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Gene Regulation by Melatonin

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ABSTRACT: **The physiological and neuroendocrine functions of the pineal gland hormone, melatonin, and its therapeutic potential critically depend on the understanding of its target sites and its mechanisms of action. This has progressed considerably in the last few years through the cloning of G protein–coupled seven-transmembrane melatonin receptors (Mel_{1a} and Mel_{1b}) as well as of nuclear receptors (RZR/RORα and RZRβ) that are associated with melatonin signaling. The transcription factor RZR/RORα appears to mediate a direct gene regulatory action of the hormone, and specific binding sites have been identified in promoter regions of a variety of genes, such as 5-lipoxygenase (5-LO), $p21^{WAF1/CIP1}$, and bone sialoprotein (BSP). The membrane signaling pathway clearly shows higher ligand sensitivity than the nuclear signaling pathway, but details of its signal transduction cascade, and target genes are presently unknown. Membrane melatonin receptors are expressed mainly in the central nervous system, whereas RZR/RORα is prominently expressed both in the periphery and the brain. The action of membrane melatonin receptors and their specific agonists have been associated with circadian rhythmicity, whereas direct effects of melatonin in the periphery, such as immunomodulation, cellular growth, and bone differentiation, mainly appear to be mediated by RZR/RORα. It is hypothesized in this review that, in some cases, RZR/RORα may be a primary target of membrane melatonin receptors.**

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) was identified as the skin-lightening ingredient of the pineal gland[1] and is the major hormone of this gland.[2] Melatonin appears to have an important role in the regulation of circadian rhythms, sleep and mood, but there is also various evidence that the pineal gland hormone is important in immunomodulation, reproduction, tumor growth, and aging.[3] The mammalian pineal gland acts as a neuroendocrine transducer for photic information from the retina via the suprachiasmatic nucleus (SCN). Melatonin appears to be a mediator of light and dark information and day length, but the circadian rhythm of melatonin secretion is directed by the SCN, that is, it is of endogenous origin.[4] Melatonin is synthesized in the pineal gland from serotonin under the control of the enzymes arylalkylamine *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT) (see FIGURE 1).[5] NAT gene expression is regulated by the SCN via β-adrenergic innervation of the pineal, which increases the cAMP level and activates the transcription factor cAMP response element (CRE) binding protein (CREB). The

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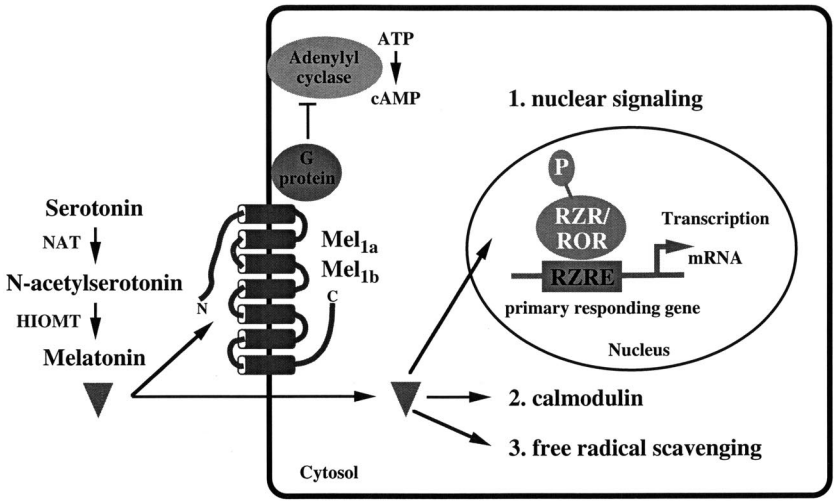

FIGURE 1. Melatonin signaling. The pineal gland hormone, melatonin, shows various molecular actions. Melatonin is synthesized in the pineal gland from serotonin. The hormone binds with high affinity in the picomolar range to the membrane receptors, Mel_{1a} and Mel_{1b}, and/or in the nanomolar range to the nuclear receptor RZR/ROR as well as to calmodulin. At even higher concentrations melatonin has also a free radical scavenging function.

NAT promoter contains several CREs that are also bound by members of the CRE modulator (CREM) family, such as the dominant repressor inducible cAMP early repressor (ICER). The diurnal regulation of NAT depends on the interplay between CREB and ICER and finally results in synthesis and release of melatonin in darkness and its inhibition by daylight. Because the biological half-life of the hormone is short, serum levels of melatonin display a clear circadian rhythm with peak levels of approximately 0.4 nM at night time.[3] However, peak serum melatonin concentrations vary considerably between individuals and depend very much on age. Standard oral doses of melatonin (1 to 5 mg), which are taken daily by hundreds of thousands of Americans, result in 10 to 100 times higher serum melatonin concentrations than the usual nighttime peak one hour after ingestion, followed by a decline within four to eight hours. Although no serious side effects have been reported with the ingestion of melatonin, in Europe melatonin has been classified as a medicine and was therefore withdrawn from general sale.

MEMBRANE MELATONIN RECEPTORS

Two membrane-associated melatonin binding sites, referred to as ML1 and ML2, can be distinguished pharmacologically by their high affinity in the picomolar range and their lower affinity in the nanomolar range, respectively (TABLE 1).[6] ML1 receptors are coupled to pertussis toxin–sensitive G proteins and belong to the family of

seven-transmembrane receptors (FIG. 1). In mammals, two types of high-affinity membrane melatonin receptors, referred to as Mel_{1a} (also named mt_1) and Mel_{1b} (also named MT_2), have now been cloned.[7,8] Both receptors show 60% homology at the amino acid level. A third membrane melatonin receptor, called Mel_{1c}, was found in amphibians but not in mammals.[9] The Mel_{1a} receptor is expressed in the pars tuberalis of the pituitary and the SCN, that is, in the presumed sites of the reproductive and circadian actions of melatonin, respectively, whereas the Mel_{1b} receptor is mainly expressed in the retina (TABLE 1). *In situ* hybridization and RT-PCR experiments suggest that the Mel_{1a} receptor appears to represent more than 99% of all melatonin membrane binding sites in the brain.[10] The Mel_{1a} receptor appears to mediate the inhibitory action of melatonin on the SCN, whereas the Mel_{1b} receptor may be involved in the phase-shifting response of melatonin.[10]

Not many details on the targets and mediators of their transduction pathways of the membrane melatonin receptors are known yet, but it appears that stimulation of these receptors decreases the intracellular level of cAMP, which then results in the change of the phosphorylation status of target proteins such as CREB.[9] In addition to the inhibition of adenylyl cyclase activity, high-affinity melatonin receptors also regulate cGMP levels through proteins upstream of the guanylyl cyclase such as NO synthase.[11] In mammals, the latter function appears to be specific for the Mel_{1a} receptor.[12] In the pars tuberalis, membrane melatonin receptors modulate the expression of the tuberalin gene, which in turn stimulates the release of prolactin from these cells.[13] Mel_{1a} receptor levels were found to be downregulated by protein kinase C (PKC).[14] Moreover, it has been suggested that second messengers, other than cAMP and cGMP, might be modulated by melatonin via Mel_{1a} receptors,[15] but a clear picture of the spectrum of biochemical signals elicited by melatonin is still lacking. Despite the insight into possible functions of melatonin membrane receptors, little is known about the molecular structure of the receptor or receptor-ligand interactions. However, elucidation of the primary structures of melatonin receptors has allowed construction of a three-dimensional rhodopsin-based model for melatonin recognition at its receptor.[16] According to this model, melatonin is recognized by specific amino acid residues in a binding pocket formed by transmembrane helices. The amino acids suggested to interact with melatonin are highly conserved within the family but are not present in other G protein–coupled receptors.

TABLE 1. Melatonin binding sites and receptors

Binding sites	Receptors	Location	Expression	Function
ML1 (high affinity)	Mel_{1a}(mt1)	Membrane	Pars tuberalis SCN	Reproduction circadian rhythm
	Mel_{1b}(mt2)	Membrane	Retina	?
ML2 (low affinity)	Calmodulin	Cytosol	Ubiquitous	Calcium signaling
	RZR/RORα (NR1F1)	Nucleus	Ubiquitous	Various
	RZRβ (NR1F2)	Nucleus	Retina, SCN pineal	Circadian rhythm?

INTRACELLULAR ACTIONS OF MELATONIN

The small lipophilic structure of melatonin suggests that it may also have intracellular binding sites and actions. Melatonin was shown to act as an intracellular scavenger of hydroxyl and peroxyl radicals and appears to protect against oxidative damage[17] (FIG. 1). In humans, the antioxidant effect probably occurs only at pharmacological melatonin concentrations, but the decrease of nighttime serum melatonin concentration that occurs with aging suggests an antiaging potential of the pineal gland hormone.[18] Melatonin was also found to bind cytosolic calmodulin and thus appears to modulate calcium signaling[19] (FIG. 1).

Most interestingly, the pineal gland hormone was also shown to bind and activate two closely related nuclear receptors, referred to as RZR/RORα[20,21] and RZRβ[22] (FIG. 1; according to the unified nomenclature of the nuclear receptor superfamily, they are now called NR1F1 and NR1F2, respectively[23]) in the low nanomolar range.[24,25] The nuclear receptor superfamily is a family of approximately 100 transcription factors that all contain a highly conserved DNA binding domain of 66 to 70 amino acids forming two zinc finger structures.[26] A further characteristic structure of nuclear receptors is a moderately conserved, carboxy-terminal ligand-binding domain that also contains dimerization and transactivation subdomains.

RZR/RORα and RZRβ show distinct spaciotemporal expression patterns, suggesting that both receptor subtypes have different functions related to cell-specific gene control mechanisms in the context of different biological processes. At least one of the four RZR/RORα isoforms is found in every tissue, but highest expression was found in peripheral blood lymphocytes (B cells, T cells, and neutrophils)[20] and skin.[27] RZR/RORα is expressed during embryonic and postnatal development of the brain in Purkinje cells of the cerebellum, in the olfactory bulb, in the dorsal root ganglia, and in the thalamus and the hippocampus.[28] Moreover, RZR/RORα upregulation was found during the differentiation of embryonic P19 cells into neurons.[29] By contrast, RZRβ is expressed only in the retina and the brain, with highest expression in the pineal gland, the SCN, the pars tuberalis of the pituitary, the hypothalamus, the thalamus, and the spinal cord.[24,30,31] The receptor shows the tendency to be expressed in regions of the brain that are involved in sensory pathways rather than in those involved in motor control.[31,32] Moreover, during embryonic development RZRβ expression in the SCN changes,[30] and in the pineal gland the expression of RZRβ was found to be regulated by cAMP in a day–night rhythm.[33] Interestingly, the RZRβ knockout mouse model shows effects on circadian rhythmicity.[34]

Nuclear receptors regulate gene transcription through the binding to specific DNA sequences, called response elements, which are located in the promoter region of their target genes.[35] Therefore, for each nuclear receptor the characterization of its response elements provides important information. RZR/RORα and RZRβ belong to the minority of the members of the nuclear receptor superfamily that are able to bind as monomers to DNA. They require the typical hexameric core-binding site RGGTCA (R = A or G) and an A/T-rich sequence 5'-flanking to this site; in particular, a T in the −1 position and an A in the −4 position appear to be crucial.[21,22] In theory, the consensus sequence for RZREs should be found every 33 kB, that is, approximately one in ten genes should, on average, carry an RZRE. Therefore, it is not surprising that RZREs have been identified in a broad variety of promoter

regions.[36,37] Apparently, the most interesting candidate genes are the proinflammatory enzyme 5-LO,[38] the cell-cycle inhibitor $p21^{WAF1/CIP1}$,[36] and BSP.[36]

RZR/ROR SIGNALING

A constitutive transcriptional activity is well known for a variety of transcription factors, such as NF-κB, AP1, or CREB. Regulation of these transcription factors is mainly achieved through phosphorylation and/or dissociation of repressor proteins. The constitutive activity of RZR/RORα and RZRβ was found to be clearly reduced by the depletion or omission of serum, indicating that serum components may either directly (as true ligands) or indirectly activate RZR/ROR.[22,24,25] Under such conditions of low constitutive activity, it was found that the thiazolidinedione CGP52608 (1-[3-allyl-4-oxo-thiazolidine-2-ylidene]-4-methyl-thiosemicarbazone)[25] and structurally related compounds[39] show, at low nanomolar concentrations (1 to 5 nM), specific activation of RZR/RORα. Those thiazolidinediones that were able to activate RZR/RORα, exhibited potent antiarthritic activity,[39] whereas close analogues were pharmacologically inactive and did not exhibit receptor activation properties. As for thiazolidinediones, low nanomolar concentrations of melatonin were sufficient for RZR/RORα and RZRβ activation. However, at high constitutive activity of RZR/RORα, a significant ligand activation has not yet been observed.[25,40,41] This suggests that RZR/RORα and RZRβ may only be mediators of nuclear melatonin signaling under restricted conditions. However, studies with melatonin responding genes that carry a RZR/ROR binding site in their promoter[38] indicated that these restrictions may only apply under artificial conditions using overexpressed receptors and heterologous promoter constructs.

Ligand binding assays with 2-[^{125}I]-iodomelatonin demonstrated specific binding to nuclear extracts of RZR/ROR-overexpressing cells and *in vitro*–translated RZR/ROR.[24,25] The thiazolidinedione CGP52608 also showed specific binding to nuclear extracts and appeared to compete with melatonin for the same binding site.[25] Interestingly, CGP52608 does not bind to membrane preparations that contain membrane melatonin receptors.[25] The K_d values of melatonin and CGP52608 binding to nuclear extracts were determined in the low nanomolar range.[24,25] Interestingly, melatonin concentrations are approximately fivefold higher in children than in adults, reaching concentrations averaging 1.4 nM,[42] which may suggest that the nuclear receptors may play a more prominent role in children than in adults.

IMMUNOMODULATORY EFFECTS OF MELATONIN

Melatonin is being considered as playing a fundamental role in immunomodulation, such as an increase of IL-2 and IL-4 production in T lymphocytes.[43] In accordance with that, melatonin receptors with a K_d value of 0.27 nM have been described in $CD4^+$ T cells[44] and monocytes.[45] Mel_{1a} receptors were initially assumed to be expressed exclusively in the brain, but sensitive RT-PCR techniques allowed the detection of the receptors also in lymphocytes.[46] However, the RZR ligand CGP52608 was shown to displace melatonin from spleen and thymus cell nuclei,[47] and in $CD4^+$

T cells, CGP52608 enhanced the production of IL-2, IL-6, and interferon-γ,[48] which suggests that these genes are regulated by melatonin via RZR/RORα. Moreover, melatonin was reported to downregulate the expression of 5-LO in cells that exclusively contain RZR/RORα.[38] In support of this finding, 5-LO mRNA expression levels were increased in the hippocampus of pinealectomized rats as compared with the sham-operated controls.[49] The enzyme 5-LO has a key role in the synthesis of leukotrienes, which mediate allergic and inflammatory reactions.[50] Inflammatory reactions are known to be associated with the generation of a large number of free radicals. Because melatonin inhibits 5-LO activity, it would tend to reduce inflammatory reactions as well as the free radical damage.

ANTIPROLIFERATIVE EFFECTS OF MELATONIN

Melatonin was shown to influence the growth of tumors and was found, in most cases, to have a protective effect. The pineal gland hormone has proved to suppress tumor growth in a number of experimental models, including undifferentiated neoplasms, sarcomas, and carcinomas. The exact mechanisms of the oncostatic action of melatonin are not known. MCF-7 breast carcinoma cells express RZR/RORα,[38] and several important proteins that regulate the cell cycle, such as $p21^{WAF1/CIP1}$, contain an RZRE within their gene promoter regions.[36] The expression of $p21^{WAF1/CIP1}$ was observed to be repressed through a dominant negative RZR/RORα mutant,[51] suggesting that RZR/RORα is an important regulator of the $p21^{WAF1/CIP1}$ gene. Moreover, the RZR/RORα ligand CGP52608 was shown to cause antiproliferative effects.[52] This evidence suggest that RZR/RORα may be the mediator of the antiproliferative effect of the pineal gland hormone. Moreover, a very interesting experimental model is the mouse mutation *staggerer.* Homozygous staggerer mice show severe cerebellar ataxia, immune defects, and reduced size. It had been shown that this mutation caries a disrupted RZR/RORα gene, that is, *staggerer* mice express an RZR/RORα protein that lacks its ligand-binding domain.[53] The *staggerer* phenotype was confirmed by the RZR/RORα knockout.[27,54] Until now, the effects of melatonin on *staggerer* mice have not yet been investigated, but such experiments may help to define the physiological role of RZR/RORα in the context of the pineal gland hormone.

Melatonin was also shown to downregulate the estrogen receptor (ER) expression[55] and to block ER activation.[56] These antiestrogenic effects appear to be mediated by membrane melatonin receptors, as the ER promoter does not contain an RZRE, and RZR/RORα was excluded as mediating antiestrogenic effects of melatonin.[56] Moreover, also TGFβ and the protooncogene c-*myc* have been shown to be upregulated by melatonin in MCF-7 cells.[57] Finally, an indirect, neuroendocrine effect of melatonin on the growth of hormone-responsive cancers may be mediated via the hypothalamic-pituitary axis through an inhibition of the release of the peptide hormones GnRH from the hypothalamus and of LH, FSH, and prolactin from the pituitary, which in turn would have repressive effects on steroid hormone production.

MELATONIN AND BONE

Melatonin has recently been shown to be capable of promoting osteoblast differentiation and mineralization of matrix in culture,[58] which suggests that the pineal gland hormone may play an essential role in regulation of bone growth. As one of the major secretory proteins of osteoblasts, BSP functions to regulate mineralization possibly by its direct interaction with cell surface integrin receptors and/or by initiating nucleation of the bone mineral, hydroxyapatite. Therefore, increased expression of BSP along with other bone marker proteins is required to induce mineralization. There is evidence that the effects of melatonin on BSP expression appear to be initiated by Mel_{1b} receptors, which were found to be expressed in osteoblasts.[58] Melatonin membrane receptors are known to reduce the cAMP levels, and the expression of RZR/RORs were found to be regulated by cAMP, at least in the pineal gland.[33] This suggests that RZR/RORα, as a primary responding gene of membrane melatonin receptors, may regulate BSP gene expression through the strong RZRE in the BSP promoter. The decreasing melatonin levels during the aging process therefore suggests that melatonin may have a significant influence on the rate of synthesis and/or maintenance of bone in the elderly.

CONCLUDING HYPOTHESIS: MEMBRANE MELATONIN SIGNALING VIA NUCLEAR RECEPTORS?

It has been postulated that, at least in mammals, direct effects of melatonin via membrane receptors are restricted to the brain.[4] The brain-specific expression of Mel_{1a} and Mel_{1b} receptors, as initially reported, supported this view. In fact, melatonin binding sites were found primarily in various regions of the brain, but in the meantime also in peripheral tissues. This would allow three cases for melatonin signaling through nuclear and membrane receptors to be distinguished: cells that express (1) membrane melatonin receptors and RZR/ROR in parallel, (2) only membrane melatonin receptors, or (3) only RZR/ROR. Inasmuch as RZR/ROR is rather ubiquitously expressed, the first case may apply to various structures of the central nervous system (SCN, retina, pars tuberalis, and the pineal gland). Until now, a tissue that only expresses membrane melatonin receptors is not known. RZR/ROR is a phosphoprotein (like, for example, CREB), and its high constitutive activity is likely to be modulated by a change of its phosphorylation status. The role of covalent modifications for the function of RZR/ROR is not yet studied, but it is tempting to speculate that it may be as important as ligand-induced activation. This leads to the idea that the phosphorylation status of RZR/ROR, that is, its constitutive activity, may be modulated by a membrane melatonin receptor, so that RZR/ROR may be a primary target gene of membrane melatonin signaling.

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