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MOLECULAR PHARMACOLOGY, REGULATION AND FUNCTION OF MAMMALIAN MELATONIN RECEPTORS

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1. ABSTRACT

Melatonin (5-methoxy-N-acetyltryptamine), dubbed the hormone of darkness, is released following a circadian rhythm with high levels at night. It provides circadian and seasonal timing cues through activation of G protein-coupled receptors (GPCRs) in target tissues (1). The discovery of selective melatonin receptor ligands and the creation of mice with targeted disruption of melatonin receptor genes are valuable tools to investigate the localization and functional roles of the receptors in native systems. Here we describe the pharmacological characteristics of melatonin receptor ligands and their various efficacies (agonist, antagonist, or inverse agonist), which can vary depending on tissue and cellular milieu. We also review melatonin-mediated responses through activation of melatonin receptors (MT_1, MT_2, and MT_3) highlighting their involvement in modulation of CNS, hypothalamic-hypophyseal-gonadal axis, cardiovascular, and immune functions. For example, activation of the MT_1 melatonin receptor inhibits neuronal firing rate in the suprachiasmatic nucleus (SCN) and prolactin secretion from the pars tuberalis and induces vasoconstriction. Activation of the MT_2 melatonin receptor phase shifts circadian rhythms generated within the SCN, inhibits dopamine release in the retina, induces vasodilation, enhances splenocyte proliferation and inhibits leukocyte rolling in the microvasculature. Activation of the MT_3 melatonin receptor reduces intraocular pressure and inhibits leukotriene B_4-induced leukocyte adhesion. We conclude that an accurate characterization of melatonin receptors mediating specific functions in native tissues can only be made using receptor specific ligands, with the understanding that receptor ligands may change efficacy in both native tissues and heterologous expression systems.

2. INTRODUCTION

In 1917, McCord and Allen found that bovine pineal extracts applied to *Rana pipiens* tadpoles caused blanching of the skin (2). This bioassay led to the isolation and discovery of melatonin in 1959 by Lerner and co-workers (3). The biosynthesis of melatonin begins with the acetylation of serotonin by N-acetyltransferase to produce N-acetylserotonin. Methylation of N-acetylserotonin by hydroxyindole-O-methytransferase forms melatonin (5-

acetyl-N-methoxytryptamine) (4, 5). In mammals, melatonin is synthesized primarily by the pineal gland and retina and is released in a circadian fashion with high levels during the night (6, 7). The circadian biosynthesis of melatonin relays photoperiodic information to the organism by defining the length of the night, which correlates with the amplitude of the endogenous melatonin profile. Melatonin modulates a myriad of physiological functions including circadian, visual, cerebrovascular, reproductive, neuroendocrine and neuroimmunological (1, 7-9). Here we will review the functions of melatonin receptors (MT_1, MT_2 and *MT_3*) and will discuss how to use pharmacological tools to investigate both the presence and physiological effects mediated by these receptors in native tissues.

3. MELATONIN RECEPTORS

The first evidence suggesting the existence of melatonin receptors originates from work done in amphibian dermal melanophores that measured the efficacy of melatonin and melatonin ligands (e.g., N-acetyl 5-hydroxytryptamine; N-acetyltryptamine) to induce pigment aggregation and established a structure-activity relationship for melatonin receptors (10). This report suggested N-acetyltryptamine as the first putative melatonin receptor antagonist. Subsequently, this bioassay was used to demonstrate that melatonin-mediated pigment aggregation was blocked by pertussis toxin suggesting activation of a G protein-coupled melatonin receptor (11). The presence of specific ^{3}H-melatonin binding sites in bovine brain membranes (12) and the inhibition of calcium-dependent release of dopamine from the rabbit retina by picomolar concentrations of melatonin (13, 14) provided evidence for the presence of melatonin receptors with a specific function in mammals. The pharmacological characterization and cloning of melatonin receptors, the discovery of selective and specific ligands for the receptors and the introduction of transgenic mice with selective deletion of MT_1 and/or MT_2 melatonin receptors are allowing the functional characterization of each melatonin receptor.

Melatonin receptors were originally classified into the ML_1 and ML_2 subtypes (15, 16) with 2[^{125}I]-iodomelatonin binding affinities in the picomolar and nanomolar range, respectively (16). cDNA's encoding melatonin receptors with ML_1-like pharmacology (Mel_{1a}, Mel_{1b}) were cloned in several vertebrate species including human (17, 18) and are now referred to as MT_1 and MT_2, respectively (19). Another receptor with ML_1-like pharmacology, the Mel_{1c} (20), is not found in mammalian species. The ML_2 melatonin site is now referred as the *MT_3* melatonin receptor; however, it is unclear whether it fulfills all the criteria for classification as a G protein-coupled melatonin receptor.

3.1. MT_1 and MT_2 melatonin receptors

3.1.1. Molecular structure

The high affinity MT_1 and MT_2 melatonin receptors are coupled to pertussis toxin-sensitive G proteins leading to the inhibition of adenylyl cyclase activity (1, 21). They are unique receptors as they show distinct molecular structures with only 60% amino acid identity and different chromosomal localization (1, 21). These receptors are 350 and 362 amino acids long, respectively, with calculated molecular weights of 39-40 kDa. The MT_1 and MT_2 melatonin receptors have two and one potential glycosylation sites in their N-terminus, respectively, and protein kinase C (PKC), casein kinase 1 (CK1), casein kinase 2 (CK2) and protein kinase A (PKA) phosphorylation sites which may participate in the regulation of receptor function (22). The molecular structure of these melatonin receptors consists of seven transmembrane (TM) helices (I-VII) linked by three alternating intracellular (IL_1, IL_2, and IL_3) and extracellular (EL_1, EL_2, and EL_3) loops. Melatonin receptors are a distinct group within the G protein-coupled receptor superfamily as they have an NRY motif (single letter amino acid code), a variant of a DRY (or ERY) that is present in intracellular loop II of all G protein-coupled receptors. This region is believed to be involved in signal transduction through G proteins (23). Interestingly, mutation of Asn 124 in the NRY motif of the MT_1 melatonin receptor led to the suggestion that this region controls receptor trafficking and cell signaling (24). In the MT_1 melatonin receptor, Gly 20 (TM VI), Val 4 (TM IV), His 7 (TM IV), Ser 8 (TM III), and Ser 12 (TM III) are essential for melatonin binding (25-27). In the MT_2 melatonin receptor, Cys 113 (in EL_1) and Cys 190 (in EL_2), two residues that are conserved in most GPCRs, are proposed to form a disulfide bond that is essential for high affinity melatonin binding (28). Melatonin receptors also have what appears to be a leucine zipper in TM IV, with 7 leucines in the MT_1 and 6 leucines in the MT_2, which may be involved in protein-protein interactions. In summary, the MT_1 and MT_2 melatonin receptors show unique structural features leading potentially to distinct binding pockets for ligand recognition.

3.1.2. Pharmacology

Melatonin receptor characterization and identification in native and/or heterologous expression systems requires knowledge of the pharmacological properties of the ligands and radioligands in the particular receptor system under study. This knowledge is essential to characterize potential therapeutic targets. Here we will define ligand efficacy, potency, affinity, selectivity and specificity, and use examples from the melatonin receptor literature to illustrate how each ligand can be characterized and used to discover functional receptors in native tissues.

3.1.2.1. Efficacy, potency and affinity

Ligand efficacy can be defined as the property of a molecule that causes the receptor to change its behavior toward the host cell (29). Ligands are classified based on their efficacy into: 1) agonist has full positive efficacy and induces a cellular response, 2) neutral antagonist has zero efficacy and produces no cellular response, and 3) inverse agonist has negative efficacy, opposite to that of the agonist. Inverse agonists abolish spontaneous receptor activity by binding to receptors uncoupled from G protein and therefore shift the equilibrium towards the free form of the receptor. Ligands showing efficacies between that of a neutral antagonist and full agonist are classified as 4) partial agonist, and those with efficacies between a neutral

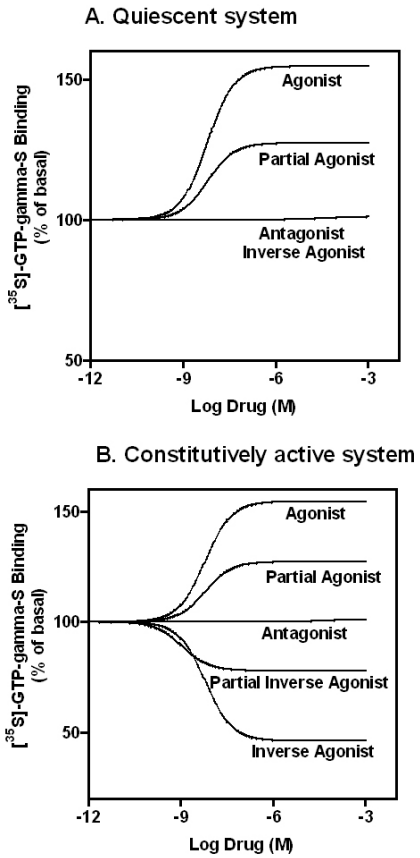

Figure 1. Effect of constitutive activity on ligand efficacy. Schematic representation of responses mediated by ligands acting as full agonist, partial agonist, antagonist, partial inverse agonist and inverse agonist on ^{35}S-GTP-gamma-S binding to G proteins. A. In a quiescent system (no constitutive activity) only the responses mediated by agonist, partial agonist and antagonist are observed. B. In systems where the receptors under study are constitutively active then partial inverse agonists and inverse agonists can induce a response by shifting the equilibrium towards the free form of the receptor.

antagonist and inverse agonist are designated as 5) partial inverse agonist (figure 1). Identification of an inverse agonist, however, is dependent on both the presence of constitutively active receptors and on the sensitivity of the experimental assay used to detect changes in receptor basal activity.

A number of assays have been used to determine melatonin receptor ligand efficacy in native tissues and in heterologous cells expressing recombinant melatonin receptors. *In vitro* pharmacological bioassays used to determine melatonin ligand efficacy include: pigment aggregation in amphibian dermal melanophores (30), inhibition of calcium-dependent release of dopamine from retina (13), melatonin potentiation of adrenergic vasoconstriction (31) and phase shifts of the circadian rhythm of neuronal firing rate in the SCN brain slice (32). Biochemical assays include forskolin-stimulation of cAMP accumulation (33, 34), GTP-shift assays (35), [^{35}S]-GTP-gamma-S binding to G proteins (36, 37), phosphoinositide turnover (38) and phosphotransferase activity (protein kinase C activity) (32).

The potency of a ligand is defined as the concentration of a drug that produces a specified effect (e.g., IC_{50}: concentration producing 50% inhibition of the maximal response measured). Potency is affected by spare receptors and/or state of receptor coupling (39). The equilibrium dissociation constant (K_B) of partial agonists or antagonists for a receptor can be determined experimentally. K_B refers to the affinity of a partial agonist or antagonist to reduce the action of an agonist ligand. The K_B is a constant for a particular ligand-receptor system, independent of the cellular background (14, 39-41).

The affinity (K_i) of a ligand for a native or a recombinant receptor expressed in heterologous cells can be determined using radioligand binding in tissue homogenates (42) or by quantitative receptor autoradiography (43). The K_i value is the apparent affinity of a ligand for a specified receptor, determined in competition studies (39).

3.1.2.2. Ligand selectivity

Selectivity refers to the propensity of a drug to bind with higher affinity to one receptor over another receptor of the same class. Ligand selectivity for two recombinant receptor types is established by determining the ratio of affinities assessed by radioligand binding. Figure 2 shows the selectivity ratios for luzindole, 4P-ADOT and 4P-PDOT for competition with 2-[^{125}I]-iodomelatonin binding (41, 44). A ligand is considered selective when the ratio of affinity is at least 100 times or greater [e.g., 4P-PDOT and 4P-ADOT, (1, 41, 44)]. Melatonin receptor ligands that are selective for the hMT_2 receptor include [$K_{i\ MT1}/K_{i\ MT2}$ selectivity ratio]: 4P-CADOT [360]; 4P-ADOT [300-1000]; 4P-PDOT [300-1500]; K185 [140]; GR128107 [110]; and 5-methoxyluzindole [130] (41, 44, 45). Ligands with affinity ratios below 100 for competition for 2-[^{125}I]-iodomelatonin binding include [$K_{i\ MT1}/K_{i\ MT2}$ selectivity ratio]: IIK7 [90]; 6-chloromelatonin [57]; luzindole [15-26]; 6,7 di-chloro-2-methylmelatonin [21]; 8M-PDOT [20]; N-acetyltryptamine [15.4]; S20098 [14]; 5-MCA-NAT [9.9]; melatonin [4.9]; GR 196429 [4.8]; N-acetylserotonin [1.2]; and 2-iodomelatonin [0.3] (41, 44, 45) (figure 3A and B). A ligand with a selectivity ratio below 100 (e.g., luzindole with an affinity ratio of 15-26) could also be used within the MT_2 sensitive range of concentrations (10 to 100 nM). At these concentrations, luzindole will competitively block only MT_2 melatonin receptors (44). It should be noted that ligand selectivity is a relative measure and that the selectivity will always be related to the range of ligand concentration used. There are currently no selective ligands available for the MT_1 melatonin receptor (44).

Selectivity can also be determined in functional studies by the relative order of ligand potency (i.e., IC_{50} or EC_{50}) or affinity (K_B) (40, 41, 46). The relative ratio of agonist potencies on different receptors is another way to determine ligand selectivity (40). This relative order of

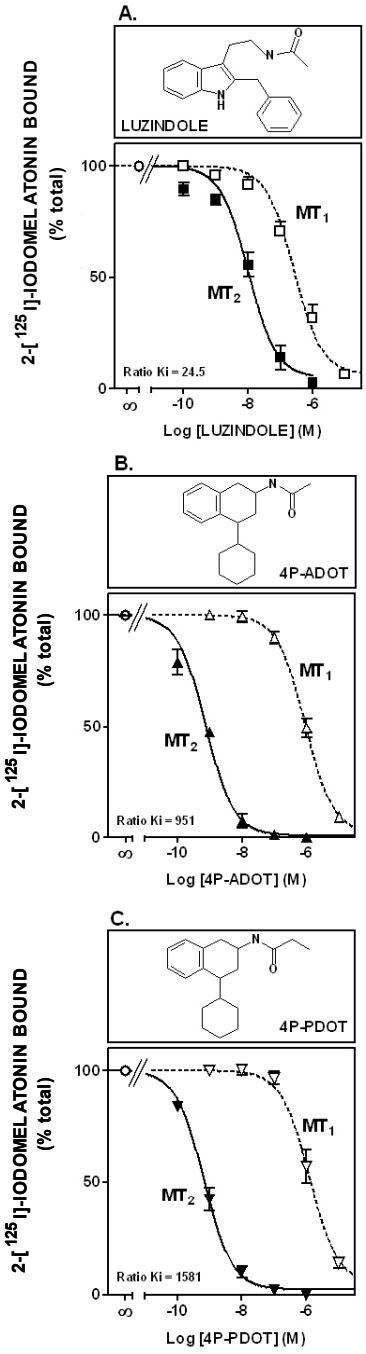

Figure 2. MT_2 melatonin receptor ligands. The melatonin receptor ligands luzindole, 4P-ADOT and 4P-PDOT competed for 2-[^{125}I]-iodomelatonin binding (100 pM) to CHO cell membranes stably expressing either hMT_1 or hMT_2 melatonin receptors. The K_i values (nM) for luzindole were 179 $\pm$ 58 (n=8) at MT_1 and 7.3 $\pm$ 2.0 (n=4) at MT_2; for 4P-ADOT were 377.7 $\pm$ 60.3 (n=5) at MT_1 and 0.4 $\pm$ 0.02 (n=3) at MT_2; for 4P-PDOT were 648 $\pm$ 222 (n=5) at MT_1 and 0.41 $\pm$ 0.04 (n=3) at MT_2. The K_i ratios (MT_1/MT_2) represent fold differences in affinity of each ligand to compete for 2-[^{125}I]-iodomelatonin binding to the hMT_1 or hMT_2 receptors. Reproduced from Dubocovich *et al.* (44) with permission

potency for agonists determined in a functional study (figure 3C) should correlate with the relative order of affinities (K_i) determined by binding to the corresponding receptor (figure 3B). Note the similarity in relative order of potency for melatonin, S 20098, 6-chloromelatonin and 8M-PDOT to compete for 2-[^{125}I]-iodomelatonin binding to the hMT_2 receptor expressed in COS-7 cells and to inhibit ^{3}H-dopamine release (41). This data demonstrates that the presynaptic melatonin heteroreceptor of rabbit retina is an MT_2 receptor (compare figures 3B and 3C).

3.1.2.3. Ligand specificity

Another consideration is the ligand specificity for the particular receptor in question. Specificity refers to the ability of a molecule to bind to one receptor rather than to receptors from other families. Specificity is generally established by screening the ligand in question in competition radioligand binding of to as many receptors and targets as possible. For example, 4P-ADOT and 4P-PDOT are selective MT_2 ligands and specific for the MT_2 melatonin receptor as they did not bind to a large number of neurotransmitter and hormone receptors (44).

3.1.3. Signaling

The best-characterized signaling transduction pathways coupled to activation of the melatonin receptors have been reported in mammalian cell lines expressing recombinant MT_1 and MT_2 receptors (figure 4). The MT_1 melatonin receptors elicit multiple cellular responses through both pertussis toxin-sensitive and -insensitive pathways. Activation of the MT_1 melatonin receptor through G_i proteins (G_{i2} and G_{i3}) inhibits forskolin-stimulated cAMP formation, protein kinase A (PKA) activity, and phosphorylation of the cAMP-responsive element binding protein (CREB) (1, 34, 47, 48) and through G_q increases phosphatidylinositol turnover and intracellular calcium (47, 49). In the mouse SCN, melatonin inhibits pituitary adenylate cyclase-activating polypeptide (PACAP)-mediated CREB phosphorylation. This effect appears to be mediated by MT_1 receptors since it is absent in the MT_1-KO mice (50). Additionally, MT_1 melatonin receptors activation stimulates c-Jun N-terminal kinase activity via both pertussis toxin-sensitive (G_i) and -insensitive (G_s, G_z and G_{16}) proteins (51). Furthermore, activation of the MT_1 melatonin receptor via the release of the beta-gamma subunit potentiates prostaglandin$F_{2\alpha}$ and adenosine triphosphate (ATP)-mediated stimulation of phospholipase C (47, 52). The MT_1 melatonin receptor increases potassium conductance by activation of G protein-coupled inwardly rectifying potassium channel (GIRK) Kir3 through a mechanism that may also involve activation by the beta-gamma subunits of the G_i protein (53). Additionally, activation of the MT_1 melatonin receptor also increases phosphorylation of MEK 1 and 2, and ERK 1 and 2 (51, 54), and increases phosphoinositide hydrolysis (38).

Activation of recombinant MT_2 melatonin receptors expressed in mammalian cells inhibits forskolin-stimulated cAMP formation (18, 55) and cGMP accumulation (55), and increases phosphoinositide

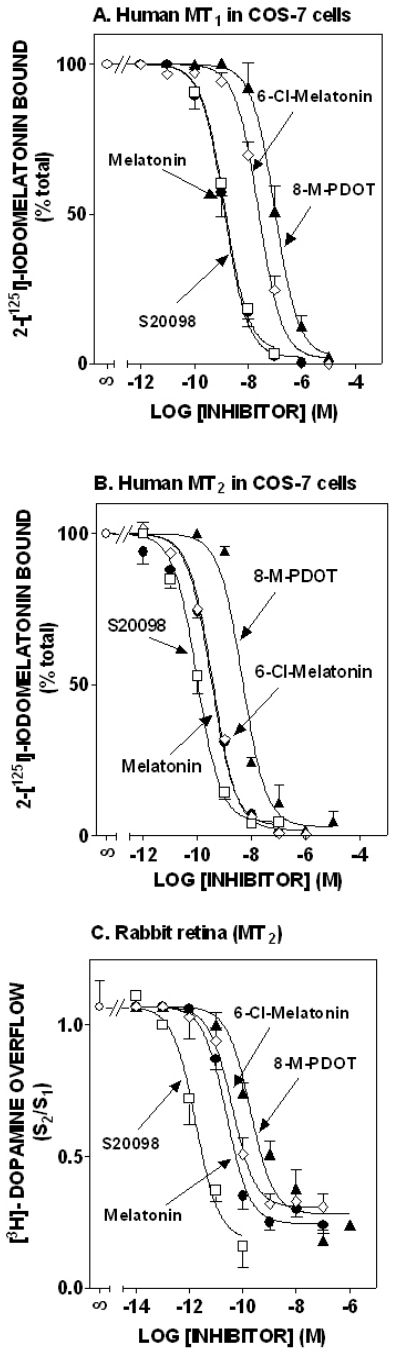

Figure 3. Competition for 2-[^{125}I]-iodomelatonin binding to recombinant hMT_1 and hMT_2 melatonin receptors and inhibition of calcium-dependent [^{3}H]-dopamine release from the rabbit retina. **A, B:** the ordinate represents 2-[^{125}I]-iodomelatonin binding expressed as percent total binding. **C.** the ordinate represents [^{3}H]-dopamine overflow elicited by field stimulation (3Hz, 2 min, 20 mA, 2 ms) above the spontaneous levels of release. Results are expressed as the ratio (S_2/S_1) obtained between the second (S_2) and the first (S_1) period of stimulation within the same experiment. Reproduced from Dubocovich *et al.* (41) with permission.

hydrolysis (38). In COS-7 cells expressing the hMT_2 melatonin receptor, melatonin induces c-Jun N-terminal kinase via pertussis toxin-sensitive (G_i) and -insensitive (G_{16}) proteins (51). Activation of a MT_2 melatonin receptors inhibits $GABA_A$ receptor-mediated function in the hippocampus (55a) and increases PKC activity in the rat SCN (32). Inhibition of PACAP-induced CREB phosphorylation in the MT_1 knockout mouse SCN appears to be mediated by the MT_2 melatonin receptor, as this effect was blocked by the competitive antagonist 4P-PDOT (1 microM) at a non-selective MT_1/ MT_2 concentration (50) and was not observed in tissue from animals with targeted disruption of both MT_2 and MT_1 melatonin receptors (56). Because 4P-PDOT did not affect PACAP-induced CREB phosphorylation in the SCN of wild type mice, the exact contribution of the MT_2 melatonin receptor in modulating this response is unclear.

3.1.4. Regulation

Melatonin receptors as members of the G protein-coupled receptor superfamily are signal transducing receptors (1). In order to maintain timely and efficient cellular responses as well to maintain cellular homeostasis, it is essential to regulate signal transduction events mediated through these receptors. Melatonin has been shown to both positively and negatively regulate its own receptors. Radioligand binding studies in the rat SCN have found an inverse relationship between receptor density and serum melatonin levels (57, 58). Exposure of Chinese hamster ovary (CHO) cells stably expressing hMT_1 melatonin receptors, but not hMT_2, to a physiological concentration of melatonin (400 pM) for eight hours followed by a sixteen hour withdrawal actually increased hMT_1 melatonin receptor binding sites and also induced a functional supersensitization of the receptor (59). Another major regulatory process is desensitization. Desensitization is the waning of receptor responsiveness following persistent agonist challenge and can be characterized by uncoupling of receptor and G protein, receptor internalization, and/or receptor down regulation (60). MT_1 melatonin receptors in the ovine pars tuberalis (61) and recombinant MT_1 or MT_2 melatonin receptors in mammalian cells (38) desensitize following long exposure (<5 hr) to melatonin (1 microM). Short exposure (10 min) to melatonin (10 nM) desensitized and internalized recombinant MT_2 melatonin receptors, however, melatonin (100 nM) had no effect on recombinant MT_1 melatonin receptors when stably expressed in mammalian cells (62). In contrast, endogenous MT_1 melatonin receptors in GT1-7 cells did internalize following short exposure to melatonin (10 nM) (63). Thus, while it appears that both MT_1 and MT_2 melatonin receptors can be desensitized following exposure to melatonin, the receptors are differentially regulated depending on the melatonin concentration (physiological versus supraphysiological), time of exposure and cellular background.

3.2. *MT_3* melatonin receptors

The putative *MT_3* mammalian receptor is widely distributed in hamster brain and peripheral tissues (16, 64). Activation of this receptor is believed to stimulate phosphoinositide hydrolysis (65, 66). The *MT_3* receptor is

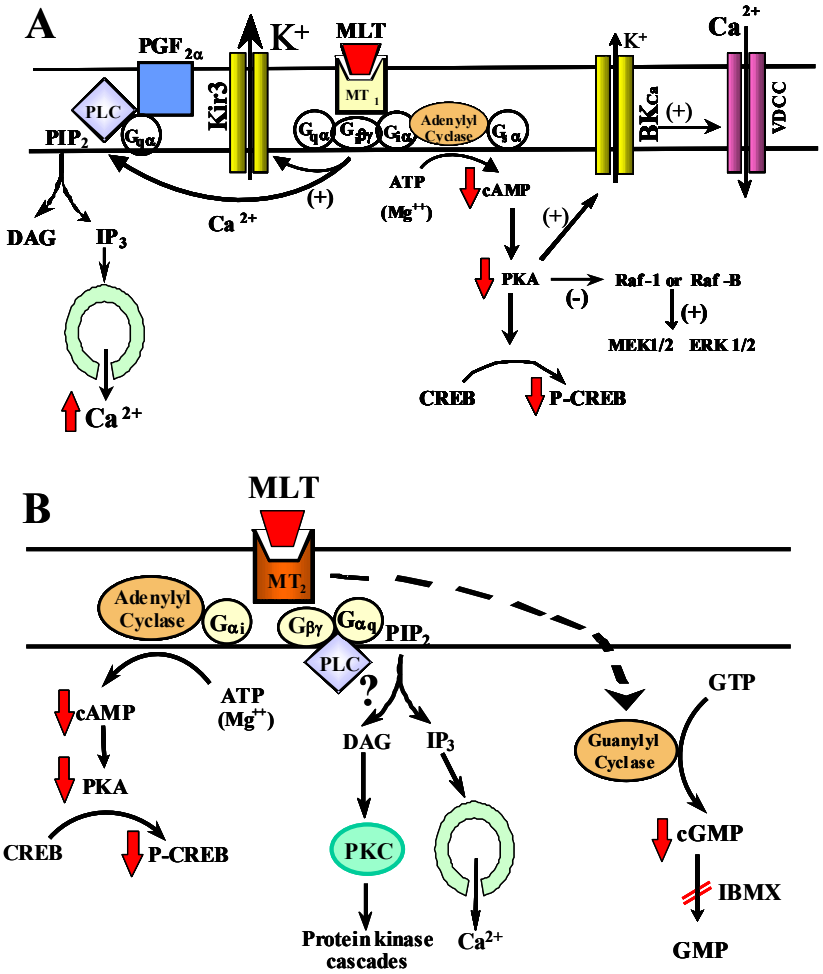

Figure 4. Putative signaling pathways activated by MT_1 and MT_2 melatonin receptors. **A:** multiple signaling pathways for MT_1 melatonin receptors coupled to G_i and $G_{q/11}$. **B:** signaling pathways coupled to MT_2 melatonin receptor activation. PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; DAG, diacylglycerol; PKA, protein kinase A; CREB, cAMP responsive element binding protein; ER, endoplasmic reticulum; VSCC, voltage–dependent K^+ channel; BK_{Ca}, calcium activated potassium channel; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$,; IBMX, isobutylmethylxantine; ATP, adenosine triphosphate; MLT, melatonin; GTP, guanosine triphosphate; GMP, guanosine monophosphate. Modified from Masana and Dubocovich (1) with permission.

activated by both melatonin and its precursor N-acetylserotonin and has a pharmacological profile (Order of affinities: 2-iodomelatonin > N-acetyl-serotonin ≥ melatonin) clearly distinct from that of the cloned mammalian receptors MT_1 and MT_2 (2-iodomelatonin ≥ melatonin >>>> N-acetyl-serotonin). 5-MCA-NAT, prazosin and N-acetyltryptamine are selective ligands for the *MT_3* melatonin receptor (44). The hypothesis suggesting that the *MT_3* site is a mammalian membrane melatonin receptor was challenged by a report suggesting that the selective *MT_3* radioligand 2-[^{125}I]-MCA-NAT binds to the quinone reductase 2 enzyme in hamster kidney membranes (67). This enzyme was cloned following its purification from hamster kidney membranes (67). Recently, it was reported that activation of the *MT_3* receptor by 5-MCA-NAT inhibits leukocyte adhesion to vascular endothelial cells (68) and decreases intraocular pressure (69). Whether the putative *MT_3* melatonin binding protein is a G protein-coupled receptor or represents a binding site for quinone reductase 2 is unclear at the present time and requires further investigation.

3.3. The same ligand can change efficacy

Evidence suggests that a given ligand can exhibit different efficacies depending on tissue or experimental system. The alpha adrenoceptor ligand oxymetazoline is a full agonist in the rat anococcygeus muscle and a partial agonist in the rat vas deferens, an effect due to differences in cellular backgrounds rather than in receptor types (39). Similarly, the beta-adrenergic receptor ligand prenalterol is a full agonist in the guinea pig trachea but a partial agonist in the guinea pig left atrium (70). Changes in receptor levels can also affect the efficacy of certain ligands. The melatonin receptor ligand 4P-CADOT is a neutral antagonist in CHO cells stably expressing low and high levels of hMT_1 melatonin receptors. On the hMT_2 melatonin receptors, 4P-CADOT is a neutral antagonist at low levels of expression but an agonist at high levels (35). Luzindole and 4P-PDOT are melatonin receptor ligands commonly used to elucidate receptors involved in melatonin-mediated physiological responses. For clarity, we will focus on how these two ligands, luzindole and 4P-PDOT, can exhibit different pharmacological efficacies on

the MT_1 and MT_2 melatonin receptors in native and recombinant systems. Melatonin is a full agonist at the MT_1 and MT_2 melatonin receptors in both native and recombinant receptors and therefore will not be included in the discussion.

3.3.1. Efficacy of luzindole and 4P-PDOT at MT_1 melatonin receptors

Initially, luzindole (31) and later on 4P-ADOT (71, 72) and 4P-PDOT (73) were found to act as competitive melatonin receptor antagonists in arterial beds as they were able to antagonize melatonin potentiation of adrenergic-mediated vasoconstriction. The affinity constants (K_B) of luzindole (K_B = 157 nM), 4P-ADOT (K_B = 302 nM) and 4P-PDOT (K_B = 200 nM) to antagonize melatonin-mediated vasoconstriction in arteries (72) correlated closely with the affinity constants (K_i) to compete for 2-[^{125}I]-iodomelatonin binding to recombinant hMT_1 melatonin receptors (179 nM, 378 and 648 nM, respectively) (44). The concept that luzindole and 4P-PDOT were in fact neutral competitive MT_1 melatonin receptor antagonists was challenged when they were tested in recombinant and native systems endowed with constitutively active MT_1 melatonin receptors. Both luzindole and 4P-PDOT are MT_1 inverse agonists when used alone at concentrations of 100 nM and above in recombinant systems where MT_1 melatonin receptors exist in a constitutively active form (33, 44, 47, 74). We were the first to report the presence of constitutively active MT_1 melatonin receptors in a native tissue, the rat caudal artery. In this preparation, both 4P-PDOT (37) and luzindole (unpublished data) acting as inverse agonists at MT_1 melatonin receptors inhibited basal ^{35}S-GTP-gamma-S binding. Tight coupling of the MT_1 melatonin receptor and G protein in the absence or presence of ligand has been proposed as a mechanism by which MT_1 melatonin receptors are constitutively active (47). In the absence of constitutively active MT_1 melatonin receptors, both luzindole and 4P-PDOT will act as competitive antagonists (1, 75). In summary, both luzindole and 4P-PDOT are MT_1 competitive melatonin receptor antagonists and/or inverse agonists depending on the relative proportion of receptors uncoupled (free form) or coupled to G proteins under basal conditions (constitutively active).

3.3.2. Efficacy of luzindole and 4P-PDOT at MT_2 melatonin receptors

Luzindole is a competitive MT_2 melatonin receptor antagonist at both native (32, 35, 41) and recombinant MT_2 melatonin receptors (33, 75). In contrast, 4P-PDOT shows different efficacies depending on the experimental systems. 4P-PDOT was originally classified as a neutral MT_2 competitive antagonist in the rabbit retina based on its ability to competitively block the inhibition of dopamine release by melatonin under conditions in which it did not alter function when used alone (41, 44). 4P-PDOT is also an antagonist at recombinant MT_2 melatonin receptors as it blocked melatonin-mediated stimulation of PI hydrolysis (38). However, this ligand was also reported to be a partial agonist at both native (68) and recombinant MT_2 melatonin receptors (33, 62, 75). It has been suggested, however, that auxiliary proteins present in different cellular backgrounds can modify the pharmacological response of ligands as shown for calcitonin and adrenomedullin (76). Also receptor dimerization can change pharmacological profiles as shown for $GABA_{B(1a)}$ and $GABA_{B(2)}$, M_2 and M_3 muscarinic, kappa and delta opioid, and SST1 and SST2 somatostatin receptors (77). A report demonstrated that both the MT_1 and the MT_2 melatonin receptors form constitutive homo- and hetero-oligomers (78). Thus different auxiliary proteins and melatonin receptor dimerization could contribute to an understanding of how both luzindole and 4P-PDOT can exhibit different pharmacological efficacies at the MT_1 and MT_2 melatonin receptors. Nonetheless, 4P-PDOT serves as an excellent example of a melatonin receptor ligand exhibiting different pharmacological efficacies at the MT_2 melatonin receptor depending on the tissue and experimental system. Therefore caution must be taken when interpreting results from functional studies mediated by ligands that are not fully characterized in the particular tissue under study.

4. PHYSIOLOGICAL RESPONSES MEDIATED BY ACTIVATION OF SPECIFIC MELATONIN RECEPTORS (MT_1, MT_2, and MT_3)

Melatonin plays a pivotal role in the adaptation of organisms to environmental and seasonal changes. Endogenous melatonin released in a circadian or seasonal fashion as well as exogenous melatonin regulates a number of physiological and behavioral responses. In this section, we will discuss the receptor mechanism(s) by which melatonin regulates circadian rhythms, endocrine functions, cardiovascular responses and the immune system (table 1).

4.1. Melatonin receptors in the central nervous system

The first demonstration of specific [^{3}H]-melatonin binding sites in bovine brain (12) was followed by the demonstration of a functional response to melatonin receptor activation in rabbit retina (13). The development of the high affinity radioligand 2-[^{125}I]-iodomelatonin for use in radioligand binding studies and receptor autoradiography allowed the identification of melatonin receptors in discrete neuronal tissues (42, 79). 2-[^{125}I]-Iodomelatonin melatonin binding sites have been localized primarily in neuronal cells in the retina, the SCN, thalamic areas, molecular layer of the cerebellum, and pars tuberalis of the pituitary in various species including human (35, 80-82). 2-[^{125}I]-iodomelatonin binds to both the MT_1 and MT_2 recombinant receptors. However, specific binding of this radioligand in mammalian native tissues appears to be restricted to MT_1 melatonin receptors (44, 83) as it is absent in the SCN and thalamic areas of mice with genetic deletion of the MT_1 receptor (83). Furthermore, the selective MT_2 melatonin receptor ligand 4P-PDOT did not compete with 2-[^{125}I]-iodomelatonin to the SCN of C3H/HeN mice (44).

Using the reverse transcriptase-polymerase chain reaction (RT-PCR), MT_1 mRNA expression was localized to the SCN, cerebellum, cerebral cortex, thalamus, hippocampus and retina, while MT_2 mRNA expression was localized to the retina, hippocampus and whole brain (17,

Table 1. Physiological responses mediated by melatonin receptors in various systems

System	Function	Receptor	Signaling	Tissue	Approach*	References
CNS	Phase shift of the circadian rhythm of wheel running activity	MT_2	UNK		4P-PDOT 4P-ADOT	44
	Phase shift of the circadian rhythm of neuronal firing rate in the SCN slice	MT_2	PKC activation	SCN	4P-PDOT MT_1-KO	32, 83
	Inhibition of PACAP-stimulated CREB phosphorylation	MT_1 MT_2 ?	Inhibition of cAMP	SCN	MT_1-KO MT_1/MT_2-KO	50, 56
	Inhibition of neuronal firing in the SCN	MT_1	Increase in K^+ conductance ?	SCN	MT_1-KO	83. 56
	Inhibition of DA release from rabbit retina	MT_2	UNK	Rabbit retina	Correlation between the K_B values for antagonists in retina and corresponding Ki values on MT_2 recombinant receptors	41
	Reduction of intraocular pressure	*MT_3*	UNK	Rabbit eye	5-MCA-NAT	69
Hypothalamic-Hypophyseal-Gonadal Axis	Inhibition of prolactin secretion	MT_1	UNK	Anterior pituitary	MT_1-KO	119
	Regulation of *Per1* gene expression	MT_1	Inhibition of cAMP	Anterior pituitary	MT_1-KO	119
Cardiovascular	Vasoconstriction	MT_1	Activation of BK_{Ca} channel	Rat caudal artery Cerebral arteries	Correlation between the K_B values for antagonists in retina and corresponding Ki values on MT_1 recombinant receptors	31, 122, 123, 125, 127
	Vasodilation	MT_2	UNK	Rat caudal artery	4P-ADOT 4P-PDOT	71, 72
Immune	Enhancement of splenocyte proliferation i.e., cell-mediated immunity	MT_2	UNK	Spleen	MT_1-KO	131

The melatonin receptors as well as the signaling pathway(s) involved in melatonin-mediated physiological responses in a particular system are indicated. SCN, suprachiasmatic nucleus; PACAP, pituitary adenylate cyclase-activating peptide; CREB, cAMP response elements binding protein; GnRH, gonadotrophin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; DA, dopamine; IL-2, interleukin 2; BKCa channel, Ca^{+2}-activated-large-conductance K^+ channel; KO, knockout; UNK, unknown. * Only studies which used selective ligands to identify melatonin receptor types are mentioned here: MT_2 (4P-PDOT and 4P-ADOT in an MT_2 concentration sensitive range), *MT_3* (5-MCA-NAT).

18, 63, 84, 85). Furthermore, *in situ* hybridization histochemistry revealed the expression of both receptors in the SCN and human cerebellum (32, 44, 81, 84, 86).

Immunocytochemistry using specific anti-MT_1 melatonin receptors antibodies in combination with *in situ* hybridization and RT-PCR revealed differences in the cellular expression of MT_1 receptors in the retina of several species. In the guinea pig and rat retina, MT_1 melatonin receptors immunoactivity was localized to both the inner and outer plexiform layers, ganglion cells, amacrine cells and horizontal cells, with no expression in photoreceptors cells (80, 87). In contrast, in the human retina, MT_1 melatonin receptors are expressed in rod photoreceptors cells (85, 88, 89). Double immunolabeling experiments with tyrosine hydroxylase and an MT_1 melatonin receptor antibody demonstrated localization of this receptor (MT_1) on dopaminergic amacrine cells of the guinea pig retina (87). In the rabbit retina, however, melatonin inhibits dopamine release through presynaptic melatonin heteroreceptors displaying a pharmacological profile similar to that of the hMT_2 melatonin receptor (41). Finally, GABAergic amacrine cells in guinea pig also express MT_1 melatonin receptors, suggesting a role for melatonin in the regulation of this neurotransmitter (87).

4.1.1. The circadian timing system

The mammalian circadian timing system formed by the retina, the intergeniculate leaftlet (IGL) and the suprachiasmatic nucleus (SCN), facilitates adaptation of an organism to environmental changes through the rhythmic regulation of physiological processes. Synchronization of the endogenous circadian clock to the 24-hour period of the sleep-waking cycle occurs by the combined actions of internal (e.g., melatonin) and external stimuli (e.g. light) (90). Light reaches the mammalian SCN through the retinohypothalamic track that projects from retinal ganglion cells to both, the IGL and SCN (91-94). The SCN are a pair of small cluster of cells located within the anterior ventral hypothalamic just above the optic chiasm. In

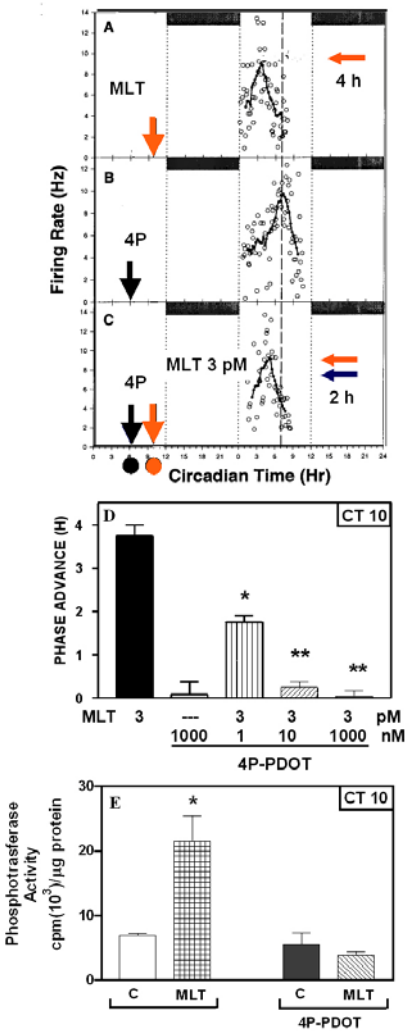

Figure 5. 4P-PDOT antagonized the melatonin-induced phase advance of the circadian rhythm of neuronal firing activity when applied to the rat SCN at CT 10. The peak in the circadian rhythm of neuronal firing in the SCN occurs near CT 7, in both untreated brain slices and in vehicle-treated controls (vertical dash line). **A:** A microdrop (1 microlitre) of melatonin (red arrow, 3 pM) applied to the SCN at CT 10 induced a ~ 4-h phase advance. **B:** The selective melatonin receptor antagonist 4P-PDOT (black arrow, 1 nM), bath-applied to the SCN by itself did not modify the peak of neuronal firing rate. **C:** 4P-PDOT (black arrow, 1 nM), bath-applied to the SCN for 1 h before a melatonin (red arrow, 3 pM) microdrop attenuated the ~ 4-h phase advance. *Open circles* represent the firing rate of individual cells. The *dark gray horizontal bar* represents subjective night. **D:** Melatonin (3 pM) applied as a microdrop at CT 10 induced ~ 4-h phase advances. Bath application of 4P-PDOT (1 microM) by itself did not induce a phase shift. 4P-PDOT bath-applied to the slice before melatonin (3 pM) at CT 10 blocked the phase advance in a dose-dependent manner. **E:** Melatonin mediated increases in PKC activity at CT 10 are antagonized by 4P-PDOT. Melatonin (3 pM) application to the rat SCN brain slice increased phosphotransferase activity by 2-fold, an effect blocked by 1 microM 4P-PDOT. Modified from Hunt *et al.* (32) with permission.

mammals, the SCN is the master clock that controls behavioral, metabolic and physiological rhythms (90, 95). The SCN also controls the circadian rhythms of synthesis and release of melatonin by the pineal gland by way of a multisynaptic pathway (96). In the absence of light cues, the SCN drives the endogenous circadian rhythm of pineal melatonin production. Light modulates the SCN and suppresses melatonin synthesis. In the absence of light, the hormone melatonin feedbacks onto the master clock to regulate circadian rhythms via activation of melatonin receptors.

4.1.2. Melatonin regulation of neuronal firing rate and circadian timing

In the mammalian SCN slice, activation of melatonin receptors mediates two distinct functional responses: acute inhibition of neuronal firing and phase shift of circadian rhythms of neuronal firing rate (table 1). Single-unit or multiunit activity recordings in the rat hypothalamic SCN slice preparation demonstrated the melatonin-mediated inhibition of neuronal firing rate (97-100). This effect appears to be mediated through activation of MT_1 melatonin receptors as it was not observed in the SCN of mice with targeted disruption of the MT_1 receptor, but it is still present in mice lacking the MT_2 receptor (56, 83). This MT_1-mediated inhibition of neuronal firing could result from an increase in potassium conductance and subsequent neuronal hyperpolarization (101) through activation of the inward rectifier potassium channel (Kir3) (53).

Melatonin-mediated phase shifts of circadian rhythms occurs at two windows of sensitivity that correspond to the hours around the day-night (dusk) and night-day (dawn) transitions (44, 102, 103). In mice, melatonin administration two hours before subjective-dusk (CT 10) (CT 12 onset of activity) phase advances the circadian rhythm of wheel running activity via the MT_2 melatonin receptor, as the selective and competitive MT_2 receptor antagonists 4P-ADOT and 4P-PDOT blocked this effect (35) (table 1). In the rat SCN brain slice, melatonin phase advances the peak of the circadian rhythm of neuronal firing at two distinct times of the day [subjective-dusk (CT 10) and -dawn (CT 23)], which coincides with the rise and fall of melatonin production (32, 104). Melatonin appears to affect the phase of the clock through a mechanism involving the activation of a PKC-dependent signaling pathway (32, 105). Using a pharmacological approach, we demonstrated that 4P-PDOT, a selective MT_2 receptor antagonist, not only blocked the melatonin-mediated phase advance of the peak of neuronal firing at both CT 10 and CT 23, but also the increase in PKC activity (32). Liu and colleagues (83) reported that in the SCN slice of mice with genetic disruption of the MT_1 melatonin receptor, melatonin applied at CT 10 phase advanced the peak of neuronal firing rate through activation of the MT_2 melatonin receptor. Taken together these reports suggest that the phase shifting effect of melatonin in the mammalian SCN is mediated by activation of the MT_2 receptor (32, 35, 83) (figure 5).

In summary, in the mammalian SCN, activation of the MT_1 melatonin receptor inhibits neuronal firing while activation of the MT_2 receptor is involved in the phase shifts

of circadian rhythms. It is therefore conceivable that drugs selective for MT_1 and MT_2 melatonin receptors could be potential therapeutic targets for the development of melatonin ligands to treat disorders involving alterations in sleep and the phase of the circadian clock (depression, blindness, delayed sleep phase syndrome) or following the rapid change in the light dark/cycle (jet travel and shift work) (106).

4.2. Regulation of the hypothalamic-hypophyseal-gonadal axis

Melatonin plays a major physiological role in the modulation of seasonal cycles of reproduction. Studies on the site(s) and mechanism(s) by which melatonin regulates reproduction have focused in the hypothalamus and pituitary as target tissues (table 1). Melatonin regulates gonadotrophin-releasing hormone (GnRH) secretion from hypothalamic neurons. GnRH in turn controls the secretion of the gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that regulate reproductive functions at the level of the gonads. In the pituitary gland, melatonin receptors are localized in the anterior part and in the pars tuberalis (107). In the neonatal rat pituitary, melatonin inhibits GnRH-induced LH release (107, 108), cAMP and cGMP accumulation (107) and the increase in intracellular Ca^{2+} (109) through activation of a pertussis toxin-sensitive G protein-coupled receptor. The type of melatonin receptor mediating these responses has not been identified.

Melatonin receptors have been reported in the ovaries using 2-[^{125}I]-iodomelatonin (110, 111). MT_1 and MT_2 melatonin receptor mRNAs were identified in human granulosa cells (112) and MT_1 melatonin receptor protein was detected using anti-human MT_1 melatonin receptor antibodies in ovaries from immature rats (111). This, together with the finding that melatonin is present in the ovarian follicular fluid suggests a direct effect of the pineal hormone in ovarian function (113, 114). Indeed, melatonin stimulates progesterone secretion by granulosa cells in culture from several species including humans (115). Nevertheless, regulation of ovarian function by melatonin may involve a complex mechanism and more than one target cell type.

Melatonin modulates reproduction in seasonal breeding animals and regulates the dynamic physiological adaptations that occur in response to changes in day length (116-118). As the duration of the dark period changes with the season, so does the duration of the melatonin acrophase, which then serves as the link between the circadian clock and peripheral tissues. In the pars tuberalis, the nocturnal secretion of pineal melatonin suppresses the expression of the clock gene *Per1* by inhibiting the cAMP dependent signaling pathway through activation of the MT_1 receptor (119) (table 1). As the levels of circulating melatonin decrease at dawn, the pars tuberalis is released from transcriptional repression, facilitating the induction of *Per1* gene expression by adenosine. Melatonin, through activation of the MT_1 melatonin receptor, also inhibits prolactin release in the pars tuberalis suggesting that gene expression serves to translate the nocturnal exposure to melatonin into a signal that regulates prolactin secretion (119). This may be a general mechanism by which the hormone melatonin regulates gene expression, thus linking the central circadian pacemaker and peripheral tissues resulting in modulation of circadian and seasonal rhythms.

4.3. Regulation of cardiovascular functions and temperature

Expression of melatonin receptors in selective mammalian vascular beds was first suggested by radioligand binding. Specific 2-[^{125}I]-Iodomelatonin binding is detected in cerebral arteries of rats, humans and non-human primates (120, 121). Expression of MT_1 (72, 122) and MT_2 (72) mRNA was demonstrated in rat caudal arteries and in cerebellar arteries (81).

Melatonin mediates both vasoconstriction and vasodilation through activation of different melatonin receptors (table 1). In the rat caudal artery, melatonin potentiates both adrenergic nerve stimulation and norepinephrine-induced contraction (31), while it does not affect vascular tone by itself. This effect occurs through the activation of MT_1 receptors present in the smooth muscle, although the role of a receptor with endothelial localization cannot be ruled out (72, 123). This potentiation is mediated by inhibition of calcium-activated potassium channels (BK_{Ca}) (123), that may result from decreases in both cAMP and phosphorylation of the channel via protein kinase A (124). Melatonin also directly vasoconstricts cerebral arteries (125), an effect blocked by the competitive melatonin receptor antagonists luzindole and S-20928, by pertussis toxin, and by blockers of the Ca^{2+}-activated-large-conductance K^+ (BK_{Ca}) channels (126, 127). Therefore, melatonin-induced contraction of rat cerebral arteries occurs through activation of G_i /G_o protein-coupled receptors and inhibition of BK_{Ca} channels.

Melatonin receptor activation also appears to induce vasodilation in rat arteries. In the rat caudal artery, melatonin-mediated potentiation of phenylephrine-induced contractions is enhanced in the presence of MT_2 selective antagonists (71). This, together with the localization of MT_2 mRNA in rat caudal arteries suggests that melatonin induces relaxation through activation of the MT_2 melatonin receptor (31, 72). Melatonin-induced vasodilation and increase in blood flow in distal skin regions may underlie the concomitant heat loss and the hypothermic effect of melatonin (128). The melatonin receptor types involved in these melatonin-mediated actions have not been determined.

4.4. Regulation of cell-mediated and humoral immune responses and inflammation

Inflammatory responses, particularly in animals living in non-tropical zones, follow daily and seasonal rhythms with enhanced immune function during short-day lengths (129). This has been correlated with high melatonin secretion during the dark-phase of the day. Indeed, several parameters of the immune system appear to be regulated by activation of melatonin receptors. Melatonin treatment enhanced both cell- and humoral-mediated responses in several species (130-132). Drazen and Nelson suggested that the MT_2 melatonin receptor is

involved in the melatonin-mediated regulation of splenocyte proliferation (i.e., cell-mediated immunity) and of serum anti-keyhole IgG concentrations (i.e., humoral immunity), as these responses are present in mice lacking functional MT_1 melatonin receptors (131) (table 1).

In mice, the circadian rhythms of experimental granulomatous inflammation is inhibited by pinealectomy and re-established by nocturnal replacement of melatonin (133). Melatonin reduces acute inflammation in rats by inhibiting leukocyte rolling in the microvasculature through activation of the MT_2 melatonin receptor and leukotriene B_4-induced leukocyte adhesion to endothelial cells through a melatonin receptor with the pharmacological characteristics of the *MT_3* site (68) (table 1). In summary, melatonin regulates inflammatory responses and several immune parameters including cell-mediated and humoral responses, providing a mechanism by which melatonin may suit organisms for adaptation to seasonal changes.

5. FUTURE TRENDS AND PERSPECTIVE

This review describes physiological responses in mammals mediated through activation of specific melatonin receptor types, i.e., MT_1, MT_2, *MT_3*. The availability of ligands with well defined pharmacological properties, advances in the molecular biology of the melatonin receptors and the creation of transgenic mice with target deletion of the MT_1 and/or MT_2 melatonin receptors are furthering our understanding of the role of melatonin and its receptors in the modulation of visual, circadian, seasonal, cardiovascular, endocrine and immune function. The challenge now is to determine the cellular and signaling mechanism(s) that transduce the ligand signal from the receptor to the effector system. The role of protein-protein interactions leading to receptor dimerization and heterodimerization, the presence of intracellular partners in signal transmission and/or through cross-talk via signal transduction cascades need to be investigated to further dissect potential disease drug targets. Studies using knockout MT_1 and/or MT_2 mice and natural MT_2 knockout hamsters suggest significant degree of redundancy as these two receptors appear to compensate for one another. Therefore, efforts need to be focused on designing selective melatonin receptor ligands to allow identification of novel targets for the treatment of disorders involving alterations in the melatonin system. Discovery of specific and selective melatonin agonists for the MT_1 and MT_2 melatonin receptors could provide novel treatments for sleep and circadian rhythms disorders with reduced side effects (106).

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