ANTIOXIDANTS & REDOX SIGNALING
Volume 37, Numbers 10–12, 2022

DOI: 10.1089/ars.2021.0275

FORUM REVIEW ARTICLE

Redox Biology of Melatonin: Discriminating Between Circadian and Noncircadian Functions

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Abstract

Melatonin has not only to be seen as a regulator of circadian clocks. In addition to its chronobiotic functions, it displays other actions, especially in cell protection. This includes antioxidant, anti-inflammatory, and mitochondria-protecting effects. Although protection is also modulated by the circadian system, the respective actions of melatonin can be distinguished and differ with regard to dose requirements in therapeutic settings. It is the aim of this article to outline these differences in terms of function, signaling, and dosage. Focus has been placed on both the nexus and the dissecting properties between circadian and noncircadian mechanisms. This has to consider details beyond the classic view of melatonin's role, such as widespread synthesis in extrapineal tissues, formation in mitochondria, effects on the mitochondrial permeability transition pore, and secondary signaling, for example, *via* upregulation of sirtuins and by regulating noncoding RNAs, especially microRNAs. The relevance of these findings, the differences and connections between circadian and noncircadian functions of melatonin shed light on the regulation of inflammation, including macrophage/microglia polarization, damage-associated molecular patterns, avoidance of cytokine storms, and mitochondrial functions, with numerous consequences to antioxidative protection, that is, aspects of high actuality with regard to deadly viral and bacterial diseases. *Antioxid. Redox Signal.* 37, 704–725.

Keywords: circadian, extrapineal, melatonin, miRNAs, mitochondria, sirtuins

Introduction

AFTER THE DISCOVERY of melatonin as a skin-lightening hormone in frogs and fish (154), the description of high-amplitude circadian rhythms of melatonin synthesis and secretion by vertebrate pineal glands (212) opened a new direction of research that was dominating this field for decades. Contrary to the other vertebrates, melatonin formation in the mammalian pineal gland was shown to be light-dependently controlled by a sympathetic input (277).

Further studies ultimately connected the mammalian pineal to the circadian pacemaker, the suprachiasmatic nucleus (SCN), which receives information on the light–dark cycle from melanopsin-containing retinal ganglion cells (21) and transmits this *via* the paraventricular nucleus, intermediolateral cell column of the upper thoracic cord, and superior cervical ganglion to the pineal (219). Further details and modulation by additional factors have been summarized elsewhere (83, 112). In the chronobiological context, melatonin was regarded as a ''troll'' among hormones that mediates the information of darkness and, thereby, contributes to the internal adjustment of body functions to environmental cyclicity, with regard to both circadian and seasonal rhythms (217–219).

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This perception of melatonin is widely present in many researchers, although it is widely incomplete. First, melatonin is produced not only by the pineal gland but also by numerous, perhaps, almost all other organs (4, 112). In particular, the frequently read statement that the pineal gland is the main site of melatonin formation has to be dropped, because extrapineal melatonin exceeds the amounts in pineal gland and circulation by orders of magnitude (24, 112, 130). This is in accordance with the finding that melatonin is poorly released from most extrapineal sources, at least, under basal conditions (94).

Moreover, high levels of extrapineal melatonin are not generally cycling in a circadian fashion, and, if so, the amplitude is often too low or poorly phased to allow chronobiological effects. For instance, its temporal pattern in the rodent Harderian gland is almost flat, with only a short and transient drop directly after light onset (127). In the gastrointestinal tract, the amplitude is either much lower than in the pineal gland or almost absent, depending on species (112). Finally, the recent demonstration of intramitochondrial melatonin synthesis in neuronal tissue did not reveal circadian variations (255). Mitochondrial melatonin formation has meanwhile been shown to occur in several different cells and tissues, such as oocytes (123), choroid plexus (213), and even outside the animals (263).

Therefore, the question arises as to whether melatonin biosynthesis has to be seen as not being primarily coupled to circadian functions, but rather originally to redox metabolism. In the latter case, the circadian role of melatonin would appear as a secondary acquisition in the course of evolution. This would not be surprising, since metabolism in general, but also oxidant formation and antioxidative metabolism are often controlled by circadian oscillators (113).

Melatonin's relationship to redox metabolism is meanwhile an established field documented by numerous publications that quantitatively exceed those dealing with circadian issues. In its early beginnings, hints were obtained, showing the nonenzymatic oxidation of melatonin to N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) by photocatalytic mechanisms (110, 114, 118, 119). The topic of melatonin's role in redox metabolism gained considerable attention and developed an enormous drive after this molecule was shown to be an exceptionally potent hydroxyl radical scavenger (258).

However, although melatonin was, thereafter, shown to be highly protective in numerous experimental settings, free radical scavenging did not appear to be physiologically relevant, because of the low levels of this compound in the circulation. At that time, investigators usually believed that such low concentrations were applicable to all tissues and subcellular compartments. To resolve the contradiction between potent antioxidative protection and physiological levels apparently too low for efficient radical scavenging, melatonin's spectrum of actions was investigated with regard to other protective mechanisms.

In fact, melatonin was shown to upregulate antioxidant enzymes (82, 222, 226). Finally, it turned out that various different mechanisms contribute to the reduction of oxidant formation by melatonin, a concept referred to as radical avoidance (82, 112). In terms of organismal metabolic economy, decreasing the production of oxidants may have advantages over detoxification of oxidants already formed. However, the earlier conclusion that melatonin's levels are too small for the latter purpose has turned out to be precocious. First, it was shown that high levels of melatonin that suffice for free-radical scavenging and convey protection do exist in organisms outside the vertebrates, as first demonstrated for a dinoflagellate (11).

Later, even higher concentrations of melatonin were measured in several plants and some nonvertebrate animals (39, 116). A contribution of direct free radical detoxification is, therefore, of biological relevance. To what extent this is also applicable to mammals and other vertebrates may require further investigation. However, findings on higher tissue levels of melatonin and of mitochondrial accumulation (222, 263) let this possibility appear in a new light.

This outline indicates that the roles of melatonin have to be differently judged with regard to low levels in the circulation and higher levels in tissues and mitochondria, and also regarding high-amplitude rhythmicity in the pineal gland and circulation or low amplitudes and virtual absence of rhythmicity in, at least, some tissues and subcellular compartments. It is the aim of this article to discriminate these different roles of melatonin, including the respective mechanisms of action.

Concentration Matters: Receptor Saturation and Oversaturation

Melatonin has been used or tested preclinically and clinically for many different purposes, such as correction of circadian malfunction (12, 50, 57, 246, 272), sleep improvement (26, 233, 238, 303), mood disorders (23, 146, 241, 243, 271), prevention of metabolic syndrome and related pathologies (28, 74, 142, 189, 191), deceleration of aging (27, 89, 90, 253), cancer prevention (126, 159, 223, 256), and, in particular, protection against oxidative stress caused by overshooting inflammation (*e.g.,* sepsis) (6, 71, 72, 59, 62, 105, 124), neuroinflammatory and excitotoxic pathologies (111, 144, 178, 251, 252), stroke and trauma (64, 216, 222, 239, 284), environmental toxins (221), and life-threatening viral diseases (121, 259).

In these highly divergent applications, melatonin was differently dosed, in adult humans, from 1 to 3 mg/day (84, 156) or even less (201) to 300 mg/day (275) or above (260), in newborns or animals in corresponding doses according to dose translation (260). The safety margin for human short-term treatment has been reported to amount to 3750 mg/day for an individual of 75 kg (192).

With regard to this extremely broad spectrum of doses that had been applied for different purposes, it seems necessary to make functional discriminations regarding modes of action. For fundamental reasons, it is immediately clear that several actions characterized by responses to low doses are explained by receptor-mediated signaling, whereas those requiring very high doses have to be explained by entirely different mechanisms. Reliable knowledge on melatonin signaling exists mainly for the G protein-coupled receptors (GPCRs) MT_1 and MT_2 (231, 232). Variations of signaling pathways regarding G protein subunits and downstream pathways have been summarized elsewhere (85).

Agonist affinities of these receptors are, of course, in accordance with melatonin concentrations in the body fluids. In blood plasma, these amount to 1 n*M* in some, but not all mammalian species, at the circadian melatonin maximum at night. However, they may be much lower in several species and also in aging humans and animals, whereas daytime values are anyway considerably decreased. According to the still relatively low nocturnal levels, pKi values of the two receptors for melatonin are typically found in the range between 9.5 and slightly above 10, that is, in good correspondence to physiological up- and down-variations around half-saturation (87, 137).

Several other proteins have also been shown to bind melatonin and some of them have been even claimed to represent low-affinity melatonin receptors. Among them, calmodulin (CaM) is the only one that has remained of actual interest. After its discovery as a melatonin-binding protein (19, 20), its capability of interacting with this ligand at reasonable concentrations was vividly discussed. However, it was shown that its affinity to melatonin is strongly increased after its interaction with CaM-activated proteins (145).

Moreover, the relevance of melatonin binding has not to be solely judged on the basis of blood concentrations. Meanwhile, it has been shown that melatonin concentrations in other body fluids and, correspondingly, in the releasing tissues often exceed blood levels by far (4, 222, 229, 257). These findings are in accordance with the demonstration of melatonin synthesis in mitochondria (123, 213, 255), an organelle known since long to also accumulate melatonin from extracellular sources (184). With regard to tissue and subcellular concentrations, melatonin signaling *via* low-affinity binding sites appears to be possible and should be systematically investigated.

Apart from CaM, several other binding sites have been discussed, but they are devoid of convincingly demonstrated receptor properties. The binding site of NRH:quinone oxidoreductase 2 (NQO2), formerly believed to represent another melatonin receptor (''MT_3''), is not specific for melatonin and lacks, according to actual knowledge, any signaling pathway. Therefore, it does meet required criteria of a receptor (112).

Members of the retinoid orphan receptor (ROR) subfamily, which have been considered in numerous publications as ''nuclear melatonin receptors,'' can presumably be excluded, with certainty in the case of the most frequently investigated RORα, which has been shown to not bind melatonin (248, 249) and whose effects in the context of melatonin require alternate explanations (100). Other putative binding sites are insufficiently characterized, in terms of either protein chemistry or/and binding properties.

As a consequence of the binding properties of MT_1 and MT_2 receptors, only doses that lead to blood melatonin levels in the physiological range or slightly above can be expected to be suitable for generating meaningful MT_1/MT_2-mediated effects. This is particularly of importance for actions that affect circadian oscillators, because their dynamics comprises sequential increases and decreases of their intrinsic functions.

However, with regard to the short half-life of melatonin in the circulation, which is mostly between 20 and 45 min (29, 46), but can be lengthened by elevating doses (8), transient receptor oversaturation may still be compatible with circadian MT_1/MT_2-effects, as far as the dynamics of saturation and desaturation is not heavily disturbed. The possibility of receptor desensitization and internalization has been discussed, but it has not been finally clarified under the various conditions (85). Of course, the duration of melatonin's actions can be extended by using slow-release formulations or choosing nonoral routes of administration (289).

However, doses of, for example, 10 mg/kg that have been frequently used for protecting mice against experimental oxidative stress would be equivalent, after dose translation, to a human dose of about 60 mg/75 kg, that is, 20 or 30 times of the officially approved quantities (3 or 2 mg/pill). Even considerably higher doses have been applied to laboratory animals and to humans (260, 275). The problem has never been the tolerability of melatonin, but one cannot expect a reasonable chronobiological action by continuously oversaturating the melatonin receptors.

In numerous studies, high doses such as those mentioned had been reported to be required. This would be implausible for an MT_1/MT_2-mediated action, which should reach its maximum at receptor saturation. Therefore, in all the cases in which melatonin treatment by very high doses was found to be required for efficacy, the conclusion can only be that other mechanisms of action beyond these GPCRs have been decisive. This conclusion is of particular importance with regard to the influences on the circadian system. In humans, the entrainment of circadian rhythms was shown to be less efficient with 10 mg melatonin than with doses in the low mg range (156). Thus, increased doses do not enhance effects in the circadian context.

Moreover, if high doses are applied, conclusions should also not be based on effects by MT_1 or MT_2 antagonists. If a compound such as luzindole is tested, one should be aware of the pKi values that are lower than those of melatonin. To obtain a meaningful effect, the antagonist dose has to be elevated above that of melatonin. In cases in which very high melatonin doses are aimed to be blocked, caution should be due regarding the toxicity of all xenobiotic antagonists, which strongly contrast with the extremely well-tolerated melatonin. Therefore, at high doses, an apparent suppression of a melatonin effect by a receptor blocker may turn out as being toxicity-induced.

These considerations allow discrimination of mechanisms involved in melatonin's antioxidant properties. First, two types of effects that are related to MT_1 and/or MT_2 signaling can be distinguished: (a) direct effects on gene regulation, for example, regarding antioxidant enzymes, and (b) indirect modulation of antioxidant parameters by influencing circadian oscillators. The circadian multioscillator system controls numerous functions of relevance to antioxidative protection, but also to oxidant generation (113).

Since the influence of melatonin on the circadian oscillator system should mainly, perhaps exclusively, occur *via* MT_1/MT_2 signaling, this would only require low doses. The same can be assumed to be the case in the regulation of antioxidant enzymes expression. Regarding the requirement of higher

doses for conveying antioxidative protection under severe conditions of exposure to toxins, sepsis, ischemia, or trauma, additional mechanisms are required for explaining melatonin's efficacy. According to actual knowledge, this may concern three different processes: (a) signaling *via* CaM, (b) binding to the mitochondrial permeability transition pore (mtPTP), and (c) scavenging of free radicals and other oxidants.

The role of CaM in melatonin's actions is only incompletely understood. In relation to anti-excitotoxic, anti-inflammatory, and antioxidant actions, the inhibition of neuronal nitric oxide synthase (nNOS) by melatonin has been interpreted in terms of this mechanism (152). Interestingly, inhibitory effects were also observed with the melatonin metabolite N^1-acetyl-5-methoxykynuramine (AMK) (58, 151). However, it has remained uncertain as to whether the effect of AMK is related to noncompetitive CaM binding, as assumed earlier (151). Because of the rather unusual kinetics of inhibition, an alternative possibility of protein modification by AMKylation has been suggested (97). The actions of melatonin may extend to other CaM-activated enzymes, such as CaM kinase II and calcineurin, which have been discussed in the context of mitigating endoplasmic reticulum stress (247).

The discovery of melatonin's inhibition of mtPTP opening (9) prompted investigators to determine the inhibition kinetics. The Ki value was reported to be in the range of 0.8 μM, that is, at a concentration much above melatonin's blood levels and MT_1/MT_2 saturation. The contribution of mtPTP to oxidant release, mitochondrial function and integrity, as well as electron flux would strongly demand a more detailed investigation of the properties of this binding site.

Finally, scavenging of free radicals represents an important field of melatonin's spectrum of actions. After the discovery of highly potent elimination of hydroxyl radicals (258, 264), the detoxification of various other radical species and nonradical oxidants was demonstrated (82, 91). Despite initial skepticism regarding the relevance of radical scavenging by melatonin, with regard to its low levels in the circulation, this mode of action has turned out to be of biological value, since by orders of magnitude higher melatonin concentrations were determined in various nonvertebrate organisms, sometimes up to the millimolar level (39, 66, 116). In vertebrates, a contribution of radical scavenging to antioxidative protection should, at least, be considered at high pharmacological doses.

Melatonin's Role in the Circadian Multioscillator System: Control of Redox-Relevant Rhythms

Without any doubt, melatonin acts on the circadian master clock, the SCN, as can be shown by its capability of phase resetting and synchronizing previously nonentrained rhythms (12–14, 79, 155, 156, 246). The original concept had only assumed circulating melatonin to be effective, which would be in accordance with observations on oral administration. However, after the discovery of melatonin release *via* the pineal recess into the third ventricle of the brain (267–269), a higher importance of this latter route for the physiological modulation of SCN function has been assumed (225, 262).

In addition to the effects in the SCN, there is substantial evidence that melatonin also influences peripheral circadian oscillators (95, 115), a field that would require more detailed information. With regard to antioxidative protection, it should also be noted that the circadian system is also involved in the avoidance of oxidative damage, even beyond melatonin's direct effects on gene expression.

In organisms as different as fruitflies and hamsters, it was shown that clock mutants led to increased protein and lipid oxidation (48, 49). Conversely, oxidative stress was shown to affect circadian rhythms. In *Drosophila*, an organism that produces extremely low amounts of melatonin, deficiency of the important radical scavenger urate (*rosy* mutation ry^{506}) caused changes in circadian patterns of antioxidant enzymes and, much more pronounced, of protein carbonyl, which was elevated by manifold in some but not all circadian phases (53).

With regard to melatonin's multiplicity of actions in both central and peripheral cellular oscillators, a look at the mechanisms of circadian gene regulation is required. The rhythmic up- and downregulation concerns countless genes, not only by gene-specific mechanisms, but also largely *via* global chromatin remodeling, which is differentially controlled by the various components of the cellular oscillators. Daily occurring chromatin remodeling is especially achieved by histone modification, such as acetylation/deacetylation and multiple methylation/demethylation of specific lysines, as summarized elsewhere (106).

Interestingly, the dynamic balance between histone acetylation and deacetylation is influenced by melatonin at the level of cellular oscillators. There is a sequential interplay between the histone acetyl transferase circadian locomotor output cycles kaput (CLOCK), a core oscillator component that also acetylates various other proteins, and the histone deacetylase sirtuin 1 (SIRT1), an accessory oscillator component that, again, acts on other proteins, too. SIRT1 is interacting with oscillator components, whereas SIRT6, which is constitutively chromatin-associated, has also been reported to interact with CLOCK:BMAL1 heterodimers at E-box containing promoters outside the oscillator (176).

SIRT6 also deacetylates histones and regulatory proteins and can be classified as a circadian output factor, because its activity is dependent, similar to that of SIRT1, on the NAD^+ cycle generated by the oscillator (Fig. 1) (175–177). However, SIRT1 and SIRT6 seem to influence different sets of genes (176). The influence of melatonin on these cycles concerns the upregulation of SIRT1 (95, 106), which is of relevance to the anti-inflammatory actions of melatonin (see subsequent section) and also to the properties of circadian oscillators, whose amplitudes are enhanced by SIRT1 (Fig. 2) (37, 240). Melatonin, being anyway a highly pleiotropic agent (112), seems to gain an even higher degree of pleiotropy *via* the circadian system, because of the multitude of circadian controlled genes (CCGs).

Several of them are co-regulated *via* response elements such as E-boxes and ROR response elements (ROREs), which are not only present in clock genes but also accessible to the respective oscillator components in various CCGs (Fig. 2) (22). The presence of either E-boxes or ROREs in different genes leads to the important consequence that CCGs with the one or the other response element are controlled by oscillators in different phases, according to the temporal peaking of the respective clock components.

In the case of ROREs, an additional complication results from the fact that ROREs are differently regulated by two oscillator proteins, that is, activation by RORα and suppression by REV-ERBα (alias NR1D1, nuclear receptor subfamily 1,

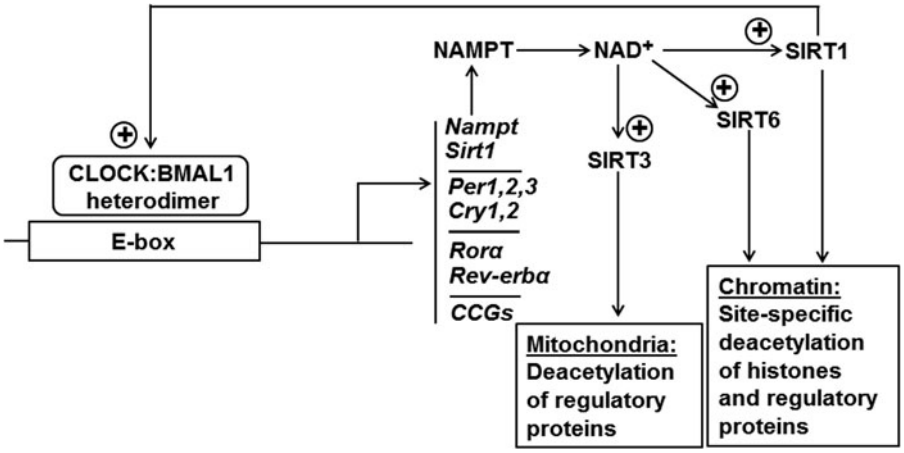

FIG. 1. E-box-dependent transcription of circadian oscillator genes and CCGs and the oscillator-mediated activation of sirtuins by NAD^+. BMAL1, brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1; CCG, circadian-controlled gene; CLOCK, circadian locomotor output cycles kaput; *Cry*, cryptochrome gene; NAMPT, nicotinamide phosphoribosyltransferase; *Per*, period gene; *Rev-erbα*, reverse-related erythroblastoma-α gene (alias *Nr1d1*, nuclear receptor subfamily 1, group D, member 1); *Rorα*, retinoic acid receptor-related orphan receptor α; SIRT, sirtuin.

group D, member 1). Promoters of more than 1000 murine genes have been shown to contain binding sites for core oscillator proteins (158). Alternately, they may be controlled *via* D-boxes, binding sites of circadian output factors such as DBP (albumin D-site-binding protein; D-box positive regulator) or E4BP4 (adenovirus E4 promoter-binding protein; D-box negative regulator, alias NFIL3, nuclear-factor interleukin 3 regulated) (281, 286, 287).

Various other output factors, accessory clock components, and clock-dependent hormones have to be added to the list of global circadian regulators (93, 203, 274, 280), but a complete list would exceed the scope of this article. In this place, it should only be mentioned that additional epigenetic regulation mechanisms have to be considered, too, such as DNA methylation and its erasure, at regulatory CpG islands in promoters but also elsewhere (106), and numerous contributions of noncoding RNAs (63, 92, 101).

These include, apart from microRNAs (miRNAs), various long noncoding RNAs (lncRNAs) of different functions (*e.g.,* miRNA sponging lncRNAs, super enhancer lncRNAs, suppressive lncRNAs such as *HOTAIR*), circRNAs (miRNA sponging circular RNAs), snoRNAs (small nucleolar RNAs, with contributions to circadian chromatin remodeling), and asRNAs (antisense RNAs), such as the *Per2* asRNA, as summarized elsewhere (101). These RNAs are of considerable importance to circadian regulation mechanisms, already from a quantitative point of view. Only in the murine liver, 604 lncRNA clusters were shown to cycle in a circadian fashion, in which a cluster comprises splice variants of the same transcript (63). A broader evaluation of more organs would presumably reveal a much higher number.

Melatonin has been shown to upregulate, in several vertebrate species and tissues, the expression of various antioxidant enzymes, such as glutathione peroxidase (GPx), glutathione

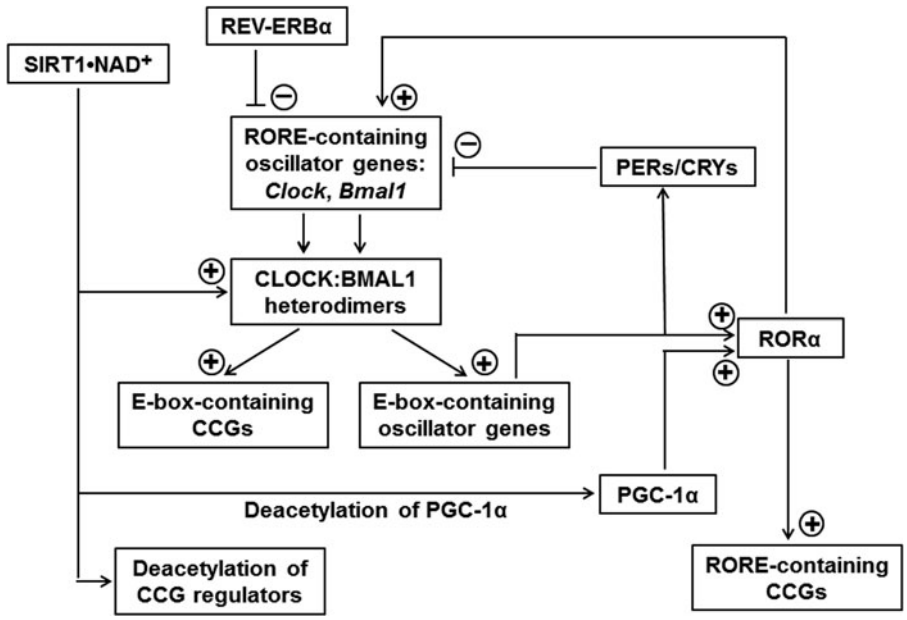

FIG. 2. The effects of SIRT1 on CCGs with E-box- or RORE-containing promoters or being under control by deacetylatable regulators. RORE, ROR response element; for others see legend Figure 1.

reductase (GR), γ-glutamylcysteine synthase, glucose-6-phosphate dehydrogenase, hemoperoxidase/catalase, Cu, Zn- and Mn-superoxide dismutases, as has been multiply reviewed (82, 112, 113, 117, 226). However, a close look at the details reveals that many of these findings cannot be generalized (113), as they are either tissue-, condition-, or species-specific or only demonstrated at mRNA levels, at the borderline of statistical significance.

Moreover, circadian cyclicity has not generally been demonstrated for all these enzymes (113), although the high-amplitude rhythmicity of melatonin may suggest a rhythmic influence on these enzymes' expression. However, one should not forget that antioxidant enzymes have to be controlled by various different factors and routes. In particular, any organism faces the necessity of adapting the expression to needs related to oxidant formation and stressful conditions.

In mammals, melatonin synthesis poorly responds to oxidative stress and is, in this sense, rather nonadaptive. On the contrary, melatonin levels may even decrease under high oxidative pressure, because of consumption by detoxification reactions, an effect first seen in a dinoflagellate that produces melatonin at micromolar levels and above (25). If this effect is already seen in a high-melatonin organism, oxidative melatonin consumption should be ever more relevant in the circulation of vertebrates. Among the antioxidant enzymes, there are especially two that reliably exhibited upregulation by melatonin and circadian rhythmicity, too, namely GPx (16, 18, 73, 78, 141, 180, 196, 198–200, 220, 226, 227, 234, 279) and GR (78, 161, 190, 200, 220, 227).

Additional data on long-term administration and deviating results in cancer will not be discussed here in detail. Circadian rhythms in the activities of these two enzymes have been most impressively demonstrated in chicken brain, in which a temporal sequence became apparent: The peak of the circadian inducer, melatonin, was followed by GPx and, a little bit later, by GR (200). The lag of the GR may reflect a need of GSSG (oxidized glutathione) reduction on increased GSH (reduced glutathione) consumption due to the higher GPx activity. The levels of GSH are of particular importance, under both circadian and toxicological aspects, since GSH is a central antioxidant. Therefore, melatonin hits a major factor by upregulating GPx, GR, and enzymes of GSH synthesis (glucose-6-phosphate dehydrogenase, γ-glutamylcysteine synthase).

In fact, the role of melatonin exceeds the modulation and rhythmicity of antioxidant enzymes. Notably, the immune system is, under inflammatory conditions, a major source of reactive oxygen species, hypochlorite and oxidizing derivatives thereof, such as taurine chloramine, and reactive nitrogen species. The immune system does not only respond to insults or is activated by processes of inflammaging, but also contains numerous factors that are under circadian control (36, 56, 107, 143, 179). Therefore, melatonin can modulate immunological functions *via* its chronobiotic properties. However, by means of its anti-inflammatory actions, it can also reduce the formation of toxic intermediates, in terms of a contribution to the principle of radical avoidance (82). For details see the subsequent section.

With regard to both antioxidant and anti-inflammatory aspects, melatonin also downregulates enzymes that indirectly contribute to prooxidant and other deleterious effects. In this regard, literature usually refers to the downregulation of lipoxygenase-5 (31, 254). However, these reports were based on actions by CGP 52608, a ligand of RORα, which is meanwhile known to not bind melatonin (100, 248, 249). Another study showed that melatonin does not downregulate lipoxygenase-5 (215). There is no good reason to assume an antioxidant effect of melatonin *via* suppression of this enzyme.

On the other hand, the lipoxygenase-5 gene was shown to carry an RORE in its promoter, and CGP 52608 was concluded to mediate suppressive effects by RORα (185). This interpretation may be entirely valid, but this means that the circadian system rather than melatonin directly controls the expression of lipoxygenase-5, since RORα is a component of cellular circadian oscillators and CCGs with ROREs are driven by their ligands, RORs and REV-ERBα (Fig. 2). Another action of melatonin was reported for lipoxygenase-12, surprisingly in the rat pineal gland (292), in which some melatonin should be normally present. Exogenous melatonin was reported to suppress 12-lipoxygenation.

However, melatonin was used at a dose of 100 mg i.v., which should have oversaturated the melatonin receptors for the time of the experiments (1 h). Experiments in cultured pineal glands showed that luzindole (0.1 m*M*) inhibited the effect by melatonin (also 0.1 m*M*). Although the experimentation may not have been perfect in terms of dosing, a suppression of lipoxygenase-12 by melatonin may still be valid, since untreated animals showed inverse circadian relationships between melatonin and the enzyme (292). Unfortunately, studies on the relationship between melatonin and lipoxygenase-12 have not been extended to other tissues.

Indirectly acting pro- and anti-inflammatory enzymes are also found in immune cells, such as inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), both of which are downregulated by melatonin. In addition to other functions, $^{\bullet}NO$ is important as a diffusible proinflammatory signal, which is, in this context, produced by iNOS in numerous tissues and various leukocytes, in particular, macrophages, microglia, and neutrophils. In the CNS, both nNOS and iNOS contribute to neuroinflammation by activating microglia and astrocytes (111).

Moreover, $^{\bullet}NO$ combines with $O_2^{\bullet-}$ (superoxide) to $ONOO^-$ (peroxynitrite), a highly reactive oxidant that can also yield hydroxyl radicals on protonation ($ONOOH \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH$) or carbonate radicals on combination with CO_2 ($ONOOCO_2^- \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-}$) and, thereby, contributes *via* its metabolites to further oxidation and nitration reactions (75, 88, 96). Importantly, iNOS is downregulated by melatonin (2, 60–62, 68, 131, 166, 265) and, thus, decreases these undesired and damaging reactions, in addition to the suppression of the proinflammatory $^{\bullet}NO$ signal. The downregulation of COX-2 in macrophages also reduces proinflammatory signaling, in this case by prostaglandins (51, 181).

Importantly, prooxidant and proinflammatory signaling share the involvement of nuclear factor κB (NF-κB), whereas antioxidant and anti-inflammatory signaling is mediated *via* nuclear factor erythroid 2-related factor 2 (Nrf2). Melatonin's effects have repeatedly been shown to be characterized by inhibition of NF-κB activation and upregulation of Nrf2, as summarized elsewhere (105, 140).

Melatonin and Inflammation: Circadian and Noncircadian Aspects

As numerous factors of the immune system are under circadian control (36, 56, 107, 112, 125, 143, 179), melatonin can

be assumed to partially modulate immunological functions because of its chronobiotic properties. However, the situation is more complex, for two reasons. First, numerous immune cells, perhaps all of them, produce melatonin (32, 34, 47, 76, 112, 148, 171, 188, 242) and the role of leukocytic melatonin as an intracrine, mitocrine, or paracrine agent is still poorly understood, because this cannot be convincingly studied by exposing these cells to exogenous melatonin. In addition to the intracellular production, many leukocytes express melatonin receptors, mostly MT_1, and, therefore, respond to extracellular melatonin (33, 70, 139, 112, 147, 171, 210).

The additionally discussed role of RORs should no longer be associated with direct actions of melatonin (cf. Refs. 248, 249), but rather be seen in the circadian context. Second, numerous studies on immunological actions of melatonin have been conducted by using supraphysiological doses and the potent anti-inflammatory actions, which are of practical medical interest (6, 35, 50–62, 71, 72, 105, 121, 124, 166, 259, 260), are beyond the circadian role.

With regard to the circadian control of immune functions and the participation of melatonin, a recently emerging aspect concerns the roles of noncoding RNAs that exceed the already mentioned lncRNAs. In particular, miRNAs are abundantly involved in the post-transcriptional regulation of gene expression. This may be seen as kind of a fine control, but is, sometimes, difficult to judge without detailed analysis, especially as a single miRNA species may have putative interaction sites in numerous mRNAs and, thus, potentially target different mRNAs (98).

Melatonin has been shown to modulate the expression of many miRNAs (102), findings that are not surprising in a highly pleiotropic regulator. The number of melatonin-controlled miRNAs will expectably further rise, as soon as more tissues will be investigated in this regard. Moreover, numerous newly discovered miRNAs have not yet been studied in the context of melatonin. Typically, the melatonin-affected miRNAs are differentially expressed when comparing deviating conditions, for example, cancer *versus* normal tissue (149).

In such cases, a substantial difficulty of interpretation remains concerning the targeted mRNAs. In the latter article, 22 differentially expressed miRNAs were reported to be putatively capable of targeting 2029 mRNAs, according to their 5′-utr sequences. Therefore, a profound understanding would require determination as to which mRNAs are expressed in which cell type and under which conditions. Another approach of analyzing the roles of miRNAs in the circadian system including the contribution of melatonin is to identify (a) targets within core and accessory clock components and (b) response elements for circadian oscillator proteins including output factors and for agents that are known to cycle with high amplitude, such as glucocorticoids.

In an initial study, this has been done for miRNAs known to be involved in the regulation of inflammation (107). In this study, 28 miRNAs were found to modulate circadian oscillator components. Among them, 10 miRNAs were identified as upregulators, 12 others as downregulators of proinflammatory cytokines. Moreover, nine miRNAs downregulated SIRT1 expression, which was in accordance with proinflammatory roles observed at cytokine levels. Three miRNAs favored macrophage polarization to the proinflammatory type M1, whereas another one favored the anti-inflammatory type M2.

Regarding the circadian control of miRNA expression, 43 miRNAs/miRNA clusters with functions in inflammation control were found to be circadian-regulated. Among them, 24 had binding sites in their promoters for canonical oscillator proteins, 35 for the important circadian output factor DBP, and 15 for the glucocorticoid receptor. Within the selection of miRNAs, nine were modulated by melatonin, five of them downregulated. Many of these miRNAs were multiply controlled by several circadian regulators.

A selection of circadian or melatonin-controlled miRNAs that also downregulate SIRT1 expression is mentioned in Figure 3. The upregulation of SIRT1 by melatonin and the suppression of several of these miRNAs by melatonin, which is also included, seem to antagonize SIRT1 downregulation (Fig. 3). Further analyses regarding miRNA-interacting RNAs revealed that the bifunctional miRNAs with regulatory roles in both immune and circadian systems were sponged by 23 circRNAs.

Some circRNAs sponged more than one miRNA species, and the number of sponged miRNAs/miRNA clusters amounted to 20. The results of this evaluation show a higher number of RNAs with a role in the circadian system, compared with those that are directly affected by melatonin. This proportion may change in the future, as soon as many more miRNAs have been investigated. In fact, new miRNAs are being continually discovered and the newest of them have not yet been evaluated for roles in both immune and circadian systems.

Although the number of miRNAs that have been shown to be directly controlled by melatonin has still remained rather low, it should be taken into account that the effects of melatonin on this category of molecules have not yet been systematically investigated. Moreover, melatonin may influence more of them indirectly, because of its chronobiotic effects on oscillators. A special aspect of potential future importance concerns the roles of circRNAs, since their sponging of miRNAs is functionally highly relevant to intraorganismal communication by exosomes and ectosomes (also known as microvesicles formed by directly budding from the plasma membrane).

Both these types of extracellular vesicular structures contain numerous regulatory molecules (98, 138, 182, 270, 291). Melatonin has already been shown to influence their contents, especially with regard to macrophage function and, thus, to inflammation control (44): Exosomes from melatonin-treated hepatocellular carcinoma cells downregulated in recipient macrophages the secretion of TNFα, IL-1β, and IL-6, whereas those from untreated cells upregulated these proinflammatory cytokines. Interestingly, melatonin was shown in another study to enhance exosome secretion by adipocytes, along with suppression of adipose inflammation, interaction of exosomes with macrophages, and favoring macrophage polarization toward M2 (164).

This treatment was associated with an SIRT1-dependent increase in the circadian rhythm of isocitrate dehydrogenase 2 (*Idh2*) mRNA levels in adipocytes. Notably, the favoring of M2 *versus* M1 polarization by melatonin seems to be a general feature that contributes to the anti-inflammatory actions of this agent, already at physiological levels (278).

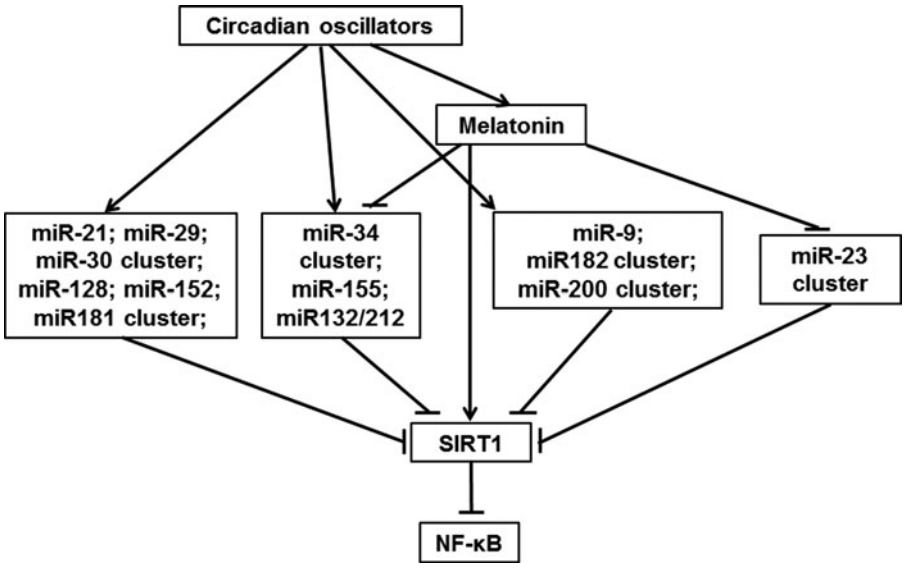

FIG. 3. Various inflammation-related microRNAs under circadian control suppress SIRT1, thereby removing the SIRT1-mediated blockade of NF-κB activation, whereas melatonin upregulates SIRT1 and downregulates several of these microRNAs. These relationships are valid for conditions of well-operating circadian oscillators, whereas strongly deviating findings are typically obtained in tumor cells, whose oscillators are often severely dysregulated (92, 95, 108). It should also be noted that many more microRNAs are under circadian control or modulated by melatonin (107). Various others have been shown to stimulate or suppress NF-κB activation or SIRT1 signaling, however, in the absence of information on circadian control (105). Moreover, NF-κB was typically negatively correlated with Nrf2, in many analyzed cases (105) (not incorporated here, for avoiding an overcomplicated scheme). The switching between NF-κB and Nrf2 reflects an important feature of proinflammatory *versus* anti-inflammatory and prooxidant *versus* antioxidant regulation. Before the discovery of the melatonin-SIRT1 relationship, it had been assumed that the influence of melatonin on the NF-κB/Nrf2 balance was independent of SIRT1. It may still be that melatonin and SIRT1 additionally act synergistically in parallel. NF-κB, nuclear factor κB; Nrf2, nuclear factor erythroid 2-related factor 2.

Extended Signaling by Melatonin *via* Sirtuins

Although melatonin's actions *via* MT_1 and MT_2 and respective G protein-mediated signaling pathways are well documented, the involvement of sirtuins in melatonin effects has opened new routes of interpretations. Unfortunately, the connections between the GPCR-mediated pathways and sirtuin expression are still poorly understood. However, the repeated demonstration of SIRT1 upregulation by melatonin in nontumor cells substantially expands the spectrum of melatonin's action and has been referred to as ''extended signaling'' (103).

The relationship between melatonin and SIRT1 is of considerable importance, for several reasons: First, SIRT1 is an accessory clock component that increases circadian amplitudes (37, 240), that is, a cellular mechanism also influenced by melatonin. Second, SIRT1 shares with melatonin anti-inflammatory and some antioxidant properties (Fig. 4) (95, 103–105). Third, both melatonin and SIRT1 have been discussed in their roles as potential anti-aging factors (104).

In the beginning of research on the relationship between melatonin and SIRT1, entirely different results had been observed, because SIRT1 was shown to be strongly downregulated by melatonin, an effect that was typical for cancer cells (95, 134, 135). However, this action turned out to be largely cancer-specific and was interpreted on the basis of a profound dysregulation of oscillators in transformed cells, a necessity because of the properties of several core oscillator components as tumor suppressor genes.

The escape of tumor cells from the suppression of tumor properties is based on downregulation of genes with antitumor properties such as *Per2*, but increased levels of *Sirt1* and *Clock*, the latter being a proliferation-promoting factor. Thus, melatonin's action of suppressing SIRT1 may be interpreted as a partial reversal of the tumor-specific dysregulation (92, 95, 108).

However, in nontumor cells, the effects of melatonin on SIRT1 are entirely different and consist, in the majority of studies, in significant upregulations, as summarized elsewhere until 2016 (95). The decisive argument for the melatonin signaling *via* SIRT1 is based on the repeated findings that various effects by melatonin are suppressed by sirtuin inhibitors such as sirtinol and EX527 or by *Sirt1* siRNA. More recent studies have confirmed this observation in various experimental systems (10, 15, 41, 67, 77, 81, 122, 157, 168, 193, 205, 209, 230, 245, 283, 285, 293–295, 297, 298).

In another study on the diabetic heart, melatonin's protective effects were found to be absent in *Sirt1*$^{-/-}$ (52). In microglia, hypoxia caused by either $CoCl_2$ or carotid occlusion, melatonin suppressed cobalt toxicity, an effect inhibited by EX527, and prevented, in the occlusion model, decreases of nuclear SIRT1 localization (183). Many more publications not summarized in this place have also reported upregulation of SIRT1 by melatonin, but they did not investigate the suppression of melatonin effects by sirtuin inhibition.

Importantly, many of the studies that demonstrated effects of melatonin *via* SIRT1 also showed upregulation of Nrf2 and downregulation of NF-κB. Collectively, the numerous

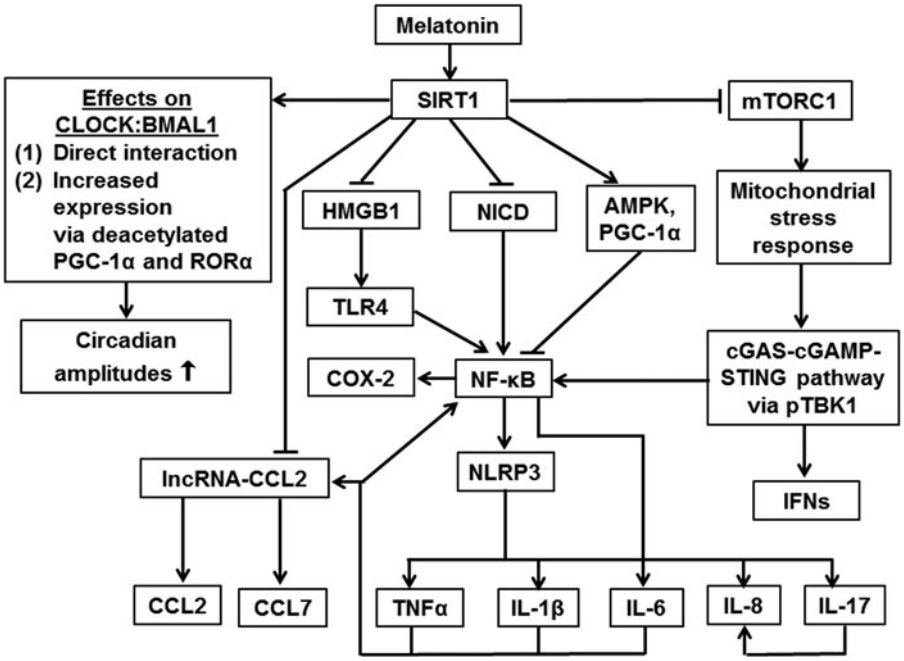

FIG. 4. Anti-inflammatory and chronobiological actions of SIRT1 under demonstrated or expected upregulation by melatonin, as abolished by SIRT1 inhibition. *Arrows* indicate positive relationships (activation or upregulation) between connected factors; blunted lines indicate inhibition or downregulation. With regard to the complexity of the immune system, this scheme cannot delineate all possible connections, factors, and differences between cell types. Melatonin may also exert some of these effects both SIRT1-dependetly and -independently. AMPK, adenosine 5′-monophosphate-dependent protein kinase; CCL, CC-chemokine ligand; cGAMP, cyclic 2′,3′ guanosine monophosphate-adenosine monophosphate; cGAS, cyclic GMP-AMP synthase; COX-2, cyclooxygenase-2; HMGB1, high-mobility group box 1; IFN, interferon; IL, interleukin; lncRNA, long noncoding RNA; mTORC1, mechanistic target of rapamycin receptor complex 1; NICD, intracellular domain of Notch; NLRP3, NLR family pyrin domain containing 3; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; pTBK1, phosphorylated TANK-binding kinase 1; RORα, retinoic acid receptor-related orphan receptor α; STING, stimulator of IFN genes; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor-α.

reports on SIRT1-mediated actions by melatonin that were observed in various tissues or cells and under entirely different conditions constitute an overwhelming body of evidence for the suggested extended signaling of melatonin, which comprises various further details of downstream pathways (Fig. 4) (104, 105). This seems to further extend to the central antagonizing roles of Nrf2 and NF-κB in the control of antioxidant and anti-inflammatory actions known for both melatonin and SIRT1.

One might ask oneself, what the significance of extended signaling *via* SIRT1 could be, especially under conditions of pharmacological doses. First, one advantage may be sought in the half-lives of the agents (109). Contrary to the short-lived melatonin, SIRT1 exhibits a half-life in the range of several hours, for example, 8 h in glomerular mesangial cells (129), and would, thus, provide longer-lasting actions. Second, especially under physiological conditions, the upregulation of SIRT1 expression may be seen as a mode by which melatonin interacts with cellular circadian oscillators. Both aspects indicate a fundamental relationship in redox biology and inflammation control.

Mitochondrial Protection

Protection of mitochondria by melatonin is well documented and has been multiply reviewed (1–3, 5, 6, 17, 30, 40, 60, 62, 68, 69, 86, 99, 112, 117, 120, 150, 160, 166, 170, 202, 211, 214, 222, 224, 228, 229, 261, 263, 266, 276). Therefore, this important part of melatonin's antioxidant actions will not be discussed in detail, but rather with focus on the distinction between circadian and noncircadian phenomena and on some aspects that have otherwise been poorly considered.

From a fundamental point of view, mitochondrial activity has to be coordinated with circadian rhythms, because of the demands by periodic metabolism resulting from, for example, locomotor activity, but also organ specifically from other metabolic rhythms. This is reflected by circadian rhythms of oxygen consumption (186, 187). This also leads unavoidably to a rhythmic mitochondrial generation of free radicals, due to electron leakage from the electron transport chain (ETC) (113).

The rhythmicity of mitochondrial activity results, to a certain extent, from the circadian variations of glycolytic and lipolytic precursors fed into the citrate cycle, but it is also a consequence of regulation mechanisms acting on these organelles. In particular, a nexus between the circadian system and mitochondria exists in the rhythmic activity of SIRT3. This latter sirtuin, which is mitochondrially localized and differs in this regard from the aforementioned SIRT1 and SIRT6, cannot directly act on cellular circadian oscillators whose components are present in the nucleus and cytoplasm, but it is driven by the cyclicity of NAD^+ (174, 177, 204).

Therefore, the activities of these sirtuins are concomitantly controlled by the same metabolite, however, with the complication that the mitochondrial NAD^+/NADH ratio is also

influenced by the activity of the mitochondrial citrate cycle. Another circadian regulation mechanism concerns the clock-controlled dynamin-related protein 1 (DRP1), which acts in this way as a circadian output factor (38, 153, 244). DRP1 is a large GTPase, which is recruited to the outer mitochondrial membrane by the mitochondrial fission factor (Mff).

By interacting with fission 1 (Fis1), it promotes fission and can lead, in the extreme, to enhanced mitochondrial fragmentation (132). Under conditions of excessive fragmentation, DRP1 may cause increased electron leakage, mtPTP opening with breakdown of the mitochondrial membrane potential ($\Delta\Psi_{mt}$), and oxidative stress (132). Moreover, DRP1 was shown to more generally control rates of oxidative phosphorylation and ATP production, since blockade or knockout of DRP1 abolished the circadian rhythms in these parameters (244).

However, DRP1 does not exhibit circadian cycles in protein concentration, but it is rather controlled at the activity level by phosphorylation and dephosphorylation at Ser637 (244). Interestingly, DRP1 phosphorylation seems to be connected to SIRT3 *via* LKB1 (liver kinase B1) deacetylation, which is followed by AMPK (adenosine 5′-monophosphate-dependent protein kinase) phosphorylation (Fig. 5) (162, 299). Corresponding mechanisms *via* deacetylation of other metabolic sensors likely exist.

With regard to melatonin's role in the interplay of Sirt3, AMPK, and DRP1, several effects including protection have been documented. Activation of SIRT3 by melatonin has been repeatedly reported (65, 80, 163, 169, 208, 228, 250, 282, 288, 290, 293, 302). In many of these studies, no upregulation of SIRT3 protein expression was observed, which is in accordance with the NAD^+-dependent activation as the decisive regulation mechanism. Effects of melatonin on AMPK and other metabolic sensors have been summarized elsewhere (90).

The suppression of DRP1 activation by melatonin has also been reported, sometimes showing the involvement of AMPK, but without demonstration of participation of SIRT3 (7, 43, 45, 136, 296, 300, 301). Another study reported the involvement of SIRT1 instead of SIRT3 as the deacetylating agent (52). This is also possible as an alternate route, since SIRT1 is present in the cytosol, where it can likewise deacetylate AMPK, with same consequences to DRP1. Notably, most studies mentioned in this paragraph have shown, in addition to the prevention of excessive mitochondrial fission, protection against oxidative stress, often associated with the prevention of apoptosis and, sometimes, anti-inflammatory actions.

Strong signaling toward mitochondrial fission, with consequences to $\Delta\Psi_{mt}$ breakdown and cell death, is especially observed under conditions of inflammation with cytokine storm, in which TNFα leads to activation of NR4A1 (nuclear receptor subfamily 4 group A member 1) (299), which initiates pAMPK dephosphorylation and, thereby, favors pDRP1 dephosphorylation by calcineurin (Fig. 6). The protective action of melatonin may be mainly attributed to the multiple anti-inflammatory effects of this agent (104, 105). The contributions of SIRT1 and, even more, SIRT3 signaling may deserve further clarification with regard to changes in NAD^+ levels, since upregulations of sirtuin expression are not *per se* indicative of higher activities.

Several additional mechanisms contribute to mitochondrial protection. The aforementioned downregulation of iNOS by melatonin is not only important for avoiding excessive peroxynitrite formation (120), but it has also been found to be decisive for the maintenance of mitochondrial function under conditions of sepsis or endotoxemia (3, 60, 62, 68, 166, 197). Recent evidence indicates that the protective effects in mitochondria are, at least partially, mediated by SIRT1 (208, 295). The maintenance of electron flux by melatonin also comprises

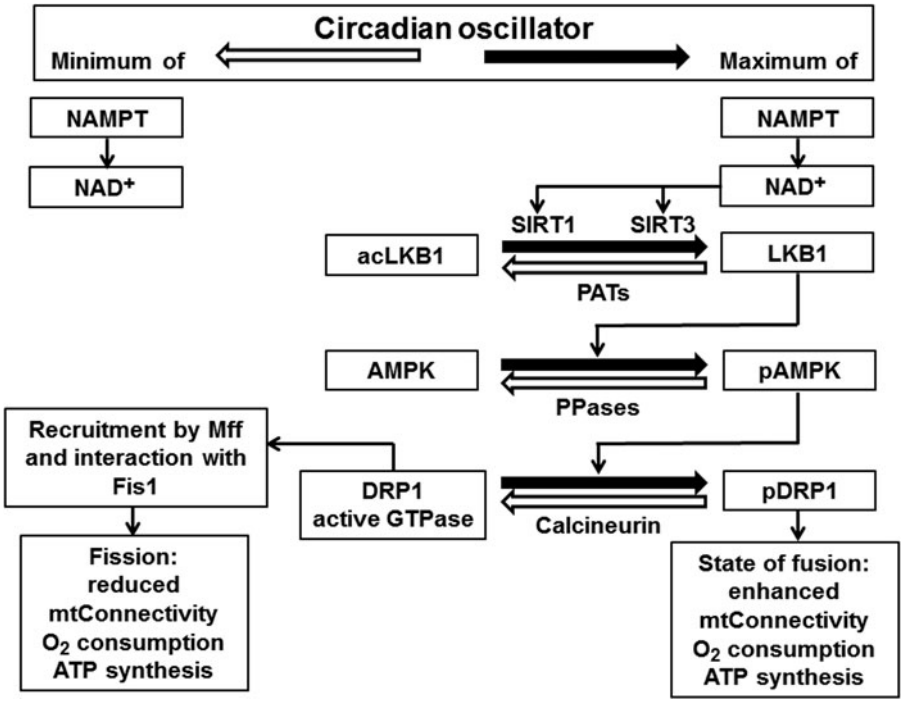

FIG. 5. The circadian oscillator drives a mitochondrial fusion/fission cycle: a comparison of conditions at circadian minima and maxima of NAD^+ levels, O_2 consumption, and ATP synthesis. Enhanced fission is associated with low, prevailing fusion with high O_2 consumption and ATP formation. $\Delta\Psi_{mt}$, mitochondrial membrane potential; AMPK, adenosine 5′-monophosphate-dependent protein kinase; DRP1/pDRP1, dynamin-related protein 1/phosphorylated dynamin-related protein 1; Fis1, mitochondrial fission 1 protein; LKB1/acLKB1, liver kinase B1/acetylated liver kinase B1 (LKB1 is part of the AMPKK complex); Mff, mitochondrial fission factor; mt, mitochondrial; PATs, protein acetyltransferases; PPases, protein phosphatases.

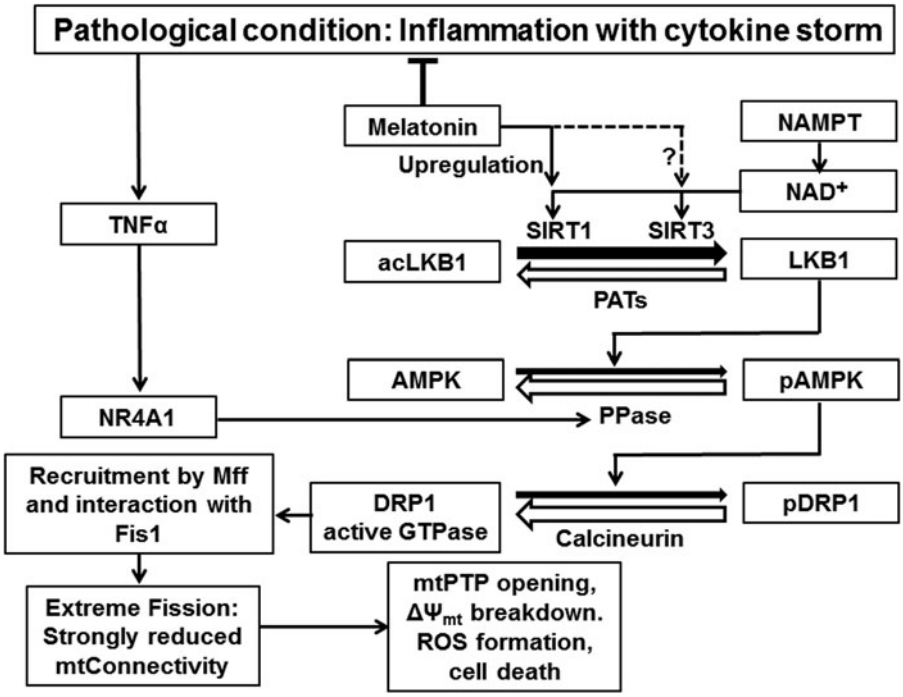

FIG. 6. Inflammation can drive mitochondria toward fission, with severe consequences to mitochondrial function and cell survival: a simplified model. The protein phosphatase upregulated *via* NR4A1 has not yet been identified; pAMPK dephosphorylation is known for PP1, PP2A, and PP2C. The model is restricted to the best understood details regarding fission and may have to be expanded by additional routes and factors. Additional mitochondrial effects of inflammation regarding $^{\bullet}$NO and its derivatives, ROS, the GSSG/GSH balance, and DAMP factors have been omitted. In this model, the counteraction by melatonin has been restricted to the mitigation of the cytokine storm, which is sufficiently documented. Upregulation of SIRT1 and, perhaps, SIRT3 by melatonin is indicated, but it remains uncertain whether this effect can prevail over the NAD^+-dependent regulation. DAMP, damage-associated molecular pattern; GSH, reduced glutathione; GSSG, oxidized glutathione; mtPTP, mitochondrial permeability transition pore; NR4A1, nuclear receptor subfamily 4, group A, member 1; ROS, reactive oxygen species.

upregulation of proteins of the ETC complexes (172, 173, 194, 195), prevention of cytochrome c-independent nonenzymatic cardiolipin peroxidation (128, 167, 202, 206, 207), and upregulation of the mitochondrial GPx subform, GPx4 (60, 62, 166, 235–237), which may be related to the observed reduction of cardiolipin peroxidation (99).

Another aspect of mitochondrial protection concerns the direct effects of melatonin on mtPTP opening. Apart from its inhibition *via* binding to a low-affinity mitochondrial site (9), more recent experiments revealed that melatonin, at reasonable concentrations, did not permanently block the mtPTP, but rather reduced the duration of opening and, thus, allowed superoxide flashes with a soon return to normal $\Delta\Psi_{mt}$ values and, thus, avoided apoptosis induction (99, 133).

Much of the data regarding mitochondrial protection have been obtained with supraphysiological doses of melatonin. Therefore, these effects are not easily attributed to the chronobiological role of melatonin. Moreover, the intracellular role of mitochondrially produced melatonin does not indicate a circadian relationship. In the seminal article on mitochondrial synthesis in neurons, the formation rates did not show any circadian fluctuation (255).

However, the consequences of chronodisruption by activation of the innate immune system with inflammation induction and resulting mitochondrial damage have indicated another, more indirect relationship to the circadian system, in terms of requirements of well-operating oscillators for preserving mitochondrial function (6). Another nexus between the circadian system, melatonin, and mitochondrial protection may be deduced from the involvement of sirtuins, as discussed in the preceding section.

Mitochondrial protection under conditions of disease-related overshooting inflammation has recently gained considerable actuality in the context of COVID-19, although the required melatonin doses are far beyond the physiological range (260). Melatonin is remarkably efficient in the treatment of various deadly viral and bacterial diseases (121, 259) and also targets mitochondria, which are subject to pathogen-associated molecular pattern- and damage-associated molecular pattern-associated cytokine storms and to hijacking by viruses that alter mitochondrial metabolism.

This may be seen as a good reason for applying melatonin with the purpose of protecting mitochondria in such diseases (261). Notably, the virus-induced Warburg effect also leads to a depletion of the cofactors needed for melatonin synthesis. Repletion of melatonin by exogenous administration is, therefore, a requirement for readjusting the availability of melatonin (261).

Conclusion

Melatonin can be applied, experimentally or therapeutically, within a remarkably broad range of concentrations. Notably, the highly divergent doses can be justified for the

specific applications and all of them have been shown to exert effects in the field of antioxidative protection. However, it is important to distinguish between different mechanisms of action that are based on their own concentration requirements. From the mechanistic point of view, lowest concentrations as attained, in humans, by doses in the low mg range are sufficient for actions *via* the MT_1 and MT_2 receptors. Somewhat higher levels may be appropriate for effects *via* complexes of CaM with CaM-activated proteins.

Concentrations in the millimolar range seem to be required for actions *via* mtPTP-associated binding site (9). The highest concentrations (daily doses of several 100 mg in humans, or 10–20 mg/kg in rodents), which may include direct scavenging of free radicals, are needed for efficiently rescuing from several acute diseases, such as viral infections, sepsis, stroke, or brain trauma (42, 121, 165, 260, 273), and they have been also applied in a chronic neurodegenerative disease (275).

These differences in dosage are often poorly considered in articles, and it seems necessary to pay more attention to this point in the future. Researchers should be aware of the problems regarding signaling mechanisms, if very high doses are required that are oversaturating MT_1 and MT_2 receptors for extended periods of time. If in such studies arguments on MT_1 and/or MT_2 involvement are forwarded, one would expect efficacy already at much lower doses.

Receptor-mediated effects of melatonin, for example, *via* G protein components such as $\alpha_{i2/3}$, α_q, or $\beta\gamma$ (85), can be of a direct or an indirect nature. Direct effects *via* MT_1 or MT_2 are typical for melatonin's chronobiotic actions and various other well-documented effects in numerous tissues (54, 55) and can also be inferred for the upregulation of antioxidant enzymes, such as GPx. However, numerous pathways other than the primary decrease of cAMP or activation of mitogen-activated protein kinase cascades have been suggested for numerous protective effects, without connecting them to the G protein-mediated changes.

As far as the circadian system is involved, the influence of melatonin on cellular oscillators may allow many additional actions by circadian output factors and various clock-controlled proteins that are acting *via* other signaling mechanisms. The multitude of miRNAs that are regulated by the circadian system and/or by melatonin introduce another degree of complexity into the signaling matrix, both intracellularly and, *via* exosomes and ectosomes, intercellularly (98, 102, 103, 105, 107, 149).

Another route of secondary signaling by melatonin concerns the actions mediated by sirtuins, especially SIRT1, with regard to the roles of this protein as a circadian oscillator component and as an anti-inflammatory and antioxidant agent (95, 103–105, 107, 109). Moreover, the influence of melatonin toward macrophage and microglial M2 polarization deserves (278) particular attention in the future, because of its numerous consequences to inflammation regulation.

Author's Contribution

This article was written by R.H., who served as the sole author.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

No funding was received for this article.

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Date of first submission to ARS Central, December 16, 2021; date of acceptance, December 31, 2021.

Abbreviations Used

AFMK = N^1-acetyl-N^2-formyl-5-methoxykynuramine
AMK = N^1-acetyl-5-methoxykynuramine
AMPK = adenosine 5′-monophosphate-dependent protein kinase
AMPKK = adenosine 5′-monophosphate-dependent protein kinase kinase
asRNA = antisense RNA
BMAL1 = brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1
CaM = calmodulin
CCG = circadian-controlled gene
CCL = CC-chemokine ligand
cGAMP = cyclic 2′,3′ guanosine monophosphate-adenosine monophosphate
cGAS = cyclic GMP-AMP synthase
circRNA = circular RNA
CLOCK = circadian locomotor output cycles kaput
COX-2 = cyclooxygenase-2
Cry = cryptochrome
DAMP = damage-associated molecular pattern
DBP = albumin D-site-binding protein
DRP1/pDRP1 = dynamin-related protein 1/phosphorylated dynamin-related protein 1
E4BP4 = adenovirus E4 promoter-binding protein
ETC = electron transport chain
Fis1 = mitochondrial fission 1 protein
GPCR = G protein-coupled receptor
GPx = glutathione peroxidase
GR = glutathione reductase
GSH = reduced glutathione
GSSG = oxidized glutathione
HMGB1 = high-mobility group box 1
Idh2 = isocitrate dehydrogenase 2
IFN = interferon
LKB1/acLKB1 = liver kinase B1/acetylated liver kinase B1
IL = interleukin
iNOS = inducible NO synthase
lncRNA = long noncoding RNA
Mff = mitochondrial fission factor
miRNA = microRNA
MT_1 = melatonin receptor 1
MT_2 = melatonin receptor 2
mTORC1 = mechanistic target of rapamycin receptor complex 1
mtPTP = mitochondrial permeability transition pore
NAMPT = nicotinamide phosphoribosyltransferase
NF-κB = nuclear factor κB
NFIL3 = nuclear-factor interleukin 3
NICD = intracellular domain of Notch
NLRP3 = NLR family pyrin domain containing 3
nNOS = neuronal nitric oxide synthase
NQO2 = NRH:quinone oxidoreductase 2
NR1D1 = nuclear receptor subfamily 1, group D, member 1
NR4A1 = nuclear receptor subfamily 4, group A, member 1
Nrf2 = nuclear factor erythroid 2-related factor 2
pAMPK = phosphorylated adenosine 5′-monophosphate-dependent protein kinase
PAT = protein acetyltransferase
Per = period
PGC-1α = peroxisome proliferator-activated receptor-γ coactivator-1α
PPase = protein phosphatase; PP1, PP2A, PP2C, protein phosphatase subforms 1, 2A, 2C
pTBK1 = phosphorylated TANK-binding kinase 1
REV-ERBα = reverse-related erythroblastoma-α
RORα = retinoic acid receptor-related orphan receptor α
RORE = ROR response element
ROS = reactive oxygen species
SCN = suprachiasmatic nucleus
SIRT = sirtuin
snoRNA = small nucleolar RNA
STING = stimulator of IFN genes
TLR4 = toll-like receptor 4
TNFα = tumor necrosis factor-α
$\Delta\Psi_{mt}$ = mitochondrial membrane potential