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Functional MT_1 and MT_2 Melatonin Receptors in Mammals

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Melatonin, dubbed the hormone of darkness, is known to regulate a wide variety of physiological processes in mammals. This review describes well-defined functional responses mediated through activation of high-affinity MT_1 and MT_2 G protein–coupled receptors viewed as potential targets for drug discovery. MT_1 melatonin receptors modulate neuronal firing, arterial vasoconstriction, cell proliferation in cancer cells, and reproductive and metabolic functions. Activation of MT_2 melatonin receptors phase shift circadian rhythms of neuronal firing in the suprachiasmatic nucleus, inhibit dopamine release in retina, induce vasodilation and inhibition of leukocyte rolling in arterial beds, and enhance immune responses. The melatonin-mediated responses elicited by activation of MT_1 and MT_2 native melatonin receptors are dependent on circadian time, duration and mode of exposure to endogenous or exogenous melatonin, and functional receptor sensitivity. Together, these studies underscore the importance of carefully linking each melatonin receptor type to specific functional responses in target tissues to facilitate the design and development of novel therapeutic agent.

Key Words: MT_1 and MT_2 melatonin receptors; MT_3 site; luzindole; 4P-PDOT; MT_1 and MT_2 knockout mice.

Introduction

The circadian release of the hormone melatonin (5-methoxy-*N*-acetyltryptamine) relays photoperiodic information to the organism by defining the length of the night, which correlates with the amplitude of the endogenous melatonin profile *(1,2)*. The biosynthesis of melatonin begins with the acetylation of serotonin by *N*-acetyltransferase producing *N* -acetylserotonin, which is then methylated by hydroxyindole-*O*-methytransferase to form melatonin (5-acetyl- *N*-methoxytryptamine) *(3,4)*. In mammals, melatonin is synthesized in a circadian fashion primarily by the pineal gland with high levels during the night *(2)*. Physiologically released pineal melatonin provides circadian and seasonal timing cues for the regulation of reproduction in seasonal breeders *(2,5,6)*, prolactin secretion *(7,8)*, and color coat *(9)*. Melatonin presence and/or synthesis has also been described in extrapineal tissues such as retina *(10)*, Harderian gland *(11)*, gastrointestinal track *(12)*, testes *(13)*, and human lymphocytes *(14)*. Locally released melatonin has been shown to modulate dopaminergic transmission in retina *(15)* and interleukin 2 production in lymphocytes *(14)*. Exogenous melatonin through activation of G protein–coupled receptors (GPCRs) in target tissues modulates circadian, cerebrovascular, reproductive, endocrine, and immune functions as well as tumor growth *(16)*. Here we review the well defined melatonin functions mediated through activation of specific melatonin receptors types (MT_1, MT_2, and MT_3) in mammalian target tissues, which are viewed as potential targets for drug discovery.

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Mammalian Melatonin Receptors

In mammals, melatonin signals through activation of at least two high-affinity G protein–coupled receptors, the MT_1 and MT_2 *(16–18)*. These are unique receptors as they show distinct molecular structures *(19)*, pharmacological characteristics *(20)*, and chromosomal localization *(21)*. The MT_1 and MT_2 receptors are 350 and 362 amino acids long, respectively, with calculated molecular weights of 39–40 kDa *(22, 23)*. These melatonin receptors have potential glycosylation sites in their N-terminus, protein kinase C (PKC), casein kinase 1 and 2, and protein kinase A (PKA) phosphorylation sites, which may participate in the regulation of receptor function as demonstrated for other G protein–coupled receptors *(24)*. The MT_1 and MT_2 melatonin receptors belong to a distinct group within the G protein–coupled receptor superfamily as they have a NRY motif, rather than the DRY (or ERY) that is present in intracellular loop II of all G protein–coupled receptors *(19)*. This region in the MT_1 melatonin receptor is involved in receptor trafficking and cell signaling *(25)*. A disulfide bond between Cys 113 and Cys 190 is essential for high-affinity melatonin binding to MT_2 and possibly to MT_1 receptors as well *(26)*. Site-directed mutagenesis revealed unique structural features within the

MT_1 and MT_2 melatonin receptors' molecular structure which show potentially distinct binding pockets for ligand recognition (for refs. *27–29*).

MT_1 and MT_2 melatonin receptors signal by coupling to heterotrimeric Gi proteins formed by α, β, and γ subunits *(16,18)*. Activation of these receptors promotes dissociation of G proteins into α and βγ dimmers, which interact with various effector molecules involved in the transmission of cell signaling *(30)*. Effector systems involved in MT_1 and MT_2 melatonin receptor signaling through G protein coupling include adenylyl cyclase, phospholipase C, phospholipase A2, potassium channels and potentially guanylyl cyclase and calcium channels (for reviews see refs. *16,18,31,* and *32*). In this review signaling pathways for MT_1 and MT_2 melatonin receptors will be described only in association with specific receptor functions in target tissues.

The putative MT_3 melatonin receptor site binds 2-[^{125}I] iodomelatonin with nanomolar affinity, displays a distinct pharmacological profile from other melatonin receptors (i.e., *N*-acetylserotonin > melatonin >> serotonin), and appears to couple to stimulation of phosphoinositide turnover *(17,33, 34)*. The MT_3 melatonin binding site was originally thought to be a G protein–coupled receptor; however, a hamster kidney protein identified as quinone reductase II binds 2-[^{125}I]iodomelatonin with the same pharmacological profile described for the MT_3 site in both hamster brain and kidney membranes *(35,36)*. The MT_3 binding site is absent in brain and kidney membranes from mice with genetic deletion of quinone reductase II *(36,37)*. The complete identity of MT_3 membrane binding sites with quinone reductase II as well as the physiological relevance of these sites remains to be determined.

The identification of the molecular structure and the pharmacological characterization of melatonin receptors, the discovery of selective and specific ligands for these receptors, and the introduction of transgenic mice with selective deletion of MT_1 and/or MT_2 melatonin receptors has facilitated the identification and localization of functional melatonin receptors in different neuronal and non-neuronal target tissues. Functional characterization of MT_1 and MT_2 melatonin receptors in native and/or heterologous expression systems requires knowledge of the pharmacological properties (affinity, efficacy, selectivity, specificity) of the ligands and radioligands in the systems under study. This knowledge becomes relevant as ligand efficacy (agonist, partial agonist, inverse agonist, competitive antagonist) at initiating a signaling response is dependent on the cellular milieu in each target tissue, including the presence of spare receptors, G protein type, and/or scaffolding molecules (for review, see refs. *16* and *38*).

Currently MT_1 and MT_2 melatonin receptor function in target tissues is derived primarily from pharmacological studies with two prototype melatonin receptor ligands: luzindole and 4P-PDOT *(20,39,40)*. Luzindole is considered a non-selective MT_1/MT_2 melatonin receptor ligand having 15–26 times higher affinity for the MT_2 receptor. 4P-PDOT is a selective MT_2 ligand as it shows over 300 times higher affinity for the MT_2 than the MT_1 melatonin receptor *(20, 40)*. Luzindole and 4P-PDOT are competitive MT_2 melatonin receptor antagonists *(20)*. However, 4P-PDOT is also a partial agonist on MT_2 melatonin receptors in the rat microcirculatory system *(41)* and in heterologous cells expressing recombinant receptors *(42)*. Luzindole and 4P-PDOT are also MT_1 melatonin receptor antagonists at 300 n*M* and higher concentration; however, they both act as inverse agonists in systems endowed with constitutive active MT_1 receptors *(43–46)*. Other selective MT_2 melatonin receptor ligands characterized as competitive MT_2 receptor antagonists include 4P-CADOT, 4P-ADOT, K185, GR128107, and 5-methoxyluzindole *(20,40,47)*. The ligand IIK7 is a selective MT_2 melatonin receptor agonist *(47)*. The melatonin receptor ligand S26131, formed by dimerization of two molecules of S20098 linked together through the methoxy substituents by three methylene groups, shows over 200-fold higher affinity for the MT_1 than the MT_2 melatonin receptor *(48)*. However, S26131 was reported to antagonize melatonin-mediated stimulation of ^{35}S-GTPγS binding to MT_1 receptors expressed in mammalian cells with only 27 times higher affinity for the MT_1 than for the MT_2 receptor *(48)*. The efficacy of this putative MT_1 melatonin receptor antagonist has not been tested in tissues expressing native melatonin receptors. The ligand 5-MCA-NAT is a high affinity and selective melatonin receptor ligand for MT_3 sites *(49)*.

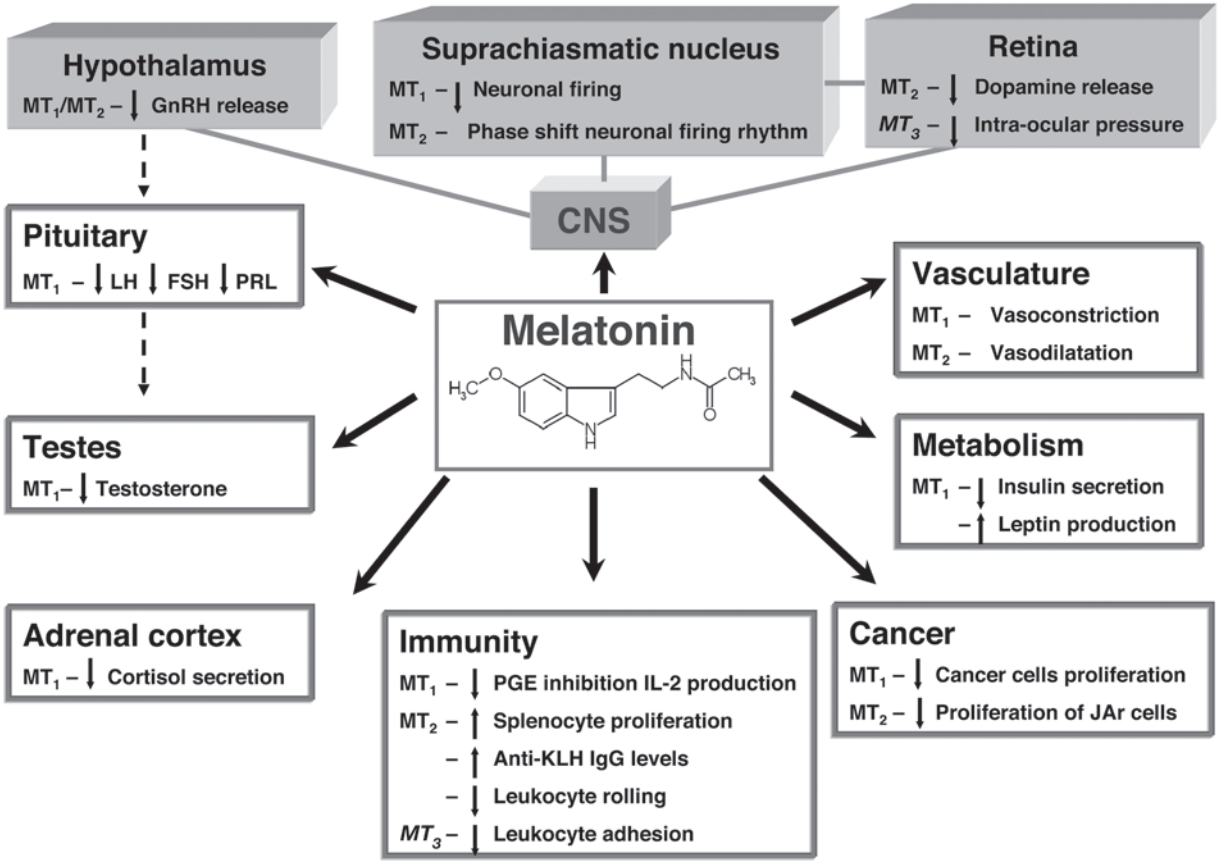
MT_1 and MT_2 Functional Melatonin Receptors in Mammals

This section will review pharmacologically or genetically characterized functional responses through activation of MT_1 or MT_2 melatonin receptors in mammalian species. MT_1 and MT_2 melatonin receptors are discretely distributed in areas of the central nervous system and peripheral target tissues. Receptor autoradiography with 2-[^{125}I]iodomelatonin binding *(50–57)*, Western analysis and immunohistochemistry with specific MT_1 and MT_2 melatonin receptor antibodies *(22,23,58–63)*; RT-PCR and/or *in situ* hybridization *(23,64)* have been used to localize MT_1 and/or MT_2 melatonin receptor proteins and mRNA expression in brain and peripheral organs. Tissues endowed with fully characterized functional MT_1 and/or MT_2 melatonin receptors include retina, suprachiasmatic nucleus, pars tuberalis, cerebral and peripheral arteries, kidney, pancreas, adrenal cortex, testes and immune cells (Table 1).

Circadian Timing System

The mammalian circadian timing system facilitates adaptation of the organism to environmental changes through

Table 1
Melatonin Membrane Receptors-Mediated Physiological Responses



The table represents the well-defined MT_1, MT_2, and *MT_3* melatonin receptor functions in the central nervous system (CNS) and peripheral tissues. The solid arrows represent the direct and the discontinuous arrows represent the indirect effects of melatonin in specific target tissues. Within the boxes, arrows represent decrease (↓) or increase (↑) of a particular function. GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; PRL, prolactine; PGE, prostaglandin E; IL-2, interleukin 2; KLH IgG, serum antikeyhole IgG. For references see text.

the rhythmic regulation of physiological processes. Signals of light and dark perceived by the retina reach the mammalian suprachiasmatic nucleus (SCN) through the retinohypothalamic track that projects from retinal ganglion cells to both the intergeniculate leaflet and the SCN *(65)*. Synchronization of the endogenous master circadian clock to the 24-h period of the sleep–waking cycle occurs by the combined actions of internal (e.g., melatonin) and external stimuli (e.g., light) *(66)*.

The mammalian retina produces melatonin locally and expresses both the MT_1 and MT_2 melatonin receptors. MT_1 melatonin receptor immunoactivity was localized to both the inner and outer plexiform layers, ganglion cells, amacrine cells, and horizontal cells in retinas from various species including humans *(58,67)*. In human retina, MT_1 melatonin receptors are expressed in rod photoreceptors cells *(59–61)*; however, they are absent from rat and guinea pig photoreceptors *(58,67)*. Double immunolabeling experiments localized MT_1 receptors on dopamine-containing and GABA-containing amacrine cells of the guinea pig retina *(58,59)*. Fujieda et al. *(58)* proposed that melatonin-mediated modulation of dopamine release occurs by enhancement of GABA activity; however, this hypothesis has not been directly tested. On the other hand, in the rabbit retina, melatonin inhibits calcium-dependent release of dopamine through activation of presynaptic melatonin heteroreceptors displaying a pharmacological profile similar to that of the human MT_2 melatonin receptor *(20)*. Luzindole and other MT_2-selective ligands competitively block melatonin's inhibition of dopamine release *(20)*; however, no direct demonstration for the presence of MT_2 melatonin receptor protein in mammalian retina has been reported (Table 1). It is conceivable that activation of MT_1 and/or MT_2 melatonin receptors in amacrine and/or ganglion retinal cells regulates

dopaminergic and GABAergic transmission and contributes to the modulation of retino-hypothamic transmission of the light–dark signal.

The SCN are a pair of approx 10,000 cells located within the anterior ventral hypothalamus just above the optic chiasm. In mammals, the SCN is the master clock that controls behavioral, metabolic, and physiological rhythms *(66,68)* including the light-dependent synthesis and release of melatonin from the pineal gland *(69,70)*. Endogenous pineal melatonin is believed to feed back onto the master clock and to regulate neuronal activity and circadian rhythms through activation of specific MT_1 and MT_2 melatonin receptors *(66)*.

The mammalian SCN and immortalized SCN 2.2 cells expresses both mRNA and protein for MT_1 and MT_2 melatonin receptors, which are localized primarily to neuronal elements *(23,40,71,72)*. In the rat and mouse SCN, the MT_2 melatonin receptor protein is not detectable by 2-[^{125}I]iodomelatonin autoradiography *(40,54,73)*. Western blot analysis, however, revealed specific immunoreactive proteins of molecular weight 37 kDa, as well as a glycosylated protein of higher molecular weight for both MT_1 and MT_2 receptors *(23)*. Activation of melatonin receptors in the mammalian SCN modulates several cellular responses. In SCN2.2 cells, melatonin inhibits forskolin-stimulated cAMP accumulation through activation of melatonin receptors as this effect was blocked by luzindole *(23)*. In mouse SCN brain slices, melatonin inhibits pituitary adelylate cyclase–activating polypeptide (PACAP)–mediated cAMP response-element binding protein (CREB) phosphorylation through activation of MT_1 melatonin receptors as this effect was not observed in preparations from mice with targeted disruption of the MT_1 receptor *(74,75)*. In the rat SCN and immortalized SCN2.2 cells, activation of the MT_2 melatonin receptor by melatonin stimulates PKC activity *(23,71)*. Together, these results demonstrate that within the SCN the MT_1 and MT_2 melatonin receptors signal through activation of multiple signaling pathways *(54)*.

MT_1 and MT_2 melatonin receptors in the mammalian SCN stimulate distinct functional responses. In SCN brain slices, activation of melatonin receptors acutely inhibits neuronal firing measured by single-unit or multiunit activity recordings *(76,77)*. This effect of melatonin is likely mediated through activation of MT_1 melatonin receptors as it was not observed in SCN from mice with targeted disruption of the MT_1 receptor, but it is still present in mice lacking the MT_2 receptor *(54,75)* (Table 1). MT_1-mediated inhibition of neuronal firing could result from an increase in potassium conductance and subsequent neuronal hyperpolarization *(78)* through activation of the inward rectifier potassium channel (Kir3) *(79)*. Neurons in the SCN are most sensitive to inhibition of neuronal firing by melatonin at dusk, suggesting a role for MT_1 receptors in altering the state of clock excitability as it shifts from day to night *(80)*. It is therefore possible that melatonin promotes sleep by inhibiting neuronal activity in the SCN and/or other areas of the limbic system. This suggests that selective MT_1 melatonin receptor agonists could be potential therapeutic agents for the treatment of insomnia and other sleep disorders *(81)*.

Melatonin phase shifts circadian rhythms at two windows of sensitivity corresponding to the day–night (dusk) and night–day (dawn) transitions *(40,82,83)*. In the rat SCN brain slice, melatonin phase advances the peak of the circadian rhythm of neuronal firing at subjective-dusk (CT 10) and -dawn (CT 23), which coincides with the rise and fall of melatonin production *(66,71,83)*. Phase shifts induced by melatonin are mimicked by phorbol ester and blocked by inhibition of PKC activity *(83)*. Furthermore, the melatonin-mediated phase shifts are blocked by the selective MT_2 receptor antagonist 4P-PDOT, and are accompanied by the abolition of increases in PKC activity suggesting the involvement of MT_2 receptors *(71)*. MT_2 but not MT_1 melatonin receptors desensitize upon exposure to physiological concentrations of melatonin *(84,85)*. Interestingly, incubation of the rat SCN brain slice with physiological concentrations of melatonin for a time mimicking the nocturnal surge (8 h) during subjective night abolished the phase advance in circadian rhythms of neuronal firing induced by melatonin applied at the dark/light transition (CT 23–2) in the rat. This treatment also abolished the stimulation of PKC in immortalized SCN2.2 cells induced by melatonin applied at CT 2 *(85)*. In rats in vivo, melatonin administration at dusk (CT 10) but not at dawn (CT 22–CT2) phase shifts circadian rhythms of rat locomotor activity *(86)*. These results suggest that nocturnally released melatonin may functionally desensitize MT_2 melatonin receptors in the SCN and preclude phase shifts at dawn *(87)*.

Physiological concentrations of melatonin significantly phase advanced the peak of the circadian rhythm of neuronal firing in the SCN brain slice from a melatonin-deficient mouse strain, the C57BL/6 *(54,88,89)*. The melatonin-mediated phase shift is pertussis toxin sensitive suggesting activation of a Gi coupled receptor *(54)*. Furthermore, the potency of melatonin to phase advance the circadian rhythm of neuronal firing was identical in the SCN brain slices obtained from C57 wild-type mice and mice with genetic deletion of the MT_1 receptor (C57 MT1KO) *(89)*. This response is blocked by MT_2 selective concentrations of the competitive receptor antagonist 4P-PDOT *(90)* (unpublished observations). Together these data suggest that in mice SCN in vitro activation of MT_2 melatonin receptors by picomolar concentrations of melatonin phase shifts the master biological clock as demonstrated in rat SCN *(71,89)* (Table 1).

In the C57BL/6 mouse, as previously shown in the C3H/HeN mouse *(40,82)*, melatonin administered at CT 10 phase advances the onset of circadian activity rhythms in vivo and accelerates the rate of re-entrainment of wheel-running activity rhythms after an abrupt advance of dark onset *(89)*. Paradoxically, in the C57 MT_1KO mouse melatonin did not shift the circadian rhythm of wheel-running activity or accelerate re-entrainment *(89)*. These studies suggest that

melatonin-mediated phase advances of circadian rhythms of neuronal firing in vitro involves activation of MT_2 receptors, while phase shifts of overt circadian rhythms in the mice in vivo may require activation of both the MT_1 and possibly the MT_2 receptor *(89)*. It is likely that in vivo molecular events regulated through activation of MT_1 receptors within the SCN or output pathways may be necessary for expression of circadian rhythms of activity. Further studies using mice with genetic deletion of MT_2 melatonin receptors in melatonin-producing C3H/HeN mice are required to elucidate these paradoxical findings. These studies suggest that MT_1 and/or MT_2 melatonin receptors are potential therapeutic targets for the development of melatonin receptor ligands to treat disorders involving alternation in sleep and circadian clock phase due to endogenous causes (e.g., depression, blindness, delay sleep phase syndrome) or to rapid changes in the light/dark cycle (e.g., jet travel, shift work) *(91)*.

Hypothalamic–Pituitary–Gonadal Axis

Endogenously released melatonin resulting from changes in day length modulates reproduction in seasonal breeders *(5,92,93)*. This effect is mediated by modulation of the hypothalamic–pituitary–gonadal (HPG) axis function through activation of melatonin receptors in hypothalamic GnRH (gonadotrophin-releasing hormone) releasing neurons, pars tuberalis of the anterior pituitary, gonadotrophs and lactotrophs of pars distalis, as well as testes and ovaries *(31,44, 94,95)*.

In laboratory rodents the effect of melatonin on the HPG axis is predominantly inhibitory. In immortalized GnRH-releasing cells expressing endogenous MT_1 and MT_2 receptors, melatonin decreased the expression of GnRH mRNA in a 24 h cyclical manner, which was blocked by luzindole *(94)*. This suggests the possibility of a direct effect of melatonin on 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. Activation of melatonin receptors expressed in neonatal rat pituitary gland inhibits GnRH-induced LH release, cAMP and cGMP accumulation, and the increase in intracellular Ca^{2+} through activation of a pertussis toxin–sensitive G protein–coupled receptor *(96–98)*. The mechanism(s) by which melatonin modulates gonadotrophin secretion from the pituitary gland is complex and appears to involve primarily activation of MT_1 melatonin receptors *(99,100)*; however, participation of MT_2 receptors *(101)* cannot be excluded.

The identification of melatonin in ovarian follicular fluid suggests a direct effect of the pineal hormone in ovarian function *(102,103)*. MT_1 and MT_2 melatonin receptor mRNAs were identified in ovarian granulosa cells and are distributed in various ovarian structures *(44,104,105)*. MT_1 melatonin receptor protein was detected in rat ovaries using specific MT_1 antisera and 2-[^{125}I]iodomelatonin binding *(44, 62,105)*. Moreover, in ovaries and granulosa cells, endogenous estrogens regulate the functional activity of melatonin receptors *(44)*. Melatonin stimulates progesterone secretion from granulosa cells in culture from several species including humans *(104,106)*. In human granulose–luteal cells melatonin increases LH and decreases GnRH receptor density *(104)*. In hamster testicular Leydig cells, through activation of MT_1 receptors, melatonin inhibits basal and chorionic gonadotropin-stimulated cAMP and androgen (testosterone and androstene 3α-diol 17β diol) production *(95)*. In this model melatonin increases local corticotrophin-releasing hormone and downregulates the expression of steroidogenic acute regulatory protein, and other key steroidogenic enzymes *(95)*. Together, these results suggest regulation of ovarian and testicular function by activation of complex mechanisms, which may involve both MT_1 and MT_2 receptors along the HPG axis targets (Table 1).

In the pars tuberalis of the pituitary gland, 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 *(107)*. 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 *(107)*. Furthermore, simultaneously during the biological night, endogenous melatonin through activation of the MT_1 melatonin receptor inhibits prolactin release in the pars tuberalis (Table 1). In this model, gene expression appears to translate the nocturnal exposure of melatonin into a signal that regulates prolactin secretion *(107)* (Table 1). This may be a general mechanism by which the hormone melatonin regulates gene expression to link the central circadian pacemaker and peripheral tissues resulting in modulation of circadian and seasonal rhythms.

Adrenal Gland

Evidence suggests a relationship between the circadian rhythm of melatonin and adrenal hormone secretion. In humans, the 24 h pattern of plasma cortisol peaks in the early morning remaining low during the night when circulating melatonin levels are maximal *(108)*. On the other hand, Demas et al. *(109)* demonstrated that the high melatonin levels, like those observed in short days, enhances aggression in male Siberian hamsters, which appears to be related to alterations in adrenocortical steroids.

Evidence showing direct melatonin receptor–mediated effects on adrenal gland is scarce. In the capuchin monkey 2-[^{125}I]iodomelatonin binding sites localize to the adult and fetal adrenal gland with specific expression of MT_1 but not MT_2 melatonin receptor mRNA *(56,57)*. In adult adrenal explants and dispersed cells, melatonin inhibited adrenocorticotropic hormone (ACTH)–induced cortisol production, which was blocked by luzindole, suggesting activation of MT_1 melatonin receptor *(56)*. Melatonin also decreased dibutyril

cAMP-induced cortisol secretion from adrenal cultured cells, which suggests an effect of melatonin beyond the membrane receptor *(56)*. Similarly, MT_1 receptor–mediated effects were observed in disperse cells from fetal capuchin monkey adrenal gland, in which melatonin inhibits ACTH and corticotropin releasing hormone–induced cortisol secretion but not dehydroepiandrosterone sulfate production. This effect was blocked by luzindole; however, neither this antagonist nor melatonin affects cortisol secretion when tested alone. Additionally, melatonin inhibited ACTH-induced increase in 3β hydroxysteroid dehydrogenase mRNA gene expression in fetal adrenal gland suggesting a selective effect of melatonin on the cortisol synthesis pathway *(57)*. Taken together it does appear that in the fetal and adult capuchin monkey adrenal cortex activation of MT_1 melatonin receptors inhibit ACTH-induce cortisol secretion (Table 1).

Endogenous maternal melatonin production appears to regulate cortisol levels in the newborn capuchin monkey. Suppression of melatonin production by chronic constant light exposure did not affect cortisol or estrogen production during pregnancy, but significantly increased blood cortisol in the newborn, which is normalized when mothers are treated with melatonin *(57)*. This is the first demonstration of a physiological role of melatonin on regulation of cortisol production during pregnancy; however, whether this effect of melatonin is mediated through activation of MT_1 receptors in adrenal cortex is not known.

Metabolism

Evidence suggests a relationship between the circadian release of melatonin and energy balance *(110)*. Melatonin appears to regulate 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 MT_1 but not MT_2 melatonin receptor mRNA and high affinity 2-[^{125}I]iodomelatonin binding sites *(55,111,112)*. Melatonin and melatonin agonists inhibit in a dose-dependent manner forskolin-stimulated insulin secretion through activation of a pertussis toxin–sensitive receptor involving Gi/Go protein coupling. The melatonin-mediated inhibition of stimulated insulin release was counteracted by incubation with the non-hydrolyzable GTP analog, GTPγS, and was antagonized by luzindole and 4P-PDOT at concentrations known to block MT_1 melatonin receptors *(55,111,112)*. However, melatonin alone failed to affect the basal level of insulin. In INS-1 β cells short treatment with melatonin through a cAMP/PKA–dependent mechanism attenuates glucagon-like peptide (GLP-1) and forskolin-stimulated insulin secretion as well as insulin promoter activity and cAMP response-element (CRE)–mediated gene expression *(112)* (Table 1). These reports provide strong evidence in support of a modulatory role of acute exposure to melatonin on pancreatic insulin secretion.

Prolonged melatonin pretreatment sensitizes the cAMP system in β cells, increasing cAMP production and insulin secretion. In rat pancreatic islets and INS-1 cells, chronic melatonin treatment increased basal levels of insulin secretion and potentiated GLP-1- and forskolin-induced insulin and cAMP production *(112)*. Photoperiodically released melatonin during subjective night was proposed to sensitize the cAMP system in β cells to potentiate insulin release upon GLP-receptor stimulation by endogenous incretin *(112)*. Regulation of the circadian release of insulin and sensitization of the cAMP system by endogenous melatonin may provide a mechanism by which high morning levels of insulin facilitates glucose disposal following nutritional stimuli *(112)*.

Melatonin also modulates rat adipocytes function through a receptor-mediated mechanism. MT_1 and MT_2 melatonin receptors are expressed in inguinal and epididymal adipocytes *(113)*. Melatonin inhibits isopropanol-stimulated lypolysis in inguinal but not epididymal adipocytes. This effect is mediated via MT_1/MT_2 melatonin receptors, as it was completely blocked by pertussis toxin, by 8-bromo-cAMP, and by the melatonin receptor antagonist S-20928 *(113)*. Furthermore, in epididymal adipocytes melatonin in the presence of insulin increases leptin secretion, and counteracts forskolin-induced inhibition of leptin secretion and mRNA expression. This effect of melatonin was completely antagonized by luzindole but not 4P-PDOT. However, insulin or melatonin alone did not modify leptin secretion suggesting cross talk between these hormones to modulate leptin production from adipocytes via MT_1 receptor activation *(114)*. Taken together melatonin appears to modulate glucose homeostasis and energy balance via its direct effect on β pancreas cells and adipocytes through activation of MT_1 receptors.

Cardiovascular System

Functional melatonin receptors are expressed in several mammalian vascular beds. MT_1 and MT_2 protein and mRNA were detected in peripheral and in cerebellar arteries of various species including humans *(115,116)*. Melatonin mediates both vasoconstriction and vasodilation through activation of distinct melatonin receptors. Melatonin potentiates adrenergic nerve stimulation and norepinephrine-induced contraction in rat caudal artery *(117)*; however, it does not affect vascular tone. Vasoconstriction appears to be mediated by decreases in cAMP-mediated phosphorylation of calcium-activated potassium channels (BK_{Ca}) through Gi/Go protein-coupled MT_1 melatonin receptors present in the smooth muscle, although participation of receptors localized in the endothelium cannot be ruled out *(115,118,119)*. In support of this conclusion, vasoconstriction of cerebral arteries induced by melatonin *(52)* is blocked by the competitive melatonin receptor antagonists luzindole and S-20928, by pertussis toxin, and by blockers of BK_{Ca} channels *(52,120)* (Table 1). MT_1 melatonin receptor localization in the arterial wall and hippocampal microvasculature of normal and Alzeheimer disease subjects suggest involvement of melatonin in the regulation of cerebral blood flow *(116)*. It is conceivable that melatonin-mediated vasoconstriction

by modulating vascular tone may attenuate diurnal fluctuations in blood pressure keeping cerebral flow constant *(121)*. It was suggested that increases in MT_1 receptor immunoreactivity in cerebral vessels from Alzeheimer subjects may result from decreases in melatonin levels associated with age and cerebral hypofunction associated with neurodegeneration *(116)*.

Melatonin receptor–mediated vasodilation was demonstrated in rat arteries. Potentiation of phenylephrine-induced contractions by melatonin in caudal arteries was enhanced in the presence of the MT_2-selective receptor antagonists 4P-PDOT suggesting blockade of a receptor involved in decreasing vascular tone, possibly the MT_2 receptor *(115,122)* (Table 1). Vasodilation and increase in blood flow induced by melatonin in distal skin regions may underlie the concomitant heat loss and hypothermic effect of this hormone *(123)*.

Melatonin is involved in the control of intraocular pressure during the daily photoperiod. Various melatonin-receptor ligands including the selective MT_3 agonist 5-MCA-NAT (5 metoxycarbonylamino-*N*-acetyltryptamine) induce inhibition of intraocular pressure in rabbits. This effect was blocked by the putative MT_3 antagonist prazosin *(124)* (Table 1). Together these results suggest 5-MCA-NAT or related ligands as potential agents to treat abnormal intraocular pressure.

Immune System And Cancer

The demonstration by Csaba and Barath in 1975 *(125)* that pinealectomy causes thymic involution and suppressed immunity established the relationships between the pineal gland and the immune system. Immune functions in certain mammals follow daily and seasonal rhythms showing an enhancement during short days, at least in laboratory conditions, which correlates with the duration of melatonin secretion *(126)*. Diurnal rhythms of experimental granulomatous inflammation in rodents are blocked by pinealectomy and are re-established by nocturnal replacement of melatonin *(127)*. Melatonin is synthesized in human lymphocytes suggesting an autocrine and/or paracrine role for these cells *(14)*. Furthermore, exogenous melatonin, both in vivo and in vitro, appear to stimulate the mammalian immune system (for review, see refs. *128* and *129*).

Melatonin membrane receptors are expressed in lymphoid cells and are involved at least in part in regulating immune responses (for review, see refs. *129* and *130*). Melatonin-mediated enhancement of mice splenic lymphocyte proliferation measured either during the day or night was blocked by the melatonin receptor antagonist luzindole *(131)*. This effect of melatonin appears to be mediated through activation of the MT_2 receptor as the increase of splenocyte proliferation (e.g., cell-mediated immunity) and anti-keyhole IgG concentration (e.g., humoral immunity) was also observed in mice with genetic deletion of the MT_1 melatonin receptor *(132)*. Melatonin reduces acute inflammation in rats by inhibiting leukocyte rolling in the microvasculature through activation of the MT_2 melatonin receptor and leukotriene B4-induced leukocyte adhesion to endothelial cells through a melatonin receptor with the pharmacological characteristics of the MT_3 site *(41)*. In human lymphocytes melatonin counteracts the inhibitory effect of prostaglandin 2 on interleukin 2 production through its MT_1 membrane receptor, but nuclear receptor cannot be ruled out *(133,134)*. Taken together, these reports suggest a role for melatonin in immunity providing a mechanism by which endogenous melatonin may participate in adaptation to seasonal changes (Table 1).

Melatonin has a direct effect on cell proliferation and cytokine secretion and hence was suggested as an oncostatic agent (for review, see refs. *135* and *136*). In animals bearing carcinogen-induced tumors, melatonin treatment reduces tumor number and size, increases tumor latency, and lowers tumor incidence. In vitro melatonin inhibits tumor cell proliferation through an effect on the cell cycle *(137)*, interaction with sex steroid–responsive pathways *(136)*, and/or perhaps in part via its free-radical-scavenging potential *(138)*.

Membrane melatonin receptors appear to be involved in melatonin's oncostatic effect. LNCaP prostate tumor and MCF-7 breast cancer cells express MT_1 melatonin receptor proteins *(139,140)*. Colon 38 cancer cells express both MT_1 and MT_2 melatonin-receptor mRNA, and MT_1 and RORα proteins *(141)*. In contrast human choriocarcinoma JEG-3 cells express only MT_2 melatonin receptors *(142)*. Histological studies in human malignant tissue show alterations in melatonin-receptor density. Human gallbladder adenocarcinoma epithelial cells expressed lower levels of MT_1 receptors *(143)*, while malignant breast epithelium expressed higher levels of MT_1 receptors than normal tissue *(144)*.

Melatonin oncostatic action appears to be mediated primarily through activation of MT_1 melatonin receptors, but an action on the MT_2 receptors cannot be excluded, as melatonin shows an antiproliferative effect in JAr cells *(137)*. Melatonin inhibited endometrial cancer in estrogen receptor–positive Ishikawa cells, which was blocked by luzindole *(145)*. Activation of MT_1 receptors induce neurite growth in N1E-115 neuroblastoma cells *(146)* and inhibits proliferation of MCF-7 cells *(140)*. Several cellular mechanisms have been suggested to mediate melatonin oncostatic effects. Perhaps the best characterized pathway is that of melatonin's suppression of linoleic acid uptake and its conversion to 13-hydroxyoctadecadienoic acid (13-HODE), which normally activates EGFR/MAPK mitogenic signaling *(147)* (Table 1).

Interestingly, overexpression of MT_1 melatonin receptors facilitates oncostatic action. Daily melatonin administration decreases the weight and volume of S-91 melanoma tumors in mice, and inhibits S-91 cells proliferation in vitro *(148)*. This effect was apparently mediated by increases in catalase and glutathione peroxidase activity. However, expression of MT_1 melatonin receptors in S-91 cells dramatically increased the inhibitory effect of melatonin on cell pro-

liferation in vitro *(148)*. Overexpression of MT_1 receptor in MCF-7 breast cancer cells results in melatonin-induced tumor growth inhibition in vivo *(149)*, as well as in melatonin-induced inhibition of tumor cell proliferation in vitro. The antiproliferative effect of melatonin was antagonized by the melatonin receptor antagonist S-20928 *(150)*. Interestingly, overexpression of MT_1 melatonin receptors in MCF-7 cells suppressed tumor formation in vivo, probably by increasing the inhibitory signal of constitutive active MT_1 receptors *(149)*. Activation of MT_1 melatonin receptors, constitutive expression as well as the involvement of ROR-α nuclear melatonin receptors may participate in melatonin's oncostatic action (for review, see ref. *135*). These data suggest immune enhancing and oncostatic effects of melatonin primarily through activation of MT_1 melatonin receptors and/or enhancement of constitutive active (Table 1).

Concluding Remarks

Novel melatonin receptor ligands and creation of transgenic mice with deletion of MT_1 or MT_2 receptors are increasing our understanding of melatonin receptor–mediated signaling to effector targets. In this review we describe well-characterized functions for the MT_1 and MT_2 receptors and for the putative MT_3 sites. These responses were characterized using mice with genetic deletion of melatonin receptors and specific and selective melatonin-receptor agonist, antagonist, and/or inverse agonist ligands with known pharmacological properties. Melatonin-mediated responses are dependent on length and time of treatment. Treatments mimicking the length of the physiological nocturnal melatonin profile differentially regulate MT_1 and MT_2 receptor function. Prolonged treatment with melatonin causes MT_1 receptor-mediated sensitization of adenylyl cyclase, which in turn potentiates CREB phosphorylation in heterologous mammalian cells, Per1 expression in pars tuberalis, and insulin secretion in INS-1 cells *(28,107,112,151)*. The sensitization of the cAMP system by nocturnal melatonin appears to be a general mechanism by which the SCN regulates MT_1-mediated responses in peripheral target tissues. By contrast, physiological levels of melatonin mimicking the nocturnal profile functionally desensitizes MT_2 receptors in the SCN perhaps as a way of translating changes in night length with the season *(85)*. Indeed, at dawn melatonin-mediated phase advances in circadian rhythms of neuronal firing in the rat SCN brain slice, as well as the stimulation of protein kinase C in immortalized SCN2.2 cells may be attenuated by endogenous melatonin secreted during the night in vivo *(86)* or by prolonged exposure to exogenous melatonin in vitro *(85)*. Furthermore, melatonin through activation of distinct receptor types expressed within the same tissue or perhaps in the same cell, exerts often distinct and/or opposite physiological responses. For instance, in the SCN melatonin inhibits neuronal firing via MT_1, but it phase shifts neuronal firing rhythms through activation of MT_2 melatonin receptors *(54,71,75)*. In the vascular system melatonin evokes opposite responses—it potentiates vasoconstriction through MT_1 and induces vasodilatation via MT_2 receptors. Melatonin-mediated effects are time dependent, with the efficacy of melatonin being probably dependent on the diurnal sensitivity of MT_1 and MT_2 melatonin receptor expression. Based on the dual function of melatonin-mediated responses it is becoming clear that melatonin receptor function should be studied in depth to facilitate discovery and development of novel agents for the treatment of sleep, circadian, metabolic, and endocrine disorders, as well as tumor cell growth.

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