mitochondrial electron flux, and, in particular, neuroprotection have been demonstrated in many experimental systems to be mediated by melatonin and its endogenous metabolites. This effect is not mediated by the known G protein-coupled melatonin receptors and thus will not be reviewed in this article.

The melatonin metabolites produced in the liver (e.g., 6-hydroxymelatonin) and in the brain (e.g., AFMK and AMK) are known to modulate a variety of functional responses possible through activation of the G-protein-coupled melatonin receptor. 6-Hydroxymelatonin competed for 2-[¹²⁵I]iodomelatonin binding to both the MT₁ and MT₂ melatonin receptors (Dubocovich et al., 1997). This metabolite also decreases in a concentration dependent manner the calcium dependent release of [³H]dopamine from rabbit retina (Dubocovich, 1985). AFMK and

AMK decrease sexual development in a prepubertal rat model (Kennaway et al., 1988). It is noteworthy that AFMK accelerates re-entrainment of the 6-hydroxymelatonin rhythm, when given at the new dark onset, after a phase advance of the light/dark cycle in male rats (Kennaway et al., 1989).

The melatonin rhythm is an important efferent hormonal signal driven by the endogenous clock, which can therefore be used as an internal synchronizer (or “internal Zeitgeber”) (Dawson and Armstrong, 1996). Melatonin can impose circadian rhythmicity upon target structures, and it also is known to act directly on the SCN to modulate the clock itself. Exogenous melatonin, given at the same time of day, can entrain physiological and behavioral rhythms (e.g., body temperature and rest-activity cycles) (Arendt and Skene, 2005). Melatonin has been reported to modulate a myriad of other functions, including visual, neuroendocrine, reproductive, neuroimmune, and vascular physiology (Arendt, 2000; Cagnacci et al., 2000; Castrillón et al., 2000; Monti and Cardinali, 2000; Maestroni, 2001; Sharkey et al., 2009). Because the duration of nocturnal melatonin secretion is directly proportional to the length of the night, this hormone also provides a signal for seasonal change. Melatonin is, indeed, the critical parameter for photoperiod integration and the induction of particular physiological responses, such as those observed in seasonal breeders (Karsch et al., 1988; Pitrosky et al., 1991; Cardinali and Pévet, 1998; Malpoux et al., 1999).

In addition to the circadian rhythm of the endogenous ligand, target tissues and physiological responses can also display daily variations in receptivity to melatonin. For example, optimal entrainment of the activity rhythm in rodents housed in constant darkness occurs when melatonin is administered at the onset of activity; this induces a phase advance in the animal's rhythm (Cardinali et al., 1997; Redman, 1997). It is possible that melatonin transmits photoperiodic information by regulating its own receptors, perhaps by altering receptor density, transduction mechanisms, and/or trafficking (Tenn and Niles, 1993; Gerdin et al., 2004a,b). Further discussion in this area is beyond this review, because it focuses primarily on the action of exogenous melatonin and drugs with therapeutic potential mediating actions through activation of MT₁ and/or MT₂ melatonin receptors.

B. Melatonin Receptor Discovery: Historical Background

The study of melatonin receptors can be traced back to 1917, when McCord and Allen demonstrated lightening of *Rana pipiens* tadpole skin by bovine pineal extracts. This bioassay was used to isolate melatonin from pineal extracts, which led to the elucidation of its chemical structure (Lerner et al., 1959). The action of melatonin to aggregate pigment granules (melanosomes) of amphibian dermal melanophores was used to 1) postulate the presence of melatonin receptors (Heward and Hadley, 1975); 2) establish the first structure-activity rela-

tionships of melatonin analogs (Heward and Hadley, 1975); and 3) demonstrate that melatonin receptors are coupled to a pertussis toxin-sensitive G-protein (White et al., 1987). It is noteworthy that the first melatonin receptor (Mel_{1c}) to be cloned was found using an expression cloning strategy of mRNA from *Xenopus laevis* melanophores (Ebisawa et al., 1994); this particular receptor has no known mammalian ortholog, but its discovery led to the cloning of several melatonin receptors from mammals, including two human receptors (Reppert et al., 1994, 1995a).

The first attempts to identify brain melatonin receptors employed [³H]melatonin as a radioligand to label binding sites in membranes from bovine hypothalamus, cerebral cortex, and cerebellum (Cardinali et al., 1979). This was followed by the discovery of the first functional melatonin receptor in a neuronal mammalian tissue, the rabbit retina (Dubocovich, 1983, 1985, 1988a). Melatonin, acting via a presynaptic heteroreceptor, inhibits retinal dopamine release, and this bioassay was used to establish the relative order of potency for a series of agonists and putative antagonists as well as to discover the first competitive melatonin antagonist, luzindole (Dubocovich, 1988a,c). Vakkuri et al. (1984) introduced the radioligand 2-[¹²⁵I]iodomelatonin as a tracer for use in melatonin radioimmunoassays. This molecule turned out to be the silver bullet of melatonin receptor research in that its selectivity and high specific activity allowed the field to move forward. Soon afterward, several laboratories simultaneously established the use of 2-[¹²⁵I]iodomelatonin as a radioligand for receptor localization (Vanecek et al., 1987) and receptor characterization in native tissues (Laudon and Zisapel, 1986; Dubocovich and Takahashi, 1987) (for reviews, see Krause and Dubocovich, 1990; Krause and Dubocovich, 1991; Morgan et al., 1994b; Sugden, 1994; and Dubocovich, 1995).

Melatonin receptors were first classified according to classic pharmacological criteria using data obtained from in vitro bioassays and radioligand binding to native tissues (Cardinali, 1981; Dubocovich, 1988a, 1995; Krause and Dubocovich, 1990; Morgan et al., 1994b). The first classification scheme distinguished two putative receptors, termed ML₁ and ML₂ receptors, on the basis of kinetic and pharmacological differences observed in 2-[¹²⁵I]iodomelatonin binding sites (Cardinali, 1981; Dubocovich, 1988a, 1995; Krause and Dubocovich, 1990; Morgan et al., 1994b). The ML₁ pharmacological profile (2-iodomelatonin > melatonin >> *N*-acetylserotonin) was exhibited by both 2-[¹²⁵I]iodomelatonin binding in mammalian retina and pars tuberalis and the functional presynaptic receptor characterized in rabbit retina (Dubocovich, 1988a, 1995; Krause and Dubocovich, 1990; Morgan et al., 1994b; Hagan and Oakley, 1995). In contrast, 2-[¹²⁵I]iodomelatonin binding to the ML₂ site (later termed MT₃) in hamster brain membranes was distinguished by another endogenous ligand, *N*-acetylserotonin, that showed equal affinity with melatonin (ML₂: 2-iodomelatonin > melatonin = *N*-acetylserotonin) (Dubocovich, 1988b, 1995; Krause and

Dubocovich, 1990; Pickering and Niles, 1990; Molinari et al., 1996).

The next milestone was the cloning of two mammalian G protein-coupled melatonin receptors (GPCRs), now termed MT₁ and MT₂ (formerly Mel1a and Mel1b), (Reppert et al., 1994, 1995a,b, 1996). 2-¹²⁵I-Iodomelatonin binding to both recombinant hMT₁ and hMT₂ melatonin receptors exhibits the general pharmacology of the ML₁ type (Reppert et al., 1996; Dubocovich et al., 1997). These two melatonin receptors were defined as unique types on the basis of their distinct molecular structure and chromosomal localization (Reppert et al., 1994, 1995a,b, 1996; Slaugenhaupt et al., 1995; Barrett et al., 1997); subsequently, distinguishing ligands were identified (Dubocovich, 1995; Dubocovich et al., 1997; Brown-ing et al., 2000; Faust et al., 2000; Audinot et al., 2003).

The mammalian melatonin binding site MT₃ (previously referred to as ML₂) also has been pharmacologically characterized. Both melatonin and its precursor *N*-acetylserotonin compete for binding of 2-¹²⁵I]iodomelatonin to MT₃ melatonin binding sites, which show a pharmacological profile distinct from mammalian G protein-coupled melatonin receptors (Dubocovich, 1995; Molinari et al., 1996; Nosjean et al., 2001). Subsequently, a protein (quinone reductase II; QR2) purified from hamster kidney was found to have a ligand binding profile identical to that of the MT₃ binding site of hamster brain (Nosjean et al., 2000). In addition, brain and kidney membranes from mice with deletion of the QR2 gene demonstrated lack of 2-¹²⁵I-5-methoxy-carbon-ylamino-*N*-acetyltryptamine binding to MT₃ sites (Mail-liet et al., 2004).

C. International Union of Basic and Clinical Pharmacology Criteria for Receptor Nomenclature and Drug Classification

Melatonin receptors are named and classified on the basis of operational and structural criteria developed by the IUPHAR Committee on Receptor Nomenclature and Drug Classification (Vanhoutte et al., 1996; Ruffolo et al., 2000). The operational criteria are fulfilled by a pharmacological profile of specific ligands at the receptor recognition site, evidence of transduction mechanisms beyond the receptor, and demonstration of endogenously expressed receptors, usually from agonist/efficacy and antagonist dissociation constants obtained in native tissues. This information, combined with structural data about the protein sequence of the receptor, allows rational classification.

The present classification of melatonin receptors evolved from deliberations of the IUPHAR Subcommittee on Melatonin Receptor Nomenclature and Classification, formed in 1995, as pharmacological, functional, and structural information about the receptors emerged (Table 1). In accordance with IUPHAR guidelines (Vanhoutte et al., 1996; Ruffolo et al., 2000), the receptors were named for their endogenous ligand melatonin,

TABLE 1
IUPHAR melatonin receptor nomenclature and classification

Nomenclature	MT ₁	MT ₂	MT ₃
Previous names	MEL _{1A} , ML _{1A} , Mel _{1a}	MEL _{1B} , ML _{1B} , Mel _{1b}	ML ₂
Structural information	7TM	7TM	
Gene/chromosome	350 aa, ^a P48039; chr 4q 35.1	362 aa, ^b P49286; chr 11q21-22	
Human	353 aa, ^c Q61184; chr 8	365 aa, ^d U57554; chr 9	
Mouse	S 26284 ^e	IIK7 (10.3) ^f	
Selective agonists	S 26131 ^e	4P-ADOT, ^{g,m} 4P-PDOT (8.8), ^{l,m} GR 128107 ^l , KI85 (9.3) ^f	<i>N</i> -Acetylserotonin, ^{g-k} 5MCA-NAT ^k Prazosin ^h
Selective antagonists			2- ¹²⁵ I]5MCA-NAT ^k , 2- ¹²⁵ I]IMLT ⁿ Leukocyte adhesion ^u
Radioligands	[³ H]IMLT, ⁿ 2- ¹²⁵ I]IMLT ^o	[³ H]IMLT, ⁿ 2- ¹²⁵ I]IMLT, ^o [³ H]4P-PDOT ^p	
Tissue Functions	Vascular vasoconstriction, ^{q-u} inhibition neuronal firing, ^v phase-shift circadian activity rhythms ^a	Vascular vasodilation, ⁱ inhibition retinal dopamine release, ^j phase-shift circadian rhythms of neuronal firing ^{n,w,v,x}	Endogenous ligands are MLT and <i>N</i> -acetylserotonin. A recent report identifies an MT ₃ binding site in hamster kidney as the enzyme quinone reductase-2. ^{dd} Antagonist potencies have not yet been established.
Comments	MT ₁ full-length cDNAs were cloned from human (350 aa), ^a mouse (353 aa), ^c sheep (366 aa), ^a rat (353 aa), ^y hamster (353 aa), ^z monkey (352), ^{ac} and partial cDNAs from cattle (257 aa) and pig (154 aa). ^{bb}	MT ₂ cDNA was cloned from human (362 aa), ^z rat (364 aa), ^y mouse (365 aa), ^d sheep (376 aa), ^{cc} monkey (362 aa), ^{aa} and partial cDNA from hamster. ^z	

aa, amino acid(s); chr, chromosome; 5MCA-NAT, 5-methoxycarbonylamino-*N*-acetyltryptamine.

^a Reppert et al., 1994. ^b Reppert et al., 1995a. ^c Roca et al., 2003. ^d Jin et al., 2003. ^e Audinot et al., 2003. ^f Faust et al., 2000. ^g Dubocovich, 1988b. ^h Lucchelli et al., 1997. ⁱ Eison and Mullins, 1993. ^j Popova and Dubocovich, 1995. ^k Molinari et al., 1996. ^l Dubocovich et al., 1997. ^m Dubocovich and Takahashi, 1987. ⁿ Dubocovich and Masana, 1999. ^o Ting et al., 1999. ^p Ting et al., 1997. ^q Masana et al., 2002. ^r Doolen et al., 1998. ^s Lotufo et al., 2001. ^t Liu et al., 1997. ^u Dubocovich et al., 2005. ^v Hunt et al., 2001. ^w Dubocovich et al., 1996. ^x Weaver et al., 2009. ^y Nishiyama et al., 2009. ^z Cogé et al., 2009. ^{aa} Nosjean et al., 2000.

which is abbreviated as “MT” using capital letters, and each particular type of receptor was denoted by a numerical subscript (i.e., MT₁, MT₂). Species orthologs are denoted by the recommended lower-case prefix (i.e., h, human; o, ovine; r, rat; m, mouse; e.g., hMT₁). Splice variants, if pharmacologically relevant, would be indicated by lowercase, subscript letters in parentheses [e.g., MT_{1(a)}]. No such variants have yet been described for either of the cloned melatonin receptors. The original melatonin receptor nomenclature and classification included the MT₃ melatonin binding site, which at the time was thought to be a GPCR.

The first nomenclature approved by the IUPHAR Nomenclature Committee for melatonin receptors was published in the IUPHAR compendium in 1998, when melatonin receptors were designated as mt₁, MT₂, and MT₃. (Dubocovich et al., 1998a). The MT₁ melatonin receptor was denoted in lower case (mt₁) because there was no evidence at the time that the native receptor was functional in mammals. Subsequent functional, pharmacological, and immunohistochemical studies, as well as studies with the MT₁ knockout (KO) mice, characterized MT₁ melatonin receptor protein in native tissues leading to the more recent classification, MT₁, MT₂, and MT₃, published in the 2000 IUPHAR Compendium (Dubocovich et al., 2000).

MT₃ (formerly ML₂) was originally included in the classification on the basis of operational criteria (Dubocovich, 1995; Molinari et al., 1996; Dubocovich et al., 1998a, 2000). Because the structure of the receptor protein was not yet established, it was referred to in upper-case italics, as dictated by IUPHAR guidelines. The MT₃ binding site has a distinct pharmacology with selective agonists and antagonists and similar affinity for two endogenous indoles, melatonin and its precursor, *N*-acetylserotonin. The characterization of MT₃ as a melatonin binding site on the enzyme QR2 led the IUPHAR Nomenclature Committee to remove the MT₃ site from the Melatonin Receptor Nomenclature and Classification. We expect the classification of melatonin receptors will continue to evolve as operational and structural data for existing receptors are further defined and possible variants and/or new receptors are characterized. This is the case for the recent cloning of the ovine MT₂ receptor, where for many years it was suspected that sheep possessed only one melatonin receptor (Cogé et al., 2009).

D. Current Melatonin Receptor Nomenclature

The current nomenclature classifies the two cloned mammalian melatonin receptors into two types: MT₁ and MT₂. Detailed pharmacological and molecular characterization and supporting scientific evidence for these receptors are described later in this review (Table 1). See also the Melatonin Receptor-IUPHAR database (Dubocovich et al., 2009).

MT₁ (formerly Mel_{1a}, MEL_{1A}, ML_{1A}) refers to the first cloned mammalian melatonin receptor (Reppert et al., 1994). It is a G_{i/o} protein-coupled receptor linked, in part, to pertussis-toxin sensitive G proteins that mediate inhibition of cAMP in both recombinant expression systems and native tissues. Functional, immunohistochemical, and genetic KO studies indicate the presence of MT₁ receptors in various tissues, including the pars tuberalis of the pituitary gland (von Gall et al., 2002a) and the SCN of the hypothalamus (Dubocovich et al., 2005; Dubocovich, 2007).

MT₂ (formerly Mel_{1b}, MEL_{1B}, ML_{1B}) refers to the second cloned mammalian melatonin receptor (Reppert et al., 1995a). It is a G_{i/o} protein-coupled receptor capable of inhibiting cAMP and cGMP production in recombinant systems and stimulating PKC activity in a native tissue, the SCN. The pharmacological profile of this receptor was initially characterized in the retina and was defined by the use of selective MT₂ melatonin receptor antagonists (4P-PDOT and 4P-ADOT) (Dubocovich et al., 1997).

It is important to note that IUPHAR nomenclature criteria are applied only to mammalian receptors because they are the most closely aligned with therapeutics. The melatonin receptor field, however, actively encompasses a variety of species and has greatly benefited from the initial characterizations and cloning of melatonin receptors that occurred using frog melanophores (Sugden, 1989; Ebisawa et al., 1994; Sugden et al., 2004). At this point, there is no official consensus on classifying nonmammalian receptors, such as the cloned Mel_{1c} subtype that is found in birds and amphibians (Reppert et al., 1995b).

II. G Protein-Coupled Melatonin Receptor Family

A. Protein Structure

The MT₁ and MT₂ melatonin receptors comprise their own subgroup within the GPCR superfamily. Both melatonin receptors have a general structural motif consisting of seven transmembrane (TM)-spanning α -helical segments connected by alternating intracellular and extracellular loops, with the amino terminus located on the extracellular side and the carboxyl terminus on the intracellular side (Fig. 3). These seven α -helical segments contain stretches of 20 to 25 predominantly hydrophobic residues that span the cell membrane. The melatonin receptors are classified with the rhodopsin/ β_2 -adrenergic receptor family (Deupi et al., 2007). Within this family, most of the sequence homology between the melatonin receptors and other G protein-linked receptors occurs within the TM domains (Fig. 4).

The human MT₁ and MT₂ melatonin receptors encode proteins of 350 and 362 amino acids, respectively. Their predicted mass is 39,374 and 40,188 Da, respectively; however, these numbers do not take into account possi-

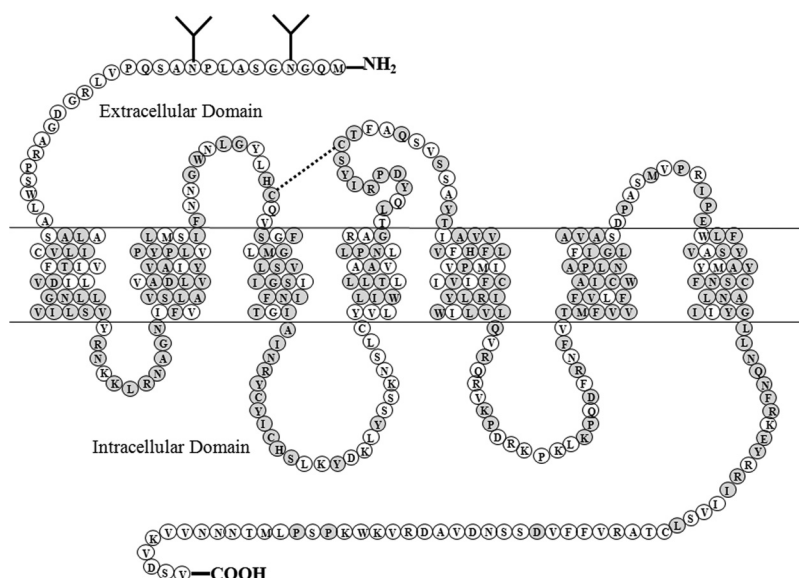


FIG. 3. Membrane topology of the hMT₁ melatonin receptor showing amino acids conserved in the hMT₂ receptor. Gray circles denote amino acids identical in the hMT₁ and hMT₂ melatonin receptors. The two glycosylation sites on the hMT₁ receptor are denoted (Y) in the N terminus. [Adapted from Reppert SM and Weaver DR (1995) Melatonin madness. *Cell* 83:1059–1062. Copyright © 1995 Elsevier Inc. Used with permission.]

ble posttranslational modifications. The amino acid homology for the human MT₁ and MT₂ melatonin receptors is approximately 60% overall and 73% within the transmembrane domains. The amino terminus of the MT₁ melatonin receptor contains two consensus sites for N-terminal asparagine-linked glycosylation, whereas that of the MT₂ shows only one site. The carboxyl tail of the two receptors contain consensus sites for casein kinase 1 α , casein kinase II, and protein kinase C as well as postsynaptic density 95/disc-large/zona occludens binding domains that may participate in membrane localization and trafficking (Hung and Sheng, 2002). Features that distinguish the melatonin receptor family from other GPCRs include a NRY motif downstream from the third transmembrane domain and a NAXIY in transmembrane domain 7, rather than DRY and NPXXY motifs, respectively (Reppert et al., 1994, 1995a; Roca et al., 1996).

B. Gene Structure and Chromosomal Localization

Molecular analyses of genomic clones show that the genes that encode the human MT₁ and MT₂ melatonin receptors are formed by two exons separated by an ~13-kilobase intron (Reppert et al., 1995a; Slaugenhaupt et al., 1995; Roca et al., 1996). The intron in the first cytoplasmic loop of the MT₁ and MT₂ melatonin receptor genes could potentially lead to alternative splice forms with distinct receptor structure, as well as operational and transduction characteristics. Such functional splice variants, however, have not yet been identified. It is noteworthy that the rat MT₂ receptor is composed of three exons, although the last exon contains no open reading frames (Ishii et al., 2009).

The melatonin receptors show distinct chromosomal localization. The MT₁ melatonin receptor was localized

to human chromosome 4q35.1 and mouse chromosome 8 (Slaugenhaupt et al., 1995; Roca et al., 1996). Slaugenhaupt et al. (1995) identified a region of syntenic conservation between distal chromosome 4 and mouse chromosome 8 that includes the genes plasma kallikrein (*KLK3*), mitochondrial uncoupling protein (*UPC*), and coagulation factor XI (*F11*) (Beaubien et al., 1991; Mills et al., 1992). By contrast, the MT₂ melatonin receptor maps to human chromosome 11q21–22 (Reppert et al., 1995a). Reppert et al., 1995 (Reppert et al., 1995a), pointed out that the hMT₂ receptor maps to a region syntenic to mouse chromosome 9 in the region of the D2 dopamine receptor (*Drd2*) and thymus cell antigen 1 (*thy*) loci (Seldin et al., 1991; Goldsborough et al., 1993).

The phylogenetic tree of the melatonin receptors (MT₁, MT₂, and Mel_{1C}) and the melatonin-related receptor GPR50 (also known as melatonin-related receptor or H9) sequences revealed that GPR50, which cannot bind melatonin, is relatively distant to the functional melatonin receptors (MT₁, MT₂, Mel_{1C}). The Mel_{1C} receptor, which is not expressed in mammals, is phylogenetically closer to the MT₂ receptor than to the MT₁ receptor. The human MT₁ receptor shows more similarities with the rodent MT₁ receptors than with the bovine, ovine, and porcine MT₂ receptors. As already observed for other GPCRs, the ovine MT₁ receptor shows significant homology with the bovine MT₁ receptor (Fig. 4).

Whereas the Mel_{1C} receptor has been found only in fish, birds, and *X. laevis*, GPR50 has only been found in eutherian mammals and not birds or fish. An in silico approach has suggested that GPR50 is the ortholog of the Mel_{1C} receptor (Dufourny et al., 2008). This conclusion is based on an analysis of the melatonin receptor family phylogenetic tree and the conserved synteny of genes surrounding the Mel_{1C} and GPR50 genes. It is

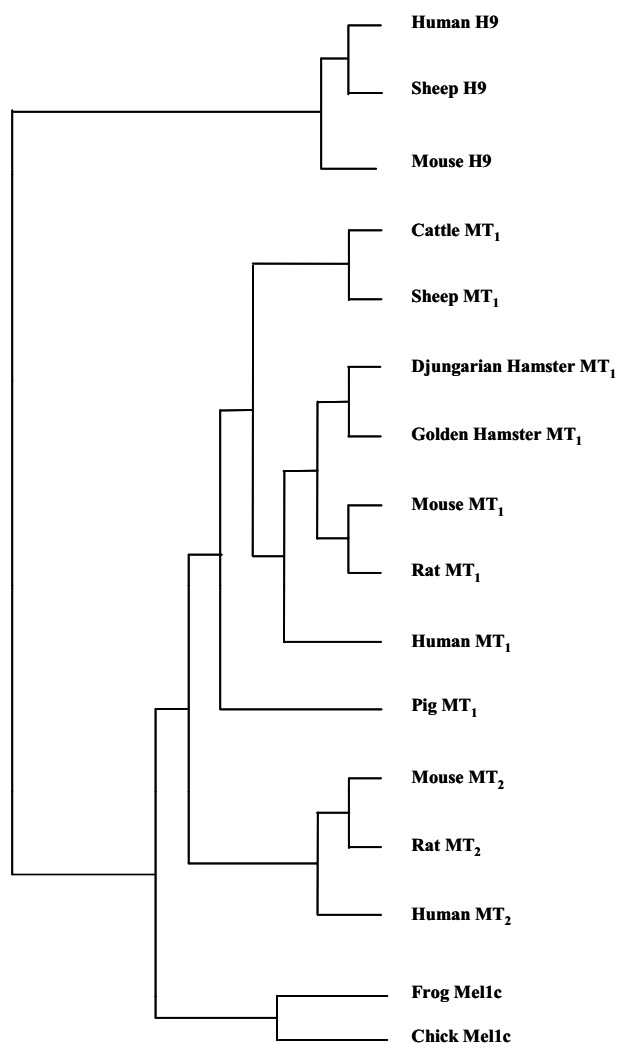


FIG. 4. MT₁ and MT₂ melatonin receptor dendrogram. Phylogenetic tree of melatonin receptor or melatonin receptor-related (GPR50, melatonin-related receptor or H9) sequences. The evolutionary distances between the different sequences were calculated with the matrix of Blosom 62 score. The tree was drawn using the Unweighted Pair Group Method with Arithmetic mean (UPGMA). GenBank accession numbers and the number of amino acids for each receptor are as follows: human H9: U52219, 613; sheep H9: U52221, 613; mouse H9: AF065145, 791; cattle MT₁: U73327, 257; sheep MT₁: U14109, 366; Djungarian hamster MT₁: U14110, 353; golden hamster MT₁: AF061158, 325; mouse MT₁: U52222, 353; rat MT₁: AF130341, 326; human MT₁: U14108, 350; Pig MT₁: U73326, 154; mouse MT₂: AY145850, 365; rat MT₂: U28218, 120; human MT₂: U25341, 362. The sequences for cattle and pig MT₁ receptors have been partially cloned.

suggested that rapid evolution of Mel_{1C} into GPR50 led to the mutation of several critical amino acids and the addition of a long C-terminal tail resulting in the loss of affinity of GPR50 for melatonin. However, formation of the GPR50/MT₁ receptor heterodimer in recombinant cells significantly reduces the affinity and potency of melatonin agonists binding for the MT₁ melatonin receptor (Levoye et al., 2006a). Recent evidence supports the idea that GPR50 expression in the Siberian hamster ependymal layer is under photoperiod control (Barrett et al., 2006). This orphan receptor may also be important in regulating energy metabolism (Ivanova et al., 2008).

The MT₂ melatonin receptor gene cloned from the Siberian or Syrian hamsters seems to be a pseudogene because it is endowed with two nonsense mutations in the coding region of the receptor cDNA. The stop codons are located in transmembrane domain V and in the second extracellular loop (Weaver et al., 1996). The Siberian and Syrian hamster are considered natural MT₂ melatonin receptor mutants.

C. Melatonin Receptor Polymorphisms

Genetic polymorphisms have been reported for melatonin receptors in human and sheep. In human, polymorphisms have been compared for both MT₁ and MT₂ in subjects with circadian rhythm sleep disorders and controls. Seven mutations were found in the MT₁ receptor, with two that resulted in amino acid changes: R54W in the first cytoplasmic loop and A157V in the fourth transmembrane domain (Ebisawa et al., 1999). Although the mutations were more common in non-24-h sleep-wake syndrome subjects than in delayed sleep phase syndrome or controls, no significant change in receptor affinity and/or density was observed when the mutants were expressed in heterologous cells. Two mutations were also reported for the hMT₂: G24E in the N-terminal domain and L66F in the first cytoplasmic loop. However, neither shows altered MT₂ receptor binding characteristics (Ebisawa et al., 2000). The effect of these mutations in melatonin receptor function has not been reported.

Melatonin secretion follows a circadian rhythm with high levels at night. By contrast, insulin release is high during the day. The drop in insulin levels at night may result from endogenous melatonin-mediated inhibition by activation of MT₁ and MT₂ in pancreatic islets (Peschke et al., 2002; Mulder et al., 2009). Recent studies have revealed an association of high fasting plasma glucose, early stage impairment of insulin secretion, and increased risk of type 2 diabetes in persons with genetic variations in the *MTNR1B* gene encoding the MT₂ melatonin receptor (Bouatia-Naji et al., 2009; Lyssenko et al., 2009; Prokopenko et al., 2009). Based on increases in MT₂ melatonin receptor mRNA expression in human pancreatic islets of subjects without diabetes with the risk allele and subjects with type 2 diabetes led to the suggestion that an increase in MT₂ receptor density may be involved in the pathogenesis of these conditions (Lyssenko et al., 2009). However, whether increases in mRNA expression reflect increases in MT₂ melatonin receptor density in pancreatic islets is not known.

Several polymorphisms have been described in the ovine MT₁ type, leading to changes in amino acids (A282D, H358R, I361V), one in extracellular loop 3 and two in the carboxyl-terminal tail. This variant receptor, which seems fully functional, has not been linked with a specific phenotype (Barrett et al., 1997). Polymorphism of MnlI restriction sites in exon II of the MT₁ receptor

was analyzed in the Mérinos d'Arles ewe in relation to the expression of reproductive seasonality (Pelletier et al., 2000). The MnlI restriction sites show an association between the homozygous genotype for the absence of a MnlI site at position 605 (–/–) and seasonal anovulatory activity. Other mutations were observed, not simultaneously, at positions 706 and 893, which resulted in the substitution of a valine by an isoleucine and of an alanine by an aspartic acid, respectively. However, in the Ile-de-France sheep breed, the two allelic forms of the MT₁ receptor gene have no direct effect on the seasonal pattern of various seasonal functions. It was suggested that the effect of this polymorphism on seasonal function seems to be dependent on the breed and/or environmental condition (Hernandez et al., 2005).

D. Molecular Structure of MT₁ and MT₂ Melatonin Receptor Ligand Binding Pockets

1. MT₁ Melatonin Receptor. A rhodopsin-based computer model has been used to propose the molecular structure of the melatonin receptor binding site (Navajas et al., 1996). This particular model has advantages over bacteriorhodopsin-based models for the melatonin receptor binding site (Sugden et al., 1995; Grol and Jansen, 1996), because bacteriorhodopsin is not coupled to G proteins, and its sequence has none of the distinctive features of the GPCR family (Baldwin, 1993). The rhodopsin-based molecular model has been investigated by site-directed mutagenesis studies, revealing that the binding site of the melatonin receptor has some similarities with those of other GPCRs of the rhodopsin/ β_2 -adrenergic receptor family. For example, His195 in putative TM5 is conserved in all of the melatonin receptors, and the position is identical to that used in the ligand binding site of many other rhodopsin-like GPCRs. The model proposes that this His residue can form a hydrogen bond with the oxygen atom of the 5-methoxy group of melatonin. Site-directed mutagenesis of the melatonin receptor (Conway et al., 1997; Kokkola et al., 1998) and studies with sulfur analogs of melatonin (Davies et al., 2004) have given support to this suggestion. The binding site model also proposes that Val192, which is located approximately one helical turn above the His195 facing the hydrophobic binding pocket, is important for the binding of the methyl portion of the methoxy group of melatonin. Val192 is analogous to a residue in the β_2 -adrenergic and 5-HT receptors that is important in ligand binding (Strader et al., 1989; Ho et al., 1992; Kao et al., 1992). In addition, Met107 in TM3 and Ser280 and Ala284 in TM7, which were proposed to be important for the binding of the *N*-acetyl group of melatonin, do not seem to directly participate in melatonin receptor activation (Kokkola et al., 1998). Thus, computer modeling has revealed one site that is important for melatonin binding, but the other residues/domain(s) of the melatonin receptor, which are critical for ligand binding, have yet to be identified.

A second method for determining residues that are important for receptor binding and activation has been to modify amino acid residues conserved in the rhodopsin-like GPCRs. Protonation of the aspartic/glutamic acid in the highly conserved D/ERY motif at the cytoplasmic side of TM3 is believed to be involved in activation of the rhodopsin-like GPCRs. The binding of the ligand causes the Asp/Glu to become unprotonated, resulting in receptor activation, as shown most directly by the rhodopsin receptor (Arnis et al., 1994). Changing the D/E to a neutral amino acid that mimics the unprotonated state results in constitutive activation and improved coupling of many of the rhodopsin-like receptors. The melatonin receptor is unique in that it has an NRY motif instead of the D/ERY motif. Changing the NRY to an ARY (mimicking the unprotonated, activated state) actually decreases binding to such an extent that it is impossible to measure receptor activation (Nelson et al., 2001). Changing the melatonin receptor NRY motif to the D/ERY motif modestly decreases the binding affinity (2-fold) and decreases the capacity for melatonin to activate the receptor (Nelson et al., 2001). Thus, unlike other rhodopsin-like GPCRs, the melatonin receptor seems to not require deprotonation of the NRY motif to be active, and Asn is needed for optimal ligand binding and receptor activation.

Pro267 is a highly conserved amino acid in rhodopsin-like GPCRs. This proline residue occurs in the center of TM6, causing a kink in the center of the α -helix. Mutating the proline to an alanine results in constitutive activity of the yeast α -factor and β_2 -adrenergic receptor (Konopka et al., 1996), presumably by making the alpha helix less "kinked." However, when the corresponding residue in the MT₁ melatonin receptor, α 253, is mutated to Ala, constitutive activity of the melatonin receptor is not seen and, in fact, the receptor affinity is decreased by severalfold (Kokkola et al., 1998).

G-protein coupled melatonin receptors have two conserved cysteines (Cys127, Cys130) between helix III and the second intracellular loop, a region important in receptor/G-protein coupling. Indeed, mutation of Cys127 and Cys130 to Ser in the MT₁ receptor revealed that these cysteines are necessary for normal G protein activation and receptor trafficking (Kokkola et al., 2005).

Finally, there is an NPXXY sequence found at the end of TM7 in rhodopsin-like receptors. The Asn302 is proposed to interact with the Asp83, suggesting that TM2 and TM7 are in close proximity. Kinking and twisting of Pro303 is proposed to allow these two residues to come in contact. It is noteworthy that the melatonin receptors have retained the conserved Asp83 (Asp73) and Asn302 (Asn291) but have replaced the Pro303 with an alanine (Ala292). How the Ala292 affects the overall structural motif is not known (Table 2).

Analysis of rhodopsin binding and of extensive mutagenesis data involving the β_2 adrenergic receptor suggests that TMs 3, 5, 6, and 7, especially TM3 and/or

TABLE 2
Effect of amino acid mutations on ligand binding to the MT₁ melatonin receptor

Amino acids are represented in single-letter code with position number shown. Superscripts after the second amino acid indicate that the substituted amino acid represents the amino acid in the designated receptor at the analogous position. The position in the transmembrane domain is indicated using the numbering scheme of Ballesteros and Weinstein (1995).

Amino Acid Mutation Scheme	TM No.	Species	Expression System	Characterization	Reference
R54W	(1.59)	Human	COS cells	Heterozygous polymorphism with no phenotype. Decreased B_{\max} ($3.5\times$) and slightly increased K_d .	Ebisawa et al., 1999
S103A	(2.28)	Human	COS-7	No change in B_{\max} or K_d .	Conway et al., 2001
M107T	(3.32)	Human	COS-7	No change in B_{\max} or K_d .	Conway et al., 2001; Kokkola et al., 1998
S110A ^a	(3.35)	Human	COS-7	Decreased B_{\max} ($10\times$), increased K_d ($8\times$) and EC_{50} of cAMP production ($22\times$). No change in K_i of luzindole	Conway et al., 2001
S114A ^a	(3.39)	Human	COS-7	Decreased B_{\max} ($4\times$), increased K_d ($9\times$) and EC_{50} of cAMP production ($14\times$). No change in K_i of luzindole.	Conway et al., 2001
N124A/K	(3.49)	Human	AfT20	Decreased B_{\max} ($21\times$), tends to be retained in Golgi. No specific binding.	Nelson et al., 2001
N124A	(3.49)	Human	<i>Saccharomyces cerevisiae</i>	Increased EC_{50} for melatonin ($230\times$).	Kokkola et al., 1998
N124L	(3.49)	Human	AfT20	Decreased B_{\max} ($21\times$), tends to be aggregated near surface. No specific binding.	Nelson et al., 2001
N124D/E	(3.49)	Human	AfT20	No change in B_{\max} or K_d . Melatonin induced inhibition of cAMP (efficacy) and voltage-sensitive Ca^{2+} channels, but not Kir3.1/3.2 potassium channel activation.	Nelson et al., 2001
A157V	(4.55)	Human	COS cells	Heterozygous polymorphism with no phenotype. No change in B_{\max} or K_d .	Ebisawa et al., 1999
H195A ^a	(5.46)	Human	<i>S. cerevisiae</i>	Decreased EC_{50} ($3-6\times$). <i>N</i> -acetylserotonin gave an apparent saturable response, whereas the wild-type receptor did not saturate at the same concentrations.	Kokkola et al., 1998
H211F/I	(5.46)	Ovine	COS-7	Increase K_d ($6\times$) with melatonin. Decreased K_i ($3-15\times$) with N-NEA. No change in K_i with <i>N</i> -acetylserotonin.	Conway et al., 1997
V192T + H195A	(5.42 + 5.46)	Human	<i>S. cerevisiae</i>	No specific response	Kokkola et al., 1998
V208A	(5.42)	Ovine	COS-7	No change in K_d or in K_i of several melatonin analogs.	Conway et al., 1997
V208L	(5.42)	Ovine	COS-7	Increased K_d and K_i for several melatonin analogs ($5-12\times$).	Conway et al., 1997
A252C	(6.49)	Human	COS-7	No change in K_d or B_{\max} .	Conway et al., 2000
G258T	(6.55)	Human	COS-7	No change in K_d or B_{\max} . Specific binding drastically reduced	Gubitz and Reppert, 2000; Conway et al., 2000
A252C + G258T	(6.49 + 6.55)	Human	COS-7	No specific binding	Gubitz and Reppert, 2000
P253A	(6.50)	Human	<i>S. cerevisiae</i>	No specific response.	Kokkola et al., 1998
A202D, H342R, I347V	(ext. loop3, C-terminal)	Ovine	L-cells	Polymorphism of previously cloned ovine MT ₁ . No phenotype in vivo and fully functional in mouse. L cells as shown by high affinity binding, competition binding analysis, GTP- γ S and inhibition of cAMP.	Barrett et al., 1997
S280A	(7.38)	Human	<i>S. cerevisiae</i>	No change in apparent EC_{50}	Kokkola et al., 1998
S280F + A284G	(7.38 + 7.42)	Human	<i>S. cerevisiae</i>	No specific response	Kokkola et al., 1998

N-NEA, *N*-[2-(1-naphthyl) ethyl]acetamide.
^a Amino acid residues important for modulating binding to the MT₁ receptor (Farce et al., 2008).

TM7, are major players in ligand binding (Kobilka and Deupi, 2007; Rosenbaum et al., 2007). The TM3 domains of ClassA GPCRs contain a high number of Ser/Thr/Cys residues (seven residues/TM3). These residues form hydrogen bonds to the peptide backbone and thereby bend and twist helices (Gray and Matthews, 1984; Ballesteros et al., 2000). Thus, different hydrogen bonding states may result in different TM3 conformations that represent different functional states of the same receptor, such as liganded versus unliganded or active versus inactive (Ballesteros et al., 2001). As in other GPCRs, the Ser/Thr/Cys residues in TM3 of MT₁ are important in ligand binding at MT₁ receptors. Mutations of Ser110 and Ser114, but not Ser103, to alanine reduced melatonin binding but did not affect luzindole binding (Conway et al., 2001). These experiments give some molecular support to the experimental data from the structure-activity relationships of a series of luzindole analogs suggesting that melatonin receptor agonist and antagonist-binding sites may differ (Teh and Sugden, 1998) (Table 2). Based on these studies, a two dimensional model of the MT₁ melatonin receptor was created comprising the transmembrane domains and the potential orientation of melatonin in the binding pocket (Barrett et al., 2003). This model takes into account the interaction between melatonin's methoxy group and the conserved histidine (His195) in TM5.

A third method for determining important residues for melatonin receptor binding has been to create chimeric melatonin MT₁ and melatonin-related receptors (GPR50). Despite having 57% amino acid sequence identity with the TM domains of the MT₁ receptor, the GPR50 does not bind 2-[¹²⁵I]iodomelatonin or [³H]melatonin. It is noteworthy that two studies involving chimeric receptors suggest that TM6, extracellular loop 2, and intracellular loop 2 are critical for melatonin receptor binding to the MT₁ melatonin receptor. Replacement of transmembrane domains 1, 2, 3, 5, or 7 of the hMT₁ receptor by the corresponding TM from GPR50 induces only minor change in 2-[¹²⁵I]iodomelatonin binding affinity. On the contrary, chimeric receptors with TM 6 from GPR50 receptor display no specific binding (Conway et al., 2000; Gubitza and Reppert, 2000). Gubitza and Reppert (2000) reported that replacement of TM4 of the hMT₁ with TM4 of GPR50 did not alter binding affinity, but Conway et al. (2000) failed to detect specific binding in this mutant. To confirm that some specific amino acids of TM 6 play an important role in binding, both research groups performed point mutations in this transmembrane of the MT₁ receptor. Mutation of glycine to threonine (G258T) severely reduced both the binding and activation of the MT₁ receptor (Conway et al., 2000; Gubitza and Reppert, 2000). The mutant A252C displayed binding affinity close to that of the native hMT₁ receptor. Double mutation of glycine to threonine (G258T) and alanine to cysteine (A252C) was found to

completely inhibit binding by Gubitza and Reppert (2000) and to have no effect on binding by Conway et al. (2000). Taken together, these data do support the idea that mutation of glycine 258, which is predicted to face the hydrophilic receptor core, may be important for maintaining an appropriate MT₁ melatonin receptor structure.

2. MT₂ Melatonin Receptor. Melatonin receptors, like most other GPCRs, contain a conserved cysteine residue in extracellular loop 1 and in extracellular loop 2. Mutation of these Cys residues in rhodopsin (Karnik et al., 1988), δ -opioid (Ehrlich et al., 1998), platelet-activating factor (Le Gouill et al., 1997), and M₃ muscarinic receptors (Zeng et al., 1999) demonstrate the critical importance of this disulfide bond for the proper receptor conformation for ligand binding, receptor activation, and cell surface expression. These conserved cysteine residues, however, do not always participate in disulfide bonding, as shown previously for the β 2-adrenergic receptor (Noda et al., 1994). The disulfide bond formation between Cys113 and Cys190 residues was shown to be crucial to maintain a proper hMT₂ receptor conformation for melatonin binding without altering cell surface receptor expression (Mseeh et al., 2002). Whether this disulfide bond occurs within a single melatonin receptor or between two melatonin receptors forming a dimer remains to be determined. *N*-ethylmaleimide alkylation of Cys140 appears to contribute to changes in ligand affinity, whereas alkylation of Cys143 and Cys219 reduced binding capacity (Mseeh et al., 2002). The cysteines involved in *N*-ethylmaleimide-induced changes in affinity and receptor density are probably located in receptor regions near the melatonin binding site and/or G protein coupling region.

Key conserved amino acids (Table 3) seem to be involved in ligand binding to the MT₂ melatonin receptors as determined in binding studies after mutation to alanine. Mutation of Asn175 in TM4 or His208 in TM5 of the hMT₂ melatonin receptor significantly decreased the binding affinity for melatonin (Gerdin et al., 2003). Asn175 in TM4 seems to facilitate binding of the 5-methoxy group of the melatonin molecule to the hMT₂ melatonin receptor (Gerdin et al., 2003). Thus, His208 in TM5 in both the oMT₁ receptor (Conway et al., 1997) and the hMT₂ melatonin receptor (Gerdin et al., 2003) are critical for melatonin binding. Trp264 or Phe257 in TM6, although not critical for melatonin binding, may interact with aromatic regions of molecules such as luzindole and 4P-ADOT. Mutation of Ser123 or Ser127 in TM3 or Ser293 in TM7 of the MT2 receptor did not affect binding affinity, although equivalent serines (Ser110 and Ser114 in TM3) were reported to be critical for melatonin binding to the hMT₁ melatonin receptor (Conway et al., 2001). Thus, the binding pockets of the MT₁ and MT₂ melatonin receptors seem to share a common histidine residue (His195/208 in TM5) but also have distinct residues (Ser110/123 and Ser110/127 in TM3) necessary for

TABLE 3
Effect of amino acid mutations on ligand binding to the hMT₂ melatonin receptor

Amino acids are represented in single-letter code with position number shown. Superscripts after the second amino acid indicate that the substituted amino acid represents the amino acid in the designated receptor at the analogous position. The position in the transmembrane domain is indicated using the numbering scheme of Ballesteros and Weinstein (1995).

Amino Acid Mutation Scheme	TM No.	Expression System	Characterization	Reference
Human				
G24E	NTerm	COS-7	Heterozygous polymorphism with no phenotype. When expressed in COS-7 cells no change in K_d or K_i with melatonin.	Ebisawa et al., 2000
L66F	1.58	COS-7	Heterozygous polymorphism with no phenotype.	Ebisawa et al., 2000
C113A	ECL1	HEK293	No specific binding.	Mseeh et al., 2002
C140A	ICL2	HEK293	No change in K_d , slightly increased K_i for melatonin (1.6×), decreased B_{max} (22×).	Mseeh et al., 2002
C143A	ICL2	HEK293	No change in K_d , slightly increased K_i for melatonin (1.4×), slightly increased B_{max} (1.8×).	Mseeh et al., 2002
C190A	ECL2	HEK293	No specific binding.	Mseeh et al., 2002
C219A	5.57	HEK293	No change in K_d or K_i . Decreased B_{max} (5×).	Mseeh et al., 2002
C263A	6.47	HEK293	No change in K_d or K_i . Decreased B_{max} (31×).	Mseeh et al., 2002
C302A	7.47	HEK293	No change in K_d or K_i . Decreased B_{max} (4×).	Mseeh et al., 2002
S123A	3.35	HEK293	No change in K_d or K_i . Decreased B_{max} (5×).	Gerdin et al., 2003
S127A	3.39	HEK293	No change in K_d or K_i . Decreased B_{max} (3×).	Gerdin et al., 2003
N175A ^a	4.60	HEK293	No change in K_d , slightly increased K_i for melatonin. No change in B_{max} .	Gerdin et al., 2003
H208A ^a	5.46	HEK293	Increased K_d and K_i for melatonin. No change in B_{max} .	Gerdin et al., 2003
F257A	6.41	HEK293	No change in K_d or K_i . No change in B_{max} .	Gerdin et al., 2003
W264A	6.48	HEK293	Decreased K_d , no change in K_i . Decreased B_{max} (22×).	Gerdin et al., 2003
S293A	7.38	HEK293	No change in K_d or K_i . No change in B_{max} .	Gerdin et al., 2003
V204A ^a	5.42	HEK293	No specific binding.	Mazna et al., 2004
V205	5.43	HEK293	No change in K_d . No change in B_{max} .	Mazna et al., 2004
F209A	5.47	HEK293	No change in K_d . Decreased B_{max} . No change in K_i for melatonin, luzindole or 4P-PDOT.	Mazna et al., 2004
G271T	6.55	HEK293	Not saturable.	Mazna et al., 2004
L272A ^a	6.56	HEK293	No specific binding.	Mazna et al., 2004
Y298A ^a	7.43	HEK293	No specific binding.	Mazna et al., 2004
M120A	3.32	HEK293	No change in K_d or B_{max} .	Mazna et al., 2005
G121A	3.33	HEK293	No change in K_d or B_{max} .	Mazna et al., 2005
G121I	3.33	HEK293	No change in K_d or B_{max} .	Mazna et al., 2005
V124A	3.36	HEK293	No change in K_d with decreased B_{max} .	Mazna et al., 2005
I125A	3.37	HEK293	No change in K_d or B_{max} .	Mazna et al., 2005
Y188A	ECL2	HEK293	No specific binding.	Mazna et al., 2005
Y188F	ECL2	HEK293	No specific binding.	Mazna et al., 2005
N268A ^a	6.52	HEK293	No specific binding.	Mazna et al., 2005
N268D ^a	6.52	HEK293	No specific binding.	Mazna et al., 2005
N268L ^a	6.52	HEK293	No specific binding.	Mazna et al., 2005
N268Q ^a	6.52	HEK293	No change in K_d or B_{max} .	Mazna et al., 2005
A275I	6.59	HEK293	No specific binding.	Mazna et al., 2005
A275V ^a	6.59	HEK293	No change in K_d or B_{max} .	Mazna et al., 2005
V291A ^a	7.36	HEK293	No specific binding.	Mazna et al., 2005
V291I ^a	7.36	HEK293	No specific binding.	Mazna et al., 2005
L295A ^a	7.40	HEK293	No specific binding.	Mazna et al., 2005
L295I ^a	7.40	HEK293	No specific binding.	Mazna et al., 2005
L295V ^a	7.40	HEK293	No specific binding.	Mazna et al., 2005
Hamster				
P41A	1.33	CHO-K1	No change in K_d or B_{max} . No change in EC ₅₀ or E_{max} for melatonin or 2-iodomelatonin stimulation of GTPγ ³⁵ S binding.	Mazna et al., 2008
P93A	2.57	CHO-K1	No change in K_d or B_{max} . No change in EC ₅₀ or E_{max} for melatonin or 2-iodomelatonin stimulation of GTPγ ³⁵ S binding.	Mazna et al., 2008
P95A	2.59	CHO-K1	No change in K_d or B_{max} . No change in EC ₅₀ or E_{max} for melatonin or 2-iodomelatonin stimulation of GTPγ ³⁵ S binding.	Mazna et al., 2008
P158A	4.40	CHO-K1	No change in K_d or B_{max} . No change in EC ₅₀ or E_{max} for melatonin or 2-iodomelatonin stimulation of GTPγ ³⁵ S binding.	Mazna et al., 2008
P174A	4.59	CHO-K1	No specific binding.	Mazna et al., 2008
P174G	4.59	CHO-K1	No specific binding.	Mazna et al., 2008
P212A	5.50	CHO-K1	No change in K_d or B_{max} . Decreased E_{max} for melatonin and 2-iodomelatonin, stimulation of GTPγ ³⁵ S binding.	Mazna et al., 2008
P212G	5.50	CHO-K1	No change in K_d or B_{max} . Increased EC ₅₀ for 2-iodomelatonin stimulation of GTPγ ³⁵ S binding with no change in E_{max} .	Mazna et al., 2008
P266A	6.50	CHO-K1	No specific binding.	Mazna et al., 2008
P266G	6.50	CHO-K1	No specific binding.	Mazna et al., 2008
A305P	7.50	CHO-K1	No specific binding.	Mazna et al., 2008
A305V	7.50	CHO-K1	No change for K_d or B_{max} . Increased EC ₅₀ for melatonin and 2-iodomelatonin stimulation of GTPγ ³⁵ S binding with decreased E_{max} .	Mazna et al., 2008

HEK, human embryonic kidney; CHO, Chinese hamster ovary.

^a Amino acid residues important for modulating binding to the MT₁ receptor (Farce et al., 2008).

ligand binding. Mazna et al. (2004) identified several amino acids in TM V (Val204), VI (Leu272), and VII (Tyr298) that are involved in melatonin interactions with the MT₂ melatonin receptor binding pocket. In a subsequent studies, this group demonstrated that residues Asn268 and Ala275 in TM6 as well as residues Val291 and Leu295 in TM7 are essential for 2-iodomelatonin binding to the hMT₂ receptor (Mazna et al., 2005). Mazna et al. (2008) assessed the impact of mutations on the MT₂ melatonin receptor structure by molecular dynamic simulations of the receptors embedded in the fully hydrated phospholipid bilayer and demonstrated that residues Pro174, Pro212, and Pro266 are important for the ligand binding and/or signaling of this receptor (Table 3). Taken together, the identification of sequence specific motifs may ultimately provide the molecular basis for the rational design of type specific therapeutic compounds.

Farce et al. (2008) have published models showing the predicted binding site for melatonin on the MT₁

and MT₂ melatonin receptors based on site-directed mutagenesis analysis and a three-dimensional homology modeling of the receptors using bovine rhodopsin as a template (Fig. 5). In these models, the binding space for melatonin on the MT₁ receptor seems to be relatively smaller than the space for the MT₂ receptor. The conserved histidine of TM5 (His195 for MT₁ and His208 for MT₂), which is predicted to bind to the methoxy group, seem to be common to both receptor binding sites (Gerdin et al., 2003). The amine moiety interacts with Asn175 of TM4 in the MT₂ receptor; however, a corresponding amino acid in the MT₁ receptor TM3 is not an Asn; rather, serines (Ser110 and Ser114) seem to bind to melatonin (see Fig. 5; Farce et al., 2008).

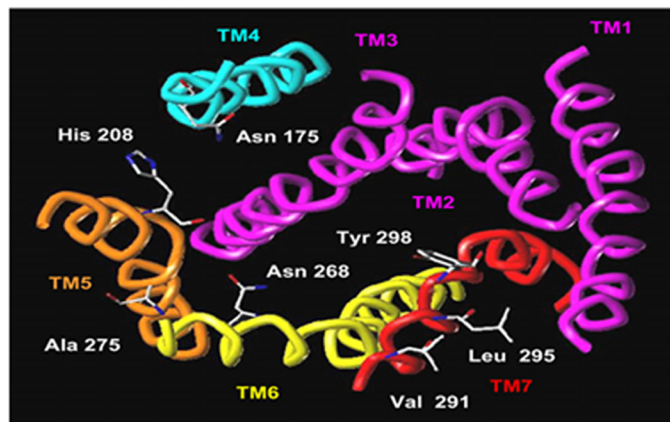
III. Cellular Signaling of MT₁ and MT₂ Melatonin Receptors

The best-known signaling pathway for melatonin receptors is inhibition of cAMP formation via pertussis toxin-

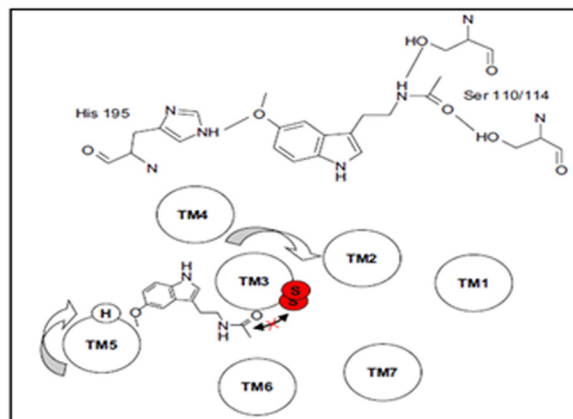
A MT₁ Model



B MT₂ Model



C MT₁ Model



D MT₂ Model

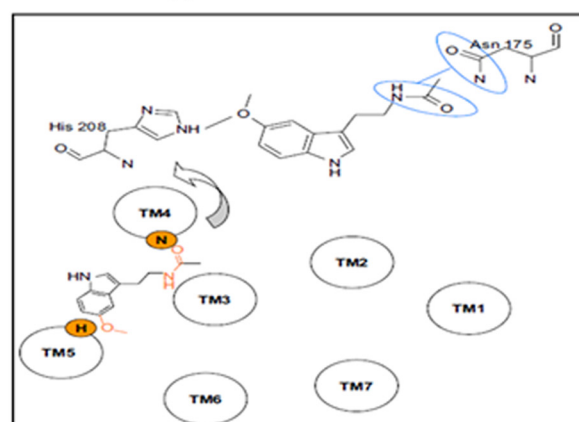


FIG. 5. MT₁ and MT₂ melatonin receptor 3 dimensional models and putative mode of binding for melatonin. A and B show critical amino acid residues for melatonin binding to the MT₁ and MT₂ melatonin receptors, respectively. The amino acids labeled in white have been defined by site-directed mutagenesis to modulate binding affinity (see Table 2 and 3). C and D show interactions among melatonin and key amino acid residues important for binding to the MT₁ and MT₂ melatonin receptors, respectively. [Adapted from Farce A, Chugunov AO, Logé C, Sabaouni A, Yous S, Dilly S, Renault N, Vergoten G, Efremov RG, Lesieur D, and Chavatte P (2008) Homology modelling of MT1 and MT2 receptors. *Eur J Med Chem* 43:1926–1944. Copyright © 2008 Elsevier Masson SAS. Used with permission.]

sensitive G proteins. Although first described in frog melanophores (White et al., 1987), melatonin-mediated decreases in cAMP have been observed in a number of mammalian tissues, including pituitary, SCN, and cerebral arteries (Capsoni et al., 1994; Morgan et al., 1994b). Pertussis toxin (PTX) sensitivity indicates the involvement of G proteins in the G_i/G_o family; however, the identity of the specific G proteins that transduce the melatonin signal in native tissues is not known. Using recombinant human receptors, adenylate cyclase inhibition has been confirmed as a signaling mechanism for both MT_1 and MT_2 melatonin receptor types (Reppert et al., 1995a). Recent studies, however, indicate that melatonin can elicit multiple recep-

tor-mediated intracellular responses. Signal transduction mechanisms shown to be associated with MT_1 and MT_2 receptors are summarized below.

A. MT_1 Melatonin Receptor Signaling

MT_1 melatonin receptors can couple to both PTX-sensitive (G_i) and insensitive ($G_{q/11}$) G proteins (Brydon et al., 1999b; Roka et al., 1999) (Fig. 6A). Activation of MT_1 melatonin receptors decreases forskolin-stimulated cAMP formation (Reppert et al., 1994; Witt-Enderby and Dubocovich, 1996; Brydon et al., 1999a; Petit et al., 1999). Protein kinase A activity and phosphorylation of the cAMP responsive element-binding protein (CREB) (Witt-

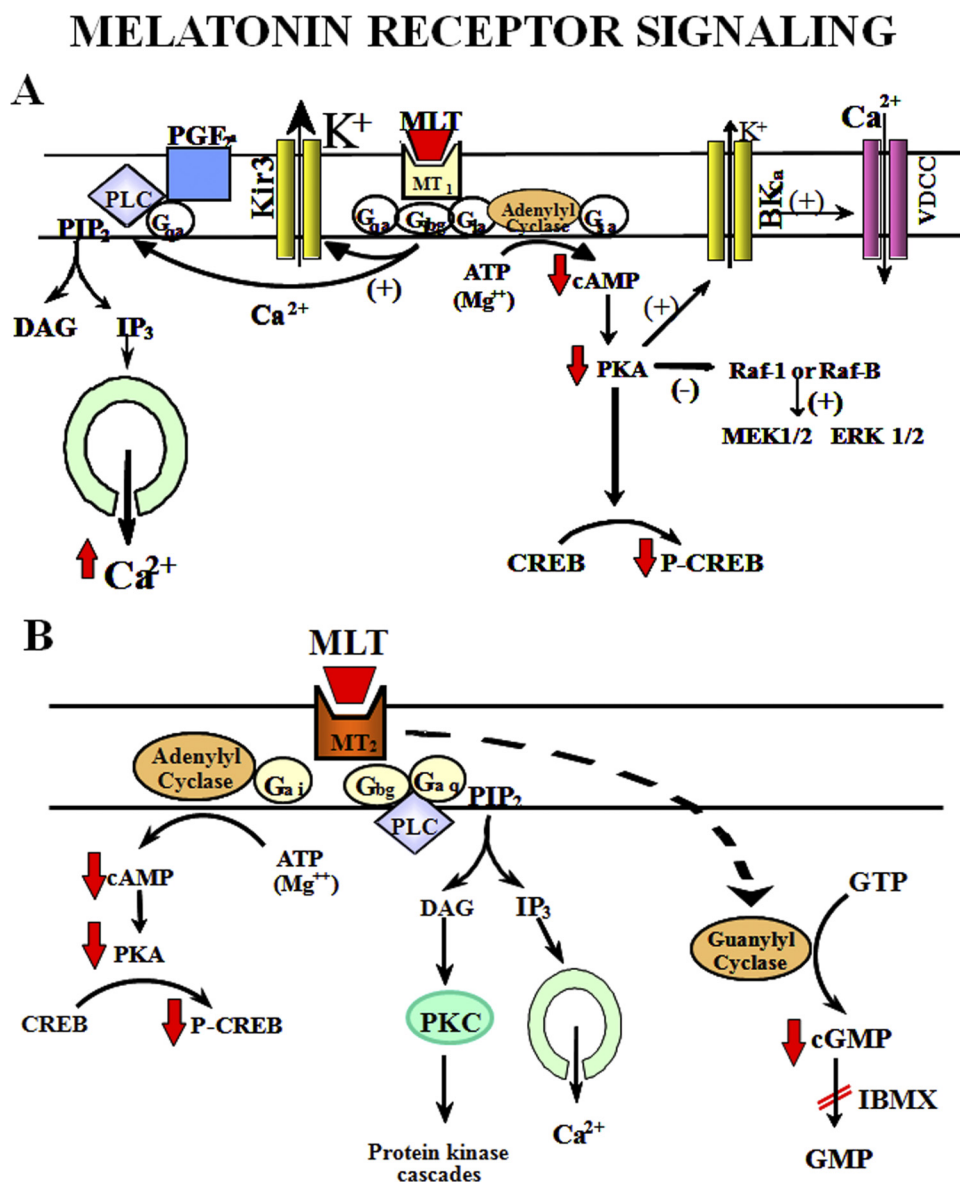


FIG. 6. MT_1 and MT_2 melatonin receptor signaling. A, melatonin (MLT) signals through activation of the MT_1 receptor via two parallel pathways mediated by the α -subunit (i.e., inhibition of cAMP formation) and the $\beta\gamma$ -subunits [i.e., potentiation of phosphoinositide turnover stimulated by a G_q -coupled receptor (R)] of G_i . B, signaling pathways coupled to MT_2 melatonin receptor activation. Melatonin-mediated phase shifts of circadian rhythms through MT_2 receptors are mediated by PKC activation (the mechanism leading to PKC activation remains putative, however). DAG, diacylglycerol; PKA, protein kinase A; R, G_q -coupled receptor (i.e., prostaglandin $F_{2\alpha}$ receptor FP and purinergic receptor P2Y) (Masana and Dubocovich, 2001). [Adapted from Masana MI and Dubocovich ML (2001) Melatonin receptor signaling: finding the path through the dark. *Sci STKE* 2001:pe39. Copyright © 2001 American Association for the Advancement of Science. Used with permission.]

Enderby et al., 1998) are also inhibited. Some authors have also proposed that the $\beta\gamma$ subunit of a PTX-sensitive G protein may mediate the potentiation of phospholipase activation by prostaglandin $F_{2\alpha}$, leading to increase in phosphoinositide turnover (Godson and Reppert, 1997) or in ATP (Roka et al., 1999). In addition, the MEK1/2-ERK1/2 pathway is stimulated by MT_1 receptors in non-neuronal cells (Witt-Enderby et al., 2000; New et al., 2003; Radio et al., 2006).

MT_1 melatonin receptors can also regulate ion fluxes and specific ion channels. Activation of endogenous MT_1 receptors in ovine pars tuberalis cells increases intracellular calcium via PTX-insensitive G proteins (Brydon et al., 1999a) (Fig. 6A). In contrast, melatonin acts via PTX-sensitive G proteins to inhibit calcium influx in neonatal rat pituitary cells (Slanar et al., 2000) and in AtT20 cells expressing MT_1 receptors (Nelson et al., 2001). Vasoconstriction seems to be mediated by decreases in cAMP-mediated phosphorylation of calcium-activated potassium channels (BK_{Ca}) through G_i/G_o protein-coupled MT_1 melatonin receptors present in the smooth muscle, although participation of receptors localized in the endothelium cannot be ruled out (Nelson and Quayle, 1995; Geary et al., 1998; Masana et al., 2002). Conversely, melatonin transiently increases BK_{Ca} channel activity in cultured rat myometrial cells (Steffens et al., 2003), an effect that can be blocked by PTX as well as by inhibition of protein kinase A activity. Inward-rectifier potassium channels (Kir) are also activated by melatonin. MT_1 melatonin receptors expressed in *X. laevis* oocytes (Nelson et al., 1996) or AtT20 cells (Nelson et al., 2001) activate Kir3 inward-rectifier potassium channels through a PTX-sensitive mechanism that may involve $\beta\gamma$ subunits of G_i proteins. Activation of Kir3 channels may underlie melatonin-mediated increases in potassium conductance (Jiang et al., 1995) and may be the mechanism by which melatonin inhibits neuronal firing in the SCN (Mason and Brooks, 1988; Shibata et al., 1989; Stehle et al., 1989). Hyperpolarization of neonatal pituitary cells may also be mediated by activation of MT_1 melatonin receptors (Vanecek and Klein, 1992). Thus the data available indicate that activation of MT_1 melatonin receptors elicits a variety of tissue-dependent signaling responses.

B. MT_2 Melatonin Receptor Signaling

Recombinant MT_2 melatonin receptors have also been shown to couple to inhibition of cAMP formation (Reppert et al., 1995a; Petit et al., 1999) (Fig. 6B). In addition, activation of MT_2 melatonin receptors also can lead to inhibition of cGMP formation (Petit et al., 1999). In the SCN, melatonin increases PKC activity through activation of MT_2 melatonin receptors, because this response is blocked by the selective MT_2 receptor antagonist 4P-PDOT (Hunt et al., 2001). This finding suggests that MT_2 melatonin receptors interact with the phospholipase C/diacylglycerol signaling pathway (McArthur et al., 1997). In the retina, MT_2 melatonin receptors inhibit neurotransmitter release through a mechanism that probably involves intracel-

lular calcium regulation (Dubocovich, 1995). Human myometrium from both pregnant and nonpregnant women expresses both MT_1 and MT_2 melatonin receptors (Schlabritz-Loutsevitch et al., 2003). In this study, 4P-PDOT blocked the melatonin-induced inhibition of cAMP signaling in cultured myometrial cells from nonpregnant women, suggesting the involvement of the MT_2 melatonin receptor. Recent data confirmed the involvement of MT_2 melatonin receptor in the action of melatonin on human myometrial smooth muscle cells and further demonstrated the involvement of PKC in MT_2 melatonin receptor signaling (Sharkey and Olcese, 2007; Sharkey et al., 2009).

C. Melatonin Receptor Regulation

Regulation of signal transduction events is essential for maintaining timely and efficient cellular responses and homeostasis. Activation of GPCRs leads to changes in receptor sensitivity (desensitization, sensitization, internalization) and trafficking, leading to changes in ligand efficacy (Ferguson, 2001). The MT_1 and MT_2 melatonin receptors are differentially and distinctly regulated by physiological (30–400 pM) and supraphysiological (1–1000 nM) concentrations of melatonin. Physiological concentrations of nocturnal melatonin (100–400 pM) are already well above the potency (EC_{50}) for the melatonin receptors, which are activated by picomolar concentrations of melatonin (Reppert et al., 1996; Dubocovich et al., 1997). Daytime concentrations typically fall below 30 pM and yet they can still induce activation and desensitization of melatonin receptors upon prolonged exposure to the hormone (~ 8 h) (Gerardin et al., 2004b). Blood melatonin levels after administration of an oral dose of 0.3 mg are similar to endogenous levels found in humans at night (Dollins et al., 1994). However, oral doses of melatonin or other ligands at ≥ 1 mg may increase blood levels several times above the concentration necessary to activate melatonin receptors and therefore may alter receptor sensitivity (Dollins et al., 1994; Vachharajani et al., 2003; Mulchahey et al., 2004; Karim et al., 2006). h MT_1 melatonin receptors expressed in heterologous mammalian cells show no observable changes in melatonin-receptor density, affinity, or functional sensitivity after exposure to physiological concentrations of melatonin for a period of time that mimics normal nocturnal exposure (i.e., 8 h) (Gerardin et al., 2004b). By contrast, exposure to supraphysiological concentrations of melatonin (100 nM) increases MT_1 receptor density and decreases receptor affinity, but there is no detectable internalization or loss of MT_1 melatonin membrane receptors in CHO cells (MacKenzie et al., 2002; Gerardin et al., 2003, 2004b). In contrast, rapid arrestin-dependent internalization of the MT_1 melatonin receptor was demonstrated in GT1–7 neurons after short-term exposure to melatonin (Roy et al., 2001). The GT1–7 cells express

low levels of endogenous MT₁ melatonin receptors and thus the presence of endogenous signaling partners different from those found in CHO and human embryonic kidney 293 cells and/or low level of constitutively active MT₁ receptors may have facilitated MT₁ melatonin receptor internalization (Dubocovich and Masana, 1998; Roka et al., 1999; Kokkola et al., 2007). Exposure to melatonin functionally desensitizes MT₁-mediated inhibition of cAMP production (Hazlerigg et al., 1993; Witt-Enderby et al., 1998; Jones et al., 2000) and stimulation of PI hydrolysis (MacKenzie et al., 2002). At high concentrations, melatonin decreases cell proliferation and transformation via activation of either hMT₁ or hMT₂ receptors expressed in NIH-3T3 cells (Jones et al., 2000). Long-term treatment of rats with melatonin did not affect modulation of neuronal firing in the SCN mediated through activation of MT₁ receptors (Ying et al., 1992). Thus, ligand alteration of MT₁ receptor function may vary with ligand concentration and cell type, but this is an important consideration for understanding the consequences of rhythmically changing hormone levels as well as therapeutic dosing.

Exposure of human MT₂ receptors in CHO cells to physiological concentrations of melatonin induced a concentration- and time-dependent receptor desensitization and internalization (Gerdin et al., 2004b). MT₂ melatonin receptor recovery after melatonin-mediated desensitization/internalization is partially dependent on new protein synthesis. MT₂ receptor resensitization after exposure to physiological levels of melatonin takes up to 8 h, whereas exposure to supraphysiological concentrations of melatonin induced a more pronounced desensitization and slower recovery, taking up to 24 h to reach pre-exposure levels (Gerdin et al., 2004b). The relation between desensitization and/or internalization of MT₁ and MT₂ melatonin receptors by the circadian production of melatonin may promote changes in melatonin receptor function in the SCN. Melatonin via activation of MT₂ receptors enhances differentiation in human adult mesenchymal stem cell osteoblasts (Radio et al., 2006). The reduction in MT₂-mediated decreases in alkaline phosphatase enzyme activity occurs when MT₂ receptors are fully desensitized, suggesting that decreases in receptor sensitivity is a necessary step in human adult mesenchymal stem cell differentiation into an osteoblast (Radio et al., 2006).

Given the potential involvement of MT₁ and MT₂ receptors in phase-shifting circadian rhythms in mammals, persistent desensitization of these receptors by supraphysiological levels of melatonin could affect circadian rhythmicity and sleep (Gerdin et al., 2004b; Wurtman, 2006). Phase advance of circadian rhythm of neuronal firing in the rat SCN brain slice by melatonin applied at circadian time (CT) 23 (CT12 is onset of activity in nocturnal animals) is mediated through activation of MT₂ receptors, which are functionally desensitized by exposure to physiological levels of melatonin (300 pM) for a length of time mimicking the nocturnal surge (8 h). Concurrent ex-

posure of the SCN brain slice to both melatonin and the MT₂ antagonist 4P-PDOT blocks the functional desensitization of MT₂ receptors (Gerdin et al., 2004b). Together these results suggest that although both the MT₁ and MT₂ receptors can be desensitized by exposure to melatonin, the receptors are differentially regulated depending on melatonin concentration (physiological versus supraphysiological), time of exposure (e.g., short versus long), cellular background (Audinot et al., 2003), and receptor state (quiescent versus constitutive) (Roy et al., 2001; Gerdin et al., 2004a,b; Kokkola et al., 2007).

IV. MT₁ and MT₂ Melatonin Receptors: Structure-Activity Relationships and Selective Ligands

A. Ligand Selectivity

Advances in medicinal chemistry led to the discovery of new molecules that are specific and selective ligands for melatonin receptors (Fig. 7A). MT₁ and MT₂ melatonin receptors show picomolar affinity for the radioligand 2-[¹²⁵I]iodomelatonin and are characterized by the following general rank order of pharmacological affinities: 2-iodomelatonin ≥ melatonin ≫ *N*-acetylserotonin ≫ serotonin, reported for COS-7 cells transiently transfected (Dubocovich et al., 1997) or NIH-3T3 (Nonno et al., 1999) and CHO cells (Browning et al., 2000) stably expressing the recombinant hMT₁ and hMT₂ receptors (Fig. 7, B and C). 2-Iodomelatonin and ramelteon show between 3 and 10 times higher affinity than melatonin on the MT₁ receptor, but both have similar affinity to melatonin on the MT₂ receptor (Dubocovich et al., 1997; Kato et al., 2005). Conversely, melatonin and 6-chloromelatonin have similar affinity on the MT₂ melatonin receptor, whereas on the MT₁ receptor, 6-chloromelatonin has 10 to 20 times lower affinity than melatonin (Dubocovich et al., 1997; Browning et al., 2000). However, ligands need approximately 50 to 100 times higher affinity/potency for one receptor type relative to the other to be considered selective (Dubocovich et al., 2000). By these criteria, melatonin analogs have been developed that show distinct selectivity for either the MT₁ or MT₂ melatonin receptor types and are discussed below, along with known structure-activity relationships.

B. Structure-Activity Relationships

The discovery of ligands selective for melatonin receptor types has been hindered by the low receptor density and heterogeneity of receptor types in native tissues and by the relatively few dependable models available in which a functional response can be ascribed to one receptor type. However, much has been learned about the structural features of the melatonin molecule that are necessary for binding and activation of its receptor. More recently with the wider use of cells expressing recombinant hMT₁ and hMT₂ receptor types, progress has been made toward developing receptor type-selective

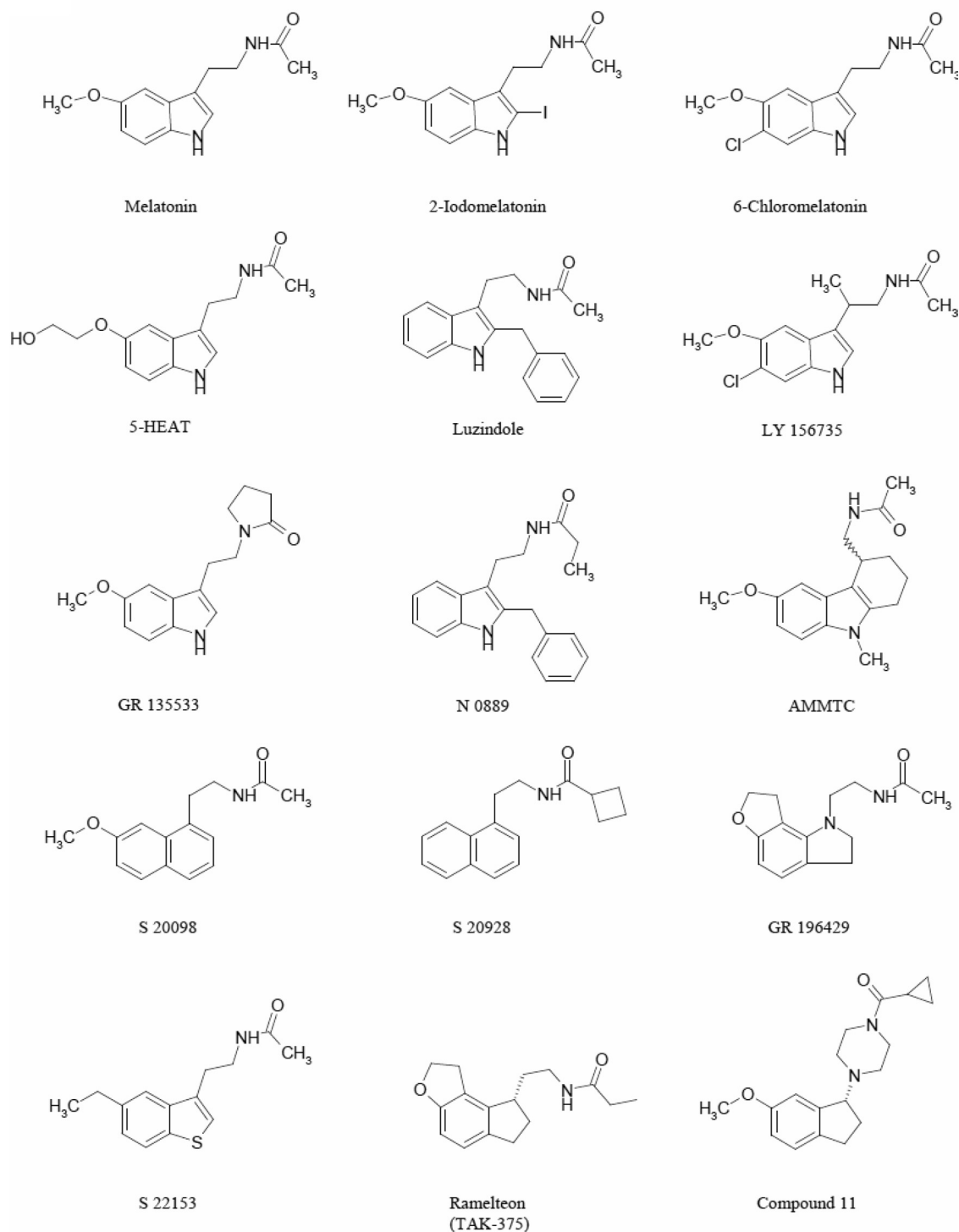
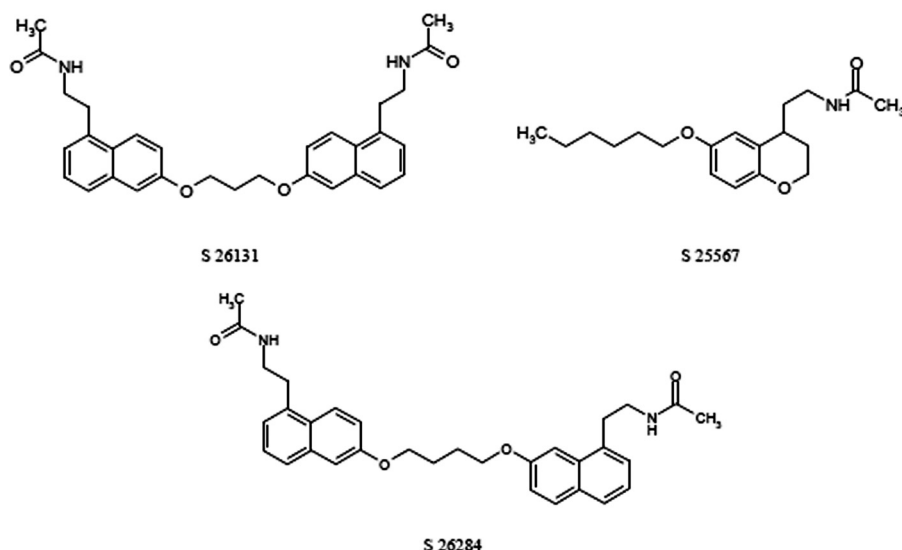
A**CHEMICAL STRUCTURES OF NON-SELECTIVE MT₁/MT₂ LIGANDS**

FIG. 7. Chemical structure of melatonin receptor ligands. A, chemical structures of nonselective MT₁/MT₂ ligands.

tive ligands. Early studies of structure-activity relationships were drawn from data obtained in native tissues. Here we will first briefly review studies carried out in native tissues and thereafter will summarize structure-activity relationships in recombinant systems (Table 4).

Melatonin has no ionizable groups at physiological pH and is a lipophilic molecule readily able to cross

the plasma membrane. Evidence suggests that the *N*-acetyl and 5-methoxy groups of melatonin are important for both receptor binding and activation. Removal of either *N*-acetyl or 5-methoxy groups leads to a dramatic reduction in 2-[¹²⁵I]iodomelatonin binding affinity (Dubocovich and Takahashi, 1987; Sugden et al., 1997), whereas 5-hydroxytryptamine (i.e., loss of

B CHEMICAL STRUCTURES OF SELECTIVE MT₁ MELATONIN RECEPTOR LIGANDSFIG. 7. Continued. B, chemical structures of selective MT₁ melatonin receptor ligands.

both *N*-acetyl and 5-methoxy groups) does not bind to melatonin receptors. Analogs with *N*-acyl groups larger than *N*-acetyl often show improved binding affinity and agonist potency.

A number of general principles have been discovered that apply to both melatonin receptors. The 5-position on the indole ring is optimal for the methoxy group, because moving it to position 4, 6, or 7 leads to a dramatic loss of affinity, although compounds with a halogen at the 5-position do retain high affinity (Mor et al., 1998). The relative position of the methoxy group and the *N*-acetylaminomethyl side chain seems to be an important determinant of affinity (Depreux et al., 1994; Langlois et al., 1995; Garratt et al., 1996). The indole ring is not essential for ligand binding because it can be replaced by various other aromatic systems such as naphthalene, benzofuran, benzothio-*phene*, or benzocycloalkene rings (Depreux et al., 1994; Leclerc et al., 1998; Fukatsu et al., 2002). If methoxy and *N*-acetylaminomethyl groups are positioned appropriately on these aromatic scaffolds, ligands with high affinity can result. Although one role of the indole nucleus is to hold the important functional groups in the proper positions, it may also play a role in ligand binding. A number of analogs with substitutions in positions 2 and 6 of melatonin (or the analogous position of ligands based on other aromatic ring systems) have been made. Substitution of a methyl, phenyl, or halogen at position 2 of melatonin can increase receptor affinity by up to 10-fold (Spadoni et al., 1993; Garratt et al., 1994a,b, 1995), and combination of an optimal *N*-acyl group with a 2-halogen substitution gives ligands with extraordinarily high affinity (e.g., 2-iodo-*N*-butanoyl-5-methoxytryptamine, *pK_i* 10.8) (Sugden and Rowe, 1994). One effect of the 2-po-

sition substituent seems likely to be to “push” the *N*-acetylaminomethyl side chain into the preferred conformation for interaction with the receptor. In addition, there is evidence that substituents in the C-2 position can interact directly with the receptor, leading to increased ligand affinity (Mathé-Allainmat et al., 1996; Spadoni et al., 1997). In vivo melatonin is degraded rapidly, primarily in the liver by 6-hydroxylation followed by conjugation and excretion in the urine. Some 6-position analogs have been synthesized with the aim of retarding metabolism (Clemens and Flaugh, 1986). A halogen substituent at the 6-position reduces affinity only slightly, whereas the affinity of 6-hydroxymelatonin is reduced by only 5- to 10-fold, and 6-methoxymelatonin by more than 100-fold (Sugden et al., 1995).

Melatonin is a rather flexible molecule with a number of bonds around which rotation is possible. The conformation melatonin adopts when interacting with its receptors has been debated. Evidence from conformationally constrained and stereoselective analogs (Jansen et al., 1996; Beresford et al., 1998; Davies et al., 1998; Leclerc et al., 1998; Jellimann et al., 2000) has provided information on the orientation likely to be adopted by the 5-methoxy and *N*-acetylaminomethyl groups when interacting with the receptor. Some of these ligands [(–)- and (+)-AMMTC] have been used as tools in experiments designed to determine whether a biological response is really mediated by melatonin receptor activation (Ting et al., 1997). Others have aided the development of receptor models of the interactions between ligand and receptor (Mor et al., 1999), and continue to guide the synthesis of novel melatonin receptor analogs.

C

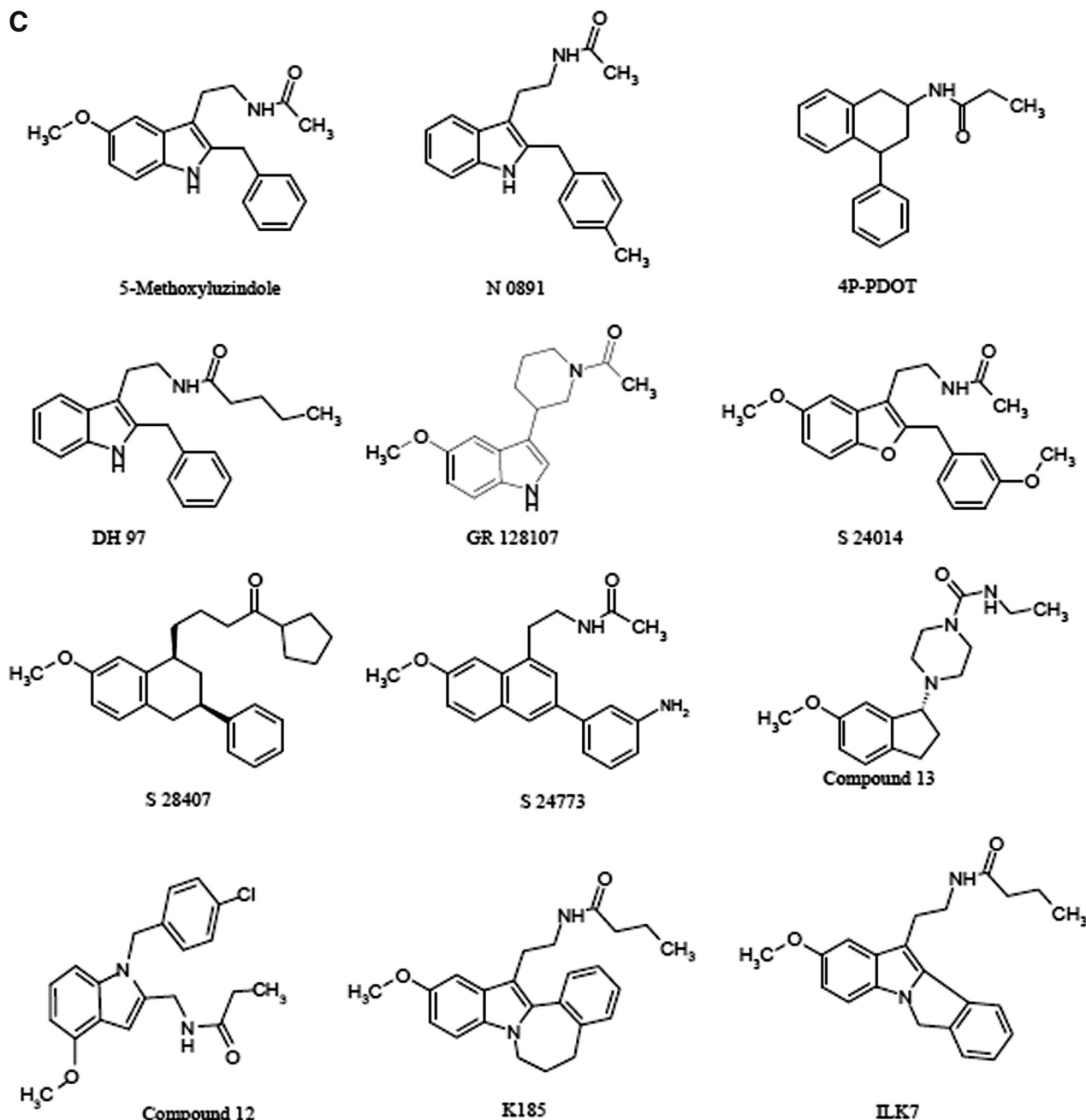


FIG. 7. Continued. C, chemical structures of selective MT_2 melatonin receptor ligands. Chemical names (see also the abbreviations list at the bottom of the first page of the article): compound 11, 1-(cyclopropylcarbonyl)-4-[(1*R*)-6-methoxy-2,3-dihydro-1*H*-inden-1-yl]piperazine; compound 12, *N*-[(1-*p*-chlorobenzyl-4-methoxy-1*H*-indol-2-yl)methyl]propanamide; compound 13, (*R*)-4-(2,3-dihydro-6-methoxy-1*H*-inden-1-yl)-*N*-ethyl-1-piperazine carboxamide; DH 97, *N*-pentanoyl-2-benzyltryptamine; GR 128107, 3-(1-acetyl-3-piperidinyl)-5-methoxyindole; GR 135533, 3-(*N*-ethyl-2-pyrrolidinone)-5-methoxyindole; GR 196429, *N*-(2-[2,3,7,8-tetrahydro-1*H*-furo[2,3-*g*]indol-1-yl]ethyl)acetamide; IIK7, *N*-butanoyl-2-(2-methoxy-6*H*-isoindolo [2,1-*a*]indol-11-yl)ethanamine; K185, *N*-butanoyl-2-(5,6,7-trihydro-11-methoxybenzo[3,4]cyclohept[2,1-*a*]indol-13-yl)ethanamine; luzindole, 2-benzyl-*N*-acetyltryptamine; LY 156735, *N*-[2-(6-chloro-5-methoxy-1*H*-indol-3-yl)propyl]acetamide; N 0889, 2-benzyl-*N*-propionyl-acetyltryptamine; N 0891, 2-(*p*-methyl-benzyl)-*N*-acetyltryptamine; S 20098, *N*-(2-[7-methoxy-1-naphthalenyl]ethyl)acetamide; S 20928, *N*-[2-naphth-1-yl-ethyl]-cyclobutyl carboxamide; S 22153, *N*-[2-(5-ethylbenzo[*b*]thiophen-3-yl)ethyl]acetamide; S 24014, *N*-[2-(2-(3-methoxybenzyl)-5-methoxy benzo[*b*]furan-3-yl)ethyl]acetamide; S 24635, *N*-[2-(5-carbamoylbenzofuran-3-yl)ethyl]acetamide; S 24773, *N*-[2-(3-(3-aminophenyl)-7-methoxy-1-naphthyl)ethyl]acetamide; S 25726, *N*-methyl-3-(2-[(cyclopropylcarbonyl)amino]ethyl)benzo[*b*]furan-5-yl)carbamate; S 25567, (*R,S*)-*N*-[2-(6-hexyloxy-3,4 dihydro-2*H*-1-benzopyran-4-yl)ethyl]acetamide; S 26284, *N*-(2-[7-[4-[(8-[2-acetylaminooxy]ethyl]-2-naphthyl)oxy]butoxy]-1-naphthyl)ethyl)acetamide; S 26553, *N*-methyl-1-[1-(2-(acetylaminooxy)ethyl)naphthalen-7-yl]carbamate; S 27533, *N*-[2-(5-methoxy-1-methyl-4-nitroindol-3-yl)ethyl]acetamide; S 28407, *N*-[2-(7-methoxy-3-phenyl-1,2,3,4-tetrahydronaphthalen-1-yl)ethyl]cyclobutyl carboxamide; TAK-375, (*S*)-*N*-[2-(1,6,7,8-tetrahydro-2*H*-indeno[5,4-*b*]furan-8-yl)-ethyl]propionamide.

TABLE 4
Pharmacological profile of ligands with agonist/partial agonist efficacy on hMT₁, hMT₂ and native melatonin receptors
pK_i, pEC₅₀ and pIC₅₀ values were obtained as detailed in footnotes.

	2-[¹²⁵ I]IMLT Binding (COS-7 Cells) ^a			CHO Cells				Inhibition [³ H]Dopamine Release in Rabbit Retina ^d (pIC ₅₀)		
	hMT ₁ (pK _i)	hMT ₂ (pK _i)	K _i Ratio (MT ₁ /MT ₂)	Stimulation GTP-γS Binding ^b		Inhibition-Forskolin Stimulated cAMP Accumulation ^c				
				hMT ₁ (pEC ₅₀)	hMT ₂ (pEC ₅₀)	pEC ₅₀ Ratio (MT ₁ /MT ₂)	hMT ₁ (pEC ₅₀)		hMT ₂ (pEC ₅₀)	pEC ₅₀ Ratio (MT ₁ /MT ₂)
2-Iodometatonin	10.2	9.7	0.3	9.7	9.8	1.1	11	10.1	0.1	11.2
S 20098	9.1	10.2	14	8.8	10	16	9.4	10.3	7.9	12
Melatonin	9.1	9.8	4.9	8.6	9.8	4.5	9.5	9.7	1.6	10.7
GR196429	8.6 ^e	9.3 ^e	4.7 ^e				8	9.0	12.6	10.5
6-Chlorometatonin	7.9	9.7	57	8.3	9.3	9.6	8.1	9.8	50	10.4
6-Hydroxymelatonin	9.4	8.3	7.3				7.2	7.4	0.6	8.7
5-Methoxyluzindole	7.5	9.6	130				N.D.	N.D.		1.3
8M-PDOT	7.2	8.5	20				N.D.	N.D.		9.3
N-Acetylserotonin	6.7	6.7	1.2				5.7	6.6	7.9	7.3
5-MCA-NAT	5.6	6.6	10				N.D.	N.D.		N.D.
IIK7	8.4 ^f	10.3 ^f	90				8.7 ^f	10.3 ^f	44	N.D.

IMLT, iodometatonin; N.D., not determined.

^a Dubocovich et al. (1997); COS-7 cells transiently transfected.

^b Audinot et al. (2003).

^c Browning et al., (2000); CHO cells stably transfected.

^d Dubocovich (1985, 1995); inhibition of calcium dependent release of dopamine elicited by electrical stimulation.

^e M. L. Dubocovich, unpublished observations; CHO cells stably transfected.

^f Faust et al. (2000); NIH 3T3 cells stably transfected.

C. Selective MT₁ and MT₂ Melatonin Ligands

Although many of the melatonin receptor ligands that have been synthesized have little receptor selectivity between MT₁ and MT₂, some progress has been made in delineating differences in the structure-activity relationships of the melatonin receptor types. Substitution of the 2-position with a benzyl group on the indole nucleus has an influence on selectivity. Indeed, luzindole was the first ligand reported to have a higher affinity for the MT₂ type and its substituted analogs. N 0891, 5-methoxyluzindole, and DH97 are MT₂-selective (Dubocovich et al., 1997; Teh and Sugden, 1998) (see Fig. 1 for full chemical names of all numbered compounds mentioned herein). A substitution with a phenyl group on 2-acetamidotetralin analogs led to one of the most MT₂ selective ligands, 4P-PDOT with pK_i of 6.3 for hMT₁ and pK_i of 8.8 for MT₂ (Dubocovich et al., 1997). Within a series of analogs in which a 2-phenyl group was attached at the ortho position to the indole N- by 1, 2, or 3 methylene groups, several MT₂-selective analogs (IIK7, K185) were identified (Sugden et al., 1999; Faust et al., 2000) indicating that the changes in ring size engendered by the N-indole-2-phenyl bridge were more readily accommodated by the MT₂ site. These results have led to the first structure-affinity relationship for MT₂ selectivity. An additional pocket at the MT₂ receptor should be present and positioned out of the plane of the aromatic nucleus of melatonin, which would not be present at the MT₁ receptor. This hypothesis is supported by a three-dimensional quantitative structure-activity relationship comparative molecular field analysis and statistical analysis (Rivara et al., 2003). Binding studies performed on the cloned receptors confirmed the MT₂ selectivity. Other publications (Wallez et al., 2002; Audinot et al., 2003; Yous et al., 2003) reported selective MT₂ ligands with a benzyl substituent in the 2-position of benzofuran bioisosters (S 24014) or a phenyl substituent in the 3-position of tetrahydronaphthalenic (S 28407) or naphthalenic (S 24773) bioisosteres. Following the same rationale, Spadoni et al. (2001) synthesized some melatonin derivatives, 2-acylaminoalkylindoles with a substitution by a benzyl in the 1-position (compound 12), that are selective for the MT₂ receptor type. Two other selective MT₂ ligands have been reported with rigidification of the side chain of melatonin with a piperidine amide chain (GR 128107) or piperazine amide chain (compound 13) (Dubocovich et al., 1997; Mattson et al., 2003). The structure-affinity relationships for MT₂ selectivity have not been explored in these series. Most MT₂ receptor-selective ligands are either antagonists or partial agonists.

The first selective MT₁ ligands were described in 2003. Their main structural feature is the presence of a bulky substituent instead of the methoxy group in the 5-position. All of them except one are dimeric derivatives in which two molecules of S 20098, the naphthalenic

analog of melatonin, are linked together through their methoxy substituent by a polymethylene side chain (Descamps-François et al., 2003). The number of methylene groups in the linking chain varied from 2 to 8; the highest selectivity ratio was for three methylene groups (S 26131). These alterations in selectivity decrease the affinity for the MT₂ type. Only one analog has been examined in a functional assay and was shown to be an antagonist with ~30- to 40-fold MT₁ selectivity. The only other selective ligand described (S 25567), which is not a dimeric derivative, bears a long alkoxy side chain at the 6-position of a benzopyran analog (Audinot et al., 2003). These MT₁-selective ligands are antagonists or partial agonists.

D. Ligand Efficacy in Native Tissues

This section focuses only on ligands for which efficacy has been reported for native mammalian melatonin receptors either in vivo or in vitro. (Dubocovich et al., 1997; Mattson et al., 2003).

1. *Agonists.* No selective MT₁ melatonin receptor agonist has been reported to date. Compound 13 [(*R*)-4-(2,3-dihydro-6-methoxy-1*H*-inden-1-yl)-*N*-ethyl-1-piperazine-carboxamidefumarate] is an agonist with selectivity for MT₂ melatonin receptors (hMT₁/hMT₂ affinity ratio = 117). Mattson et al. (2003) reported that Compound 13 had lower vasoconstrictor efficacy in rat caudal arteries compared with an equimolar concentration of melatonin. Compound 13 was also found to phase advance the circadian running-wheel activity of rats when given at dose of 1 to 56 mg/kg. However, in vivo receptor selectivity is difficult to attain because of the high affinity of melatonin ligands and the need to administer these ligands at very low doses. Even when agonists show some selectivity in in vitro assays, a low dose in vivo may activate both MT₁ and MT₂ receptors (see further discussion under section V.B.1).

2. *Antagonists/Partial Agonists.* A number of antagonists/partial agonists have been identified using native tissues, and several show selectivity for MT₂ receptors. Structural modifications of melatonin or melatonin bioisosteres that seem to predispose to antagonist action include removal of the 5-methoxy group (e.g., luzindole, 2-benzyl *N*-acetyltryptamine) and the 4-phenyl substituted tetralines (e.g., 4P-PDOT), or the naphthalenic bioisostere (e.g., S 20928) (Dubocovich et al., 1997; Conway et al., 2000; Audinot et al., 2003). Luzindole was the first ligand described as a competitive melatonin receptor antagonist (Dubocovich, 1988a), and it has been used extensively in the field to validate melatonin receptor action, although it is relatively nonselective of receptor type (MT₁/MT₂ affinity ratio = 16–26). Luzindole was the first antagonist used to demonstrate the presence of melatonin receptors mediating inhibition of dopamine release in rabbit retina (Dubocovich, 1988a; Dubocovich et al.,

1997) and the phase shift of circadian rhythms in the rodent SCN (Dubocovich, 1988a; Hunt et al., 2001). S 20928, which also behaves as an antagonist in the rat SCN (Ying et al., 1996), was used to demonstrate that the duration of melatonin-receptor exposure per day determines the onset of seasonal obesity in garden dormice (Le Gouic et al., 1996). The replacement of the 5-methoxy group by an ethyl group that prevents hydrogen bonding with the receptor led to the benzothiophene antagonist S 22153 (Audinot et al., 2003). This nonselective melatonin antagonist is able to block the phase advancing effect of melatonin on running-wheel activity in mice (Weibel et al., 1999) and the anxiolytic-like properties of melatonin (Kopp et al., 1999). Moreover, in Syrian hamster, S 22153 implants dissociate different aspects of the photoperiodic responses (Pitrosky et al., 2003) (Table 5).

4P-PDOT was the first available selective MT₂ melatonin receptor antagonist and it has been used in many studies as a pharmacological tool to demonstrate the involvement of the MT₂ receptor type in physiological function. In particular, it has been used to demonstrate the involvement of MT₂ receptors in mediating phase advances of circadian rhythm of neuronal firing in the SCN circadian clock (Dubocovich et al., 1998a; Hunt et al., 2001) and in inhibiting dopamine release in retina (Dubocovich et al., 1997). Among the other selective MT₂ ligands described, the activity on native mammalian tissues has been evaluated only for GR 128107, in which the secondary amide of the *N*-ethylamino side chain has been replaced with a constrained tertiary amide (Dubocovich et al., 1997). GR 128107 was reported to antagonize melatonin-induced inhibition of dopamine released from rabbit retina (Dubocovich et al., 1997). There are no published data available regarding MT₁ selective antagonists on native tissues.

Whereas luzindole and 4P-PDOT are competitive antagonists in native tissues, their pharmacological effects are complex in recombinant system and non-neuronal tissues. 4P-PDOT and its congener 4P-ADOT are neutral antagonists in native tissues that express a low density of melatonin receptors (Dubocovich et al., 1997, 1998a).

4P-PDOT was shown to act as a partial agonist/agonist on the inhibition of leukocyte rolling in the microcirculation (Lotufo et al., 2001) and in recombinant systems (Nonno et al., 1999; Browning et al., 2000). Moreover, it was shown using recombinant systems that the affinity ratios of 4P-PDOT (MT₁/MT₂: 66–22,000) can vary depending on the level of receptor expression or signaling pathways in each recombinant system (Dubocovich et al., 1997; Dubocovich and Masana, 1998; Nonno et al., 1999; Browning et al., 2000; Audinot et al., 2003). Results obtained with these and other less characterized ligands should be interpreted with caution, because doses used in vivo may lead to blood levels that block both MT₁ and MT₂ receptors (Dubocovich et al., 1998).

TABLE 5

Pharmacological profile of partial agonists/antagonists on hMT₁, hMT₂, and native melatonin receptors

All values are from Dubocovich et al. (1997) except as noted otherwise.

Melatonin Receptor Ligands	2-[¹²⁵ I]MLT Binding			³ H]DA Release in Rabbit Retina MT ₂ (pA ₂)	GTPγS Binding		
	Ratio hMT ₁ /hMT ₂	hMT ₁ pK _i	hMT ₂ pK _i		hMT ₁ K _B	hMT ₂ K _B	Ratio hMT ₁ /hMT ₂
4P-CADOT	362	6.5	9.1	9.3			
4P-ADOT	330	6.2	8.9	8.8			
4P-PDOT	311	6.3	8.8	9.5			
K185 ^a	132	7.2	9.3	N.D.			
5-Methoxyluzindole	130	7.5	9.6	10.2			
S 24014 ^b	125	7.5	9.6		7.3	8.8	30
GR 128107	113	7.0	9.1	10.2			
N 0891	91	6.1	8.0	7.7			
DH97 ^c	89	6.1	8.0	N.D.			
N 0890	56	6.2	7.9	7.5			
N 0889	31	6.8	8.3	7.8			
Luzindole	16	6.8	8.0	7.7			
N-Acetyltryptamine	15	6.7	7.9	8.0			
GR 135533	15	6.5	7.7	8.6			
S 20928 ^b	3.1	7.95	8.22		7.1	7.9	7.1
S 22153 ^b	1.9	8.85	8.15		8.5	7.9	2.5
5-HEAT ^d	0.2	7.8	7.1	N.D.			
S 25567 ^b	0.16	8.5	7.7		8.4	7.7	0.23
S 26284 ^b	0.01	8.85	7		8	7.4	0.28
S 26131 ^b	0.01	8.85	6.9		8.3	6.8	0.04

IMLT, iodomelatonin; DA, dopamine; 4P-CADOT, 4-phenyl-2-chloro-acetamidotetraline; N.D., not determined; 5-HEAT, 5-hydroxyethoxy-N-acetyltryptamine.

^a Values from Faust et al., 2000.^b Audinot et al., 2003.^c Teh and Sugden, 1999.^d Nonno et al., 2000.

3. Inverse Agonists. Recombinant MT₁ melatonin receptors expressed at physiologically relevant levels are capable of constitutive activity, producing spontaneous regulation of effectors in the absence of agonist activation (Roka et al., 1999). Inverse agonists stabilize the free/uncoupled form of the receptor and are used to detect constitutive activity by reducing agonist-independent receptor activity (Browning et al., 2000). In this regard, a few of the melatonin ligands described above were also shown to reduce agonist-independent MT₁ melatonin receptor activity. As inverse agonists, luzindole and 4P-PDOT have higher apparent affinity for the hMT₁ melatonin receptor in the presence of GTP (Dubocovich and Masana, 1998), decreasing basal guanosine 5'-O-(3-[³⁵S]thio)triphosphate binding (Glaser et al., 1998) and increasing cAMP formation (Browning et al., 2000) in CHO cells expressing the hMT₁ receptor at high density.

4. Dimers/Heterodimers. The three members of the melatonin receptor gene family expressed in humans, MT₁, MT₂, and related orphan receptor GPR50, have been shown to dimerize in transfected human embryonic kidney 293 cells (Levoye et al., 2006b). It is noteworthy that these proteins not only self-associate to form stable homodimers but can also form heterodimers in cells coexpressing various gene products (Ayoub et al., 2002, 2004; Levoye et al., 2006a). Several lines of evidence indicate that the formation of melatonin receptor heterodimers may also have a pronounced impact on receptor function. A modified pharmacological profile has been observed for MT₁/MT₂ heterodimers compared with the corresponding homodimers (Ayoub et al., 2004). The propensity of MT₁/MT₂ heterodimer formation is

similar to or even higher than that of the corresponding homodimers. Both the MT₁ and MT₂ binding sites are functional within the heterodimer. The two binding sites within the heterodimer maintain their respective selectivity for MT₁- and MT₂-selective ligands. Furthermore, the ligand-interaction profile of the MT₁/MT₂ heterodimer determined is distinct from that of the MT₂ homodimer. Although further studies will be necessary to firmly establish the existence of such complexes in tissues and their physiological relevance, the existence of such complexes may be anticipated. A widespread coexpression of melatonin receptors in several tissues such as the retina and different regions of brain has indeed been reported by several groups (Dubocovich, 1983; Reppert et al., 1994; Dubocovich et al., 1998a; Savaskan et al., 2002a,b, 2005; Wu et al., 2007). Engagement of MT₁ into heterodimers with the orphan GPR50 completely abolished melatonin binding to MT₁ and coupling of this receptor to G_i proteins and β-arrestins (Levoye et al., 2006a). This may represent an interesting regulatory mechanism for melatonin function (Jockers et al., 2008).

V. MT₁- and MT₂-Mediated Functional Responses

The identification of functional responses mediated by MT₁ melatonin receptors has been hampered by the lack of specific and selective MT₁ melatonin receptor agonists and antagonists. Nevertheless, the use of molecular, pharmacological, and immunohistochemical approaches in conjunction with nonselective MT₁/MT₂ melatonin receptor ligands and the use of tissues from animals with genetic deletion of the MT₁ and/or MT₂ melatonin

receptors has allowed the identification of several MT₁ and MT₂ melatonin receptor-mediated functional responses (see Table 6).

Evidence suggests that endogenous melatonin may act to modulate melatonin-mediated functions; however, removal of the pineal gland or genetic deletion of either the MT₁, the MT₂, or both the MT₁ and MT₂ melatonin receptors have provided minimal evidence for a functional role of endogenous melatonin. Early studies by Quay (1968, 1970a,b) demonstrated that pinealectomy accelerates the rate of re-entrainment after a shift in the photoperiod, but did not alter re-entrainment either when animals are kept in a light/dark cycle or are free running in constant conditions. These results suggested a role for an endogenous pineal product (e.g., melatonin) on circadian entrainment; however, the mechanism of this phenomenon is still unclear. Mice with genetic deletion of the MT₁, MT₂, or MT₁/MT₂ melatonin receptors show no circadian phenotype in experiments reported so far (Liu et al., 1997; Jin et al., 2003; Dubocovich et al., 2005; Dubocovich, 2007). Mice lacking the MT₁ melatonin receptor display depression-like behavior in the swimming test and deficits in sensory gating as demonstrated in the acoustic startle/prepulse inhibition (Weil et al., 2006). The MT₂ receptor knockout mice do not exhibit melatonin-mediated hippocampal long-term potentiation (Wang et al., 2005) or luzindole-mediated decrease in immobility in the swimming test (Sumaya et al., 2005) compared with wild-type (WT) mice. Yasuo et al. (2009) reported that the MT₁ melatonin receptor in the mice is directly involved in transmitting photoperiod information and affecting reproductive function. These results then go along with the discovery that the Siberian hamster, a seasonal breeder in which reproductive function responds to changes in photoperiod, does not express the MT₂ melatonin receptor (Weaver et al., 1996). Whether these phenotypes are due to lack of activation of melatonin receptors and/or to indirect alterations in brain neuronal pathways remains an open question.

A. Melatonin Receptor Expression

In mammals, the MT₁ and MT₂ receptors seem to mediate the physiological effects of endogenous and exogenous melatonin (Reppert et al., 1994, 1995a; Liu et al., 1997; Masana et al., 2002; Dubocovich et al., 2005). As discussed above, these receptors show distinct molecular structures, chromosomal localizations, and pharmacological profiles (Reppert et al., 1994, 1995a; Slauenhaupt et al., 1995; Dubocovich et al., 1997).

Melatonin receptors have been localized in the human brain and peripheral tissues using receptor autoradiography with 2-[¹²⁵I]iodomelatonin, mRNA expression by RT-PCR, and in situ hybridization as well as immunohistochemistry. MT₁ and MT₂ melatonin receptors mRNA has been amplified from human brain cerebellum, occipital cortex, parietal cortex,

temporal cortex, thalamus, frontal cortex, hippocampus, and SCN (Mazzucchelli et al., 1996). Beresford et al. (1998) characterized the pharmacological profile of 2-[¹²⁵I]iodomelatonin binding in post mortem human cerebellar membranes. The profile of this site is identical to that of the MT₁ human recombinant melatonin receptor expressed in either COS-7 or CHO cells (Dubocovich et al., 1997; Beresford et al., 1998). Using in situ hybridization histochemistry, MT₁ mRNA was localized to cerebellar granule cells and basket-stellate cells (Mazzucchelli et al., 1996; Weaver and Reppert, 1996; Al-Ghoul et al., 1998). MT₁ mRNA also has been localized in the retina and SCN of human postmortem brain, consistent with the important role of these structures in biological rhythmicity (Reppert et al., 1994, 1995a; Mazzucchelli et al., 1996; Weaver and Reppert, 1996). Within the retina, MT₁ mRNA and protein have been localized to ganglion, amacrine, and photoreceptor cells using RT-PCR and/or immunohistochemistry (Savaskan et al., 2002a; Scher et al., 2002, 2003). MT₁ receptor-like immunoreactivity appears higher in ganglion and amacrine cells from retinas of subjects with Alzheimer's disease, although immunoreactivity was decreased in photoreceptor cells (Savaskan et al., 2002b). MT₁ immunoreactivity in the hippocampus was also increased in Alzheimer's disease subjects (Savaskan et al., 2001).

MT₂ mRNA has been found in human retina, hippocampus, and whole brain (Reppert et al., 1994, 1995a; Weaver and Reppert, 1996). MT₂ melatonin receptor immunoreactivity was localized to ganglion and bipolar cells in the inner nuclear layer of the retina, and to the inner segments of the photoreceptor cells. In addition, cellular processes in inner and outer plexiform layers of the retina were strongly positive for MT₂ (Savaskan et al., 2007). Within the hippocampus, MT₂ receptors are found in hippocampal pyramidal and granular neurons (Savaskan et al., 2005). In contrast to MT₁ receptors, the expression of MT₂ receptors was reduced in subjects with Alzheimer's disease in both hippocampal and retinal tissue (Savaskan et al., 2005, 2007). In the cerebellum, the location of MT₂ receptor mRNA is distinct from that of MT₁ receptors; MT₂ expression was restricted to Bergmann glial cells and astrocytes (Al-Ghoul et al., 1998).

MT₁ and MT₂ receptor mRNAs are also found in non-neural human tissues. Using RT-PCR, mRNA for both types has been amplified from human fetal kidney (Drew et al., 1998), granulosa cells (Niles et al., 1999), PAZ6 adipocytes (Brydon et al., 2001), and coronary arteries (Ekmekcioglu et al., 2001a). MT₁ melatonin receptors were localized by immunocytochemistry to both adult and fetal human kidney (Song et al., 1997; Drew et al., 1998). Ekmekcioglu et al. (2001b) observed a 24-h variation in the expression of the MT₁ melatonin receptors in coronary arteries derived from patients with coronary heart disease. MT₁ receptors were also found in cerebral blood vessels and the central vessels of human

TABLE 6
Melatonin receptor-mediated functional responses in native tissues

Modified from Dubocovich et al. (2003).

Function	Receptor	Signaling	Tissue	Approach	References
CNS	Inhibition—neuronal firing in the SCN	Increase in K ⁺ conductance?	SCN	MT ₁ -KO	Liu et al., 1997; Jin et al., 2003
	Inhibition—PACAP-stimulated CREB phosphorylation	cAMP Inhibition	SCN	MT ₁ -KO	von Gall et al., 2000
	Phase shift—circadian rhythm of neuronal firing rhythms in the SCN brain slice	UNK	SCN	MT ₁ MT ₂ -KO	Jin et al., 2003
	Phase shift—circadian rhythm of wheel activity	PKC activation	SCN	MT ₁ -KO	Liu et al., 1997;
		UNK		4P-PDOT	Dubocovich et al., 2005
Hypothalamic-Hypophyseal-Gonadal Axis	Inhibition—dopamine release from rabbit retina	UNK	Rabbit retina	Luzindole	Hunt et al., 2001
	Inhibition—long term potentiation	UNK	Hippocampus	MT ₁ -KO	Dubocovich et al., 2005
		cAMP/PKA Inhibition		MT ₂ -KO	
				Luzindole	
				4P-PDOT	
Cardiovascular	Inhibition—prolactin secretion	UNK	Anterior pituitary	MT ₁ -KO	von Gall et al., 2002a
	Regulation of <i>Per1</i> gene expression	cAMP Inhibition	Anterior pituitary	MT ₁ -KO	von Gall et al., 2002a
	Inhibition—Testosterone secretion	cAMP Inhibition	Leydig cells in testis	Luzindole	Frungieri et al., 2005
	Inhibition—Cortisol secretion	Corticotropin/CRH inhibition	Adrenal from Capuchin monkey	MT1 antibody	Torres-Farfan et al., 2003, 2004
	Regulation of oxytocin receptor gene expression and contractility in uterus	PLC, PKC activation	Human myometrium	Luzindole	Sharkey and Oleese, 2007;
Immune	Regulation of photoperiodic information	Type 2 and 3 deiodinase	Pars tuberalis	4P-PDOT	Sharkey et al. 2009
	Vasodilation	UNK	Rat caudal artery	MT ₁ -KO	Yasuo et al., 2009
		Activation of BK _{Ca} channel	Cerebral arteries	MT ₂ -KO	
				MT ₁ MT ₂ -KO	
				Luzindole	Krause et al., 1995; Geary et al., 1997, 1998; Viswanathan et al., 1997; Ting et al., 1999
Metabolism	Increase—splenocyte proliferation (i.e., cell-mediated immunity)	UNK	Spleen	4P-ADOT	Doolen et al., 1998;
	Increase—serum anti-keyhole IgG concentrations (i.e., humoral immunity)	UNK	T cells	4P-PDOT	Masana et al., 2002
	Inhibition of leukocyte rolling	UNK	Microvasculature	MT ₁ -KO	Drazen and Nelson, 2001
	Inhibition—insulin secretion	cAMP inhibition	Pancreatic β-cells	MT ₁ -KO	Drazen and Nelson, 2001
		GLP-1 inhibition		MT ₂ -KO	
Increased leptin production	Inhibition—insulin secretion	cAMP Inhibition	Pancreatic β-cells	4P-PDOT	Lotufo et al., 2001
				4P-PDOT	
				Luzindole	Peschke et al., 2000, 2002; Kemp et al., 2002; Mühlbauer et al., 2009
				MT ₁ -KO	
				MT ₂ -KO	
Increased leptin production	Inhibition—insulin secretion	cAMP Inhibition	Pancreatic β-cells	MT ₁ MT ₂ -KO	Mühlbauer et al., 2009
				MT ₁ -KO	
				MT ₂ -KO	
				MT ₁ MT ₂ -KO	
				4P-PDOT	Alonso-Vale et al., 2005

TABLE 6.—continued

Function	Receptor	Signaling	Tissue	Approach	References
Cancer					
Inhibition—cancer cell proliferation	MT ₁	cAMP Linoleic acid uptake 13-HODE production	Breast tumor Rat hepatoma	4P-PDOT Luzindole S20928	Dauchy et al., 2003; Blask et al., 2005a,b
Inhibition—cell proliferation	MT ₂	cAMP Linoleic acid uptake 13-HODE production	JAr cells Rat hepatoma	Luzindole 4P-PDOT	Shiu et al., 1999; Dauchy et al., 2003

UNK, unknown/undetermined etiology; PKA, protein kinase A; CRH, corticotropin-releasing hormone; PLC, phospholipase C; GLP-1, glucagon-like peptide; 13-HODE, (±)-13-hydroxy-9Z,11E-octadecadienoic acid.

and macaque retinas (Savaskan et al., 2002a; Scher et al., 2002, 2003). MT₁ receptor levels were increased in cerebral blood vessels of subjects with Alzheimer's disease (Savaskan et al., 2001).

Mouse and rat brain (e.g., hippocampus), retina, and peripheral tissues such as heart, lung, liver, and kidney (Naji et al., 2004; Sallinen et al., 2005) express mRNA for both MT₁ and MT₂ melatonin receptors. mRNAs for both receptor types also have been demonstrated in rat vasculature, where MT₁ receptors mediate vasoconstriction and MT₂ receptors mediate vasodilation (Doolen et al., 1998; Masana et al., 2002).

In the SCN of the C3H/HeN mouse, MT₁ and MT₂ receptor mRNAs were localized by in situ hybridization (Dubocovich et al., 1998a). MT₁ and MT₂ melatonin receptor mRNA and protein have been reported in the mammalian SCN (Reppert et al., 1988; Siuciak et al., 1990; Dubocovich et al., 1998a; Hunt et al., 2001; Rivera-Bermúdez et al., 2004). However, the MT₂ melatonin receptor protein in the SCN is expressed at such a low level that it is undetectable by 2-[¹²⁵I]iodomelatonin binding (Liu et al., 1997; Dubocovich et al., 1998a; Poirel et al., 2002).

Melatonin receptor gene expression can change markedly in neural and peripheral tissues over the lifespan of an organism (Davis, 1997). In addition, 24-h variations in MT₁ receptor mRNA expression, as well as repressed MT₁ receptor transcription, under short photoperiod conditions have been reported (Masana et al., 2000; Schuster et al., 2001; Poirel et al., 2002). Little is known, however, about the mechanisms that drive these changes in melatonin receptor gene expression. The transcription factors Pitx-1 and Egr-1 have been shown to regulate the MT₁ receptor expression in both rats and sheep (Johnston et al., 2003b, 2007).

B. Melatonin Receptor Function

1. Central Nervous System. In the SCN, functional MT₁ and MT₂ receptors have been characterized in pharmacological studies using the two melatonin receptor antagonists luzindole and 4P-PDOT as well as in studies of mice with genetic deletion of either the MT₁ or/and MT₂ receptors (von Gall et al., 2000, 2002a; Jin et al., 2003; Dubocovich et al., 2005). Melatonin applied in vitro to rodent SCN brain slices inhibits neuronal firing in a concentration-dependent manner (Shibata et al., 1989; Liu et al., 1997). Melatonin inhibits neuronal firing in SCN brain slices from wild-type and MT₂ KO mice but not MT₁ KO mice (Liu et al., 1997; Jin et al., 2003). Together, these data suggest that the MT₁ receptor mediates the inhibitory effects of melatonin in the SCN (Liu et al., 1997). In the SCN, melatonin, through activation of MT₁ melatonin receptors, inhibits pituitary adenylate cyclase-activating polypeptide-stimulated phosphorylation of the transcription factor CREB, an early event in the signaling cascade leading to phase-shifts of the endog-

enous clock (von Gall et al., 2000; Jin et al., 2003). These effects of melatonin were not observed in the MT₁ KO mice.

Melatonin released from the pineal gland after a circadian rhythm with high levels at night may exert feedback onto the SCN and activates MT₁ and MT₂ receptors to phase shift local and overt circadian rhythms (Gillette and Mitchell, 2002). To determine the melatonin receptor type (MT₁ and/or MT₂) involved in phase shifting circadian rhythms in the SCN, circadian rhythms of neuronal firing in vitro and overt activity rhythms in vivo were measured (Liu et al., 1997; Dubocovich et al., 2005; Dubocovich, 2007). Melatonin (1–10 pM) given at CT10 phase advances, by approximately 3 to 4 h, the circadian rhythms of neuronal firing in rat and mouse SCN. This effect was blocked by MT₂-selective concentrations of the competitive melatonin receptor antagonist 4P-PDOT and was absent in SCN from mice with genetic deletion of the MT₂ receptor (Hunt et al., 2001; Dubocovich et al., 2005; Dubocovich, 2007). The magnitude of the phase shift induced by melatonin at 10 pM in the SCN brain slice from WT mice and MT₁ KO mice was identical. Together, these results suggest that the MT₂ melatonin receptor mediates phase shifting of circadian rhythms of neuronal firing in the SCN in vitro (Liu et al., 1997; Dubocovich et al., 2005). Liu et al. (1997) reported that the phase shift of neuronal firing rhythms induced by the MT₁/MT₂ melatonin agonist 2-iodomelatonin (10 pM) was of smaller magnitude in the SCN brain slice from MT₁ KO mice than in that from the WT mice, suggesting a potential role for MT₁ melatonin receptor activation. It is conceivable that in this experimental paradigm, 2-iodomelatonin phase shifted circadian rhythms through the activation of the MT₁ (Liu et al., 1997). Recent evidence suggests that melatonin at 1 nM phase advanced circadian rhythms of neuronal firing in the mouse SCN brain slice through activation of MT₁ melatonin receptors (R. L. Hudson and M. L. Dubocovich, unpublished observations). Together, these results show that, at least under the experimental conditions used in the mouse, both the MT₁ and MT₂ receptors mediated phase advances, with the shift induced through activation of the MT₂ receptor being significantly larger (Liu et al., 1997; R. L. Hudson and M. L. Dubocovich, unpublished observations). It is noteworthy that in the Siberian hamster, which does not encode a functional melatonin MT₂ melatonin receptor, melatonin at 1 nM induced a robust phase shift of the circadian rhythm of neuronal firing rate in the SCN slice (Weaver et al., 1996). Whether this response is mediated by the MT₁ melatonin receptor, which is functional in the hamster, or a receptor not yet characterized is an open question.

Studies intended to assess the melatonin receptor type involved in phase-shifting circadian rhythms in an

in vivo mouse model led to unexpected results. In this model, melatonin treatment phase-shifted circadian rhythms of wheel-running activity in the WT and MT₂KO mice kept in constant dark when given 2 h before onset of activity but not in the MT₁KO or MT₁MT₂KO mice, suggesting that in vivo activation of the MT₁ receptors phase advances overt circadian rhythms (Dubocovich et al., 2005; Dubocovich, 2007). Melatonin accelerated re-entrainment to an advance in dark onset through activation of the MT₁ receptor (Dubocovich et al., 2005). Together, these results suggest the involvement of an MT₁ melatonin receptor in the phase-shifting process in vivo. These paradoxical findings (i.e., phase shift by MT₂ activation in the SCN in vitro and by MT₁ activation in vivo) are difficult to explain at the present time, although it is conceivable that distinct melatonin receptors are necessary to phase-shift circadian rhythms of activity and neuronal firing rhythms. Participation of both the MT₁ and MT₂ receptors to phase shift circadian rhythms within the SCN cannot be excluded (Liu et al., 1997). The selective MT₂ melatonin receptor antagonist 4P-PDOT blocked the melatonin-mediated phase advance of circadian activity rhythms induced by melatonin given in vivo at CT 10 (Dubocovich et al., 1998b), suggesting that melatonin-mediated phase advances were mediated by activation of MT₂ melatonin receptors. However, the doses of 4P-PDOT (90 µg/mouse s.c.) used in the above-mentioned report (Dubocovich et al., 1998b) may have raised levels of drug in the circulation to micromolar concentrations that might have blocked both MT₁ and MT₂ melatonin receptors. Mattson et al. (2003) concluded that a synthetic indanyl piperazine (compound 13) with approximately 117 times higher affinity for the MT₂ receptor (K_i = 200 nM for MT₁; 1.7 nM for MT₂) phase-shifted circadian activity rhythms in the rat through activation of MT₂ receptors using doses of 1 to 56 mg/kg. These doses would probably reach blood concentrations of at least 1 µM, which would fully activate both the MT₁ and MT₂ receptors, thereby acting as a nonselective agonist. Further research to determine the specific mechanisms for phase shifting in the SCN is needed to explain these results, as well as to help determine how best to therapeutically target the MT₁ and MT₂ receptors for treatment of sleep and circadian rhythm disorders.

The first functional MT₂ melatonin receptor characterized in a native tissue was the melatonin presynaptic heteroreceptor that modulates the calcium-dependent release of dopamine from rabbit retina (Dubocovich, 1985, 1995; Dubocovich et al., 1997, 1998a). The signaling pathway of the rabbit retina receptor is not known. However, in other tissues, the MT₂ receptor seems to be linked to PKC activation; in the rat SCN, activation of an MT₂ melatonin receptor, possibly via Gβγ stimulates PKC (McArthur et al., 1997; Hunt et al., 2001). This

response is blocked by 4P-PDOT, a selective MT₂ receptor antagonist.

Melatonin inhibits, in a concentration-dependent manner (IC₅₀ = 100 nM), long-term potentiation and excitability induced by stimulation of the Schaffer collaterals in the cornu ammonis 1 dendritic layer in the mouse hippocampal brain slices (Wang et al., 2005). Luzindole and 4P-PDOT blocked this inhibitory effect of melatonin. Furthermore, the inhibitory effect of melatonin was impaired in the MT₂KO but not in the MT₁KO mouse. The protein kinase A inhibitor 5-isoquinolinesulfonamide (H-89) mimicked the inhibitory effect of melatonin on long-term potentiation, whereas forskolin counteracted melatonin-mediated inhibition (Wang et al., 2005). In summary, in the mouse hippocampus, melatonin inhibits long-term potentiation through activation of the MT₂ melatonin receptor and inhibition of the adenylyl cyclase-protein kinase A pathway.

More recently it has been reported that exogenous melatonin could increase the survival of neuronal progenitor cells and postmitotic immature neurons in the hippocampus of adult mice (Ramírez-Rodríguez et al., 2009), consistent with melatonin actions on proliferative activity in the rat dentate gyrus and embryonic neural stem cells (Kim et al., 2004; Moriya et al., 2007). These findings are particularly interesting in view of the recent reports that melatonin can reduce learning and memory deficits in mice models of Alzheimer disease (Feng et al., 2004; Olcese et al., 2009).

2. Hypothalamic-Pituitary-Gonadal Axis. Endogenously released melatonin resulting from changes in day length modulates reproduction in seasonal breeders in part through activation of melatonin receptors in the hypothalamic-pituitary-gonadal axis. (Reiter, 1980; Tamarin et al., 1985; Vanecek, 1998; Malpoux et al., 2001; Roy et al., 2001; Soares et al., 2003; Frungieri et al., 2005). In immortalized GnRH-releasing cells, activation of endogenous MT₁ and MT₂ receptors decreased the expression of GnRH mRNA in a 24-h cyclical manner, which was blocked by luzindole (Roy et al., 2001). In the neonatal rat pituitary gland, melatonin inhibits GnRH-induced LH release, cAMP and cGMP accumulation, and increases in intracellular Ca²⁺ through activation of a pertussis toxin-sensitive GPCR (Martin et al., 1980; Vanecek and Vollrath, 1990; Vanecek and Klein, 1995). The mechanism(s) by which melatonin modulates pituitary gonadotropin secretion involves activation of MT₁ melatonin receptors (Johnston et al., 2003a,b); however, participation of MT₂ receptors (Balík et al., 2004) cannot be excluded.

Regulation of ovarian and testicular function by melatonin may involve activation of both MT₁ and MT₂ receptors along the hypothalamic-pituitary-gonadal axis. Melatonin may also exert direct effects on ovarian function, because it is found in ovarian follicular fluid (Brzezinski et al., 1987; Rönnberg et al., 1990). Specific 2-[¹²⁵I]iodomelatonin binding, as well as MT₁ and MT₂ melatonin receptor mRNAs and MT₁ melatonin receptor protein, was identified in

various ovarian structures (Niles et al., 1999; Clemens et al., 2001; Woo et al., 2001; Soares et al., 2003). Endogenous estrogens regulate the functional activity of melatonin receptors (Soares et al., 2003), whereas melatonin stimulates progesterone secretion from granulosa cells in several species including humans (Schaeffer and Sirotkin, 1995; Woo et al., 2001). In human granulosa-luteal cells, melatonin increases LH and decreases GnRH receptor density (Woo et al., 2001). In hamster testicular Leydig cells, melatonin inhibits basal and chorionic gonadotropin-stimulated cAMP and androgen (testosterone and androstene 3 α -diol 17 β -diol) production through activation of MT₁ receptors (Frungieri et al., 2005).

Prolonged treatment with melatonin causes sensitization of MT₁ melatonin receptor signaling upon withdrawal involving potentiation of adenylate cyclase and CREB phosphorylation, leading to increases in gene expression or hormone secretion. Nocturnal melatonin secretion regulates gene expression through heterologous sensitization of adenylate cyclase-linked GPCRs, thereby coupling the central circadian pacemaker with the circadian regulation of peripheral tissues, which results in modulation of circadian and seasonal rhythms. The nocturnal secretion of pineal melatonin suppresses the expression of the clock gene *Per1* in the pars tuberalis of the pituitary gland, by inhibiting the cAMP dependent signaling pathway through activation of the MT₁ receptor (von Gall et al., 2002a). At dawn, when circulating melatonin levels decrease, the pars tuberalis is released from transcriptional repression, facilitating the induction of *Per1* gene expression by heterologous sensitization of adenosine A2b receptors (von Gall et al., 2002a). Furthermore, simultaneously during the biological night, endogenous melatonin, through activation of the MT₁ melatonin receptor, inhibits prolactin release in the pars tuberalis (Table 6). This may be a general mechanism by which the circadian production of melatonin provides links to the central circadian pacemaker and peripheral tissues, imparting the signal of darkness.

A relationship between the circadian rhythm of melatonin and adrenal hormone secretion has been described in mammals. In humans, plasma cortisol peaks in the early morning but remains low during the night when circulating melatonin levels are high (Weitzman et al., 1971). Adult and fetal adrenal gland of the capuchin monkey expresses 2-[¹²⁵I]iodomelatonin binding sites and MT₁ but not MT₂ melatonin receptor mRNA (Torres-Farfan et al., 2003, 2004). Melatonin inhibited corticotropin-releasing hormone- and adrenocorticotropin-induced cortisol production and decreased dibutyl cAMP-induced cortisol secretion from adrenal cultured cells. This MT₁-mediated effect was blocked by luzindole.

In several mammalian species, changes in photoperiod regulate reproduction via the duration of the melatonin signal known to encode the length of the night (Bartness et al., 1993). Reproduction in ham-

sters is regulated by melatonin-mediated photoperiodic signals; however, this species does not express functional MT₂ melatonin receptors (Weaver et al., 1996). Although reproductive responses are assumed to be mediated by activation of MT₁ melatonin receptors, direct evidence for this assumption was missing. Yasuo et al. (2009) recently reported differential regulation of photoperiod-induced changes in *Diodinase 2* and *Diodinase 3* expression (Ono et al., 2008) in C3H mice with genetic disruption of the MT₁, MT₂, or MT₁MT₂ melatonin receptors. These studies provided evidence for distinct functions for the MT₁ and MT₂ melatonin receptors in the regulation of photoperiodic responses in pars tuberalis and confirmed that melatonin through activation of the MT₁ melatonin receptor transmit photoperiod information to the hypothalamus-hypophyseal axis to regulate reproductive function (Yasuo et al., 2009).

3. Cardiovascular System. A number of studies point to a role for melatonin in cardiovascular regulation (Krause et al., 1999). MT₁ and MT₂ protein and mRNA are expressed in peripheral and cerebral arteries of various species, including humans (Savaskan et al., 2001; Masana et al., 2002). Activation of MT₁ and MT₂ receptors mediates vasoconstriction and vasodilation, respectively. In the rat caudal artery, which is important for thermoregulation, melatonin causes vasoconstriction, directly contracting pressurized arterial segments (Evans et al., 1992) and potentiating contraction induced by adrenergic nerve stimulation or norepinephrine (Krause et al., 1995; Geary et al., 1998). Various melatonin receptor analogs act as full or partial agonists in the rat caudal artery model, the enantiomers of the melatonin agonist *N*-acetyl-4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole showing a difference (400-fold) in vasoconstrictor potency matching the known difference in melatonin receptor affinity of the two enantiomers (Krause et al., 1995; Ting et al., 1997). Vasoconstriction seems to be mediated by MT₁ receptors because it is not blocked by the MT₂ selective antagonist 4P-PDOT (Doolen et al., 1998; Masana et al., 2002). In fact, vasoconstrictor effects of melatonin in caudal arteries are further enhanced in the presence of 4P-PDOT, consistent with the blockade of MT₂ receptors that mediate vasodilation (Doolen et al., 1998; Masana et al., 2002), although MT₂ mRNA has not been detected in all studies (Table 1). Vasodilation and an increase in blood flow induced by melatonin in distal skin regions may be responsible for the hypothermic effect of this hormone in humans (Kräuchi et al., 2000).

Melatonin-mediated vasoconstriction in cerebral arteries is blocked by the competitive melatonin receptor antagonists luzindole and S 20928, by pertussis toxin, and by blockers of BK_{Ca} channels (Viswanathan et al., 1990; Geary et al., 1997; Mahle et al., 1997) (Table 1). MT₁ melatonin receptors in cerebral arteries and hippocampal microvasculature seem to regulate blood flow (Savaskan et al., 2001). It is suggested that melatonin-

mediated vasoconstriction enhances cerebrovascular autoregulation, thereby keeping cerebral blood flow constant in the face of diurnal fluctuations in blood pressure (Régrigny et al., 1998).

4. Immune System. Melatonin receptors expressed in lymphoid cells seem to be involved in regulating immune responses (for review, see Guerrero and Reiter, 2002; Skwarlo-Sonta et al., 2003). Luzindole blocks melatonin-mediated enhancement of splenic lymphocyte proliferation in mice independent of time of day (Drazen et al., 2001). This effect of melatonin seems to be mediated through activation of the MT₂ receptor, because the effect of melatonin on splenocyte proliferation (e.g., cell-mediated immunity) was also observed in mice with genetic deletion of the MT₁ melatonin receptor (Drazen and Nelson, 2001). In rat microvasculature, MT₂ activation also reduces acute inflammation by inhibiting leukocyte rolling (Lotufo et al., 2001). In human lymphocytes MT₁ receptor activation counteracts the inhibitory effect of prostaglandin E₂ on interleukin 2 production (Carrillo-Vico et al., 2003; Carrillo-Vico et al., 2005). Activation of MT₂ receptors modulates immune responses, providing a mechanism by which endogenous melatonin may participate in adaptation to seasonal changes (Table 1).

5. Metabolism. A relationship between the circadian release of melatonin and energy balance has been described (Barrenetxe et al., 2004). Melatonin modulates glucose homeostasis mainly via changes in insulin secretion and leptin production. Pancreatic islets and INS-1 insulinoma cells, a model of pancreatic β cells, express high-affinity 2-[¹²⁵I]iodomelatonin binding sites and MT₁ and MT₂ melatonin receptor mRNA (Peschke et al., 2000, 2002; Kemp et al., 2002). The expression of MT₁ and MT₂ receptors has also been reported for human pancreatic tissue (Peschke et al., 2007). Activation of MT₁ melatonin receptors inhibits cAMP-stimulated insulin secretion through pertussis toxin sensitive-G_i/G_o protein coupling. (Peschke et al., 2000, 2002; Kemp et al., 2002). In INS-1 insulinoma cells, melatonin attenuates glucagon-like peptide (GLP-1), forskolin-stimulated insulin secretion, insulin promoter activity, and CREB-mediated gene expression (Kemp et al., 2002) (Table 1).

Prolonged melatonin pretreatment of pancreatic β -cells, via activation of MT₁ melatonin receptors and cross-talk with GLP-1 receptors, sensitizes the cAMP signaling system, increasing cAMP production and insulin secretion. In rat pancreatic islets and INS-1 cells, long-term melatonin treatment increased basal levels of insulin secretion and potentiated GLP-1- and forskolin-induced insulin and cAMP production (Kemp et al., 2002). Endogenous melatonin released during the night seems to sensitize the cAMP system in β -cells, leading to potentiation of insulin release upon GLP-1 receptor stimulation by endogenous incretin (Kemp et al., 2002). The sensitization of the cAMP system by endogenous melatonin may influence the circa-

dian profile for insulin release and could provide a mechanism by which high morning levels of insulin facilitate glucose disposal after nutritional stimuli (Kemp et al., 2002). Melatonin has also been shown to act via the MT₂ receptor in pancreatic β -cells to inhibit cyclic GMP signaling (Stumpf et al., 2008, 2009).

Melatonin receptor activation modulates rat adipocyte function via activation of MT₁ and MT₂ melatonin receptors expressed in inguinal and epididymal adipocytes (Zalatan et al., 2001). In epididymal adipocytes, melatonin in the presence of insulin increases leptin secretion and counteracts forskolin-induced inhibition of leptin secretion and mRNA expression through activation of a melatonin receptor. This suggests cross-talk between these hormones to modulate leptin production from adipocytes via MT₁ receptor activation (Alonso-Vale et al., 2005). In human adipocytes, activation of MT₂ melatonin receptors modulates glucose uptake (Brydon et al., 2001). In summary, melatonin seems to modulate glucose homeostasis and energy balance via its direct effect on pancreatic β cells and adipocytes through activation of MT₁ and MT₂ melatonin receptors. Furthermore, melatonin inhibits insulin secretion in isolated islets and beta cells from rodents and human through activation of MT₁ and/or MT₂ melatonin receptors (Mühlbauer and Peschke, 2007; Lyssenko et al., 2009; Mühlbauer et al., 2009). Mühlbauer et al. (2009) reported that insulin secretion from isolated islets of melatonin receptor MT₁, MT₂, and MT₁/MT₂ knockout mice was modulated by melatonin. Together these data suggest that both the MT₁ and MT₂ receptor may be involved in the pathogenesis of some form of diabetes.

6. Cancer. Melatonin decreased cancer cell growth by affecting cell proliferation and cytokine secretion, reducing tumor number and size, increasing tumor latency, and lowering tumor incidence (for review, see Pawlikowski et al., 2002; Sánchez-Barceló et al., 2003). Melatonin inhibits cell proliferation through receptor-mediated effects on the cell cycle (Shiu et al., 1999), interaction with sex steroid-responsive pathways (Sánchez-Barceló et al., 2003), and/or perhaps in part via its free radical scavenging potential (Reiter et al., 2001).

MT₁ and/or MT₂ melatonin receptors are expressed on LNCaP prostate tumor cells, MCF-7 breast cancer cells (Xi et al., 2001; Ram et al., 2002), colon 38 cancer cells (Karasek et al., 2002), human choriocarcinoma JEG-3 cells (Shiu et al., 2000), human gall bladder adenocarcinoma epithelial cells (Aust et al., 2004), and malignant breast epithelium cells (Dillon et al., 2002). The oncostatic action of melatonin seems to be mediated primarily through activation of MT₁ melatonin receptors, but an action on the MT₂ receptors cannot be excluded (Shiu et al., 1999, 2000). Melatonin inhibited endometrial cancer cell growth in in vitro studies with estrogen receptor-positive Ishikawa cells (Kanishi et al., 2000). It induced neurite growth in N1E-115 neuroblastoma cells (Bordt

et al., 2001) and inhibited proliferation of MCF-7 cells (Ram et al., 2002). Several cellular mechanisms have been suggested to mediate melatonin's oncostatic effects. The best-characterized cellular pathway is the suppression of linoleic acid uptake and its conversion to 13-hydroxyoctadecadienoic acid, which normally activates EGFR/MAPK mitogenic signaling (Blask et al., 2002, 2005a). Endogenous nocturnal levels of melatonin attenuate linoleic acid uptake, and its conversion to 13-hydroxyoctadecadienoic acid decreases cell proliferation and cAMP through a melatonin receptor-mediated effect (Blask et al., 2005a).

MT₁ melatonin receptor overexpression facilitates melatonin-mediated oncostatic action. Melatonin administration decreases the weight and volume of S-91 melanoma tumors in mice in vivo, and melatonin inhibits S-91 cell proliferation in vitro (Kadekaro et al., 2004), which was dramatically potentiated by expression of MT₁ melatonin receptors (Kadekaro et al., 2004). Overexpression of MT₁ receptor in MCF-7 breast cancer cells facilitates melatonin-mediated tumor growth inhibition in vivo and in vitro (Collins et al., 2003) through a melatonin receptor mediated mechanism (Yuan et al., 2002). Overexpression of MT₁ melatonin receptors in MCF-7 cells seems to suppress tumor formation in vivo, probably by increasing expression of constitutively active MT₁ receptors (Collins et al., 2003). Together, these data suggest that oncostatic effects of melatonin occur primarily through activation of MT₁ melatonin receptors and/or enhancement of constitutive receptor activity (Table 1).

VI. Melatonin receptors as therapeutic targets

Melatonin receptor agonists currently on the market or in advanced stages of development are all MT₁/MT₂-nonselective melatonin receptor agonists. These agonists are indicated or being developed for a number of conditions ranging from insomnia and circadian entrainment to depression and seasonal affective disorder. Melatonin and the synthetic melatonin agonists are generally devoid of the common side effects frequently observed with sleep medication (e.g., impairment of learning, memory, or motor function).

A. Agomelatine

The nonselective melatonin agonist agomelatine (S 20098) has been studied extensively using in vitro and in vivo models (Racagni et al., 2007) (Fig. 7A). This high-affinity MT₁/MT₂ melatonin receptor agonist is also a 5-HT_{2C} serotonin receptor antagonist (Conway et al., 2000; Audinot et al., 2003; Millan et al., 2003). The European Medicines Agency recently granted approval to agomelatine for major depression. In addition, agomelatine promotes sleep in human volunteers and phase-shifts circadian rhythms (Cajochen et al., 1997).

Agomelatine, a novel and efficacious antidepressant, shows a unique pharmacological profile with high efficacy on sleep, circadian rhythm dysfunction, and depression (Dubocovich, 2006; Ghosh and Hellewell, 2007; Montgomery and Kasper, 2007; Olié and Kasper, 2007). Agomelatine was found to be significantly more effective than placebo in the treatment of major depression, with significantly reduced depression scores in the 17-item Hamilton Rating Scale for Depression and several other criteria (Lôo et al., 2002, 2003) after 2 weeks of treatment. Agomelatine showed higher efficacy than paroxetine, a specific serotonin-reuptake inhibitor, and both were effective in the treatment of anxiety symptoms in depression (Lôo et al., 2003). Agomelatine's profile of adverse effects, including discontinuation symptoms, is comparable with that of placebo and less than that of paroxetine (Judge et al., 2002; Lôo et al., 2003; Montgomery et al., 2004). Agomelatine seems to have an advantage over other current antidepressant treatments in that it promotes sleep (Cajochen et al., 1997; Guilleminault, 2005), entrains circadian rhythms (Kräuchi et al., 1997; Quera-Salva et al., 2005), and shows anxiolytic effects and a faster onset of therapeutic effectiveness (Lôo et al., 2002; Montgomery et al., 2004; Kennedy, 2005). It also lacks the prominent side effects shown by most classes of antidepressants, such as sexual dysfunction, gastrointestinal reactions, and discontinuation symptoms (Lôo et al., 2002; Millan et al., 2003; Montgomery et al., 2004).

B. Ramelteon

Ramelteon is a novel, high-affinity, nonselective MT₁/MT₂ receptor agonist with little affinity for other GPCRs that was developed for the treatment of insomnia and circadian sleep disorders (for reviews, see Cajochen, 2005; Miyamoto, 2009) (Fig. 7A). In freely moving cats (Miyamoto et al., 2004) and monkeys (Yukuhiro et al., 2004), ramelteon promotes sleep, showing higher efficacy than melatonin. Fisher et al. (2008) demonstrated that both melatonin and ramelteon administration (10 mg/kg i.p.) had an acute, short-lasting, sleep-promoting effect in the rat, as determined using implanted radiotelemetry transmitters to record electroencephalogram and electromyogram parameters. Ramelteon received approval from the FDA in 2005 for the treatment of insomnia characterized by difficulty in sleep onset, and it is the only hypnotic drug indicated for long-term treatment. Ramelteon does not affect learning, memory, or motor function and is devoid of rewarding properties (Meier et al., 1988). In humans with primary chronic insomnia, this novel melatonin receptor agonist decreases the latency to sleep and increases total sleep time and sleep efficiency (Erman et al., 2006).

C. PD 6735 (LY 156735)

PD 6735 is a close indole analog of melatonin that shows high affinity for both MT₁ and MT₂ receptors. In subjects with moderate to severe insomnia, PD 6735 given for 2 consecutive days reduced sleep latency as determined by polysomnography and reduced subjective sleep latency (Fig. 7C). PD 6735 did not negatively affect other sleep parameters or produce morning-after psychomotor impairment (Mulchahey et al., 2004; Zemlan et al., 2005). PD 6735 did not cause hypothermia, hypertension, or bradycardia.

D. Circadin

Circadin has recently been approved for marketing in Europe as a prolonged-release melatonin formulation that mimics the physiological secretion profile of melatonin. The clinical development of Circadin is based on the controlled clinical studies by Garfinkel et al. (1997), Leger et al. (2004), and Haimov et al. (1995), which showed that it improves sleep quality in elderly insomnia patients. Circadin was found to be effective in improving quality of sleep and sleep latency and in improving daytime function. It was not associated with memory impairment or decreased vigilance, and it had no significant withdrawal symptoms. In exploratory studies, Circadin also improved sleep quality in children with sleep-wake cycle disorders as shown in studies by De Leersnyder et al. (2003).

VII. Conclusion

This review describes the molecular, pharmacological, and functional properties of the two mammalian G-protein-coupled melatonin receptors (i.e., MT₁ and MT₂). The availability of melatonin receptor ligands with well defined pharmacological properties and selectivity, advances in the molecular biology of the melatonin receptors, the discovery of novel cellular and signaling mechanisms transducing effector responses, and the creation of transgenic mice with targeted deletion of the MT₁ and/or MT₂ melatonin receptors have increased our understanding of the role of melatonin and its receptors in the modulation of visual, circadian, seasonal, cardiovascular, endocrine, and immune function as well as cancer cell growth.

The cellular and molecular mechanisms by which circadian and seasonal changes in melatonin levels regulate receptor functioning are not fully understood. Studies on melatonin regulation of cellular trafficking and receptor oligomerization will provide new insights into the hormone signaling pathways. The role of protein-protein interactions leading to receptor dimerization and heterodimerization, the presence of intracellular partners in signal transmission, cross-talk via signal transduction cascades, and the role of constitutively active receptors in effector responses all need to be further investigated to understand better the physiological role

of the melatonin receptors and the development of drugs with therapeutic potential. The identification of novel targets for the treatment of disorders involving alterations in the melatonin system should provide new opportunities for therapeutic targets. Discovery of specific and selective agonists and antagonists for the MT₁ and MT₂ melatonin receptors would provide additional tools for the study of melatonin function and the design of novel therapeutics. The discovery of constitutively active MT₁ melatonin receptors opens up the additional possibility of developing inverse agonists as therapeutic tools. A unique feature of the melatonin message that must always be taken into account is that it is time-dependent. Effects of melatonin and melatonin-related drugs are affected by time of day and duration of exposure, probably because of changes in the diurnal sensitivity and/or efficacy of MT₁ and MT₂ melatonin receptors. Treatments mimicking the length of the physiological nocturnal melatonin profile may differentially regulate MT₁ and MT₂ receptor function. Furthermore, through activation of distinct receptors types expressed within the same tissue or perhaps in the same cell, melatonin may exert distinct and/or opposite physiological responses. Our current knowledge on the mechanism(s) by which melatonin affects physiology through activation of specific membrane receptors is exciting but still in its infancy. Better understanding of these processes will facilitate discovery and development of melatonergic agents for the treatment of sleep, circadian, metabolic, and endocrine disorders, as well as tumor cell growth.

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