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Preprint – Recent Patents on Endocrine, Metabolic & Immune Drug Discovery
Vol. 5 Nº 2, 2011

Publicado en *Recent Patents on Endocrine, Metabolic & Immune Drug Discovery, 5 (2), 109-123, 2011*

Melatonin, Immune Function and Cancer

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Abbreviated title:

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Abstract

Melatonin is a natural substance ubiquitous in distribution and present in almost all species ranging from unicellular organisms to humans. In mammals, melatonin is synthesized not only in the pineal gland but also in many other parts of the body, including the eyes, bone marrow, gastrointestinal tract, skin and lymphocytes. Melatonin influences almost every cell and can be traced in membrane, cytoplasmic, mitochondrial and nuclear compartments of the cell. The decline in the production of melatonin with age has been suggested as one of the major contributors to immunosenescence and development of neoplastic diseases. Melatonin is a natural antioxidant with immunoenhancing properties. T-helper cells play an important role for protection against malignancy and melatonin has been shown to enhance T-helper cell response by releasing interleukin-2, interleukin-10 and interferon-γ. Melatonin is effective in suppressing neoplastic growth in a variety of tumors like melanoma, breast cancer and ovarian and colorectal cancer. As an adjuvant therapy, melatonin can be beneficial in treating patients suffering from breast cancer, hepatocellular carcinoma or melanoma.

Key words: melatonin, melanoma, immune therapy, oxidative stress, breast cancer, gastrointestinal cancer, colorectal cancer, cytokines, T-helper cells.

Introduction

Melatonin is a natural substance that has been identified in all major living species, including bacteria and other unicellular microorganisms, plants and animals, as well as in humans [1,2]. It is possible that the first function of melatonin in phylogeny was related to its activity as a direct and indirect antioxidant. Interestingly enough, several herbs that contain high levels of melatonin have been used by Chinese since ancient times to retard ageing and to treat diseases associated with the generation of free radicals [3].

Both ageing and free radical generation are major factors involved in all steps of carcinogenesis, including initiation, promotion and progression of neoplastic disease [4]. The ubiquitous distribution of melatonin in Nature is compatible with the view that it can be one of the natural molecules that are effective in treating neoplastic [5-9] as well as degenerative diseases [10,11].

Melatonin is normally synthesized and secreted during the dark phase of daily photoperiod. Though it is produced primarily in the pineal gland, melatonin is also synthesized in other organs like the retina, bone marrow cells, gastrointestinal (GI) tract, lymphocytes, platelets and skin (see for ref. [2]). Reports on plasma melatonin levels among subjects of different age groups reveal a decrease in melatonin production with advanced age [12].

Ageing is associated with a decline in immune function known as immunosenescence. This leads to increased susceptibility to infectious diseases and cancer. The decline in circulating levels of hormones associated with ageing such as dehydroepiandrosterone, estradiol, growth hormone and melatonin have been suggested to contribute to immunosenescence [13]. Pineal ablation, or any other experimental procedure that inhibits melatonin synthesis and secretion, induces a state of immunodepression, which is partly counteracted by melatonin administration [6,14-16]. The immunoenhancing action of melatonin is demonstrable in a variety of animal species and in humans.

Among the various functions attributed to melatonin in the control of the immune system, antitumor defense assumes a primary role [17-20]. The nighttime

physiological surge of melatonin in the blood or extracellular fluid has been suggested to serve as a "natural restraint" for tumor initiation, promotion and/or progression [5]. Melatonin was demonstrated to be oncostatic for a variety of tumor cells like breast carcinoma [21-23], ovarian carcinoma [24], endometrial carcinoma [25], human uveal melanoma cells [26], prostate tumor cells [27] and GI tumors [28,29].

Melatonin biosynthesis

Melatonin is synthesized from the amino acid tryptophan via its conversion to serotonin. Serotonin is then acetylated to form N-acetylserotonin by the enzyme arylakylamine *N*- acetyltransferase (AANAT). N-acetylserotonin is converted into melatonin by the enzyme hydroxyindole-O-methyltransferase (HIOMT). The enzymatic machinery for melatonin biosynthesis was first identified by Axelrod in the pinealocytes [30] and has been subsequently identified in the retina, bone marrow cells, GI tract, skin, lymphocytes and platelets (see for ref. [2]). Pineal melatonin production exhibits a circadian rhythm with low levels during daytime and high levels during night. This circadian rhythm occurs in all living organisms irrespective of whether they are diurnally or nocturnally active.

The regulation of pineal melatonin biosynthesis by ambient illumination is mediated by the retinohypothalamic tract that projects from the retina to the suprachiasmatic nucleus (SCN), the major circadian oscillator [31]. Special photoreceptive retinal ganglion cells are the origin of that retinohypothalamic projection [32] (Fig. 1). These ganglion cells contain a special photosensitive pigment, known as melanopsin, which is involved in the phototransduction mechanism [33].

Nerve fibers from the SCN project to a multisynaptic descending pathway that passes through the paraventricular nucleus, medial forebrain bundle and reticular formation and makes synaptic connections with intermediolateral cells of the cervical spinal cord. From there, preganglionic fibers project to the superior cervical ganglia where postganglionic sympathetic fibers innervating the pineal gland are located, regulating pineal melatonin synthesis by releasing norepinephrine (NE) at their postganglionic nerve terminals [31].

The release of NE from pineal nerve terminals occurs during nighttime. NE, by binding to β-adrenergic receptors at the pinealocyte membrane, activates G-protein subunits to stimulate adenylate cyclase activity and the subsequent cyclic AMP (cAMP) production. The increase of cAMP promotes the synthesis of enzymes involved in melatonin biosynthesis [34].

Circulating melatonin derives almost totally from the pineal gland, as shown by the fact that undetectable melatonin levels are found after pinealectomy. After its release, melatonin is bound to albumin [35] and reaches all tissues within a very short period [36,37]. Melatonin's half-life is biexponential with a first distribution half-life of 2 min and a second of 20 min.

Melatonin metabolism occurs mainly in the liver, where it is first hydroxylated in the C6 position and then conjugated with sulfate and excreted as 6-sulfatoxymelatonin. In many cells it is converted into cyclic 3-hydroxymelatonin after scavenging two hydroxyl radicals [38]. Melatonin is also metabolized into kynuramine derivatives [39]. It is interesting to note that the antioxidant properties of melatonin are shared by some of their metabolites like N^1-acetyl-5-methoxykynuramine (AMK) and N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) [40]. Thus melatonin gives rise to a cascade of antioxidant molecules that multiply the free radical scavenger effect (Fig. 2).

As melatonin diffuses through all biological membranes with ease, it is localized in membrane, cytoplasmic, mitochondrial and nuclear compartments [41]. Depending upon its production site and target organ, melatonin acts as a hormone, autacoid, chronobiotic, hypnotic, immunomodulator or as a biological modifier.

Melatonin receptors

Melatonin exerts some of its actions through interaction with MT_1 and MT_2 receptors [42,43]. These membrane receptors have seven intramembrane domains and belong to the superfamily of G- protein coupled receptors. A third binding site, identified initially as MT_3, was subsequently characterized as the enzyme quinone reductase 2 [44].

Many G protein-coupled receptors, including the MT_1 and MT_2 receptors, exist in living cells as dimers. The relative propensity of the MT_1 homodimer and MT_1/MT_2 heterodimer formation are similar whereas that of the MT_2 homodimer is 3-4 fold lower [45,46]. It is of interest that a receptor that shares 45% of the amino acid sequence with MT_1 and MT_2 but does not bind melatonin (called GPR50, [47]), abolishes high affinity binding of the MT_1 receptor through heterodimerization [48,49]. Thus the GPR50 receptor may have a role in melatonin function by altering binding to the MT_1 receptor.

Melatonin also acts by binding to cytoplasmic proteins like the calcium binding protein calmodulin [50] or tubulin [51], and to nuclear receptors like RZR/ROR [52]. The melatonin receptor present in the skin has been identified as MT_1 [53]. MT_2 receptors have been detected in neonatal keratinocytes, and in cutaneous melanoma cell lines as well as in normal and malignant uveal melanocytes [54].

The decrease in cAMP production caused by melatonin via MT_1 and MT_2 receptor interaction decreases the uptake of linoleic acid, an essential fatty acid, by affecting a specific fatty acid transporter [55]. Linoleic acid can be oxidized to 13-hydroxyoctadecadienoic acid by 15-lipoxygenase, serving as an energy source for tumor growth and tumor growth-signaling molecules. Inhibition of linoleic acid uptake by melatonin is regarded as a mechanism of its antiproliferative effects [55].

Some studies have also suggested modulations in the expression and function of nuclear receptors, RZR/ROR, as the mechanism for biological effects of melatonin. By binding to nuclear receptors, melatonin alters the transcription of several genes that play a role in cellular proliferation (e.g., 5-lipoxygenase, p21, or bone sialoprotein) [56].

Another mechanism of the biological effects of melatonin may be its ability to modulate intracellular calcium and calmodulin activity. Calcium-activated calmodulin is involved in the initiation of the S and M phases of the cell cycle, cell cycle–related gene expression, and the reentry of quiescent cells from G_0 back into the cell cycle [55]. Melatonin has been shown to increase calmodulin degradation due to direct binding as well as causing redistribution of calmodulin, thereby inhibiting cell cycle progression [50].

Melatonin also serves as a potent modulator of gene transcriptional activity. Conventional approaches have allowed identification of a large number of genes, targeted by melatonin centrally (in brain structures, most importantly in SCN and in pars tuberalis of the hypophysis), or in peripheral tissues. Discovering the mechanisms of melatonin interaction with clock genes (*Per*, *Clock*, *Bmal* and others) could be considered as one of the major achievements of these studies. It has been hypothesized that melatonin mediate seasonal photoperiodic control via the phasing of expression of clock genes in the pars tuberalis, with a length of the melatonin signal decoded in target tissues in a form of the clock gene expression profile signatures ("internal coincidence model" [57]).

Progress in a development of DNA microarray technology has increased the list of possible melatonin targets in peripheral tissues. Microarray-based screening of about 8000 rat cDNA clones have led to the identification of a limited group of genes with expression in rat neural retina and retinal pigmentary cells that were changed significantly by melatonin [58]. In neural retina, treatment with melatonin stimulated the expression of 6 genes and repressed the expression of 8 genes, while in retinal pigmentary cells 15 genes were up-regulated and 2 were down-regulated. Among these genes, some with important physiological functions were present. For example, melatonin down-regulated gene expression of integrin and integrin-associated protein-encoding genes in rat retina, while the cAMP response element binding protein (CREB) gene was up-regulated in retinal pigmentary cells [58].

In mice administered melatonin in drinking water, total RNA purified from cardiac tissue was used to synthesize isotope-labeled probes which were subsequently hybridized to microarrays [7]. Analysis of the microarray data indicated a limited group of transcripts (212, <1.4% of the clones screened) with significantly altered cardiac gene expression. Among these, 146 genes were up-regulated and 66 down-regulated.

Although melatonin affected the expression of a wide spectrum of genes, its primary effectors tended to be associated with the genes controlling the cell cycle, adhesion, and transport. This finding is in agreement with the established data on the effect of melatonin on cell proliferation, apoptosis, and adhesion. Notably,

melatonin has also demonstrated a pronounced effect on the expression of genes related to oncogenesis (e.g., *Mybl1*, *Rasa1*, *Mllt3* and *Enigma homolog 2*) and calcium metabolism (*Kcnn4* and *Dcamkl1*) [7].

A significant effect of melatonin on expression of mitochondrial genes was also revealed, like genes encoding 16S ribosomal RNA (*mt-Rnr2*), cytochrome C oxidase subunits I and III (*mt-Co1*, *mt-Co3*) and NADH dehydrogenase 1 (*mt-Nd1*) (all up-regulated) and ATP synthase subunit 6 (*mt-Atp6*; down-regulated) [7]. This finding supports previous observations of the direct effect of melatonin upon the expression of mitochondrial genome-encoded genes in the brown adipocytes of Siberian hamster [59].

Aging, immune function and cancer

That the levels of immunity is a predictor of individual longevity in human beings has been suggested by several epidemiological studies like OCTO and NONA [60] which reveal the existence of "immunological risk phenotypes" that can predict the life span in the elderly [61-63]. Longer life in centenarians has been associated with high natural killer (NK) cell number, augmented interferon (IFN)-γ production and phagocytosis [64,65]. The age-associated increases in NK cells were interpreted as a compensatory response to overcome the decreased immune function that could otherwise trigger neoplastic growth [66].

Studies of knockout mice have shown the important role of immune system in controlling the spontaneous generation of tumors. Nearly 50% of aged IFN-$\gamma^{-/-}$ or perforin $^{-/-}$ mice developed lymphomas, lung adenocarcinoma or sarcoma [67]. Immune changes during aging may result in tumor growth since the incidence of metastatic cancer at autopsy peaks at 75- 90 years and has been shown to decline in 95-99 year old and centenarians [68]. That the personality and the emotional state of the individual can influence the course of illness by altering the immune function has been well documented [69,70].

The understanding of the immune changes in the elderly can provide new insights into the complex relationship between immunity and cancer [71,72]. In this respect, the decline in the production of melatonin with aging was suggested

to play an important role in triggering immunosenescence, especially age-associated neoplastic diseases [6].

Any search for a therapeutic agent that can improve the quality of life in the elderly depends upon the identification of substances that have both antioxidant and immunoenhancing qualities. As melatonin has been identified as a natural antioxidant with immunoenhancing properties, it has the potential of becoming an effective therapeutic substance in preventing or arresting neoplastic growth.

Melatonin in immune mechanisms

There are many natural mechanisms that protect against carcinogenesis and they fall into two main categories, immune and non-immune. Among the former, immunosurveillance has been suggested as one of the major processes by which cancerous cells are detected and eliminated [73]. The activation of lymphocytes and monocytes/macrophages by melatonin can be one of the major mechanisms in preventing tumor development [20]. Melatonin has a significant immunomodulatory role in the immunocompromised state [74]. The age-related impairment of the immune system first appears around the sixth decade of age coinciding with a normal decrease in plasma melatonin concentration. Aging is associated with a decline in immune function that predisposes to increased incidence of cancer and infectious and neurodegenerative diseases like Alzheimer's disease.

The diurnal and seasonal changes in the immune function correlate with melatonin biosynthesis and secretion [75]. In addition, the synthesis of melatonin by human lymphocytes [76] lend support to the hypothesis that melatonin has a role in the regulation of immune function. Other studies demonstrated that the melatonin synthesized by human T cells contributes to regulation of interleukin (IL)-2 production acting as an intracrine, autocrine and/or paracrine substance [77].The presence of high levels of melatonin in cultured rat thymocytes and expression of mRNAs encoding for AANAT and HIOMT in the rat and human thymus cells support that melatonin is also synthesized by thymocytes [78].

Seasonal changes of melatonin secretion are observed in human beings [79] and it is suggested that melatonin has significant role in immune modulation during different seasons of the year [80]. The role of melatonin as a possible mediator of seasonal changes effects on immune function has been well documented [81,82].

Melatonin receptors are detectable in the monocyte/macrophage lineage [83]. Administration of melatonin increases the production of both monocytes and NK cells in bone marrow and spleen within 7-14 days of treatment [84]. As both cell types are components of the non-specific immune system, the findings suggest that melatonin can be effective in arresting neoplastic growth and in destroying virus infected cells. Melatonin's stimulatory action on monocyte production could be due either to its direct action on melatonin receptors in monocytes or to its sensitizing action on monocytes to stimulants like IL-3, IL-4 or granulocyte-macrophage-colony stimulating factor [84,85]. By this action melatonin was able to rescue hematopoiesis from the toxic effect of cancer chemotherapy in several experimental models [86]. This evidence actually poses the basis for the therapeutic use of melatonin as an adjuvant in combination with myelotoxic anti-cancer therapeutic protocols.

NK cells play an important role in immunosurveillance against neoplasia and virus infected cells [87,88]. Acute administration of melatonin increased NK cell responsiveness to IFN-γ while its chronic administration not only augments NK cell activity but also increases the number of NK cells in circulation [89]. The increased NK cell number brought out by melatonin is attributed to an increased production of cytokines like IL-2, IL-6, IL-12 and IFN-γ from T helper (Th)-1 lymphocytes and from monocytes [84,90]. The presence of melatonin receptors on T lymphocytes explains melatonin's action in releasing cytokines that enhance the NK cell activity and augment NK cell number. By activating Th-1 cells melatonin enhances the production of IFN-γ [91]. Melatonin's immunoenhancing effect depends not only upon its ability to enhance the production of cytokines but also upon its antiapoptotic and antioxidant actions (Fig. 2).

Melatonin and T lymphocytes

Th lymphocytes play an important role for protection against malignancy, by recruiting cells of the immune system and by activating antigen-specific effector cells [92,93]. Importance has been given to the stimulation of $CD4^+$ Th cell in cancer chemotherapy. $CD4^+$ lymphocytes secrete IFN-γ and tumor necrosis factor (TNF)-α that activate and regulate cytotoxic T cell responses. Melatonin treatment augmented $CD4^+$ cells in lymph nodes of rats [94]. Th-1 cells directly kill tumor cells by releasing cytokines that activate "death" receptors on the tumor cell surface [93]. Melatonin also favors Th-2 responses: it not only stimulates the release of IFN-γ and IL-2 but also of IL-10 [95].

In immunodepressed states, melatonin's immunoenhancing action is restricted to T-lymphocytes [14]. Suppression of nocturnal melatonin rise in mothers with mastitis was highly correlated with increased TNF-α secretion from immunocompetent cells in calostrum [96]. Since the proinflammatory cytokine inhibits nocturnal pineal melatonin production [97], the results suggest that during the response to an injury the production of melatonin can be transiently shifted from an endocrine (pineal) to a paracrine (immunocompetent cells) source [98].

Immune function and skin melanoma

Melanocytes in the skin are involved in the production of melanin pigment; in the adult skin, they exhibit only intermittent mitotic activity. Under certain conditions, such as exposure to UV radiation or during the process of wound healing, their mitotic activity increased several-fold. That proliferative response depends upon the delicate balance between positive and negative cell cycle regulatory signals and alteration of these signals can result in uncontrollable cell division and malignancy [99].

UV radiation of the skin promotes the production and release of photoprotective melanin pigments via the α-melanocyte-stimulating hormone receptor system and the p53 transcription factor. This activates the cAMP pathway to initiate a series of events that culminate in increased melanin synthesis [99]. The UV-irradiated melanocytes also express elevated

concentrations of inhibitor kinase (INK) 4 cyclin-dependent kinases (cdk) inhibitor, $p16^{ink4a}$. This inhibits the cyclin D3/cdk4, a complex that is essential for cell cycle progression through G2 phase to meiosis. Inhibition of this phenomenon by $p16^{inka}$ delays the G2 phase progress, thus promoting melanoma [99].

The fact that melanoma cells express antigens that can be recognized by T cells, that T cells can destroy melanoma cells, and that exposure of the skin to UV radiation causes immunosupression suggests that the immune response has an important effect in melanoma development [100]. Cytotoxic T cells that specifically destroy melanoma cells have been identified in the blood of patients with melanoma [101]. UV radiation enhances the growth of melanoma by inhibiting the infiltration of melanoma by T cells [102].

The magnitude of the T cell response determines the survival rate of patients suffering from primary melanoma. In a study of 285 patients with primary melanoma [96], patients who had a brisk infiltrate showed a 5-year survival of 77 % while in those who had a non-brisk infiltrate a 53 % survival was found; patients who had no infiltrate had a 5-year survival of 37 %. Ten-year survival rates were 55%, 45%, and 27%, respectively [92]. The main T cell type infiltrating early lesions of a superficial spreading melanoma was identified as $CD4^+$. In the more advanced metastatic lesions, $CD8^+$ cells dominated the scene [103].

With regard to cytokine involvement, IL-2 treatment caused either complete or partial regression in 7 % and 10 % of patients, respectively; IL-2 caused protection probably by activating NK cells [108]. IL-6, although proinflammatory, has been shown to inhibit growth of melanoma cells by its direct action during the early phase of growth, whereas in the advanced metastatic lesions it did not have any significant effect [104]. An inverse relationship between IL-6 production and growth of murine melanoma has been reported. Overexpression of IL-6 by transfection of murine melanoma reduced their growth [105]. IL-10 administration either inhibited or enhanced melanoma development [100]. IL-10 retarded melanoma growth by preventing macrophage production of angiogenic factors.

Treatment of melanoma patients with IFN-α increased their survival [106]. IFN-α not only caused infiltration of $CD4^+$ lymphocytes and induced tumor

regression but also increased the formation of antimelanoma cytotoxic T lymphocytes [107], showing thereby that it participates in the suppression of melanoma growth via its immunoenhancing properties [100]. The fact that natural $CD4^+$, $CD25^+$ regulatory T cells influence tumor immunity in cancer patients has gained much support in recent years [108]. A number of studies have found increased frequencies of $CD4^+$, $CD\ 25^+$ T cells in the peripheral blood of patients suffering from melanoma [109,110] and also in other cancers like breast cancer [111], GI cancer [112] and ovarian cancer [113].

The importance of altering the balance of tumor-specific effector cells and T cells for improving the immunotherapeutic strategies in human tumors has been recognized [114]. This constitutes the immunological basis for therapeutic cancer vaccination [115]. Indeed, melanoma remains as the model for development of immunotherapy. In cancer, the primary aim is to eradicate the existing disease and the vaccines are intended to be therapeutic and not preventive. In a phase III trial of patients with melanoma, vaccine treatment with autologous dendritic cells that contain heat-shock protein gp96-peptide has showed promising results for patients´ survival [115]. Passive immunotherapy by adaptive transfer and manipulation of T cell responses is a successful approach for treatment of melanoma and other cancers [116]. In a largest clinical trial involving 155 patients with hepatocellular carcinoma, patients who received IL-2 and autologous activated lymphocytes showed overall improvement and longer survival. The enhancement of anti-tumor responses is thus beneficial in reducing the recurrence after curative resection [116].

Melatonin in melanoma

Melatonin acts as a protective agent against damage induced by UV radiation in the human skin [117]. Melatonin is also radioprotective against X-ray induced skin damage in the albino rat [118]. The radioprotective action of melatonin is attributed to its antioxidant properties [119] via direct radical scavenging properties and stimulation of antioxidant enzymes as demonstrated in human skin fibroblasts [120] (Fig. 2).

Melatonin has oncostatic properties in melanomas and tumors of epithelial origin [121,122]. The ability of melatonin to stimulate IL-2 production and to enhance its antitumor activity has been tested both in experimental animals and in clinical trials. Melatonin on its own exerted a significant antitumor effect but when combined with IL-2 it potentiated the antitumor effect of IL-2 in an additive manner [123,124]. In cancer patients both T and NK cells are generally depressed, and since melatonin can augment the production of T lymphocytes and NK cells via IL-2 increase, melatonin administration could be a useful adjuvant therapy to impair tumor growth [125].

Melatonin administration along with IL-2 and naltrexone in patients with untreatable metastatic melanoma increased Th-1 and suppressed Th-2 responses, a reportedly favorable result in anticancer treatment [126-129]. In the studies by Lissoni *et al.* it was found that advanced neoplasms resistant to IL-2 responded well to IL-2 therapy after the concomitant administration of melatonin [130-133]. Patients who received both IL-2 and melatonin exhibited a significantly higher number of lymphocytes, T lymphocytes, NK cells and $CD4^+$ cells than those receiving IL-2 alone. A further study using IL-2 along with melatonin and cisplatin demonstrated that it was the most effective immunotherapeutic way for treating metastatic melanoma. In that study the combination of melatonin with IL-2 was proved to be successful after failure of a first line therapy with decarbazine and IFN-α. Melatonin not only suppressed tumor growth but also suppressed significantly the toxicity of chemotherapeutic drugs and potentiated their anticancer cytotoxicity [130-133].

In a study aiming to determine location and intensity of expression of MT_1 melatonin receptors and of Ki-67 proliferation-associated antigen in dermal melanoma, material from 48 cases of dermal melanoma, including 38 primary tumors and 10 metastatic lymph nodes was examined [134].Expression of MT_1 receptor was more pronounced in primary tumors than in related metastatic lymph nodes. Depth of tumor infiltration demonstrated a moderate positive correlation with the intensity of MT_1 expression and a strongly positive correlation with the expression of Ki-67 antigen. In both primary tumors and

metastatic lymph nodes, a weak correlation was found between the expression of MT_1 receptor and the expression of Ki-67 antigen [134]

Melatonin was effective in inhibiting cell proliferation of S-91 murine melanoma cells, under both *in vitro* and *in vivo* conditions [135]. Melatonin exerted its antiproliferative action by increasing the expression of MT_1 receptor and also by increasing the activity of antioxidant enzymes. Early studies demonstrated that melatonin can act directly at the cellular level to inhibit the proliferation of PG 19 and B16BL6 mouse melanoma cells in culture [136]. The antiproliferative action of melatonin is dose-dependent [137]. With the highest melatonin concentration employed (19356 pg /cell) the cancer cells became undetectable at day 5 of treatment. The total elimination of cancer cells observed in this study was the first of this kind reported in the scientific literature.

The disruption of circadian rhythmicity becomes significant as a tumor progresses, whereas the incidence of cancer augments after disruption of the circadian system. In a study to test whether body temperature rhythms are impaired by tumor progression, and to what extent exogenous melatonin restricts tumor growth and restores circadian rhythmicity, C57 mice were subcutaneously inoculated with melanoma cells [138]. Animals were then submitted to 12:12 light-dark (LD) cycles or to continuous light (LL), with or without melatonin administration (2 mg/kg/day). Under LD light conditions, the body temperature rhythm exhibited a marked reduction and increased phase instability as the tumor progressed. Melatonin administration increased the body temperature rhythm amplitude and phase stability, reduced tumor weight and prevented intraperitoneal dissemination when administered in the subjective night [138].

The effect of melatonin on the growth of uveal melanoma cells has also been examined. Hu and his coworkers [139], by using cultured human uveal melanoma cells, found that melatonin (0.1-10 nM) inhibited the growth cells in a dose-dependent manner. Growth inhibition occurred at a concentration of 2 nM, the physiological levels found in aqueous humor. High affinity melatonin binding sites occurred in SK- Mel 28 human melanoma cell lines. In these cells use of luzindole, a selective blocker of MT_2 receptors reversed the anti-proliferative and melanogenic effects of melatonin [140]. In human melanoma cells SK-MEL-1, the

antiproliferative effects of melatonin were associated with an alteration in the progression of the cell cycle and also with an increase in tyrosinase activity, a key regulatory enzyme of melanogenesis [141]. Antagonists for melatonin membrane receptors (luzindole and 4-P-PDOT) and the general G-coupled receptor inhibitor, pertussis toxin, did not prevent the melatonin-induced cell growth arrest; this suggests a mechanism independent of G-coupled membrane receptors. The p38 mitogen-activated protein kinase signaling pathway seems to play a significant role in cell growth inhibition by melatonin [141].

Serum melatonin levels in patients with melanoma were higher than those of normal individuals [142]. Similarly, another study reported high serum melatonin levels in patients with choroidal melanoma; in this case melatonin levels decreased after enucleation by transpupillary thermotherapy [143]. The increase of serum melatonin levels seen in patients with choroidal melanoma was linked to the growth inhibitory effect melatonin on human melanoma cells as demonstrated under in vitro conditions [143].

Melatonin in breast cancer

Melatonin is oncostatic and antiproliferative in breast cancer [144,145]. Studies using MCF-7 human breast cancer cells demonstrated that physiological concentrations of melatonin inhibit cell proliferation. As the melatonin's growth inhibitory effect was abolished by MT_1 receptor antagonism, the MT_1 receptors detectable in MCF-7 cells were identified as functional receptors responsible for transducing growth inhibitory effect of melatonin [146]. As the antiproliferative effect of melatonin is also a serum dependent phenomenon, the interaction of melatonin with a factor in the serum has been postulated for its antiproliferative action.

Melatonin not only blocks the mitogenic effects of estradiol but is also able to counteract the estradiol-induced invasiveness of MCF-7 cells [147]. In vitro experiments with the ER- positive MCF-7 human breast cancer cells demonstrated that melatonin at physiological concentrations (1 nM) inhibited the cell proliferation in the presence of serum or estradiol and increased the expression of p53 and p21WAF1 proteins, which modulate the length of cell cycle [148]. There is

indication that melatonin could exert its antitumoral effects on hormone-dependent mammary tumors by down-regulating the sulfatase pathway of the tumoral tissue [149]. Since melatonin binds to calmodulin in a Ca^{2+} dependent fashion, calmodulin was implicated in the antiestrogenic effects of melatonin. Melatonin acts as a calmodulin antagonist inducing conformational changes of the ERα-CaM complex thus impairing binding of the ERα-CaM complex to DNA and thereby transcription [150]. This has been suggested as the mechanism by which melatonin exerts oncostatic and antiproliferative actions.

In recent years increased breast cancer risk in women associated with work at night–shifts has been attributed to the low melatonin levels following light-induced inhibition of melatonin synthesis [151-157]. The protective role of melatonin in mammary carcinogenesis was also suggested by studies in postmenopausal women with advanced breast cancer who have diminished urinary levels of melatonin as compared to controls [158]. The inhibitory action of melatonin on mammary carcinogenesis has been attributed to effect of melatonin on immune modulation [159]. Indeed, disturbances of immune mechanisms have been documented in experimental models of mammary cancer. For example, the absence of the cytosolic protein Nod1 in MCF-7 cells correlated with tumor growth, an increased sensitivity to estrogen induced cell proliferation, and a failure to undergo Nod1–dependent apoptosis [160].

IL-2 and chemotherapy are employed for treatment of metastatic breast cancer [161]. IL-2 is used to achieve an increased efficacy of increasing NK cells and cytolytic function and, in combination with IFN-α and chemotherapy, as an adjuvant treatment in high- risk breast cancer [162]. The link between melatonin and the immune system in cancer has been explored in phase II studies with melatonin causing increase of some cytokines and amplification of objective responses to cytokine in patients [163].

A correlation between tumor size and the nocturnal amplitude of melatonin secretion was noted in some studies. Peak nocturnal amplitude of melatonin was reduced in 50% of patients with primary breast cancer and was inversely correlated with tumor size [164]. The nocturnal amplitude of the 6-sulfatoxymelatonin concentration was found lower in patients with primary

breast cancer. The circadian rhythm of nocturnal melatonin production may represent a "regulatory signal" for the carcinogenic process; it may exert a "natural restraint" on tumor initiation, promotion, and/or progression [165,166].

Melatonin in ovarian, endometrial, and other cancers of the female reproductive tract

Low melatonin secretion has been reported in patients with endometrial cancer, but not in those with non-invasive ovarian cancer or squamous cervical cancer [167]. In vitro, an ovarian adenocarcinoma cell line (BG-1) exposed to melatonin (1-100 nM) showed a 20-25 % reduction in cell number [24]. In another study application of melatonin to ovarian carcinoma cell cultures revealed that three out of seven ovarian cell cultures were affected by melatonin in different ways melatonin [168]. Cells of one tumor were inhibited by 90% at 10 nM, while in another the growth inhibition was by 30 % at a concentration 0.1 – 1000 nM; a third specimen was stimulated up to 30% by 100 nM melatonin. The variability in the response was attributed to the presence of some unknown tumor condition likely to modify the melatonin response[168].

Melatonin did not exert antiproliferative effects on ovarian cancer cell lines at 0.001 nM - 1 μM concentrations but enhanced the sensitivity to cisplatin in two ovarian cell lines [169]. Results were interpreted as indicating that melatonin may play a role in the control of telomerase activity and the suggestion was made that the resistance of ovarian cancer to cisplatin could be overcome by the administration of melatonin.

In ovarian cancer patients, IL-2 treatment has been employed [170]. For example, in the analysis of six studies of i.p. immunotherapy in ovarian cancer, 21 individual responses to IL-2 treatment were reported out of 69 patients showing a 22% of clinical efficacy [171]. Since melatonin increases the production of IL-2, the prospective therapeutic role of melatonin in cancer is that it may well act as a modulator of IL-2 and IFN-γ production by Th1 cells. Melatonin has the possibility of being used as a novel oncostatic adjuvant agent [172,173].

Melatonin in hepatocellular carcinoma

Hepatocellular carcinoma is the cancer of the liver found after hepatitis B and hepatitis C infection, as well as in conditions associated with alcohol abuse [116]. Many immunotherapeutic procedures were employed for treating hepatocellular carcinoma, like the use of cytokines or transfer of autologous-activated lymphocytes. Intratumor injection of recombinant adenoviral vectors that induce the local release of interleukin –12 has also been employed [174]. Improvement in overall recurrence-free survival was seen in 155 hepatocellular carcinoma patients after immunotherapy with IL-2 and α CD3 [175]. In another study carried out on stage III and IV inoperable patients, IL-2 administered along with IFN-γ and transarterial chemotherapy brought about tumor size reduction in 14 out of 20 patients [176].

Melatonin induces cell cycle arrest and apoptosis in hepatocarcinoma HepG2 cell line [177,178]. In 100 patients with inoperable advanced primary hepatocellular carcinoma, transcatheter arterial chemoembolization (TACE) was used alone or associated with melatonin [179]. The effectiveness rate of TACE or TACE + melatonin was 16 % and 28 %, respectively. The 0.5, 1 and 2 year survival rate in the TACE group was 82 %, 54 %, and 26 % respectively, while in the TACE + melatonin it was 100 %, 68 % and 40 %, respectively. IL-2 levels were found elevated in all these patients. The protective and treatment effect of TACE plus melatonin on liver function was attributed to enhancement of immunological function in patients [179].

Melatonin in colorectal carcinoma

Epidemiological studies of nurses engaged in night-shift work indicated an increased incidence of colorectal cancer, a finding interpreted as supporting the cancer-promoting effect of melatonin inhibition by environmental light [154,180]. Indeed, many *in vitro* and *in vivo* studies have shown that melatonin exerts antiproliferative effects on intestinal cancer. In a study on CT-26 a murine colon carcinoma–derived cell line, melatonin inhibited growth in a dose-dependent manner [181]. A statistically significant correlation was found between the

decrease in DNA synthesis and the doses of melatonin used. The growth inhibitory effect found was 22% (1 nM melatonin), 25 % (2 mM melatonin) and 47 % (3 mM melatonin) [181]. High melatonin binding sites were demonstrated in human colonic mucosa and a melatonin concentration of 467 ± pg/g of wet tissue of human colon has been reported [182]. The oncostatic action of melatonin appears to depend on both MT_2 and nuclear RZR/ ROR receptors [183]. Luzindole (a MT_1/MT_2 antagonist) but not 4-phenyl-2- propionamidotetralin (a specific MT_2 antagonist) diminishes the inhibitory effect of melatonin on murine colon 38 cancer cell growth in vitro [184].

The inhibitory effect of exogenous melatonin on colon oncogenesis was investigated using the azoxymethane/dextran sodium sulfate rat model [185]. At week 20, the development of colonic adenocarcinoma was significantly inhibited by the administration with melatonin in a dose-dependent manner. Melatonin exposure decreased mitotic and apoptotic indices in the colonic adenocarcinomas and lowered the immunohistochemical expression of nuclear factor κ B, TNF-α, IL-1β and STAT3 in the epithelial malignancies. These results may indicate the beneficial effects of melatonin on colitis-related colon carcinogenesis and a potential application for inhibiting colorectal cancer development in the inflamed colon.

Early studies on the effects of melatonin in colorectal carcinoma were based upon the immunoneuroendocrine and synergistic relationship between melatonin and IL-2 [186,186]. In a study on 24 patients with advanced cell tumors (non–small cell lung cancer, 9 patients; colorectal cancer, 7 patients; gastric cancer, 3 patients; breast cancer, 2 patients; cancer of pancreas, 1 patient; hepatocarcinoma, 1 patient; unknown tumor, 1 patient) who did not respond to previous chemotherapies, IL-2 plus melatonin was given. Melatonin was administered starting 7 days before IL-2 injection. While progress was reported in 6/24 patients, stability was reported in 14/24 patients. IL-2 in combination with melatonin seemed useful to control tumor growth in patients with advanced neoplasms [187]. In another study on 35 patients with advanced neoplasms of the digestive tract, immunotherapy with a low-dose of IL-2 plus melatonin was a well-tolerated and effective therapy. Complete response was obtained in patients with

gastric carcinoma and hepatocarcinoma. The overall response rate was 8/35 (23%) [188]. Similarly, in another study a low subcutaneous dose of IL-2 plus melatonin was found to be a second – line therapy for tumor regression and for prolonging survival of patients with metastatic colorectal cancer [189].

Measurements of melatonin levels in patients with colorectal patients and controls demonstrated a higher nocturnal concentration in patients [190]. Daytime melatonin concentrations in gut tissue of colorectal carcinoma patients was found to be 317 ± 87.8 pg/g, nearly 10 times higher than the day time levels in circulation. An increased level of melatonin in the gut has been found after surgery and it was suggested that they play a protective role against the development of colorectal cancer [190].

The interrelationship between melatonin and immune function was studied in patients with advanced GI cancer (42 patients with colorectal, gastric and pancreatic cancer) [191]. The circadian rhythm of melatonin was altered with peak melatonin level reaching at 0800 - 0900 h, with a 5-7 h-delay respecting average peak time in healthy humans. The rhythm in TNF-α and soluble TNF-α receptors (type I and type II) also indicated the existence of complex self-regulatory mechanisms between the neuroendocrine system and the cytokine network in those patients [191]. Suppression of nocturnal melatonin rise in mothers with mastitis was highly correlated with increased TNF-α secretion from immunocompetent cells phagocytes in calostrum [192].

Besides interacting with cytokines, melatonin induces apoptotic cell death in cancer cells. In a study on HT-29 human colon cancer cells, melatonin potentiated flavone–induced apoptosis [193]. The role of melatonin as pro-apoptotic agent is a new field of investigation. The pro-apoptotic action of melatonin has been documented not only in colon cancer cells [194] but also in breast cancer [195]. The mechanisms underlying the pro-apoptotic action of melatonin is still not clear. The finding that melatonin induces apoptosis uniformly in all cancer cells may have important clinical significance. It could involve free radical scavenging properties and other intracellular pathways. Indeed, the antioxidant and anti-inflammatory actions of melatonin, counteracting the oxidative status and

reducing the production of nitric oxide by cultured HT-29 cells seem to be directly involved in its oncostatic properties [196].

Current & Future Developments

Melatonin has been demonstrated to inhibit tumor development under both *in vivo* and *in vitro* conditions. There are several mechanisms by which melatonin can exert its oncostatic actions: (a) by its direct pro-apoptotic, gene-mediated, actions on tumor cells; (b) by its antioxidant actions; (d) by reducing the uptake of key factors for tumor growth and tumor growth signaling molecules (e.g., linoleic acid); (d) by enhancing immune mechanisms in body. Among these, the later mechanism has been shown to very significant for melatonin's oncostatic action.

The fact that there is an increased incidence of neoplastic diseases at an old age, concomitant with the age-associated decline in immune functions of the body, have prompted many investigators to look for the use of agents or nutrients that could enhance immune function. Of the various substances examined, melatonin received wide attention as it enhances immune function effectively in both animals and humans. Altered melatonin levels have been seen in patients suffering from melanoma, breast cancer, and colorectal cancer, among other. Melatonin suppresses growth of melanoma, breast cancer, ovarian cancer and colorectal and other GI cancers. Besides interacting directly with tumor cells through MT_1 and MT_2 melatonin receptors, melatonin stimulates NK cell activity, regulatory Th cell activity and enhances the release of cytokines like IL-2 and IFN-γ from T lymphocytes. Based on these findings observed in animals, phase II clinical trials are being undertaken wherein the administration of melatonin along with IL-2 is found beneficial in treating patients suffering from various neoplastic diseases like melanoma or colorectal cancer. Melatonin has the potentiality to become a useful oncostatic drug [197].

Patent Selection

The following recent patents are considered by the authors to be relevant in the field of melatonin and cancer. It must be noted that melatonin, as a natural product, cannot be patented itself but through its different uses.

- [198] This invention is directed to combinations of compounds useful in the treatment and prevention of cancer and inflammatory conditions or diseases.
- [199] This invention relates to the use of at least one of melatonin, a melatonin analogue, or a pharmaceutically acceptable salt thereof in the treatment of certain cancers, in particular metastatic colorectal cancer and metastatic breast cancer.
- [200] This invention relates to methods and compositions for treating cancer, the methods including the step administering, either sequentially or simultaneously, several compounds and melatonin
- [201] This invention relates to methods for preventing the development of cancer or neurodegenerative diseases by administering melatonin or N-acetylcysteine alone or in combination.
- [202] This invention proposed melatonin as a useful agent to prevent skin against damage induced by ultraviolet radiation.
- [203] This invention proposed a method of maintaining circadian rhythm of a subject comprising selectively blocking of retinal exposure of the subject to light having a wavelengths that do not inhibit melatonin synthesis at night, thus preventing the consequences of night work, in particular those of tumor growth.
- [204] This invention describes a light emitting diode lamp free of melatonin-suppressing radiation for preventing the consequences of night work in particular those of tumor growth.

Acknowledgements

D.P.C. is a Research Career Awardee from the Argentine National Research Council (CONICET), Argentina.

Conflict of interest statement and disclosure statement

S.R. Pandi-Perumal is a stockholder and the President and Chief Executive Office of Somnogen Inc., a New York Corporation. He declared no competing interests that might be perceived to influence the content of this article. All remaining authors declare that they have no proprietary, financial, professional, nor any other personal interest of any kind in any product or services and/or company that could be construed or considered to be a potential conflict of interest that might have influenced the views expressed in this manuscript.

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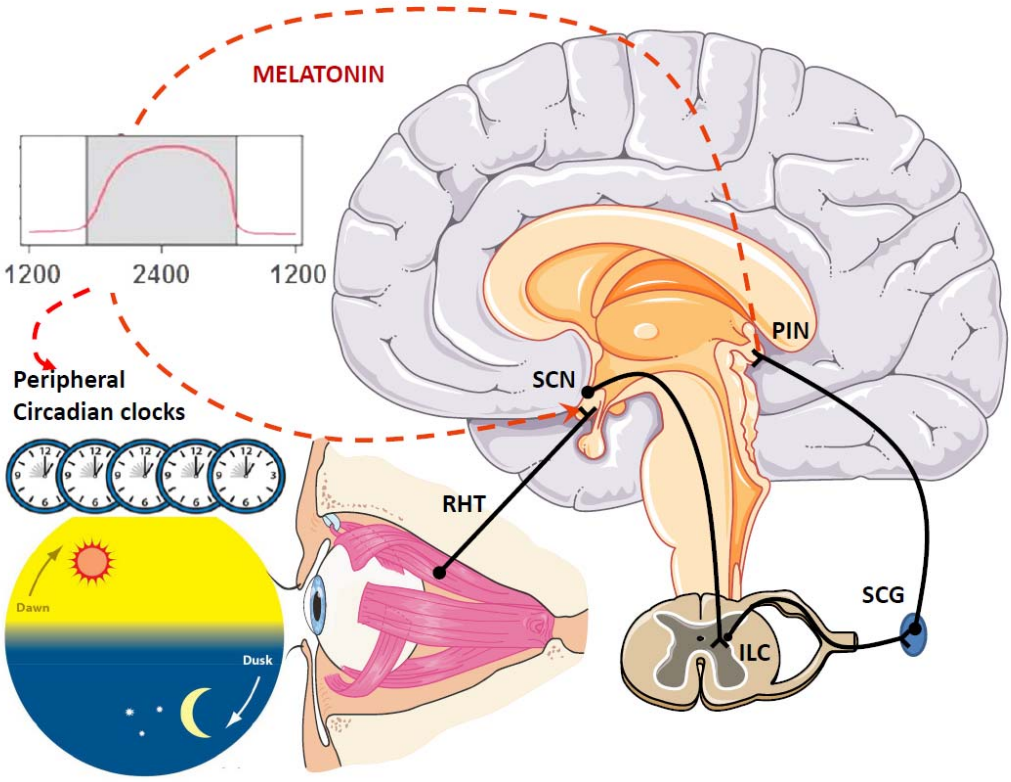
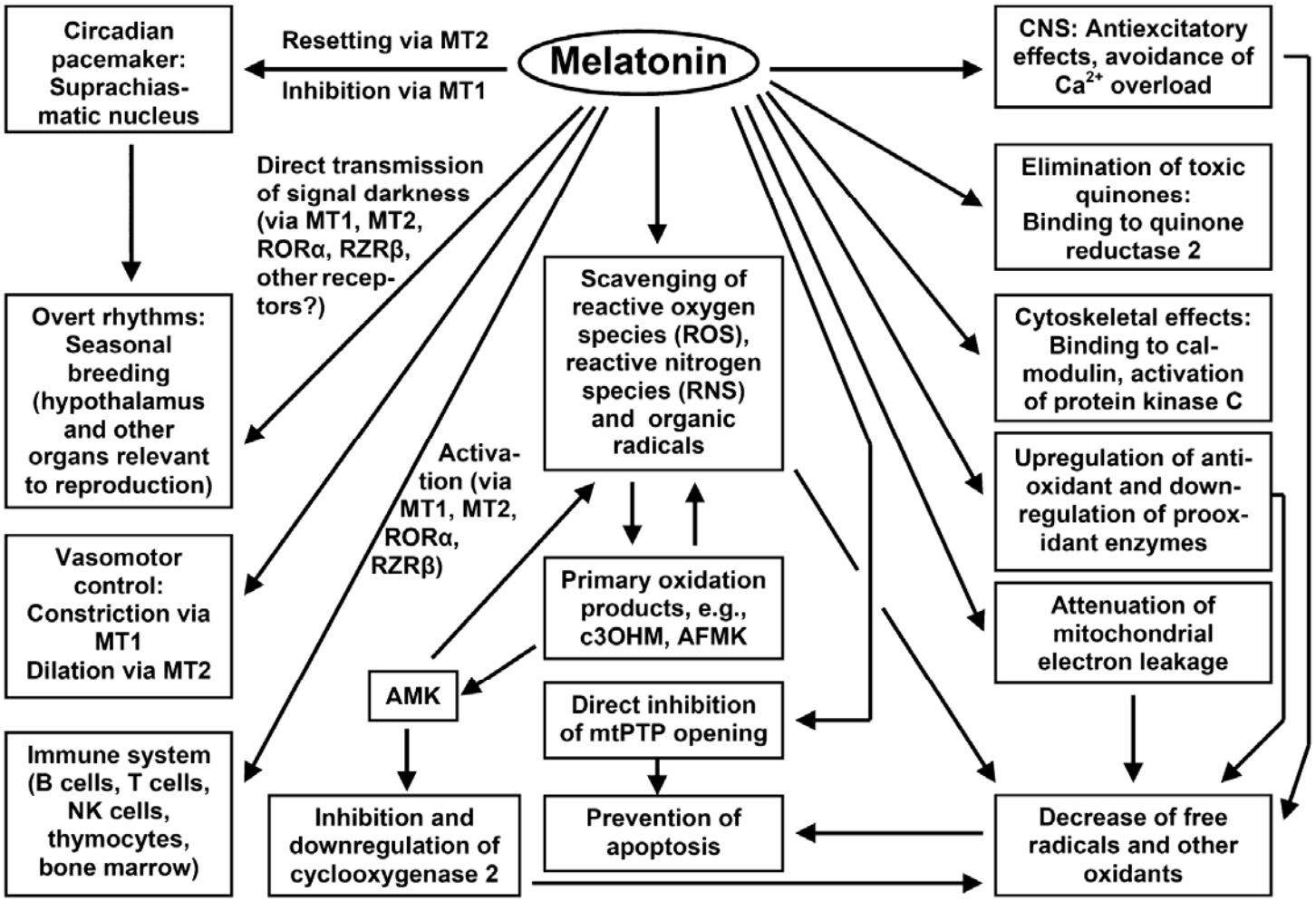
FIGURE LEGENDS

Fig. (1). Circadian regulation. Light impinging on the eye send neural signals to a population of receptive neurons in the suprachiasmatic nuclei (SCN) via the retinohypothalamic tract (RHT). The SCN in turn acting via a complex indirect multisynaptic pathway including projections to the intermediolateral column (ILC) of the cervical spinal chord and the superior cervical ganglion (SCG), sends a circadian signal to the pineal gland (PIN) regulating synthesis of melatonin. Melatonin feeds back on the SCN as well as on numerous other brain sites that contain melatonin receptors

Fig. (2). The pleiotropy of melatonin: an overview of several major actions. Abbreviations: AFMK = N^1-acetyl-N^2-formyl-5-methoxykynuramine; AMK = N^1-acetyl-5-methoxykynuramine; c3OHM = cyclic 3-hydroxymelatonin; mtPTP = mitochondrial permeability transition pore; RORα, RZRβ = nuclear receptors of retinoic acid receptor superfamily. (Reprinted with permission from [2])


MELATONIN
1200
2400
1200
Peripheral
Circadian clocks
Dawn
Dusk
RHT
SCN
PIN
SCG
ILC


Melatonin
Circadian pacemaker: Suprachias-matic nucleus
Resetting via MT2
Inhibition via MT1
Direct transmission of signal darkness (via MT1, MT2, RORα, RZRβ, other recep-tors?)
Overt rhythms: Seasonal breeding (hypothalamus and other organs relevant to reproduction)
Vasomotor control: Constriction via MT1 Dilation via MT2
Immune system (B cells, T cells, NK cells, thymocytes, bone marrow)
Activa-tion (via MT1, MT2, RORα, RZRβ)
Scavenging of reactive oxygen species (ROS), reactive nitrogen species (RNS) and organic radicals
Primary oxidation products, e.g., c3OHM, AFMK
AMK
Direct inhibition of mtPTP opening
Inhibition and downregulation of cyclooxygenase 2
Prevention of apoptosis
CNS: Antiexcitatory effects, avoidance of Ca2+ overload
Elimination of toxic quinones: Binding to quinone reductase 2
Cytoskeletal effects: Binding to cal-modulin, activation of protein kinase C
Upregulation of anti-oxidant and down-regulation of proox-idant enzymes
Attenuation of mitochondrial electron leakage
Decrease of free radicals and other oxidants