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REVIEW ARTICLE

Future Demands Concerning the Epigenetic Relevance of Melatonin and the Circadian System in Gerontology

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Abstract

Melatonin is a highly pleiotropic regulator molecule that influences numerous functions in many cell types. Its actions comprise direct and circadian oscillator-mediated effects. The levels of circulating melatonin typically decline in the course of aging. Additionally,various aging-associated diseases further decrease melatonin concentrations. With regard to its remarkably broad spectrum of actions, control mechanisms upstream and downstream of melatonin should be investigated much more in detail with regard to the contribution of epigenetic modulation. The importance of epigenetic alterations has already become evident in the fields of both gerontology and chronobiology. Therefore, it seems necessary to fill the gaps concerning corresponding processes related to melatonin, especially under the aspects of physiologic malfunctions because of aging-associated decreases of melatonin. This review outlines the findings on melatonin's epigenetic actions, as obtained to date, and sets these results in correspondence to general knowledge and many specific findings concerning circadian rhythms. These considerations focus on DNA methylation and erasure of 5-methylcytosine, on histone modification, in particular, acetylation/deacetylation and methylation/demethylation, and on the manifold roles of noncoding RNAs, especially microRNAs. With regard to melatonin's spectrum of actions in the gerontological context, emphasis is given to its contribution to circadian oscillation amplitudes, to anti-inflammatory actions and to antioxidative protection.

Keywords

Circadian, CpG islands, DNA demethylation, Epigenetics, Histone modification, Melatonin, miRNAs

Introduction

Epigenetics is a rapidly expanding field, which receives increasing attention in all areas of molecular biology and physiology. The numerous changes observed in aging organisms strongly indicate that these deviations cannot be exclusively caused by an increasing number of mutations, senescent cells and cell death, despite their indisputable contribution to senescence. With regard to the manifold processes of aging-related reprogramming, it seems likely that epigenetic mechanisms are strongly involved. While epigenetics was formerly thought to induce more or less stable changes that, by earlier definition, would be transmitted to next generations, our actual view has considerably changed towards a much more dynamic scenario, in which processes of, e.g., DNA and protein modifications can be reversed. Although the possibility of transmitting epigenetic alterations to new cell generations and to offspring shall not be generally disputed, the previously unexpected discovery of potentially important processes of reversal are in favor of a dynamics that may allow both silencing and reactivation of genes and, perhaps, a certain degree of rejuvenation or, at least, functional improvements.

Important mechanisms of reversal have been discovered in the fields of DNA methylation, histone modification and telomere attrition. DNA methylation, which is known to occur in CpG islands of promoters as well as in other DNA regions, is now known to be reversible. In terms of aging, the so-called "epigenetic drift" describes an increasing hypermethylation in promoters, but also a progressing demethylation within the entire genome, especially in CpG-poor sites [1,2]. Without discussing in this place the consequences hereof, the observation of overall hypomethylation clearly demonstrates the existence of demethylation processes. According to actual knowledge, demethylation is initiated by the ten-elev-

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en-translocation enzymes TET1, TET2, and TET3, which first hydroxylate 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), followed by conversion to 5-formylcytosine (5fC) and, thereafter, to 5-carboxylcytosine (5caC) [3-6], and is finally corrected by base excision repair [7,8].

The second area of reversible processes concerns chromatin remodeling by histone modification. Without going in this place into too many details, the focus will be laid here on histone acetylation and methylation, although numerous other histone modifications with additional or different functions also exist, such as phosphorylation, ubiquitinylation, SUMOylation, crotonylation, butyrylation, propionylation, citrullination and ADP-ribosylation [9]. Frequently observed modifications concern histone H3 acetylation (e.g., H3K9ac, H3K27ac) and mono-, di- or trimethylation (e.g., H3K4me1, H3K4me2, H3Kme3, H3K36me3, the latter being especially enriched at actively transcribed genes) [10-12]. Numerous histone acetyltransferases (HATs) are involved in chromatin remodeling, including various GNATs (Gcn5 *N*-acetyltransferases), the 60 kDa Tat interactive proteins (MYSTs), and the so-called orphan HATs [13]. Interestingly, a member of the latter group, the p300/CBP complex (CBP = CREB binding protein), exhibits a regulatory relationship to melatonin via NF-κB. Melatonin was shown to inhibit DNA binding of NF-κB, an effect that was concluded to be responsible for the downregulation of iNOS (inducible NO synthase) and cyclooxygenase 2 [11,14,15]. Another potentially important relationship concerns the HAT activity of a component of the cellular circadian core oscillator, CLOCK (circadian locomotor output cycles kaput) [16,17]. A similar complexity is observed in the histone deacetylases (HDACs), with four classes comprising HDAC1-11 [18] and additionally the sirtuins (class 3). Sirtuin subforms localized in the nucleus (SIRT1, SIRT2, SIRT6, and SIRT7) can act on histones [19,20], whereas those localized in mitochondria (SIRT3, SIRT4, SIRT5) deacetylate other substrates. It should also be noted that many histone acetylating and deacetylating enzymes additionally accept various other proteins as substrates. In the context of circadian cell biology, it is a remarkable fact that SIRT1 acts as an accessory component of the oscillator and is capable of enhancing rhythm amplitudes [21-24]. Therefore, circadian oscillators are capable of phase-dependently promoting histone acetylation via CLOCK or deacetylation via SIRT1. These rhythmic changes may be seen in the context of circadian chromatin remodeling that seems necessary in terms of daily changes in gene expression that differs between groups of circadian-controlled genes. Melatonin can be assumed to participate in these processes, in particular, as it influences both central and peripheral oscillators [25] and upregulates SIRT1 in numerous nontumor cells, especially in the context of aging [11,24,26]. Under these premises, it seems worthwhile and promising to intensify epigenetic research in the field of the circadian oscillator system and the role of melatonin as a regulatory player herein. The considerable dynamics that is meanwhile apparent in epigenetic processes sufficiently conforms to the rhythmic changes produced by circadian oscillators and melatonergic signaling. Losses of circadian amplitudes and melatonin secretion, as occurring during aging, should be taken as a good reason for identifying the epigenetic changes that are associated with these deteriorations. Studies of this type might reveal insights for strategies to reverse the reduced circadian dynamics and, thereby, to improve the physiological well-functioning of the aging subject.

Circadian Oscillators and Melatonin in the Context of DNA Modification

In the circadian context, an important role of DNA methylation was already indicated by several studies demonstrating global rhythms in 5mC abundance in various tissues [27-29]. In mouse liver, 5mC levels were increased and their rhythmicity was lost in double knockouts of *Per1*/*Per2*, two core oscillator genes [28]. In the hypothalamus of Siberian hamsters, a circadian rhythm of DNA methyltransferase was described [30]. Another hint for the relevance of circadian DNA methylation came from the observation that global 5mC patterns differ between human monozygotic twins that are discordant for diurnal preference [31]. Although such findings may be taken as a strong indication for the importance of this type of epigenetic modulation in the circadian field, they do not tell too much in mechanistic terms. This can be only achieved by specific studies on selected genes that are either part of cellular circadian oscillators or known to be circadian-driven. Moreover, it is of crucial importance to analyze the sites of methylation, whether occurring in promoters, and if so, in which response elements, or outside promoters, which may also have consequences to expression including alternate splicing. Finally, one has to be aware that CpG methylation in a promoter is not per se a sign of silencing, but that the consequences depend on the precise site, i.e., whether 5mC is present in an enhancer or in a silencer element, where methylation may prevent binding of a negative regulator protein.

Gene-specific information has meanwhile been obtained in a number of studies. As summarized earlier [11], several core oscillator components act as tumor suppressors, and various tumors and tumor cell lines exhibit hyper- or altered methylation in the promoters of *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2* and *Bmal1*. Generally, tumor cells have to silence tumor suppressor genes [32-34], and this has to be also the case for the anti-tumor factors in the circadian oscillators.

Additional information concerning the circadian role of DNA methylations have been obtained in studies on shift work and light at night (LAN). Human DNA samples from blood revealed widespread changes in long-term

shiftworkers: 3593 CpG sites were found to be hypermethylated and 1816 CpG sites hypomethylated [35]. In another, smaller report with focus on promoters of imprintable genes, hyper- or hypomethylations were detected in 20 and 30 CpG sites, respectively [36]. A recent investigation comparing 65 dayshift and 59 nightshift workers revealed differences in 3769 genes, 16,135 loci, and 7173 CpG islands, including 21 circadian genes, with greatest differences in *Per3* [37]. More specifically, hypermethylation in the *Cry2* promoter and hypomethylation in the *Clock* promoter were documented [35], two changes also known from mamma tumors and of interest to the assumed relationship between shift work and cancer. Moreover, hypermethylation with assumed silencing effects was reported for *HDAC2*, a histone deacetylase gene, and for *Mbd2* (methyl-CpG-binding domain 2), a gene encoding an important methyl-CpG reader [35], findings that indicate further connections to chromatin structure and 5mC detection in the nucleus.

Changes in the DNA methylation pattern were already detected after relatively moderate environmental changes, indicating a remarkable epigenetic dynamics, which modulates gene expression. A single night of total sleep deprivation caused increased methylation in the *Cry1* promoter and two enhancer regions of *Per1* in adipose tissue [38]. In terms of percentage, these changes were relatively small, but, with relevant site specificity, the effects on gene expression may already be substantial. More remarkably, relatively small changes in the light/dark cycle, from 24 to 22 or 26 hours light, causedalterations of promoter DNA methylation in the circadian master clock, the suprachiasmatic nucleus (SCN), of mice [39-41]. These changes were reversed by return to the previous lighting pattern, and changes were blocked by a DNA methyltransferase inhibitor. It seems that the SCN utilizes promoter methylation for adapting its cycles to the environment, in other words, for cycle plasticity.

As the circadian system also changes in the course of aging, studies on DNA methylation in oscillator genes should be of particular interest. In aging rats, considerable differences in their capacity of generating oscillations develop between different tissues [42]. In some of them,the rhythmicity appeared to be more or less unaffected by aging, whereas in others, phase advances were observed. Moreover, in a third category rhythmicity was lost. Remarkably, oscillations could be re-initiated, in these cases by forskolin. Therefore, the capacity of behaving rhythmically was retained, but not displayed in the absence of suitable internal periodic stimuli. It seems highly likely that these age-related losses as well as the observed re-initiation have an epigenetic basis. In some organs of aging mice, tissue-specific alterations in promoter methylation of oscillator genes were observed [43]. For instance, the *Per1* promoter became hypomethylated in the stomach, whereas, in the spleen, *Cry1*, *Bmal2* and *Npas2* promoters exhibited increased methylation.

Relatively much information is available on the methylation of the *Bmal1* (= *Arntl*) gene, however, mostly in a different context, although some aspects of age-related diseases have been also considered. In several ovarian cancer cell lines, methylation at *Bmal1* was associated with silencing of this gene, although this was accompanied by enhanced histone methylation (H3K27me3) [44]. In various other tumor cell lines, hypermethylation of the *Bmal1* promoter was shown to silence this gene and to suppress circadian rhythmicity [11,25,45-47]. Hypermethylation of a CpG island in the 5' region and hypomethylation in the first exon of *Bmal1* were reported for cases of bipolar disorder [48]. Changes in CpG methylation of *Bmal1* were also observed upon dietary changes [49]. Another, recent study [50] is of importance under two aspects. A rhythm of *Bmal1* methylation was described and also deviations of this rhythm were observed in brain tissue from early stages of Alzheimer's disease (AD). Circadian deviations are known in AD, but have previously mainly been seen under the aspect of progressive neurodegeneration [51,52]. With this new information, one might be inclined to consider earlier prodromal changes by epigenetic modulation. The reasons for these early alterations, e.g., because of inflammatory processes, brain insulin resistance, enhanced NO generation etc. [52], will have to be identified in the future. The epigenetic modulation of BMAL1 actions further extends from changes in its expression to the actions at BMAL1-dependent genes that carry an E-box. This BMAL1 binding site is present in *Per* and *Cry* genes as well as other oscillator and circadian-driven genes. Notably, the canonical E-box (CACGTG) contains a central CpG island and is, therefore, principally susceptible to epigenetic modulation. Interestingly, the first C in the E-box was also shown to be methylatable [53]. The consequences of non-CpG methylation for regulation by E-box-binding transcription factors deserves future attention. The relevance of *Bmal1* hypermethylation may be far reaching. It also offers alternate interpretations for reduced expression of E-box-containing genes, especially in the circadian oscillator machinery. As summarized elsewhere [25], various oscillator genes, especially *Per* and *Cry* genes, were shown to display enhanced or, at least altered promoter methylation patterns in various tumor cell lines, findings that were in agreement with the downregulation of these tumor suppressor genes. In the case of *Per2*, hypermethylation had been assumed to be responsible for the observed silencing [25,54]. In specific cases, this remains, of course, a possibility for downregulation. However, in some detailed investigations, the *Per2* promoter was instead found to be hypomethylated, despite the observed suppression of the gene [46,47]. This is, however, also explainable under conditions of silenced *Bmal1*, because the BMAL1 protein is required for *Per2* expression.

Although an increasing number of publications shows changes in DNA methylation patterns, comparably little is known about the erasure of methyl groups under corresponding conditions. Studies on Tet activities can only reveal capacities for global reductions of 5mC. What is urgently needed are gene- or promoter-specific analyses concerning factors of particular regulatory importance, including core and accessory oscillator components as well as genes involved in melatonin signaling. Frequency and distribution of 5hmC (and less, 5fC and 5caC) have been occasionally studied, also in the context of aging [6,55-57], but not involving the circadian system. Moreover, the statement that 5hmC is present at a certain site does not tell how stable this modification may be, i.e., whether it will soon undergo further metabolization and removal by base excision repair or whether it may persist and serve other functions.

Melatonin has likewise been studied with regard to effects on DNA modification. This has been done because the pineal hormone is part of the circadian system, but also because of additional properties that exist especially in some extrapineal sites that also produce this compound in substantial quantities, sometimes exceeding by far those of the pineal gland [58]. The circadian aspect of melatonin has been mainly investigated with regard to LAN, which does not only disturb the circadian oscillator system, but also depresses melatonin synthesis by the so-called photic shutoff. Moreover, the manifold protective actions and some anti-tumor properties of melatonin have been in the focus of investigations on DNA modification. Concerning antioxidative protection, the capacity of melatonin of preventing 8-hydroxy-deoxyguanosine formation by hydroxyl radicals [59,60] has been discussed [11]. If 8-hydroxy-dG (which easily turns into its tautomer 8-oxo-dG) is formed in CpG islands containing 5mC, the double modification prevents DNA repair [61]. 8-oxo-dGmay already inhibit the binding of Tet enzymes or of thymine DNA glycosylase (TDG), the key enzyme of base excision repair. It is still unclear whether this modification also interferes with 5mC reading. Moreover, the double-modified CpG islands have been assumed to promote amyloid deposit formation in the brain [61].

Anti-tumor properties of melatonin have been studied under different conditions. One of these approaches had considered the assumed tumor-promoting effects of LAN. When 4T1 breast cancer cells were inoculated into BALB/c mice, tumor growth was favored by LAN, along with changes in the global DNA methylation pattern, effects that were reported to be partially reversed by melatonin [62]. The reversal was reportedly associated with reduced growth rates of breast tumors. In the breast adenocarcinoma cells MCF-7, DNA methylation patterns were studied on a genome-wide scale [63]. In this study, melatonin was shown to have a remarkably broad impact on epigenetic modulation of gene expression. At 1 or 100 nM melatonin, 8508 and 9196 methylated promoters, as well as 5256 and 6543 methylated CpG islands were detected, respectively. Among these, 2200 and 2824 genes carried methylations in both promoters and intragenic CpG islands. In comparison to controls, 1605 and 3250 genes had hypermethylated, and 1925 and 1786 hypomethylated promoters, at 1 or 100 nM melatonin, respectively. Changes were also detected in miRNA promoters, in which 15 and 20 were found to be hypermethylated, 4 and 9 hypomethylated. Numerous affected genes have been listed, which are downregulated by hypermethylation or upregulated by hypomethylation at 1 nM melatonin, among them many that are cancer-related or encode important signaling molecules [63]. Melatonin signaling was also reported to be changed in cancer cells. The melatonin receptor gene *MTNR1A*, which encodes the receptor protein $MT_1$and is downregulated in C6 rat glioma cells, was strongly upregulated by valproic acid, in conjunction with changes in the mRNA levels of the 5mC reader methyl-CpG binding protein 2 (MeCP2) [64]. The mechanistic connection to DNA modification deserves further clarification, whereas a relationship to histone modification seems to be likely, because of HDAC inhibitor properties of valproic acid.

Chromatin Remodeling by Circadian Oscillators and Melatonin

DNA methylation and histone modification seem to be processes that are, at least, partially interrelated. This is insofar meaningful as the accessibility of a promoter at the DNA level should not be prevented for extended periods by inhibitory posttranslational modifications at the nucleosomes. With regard to circadian rhythmicity, cyclic changes in histone modification are a prerequisite for daily repeated chromatin remodeling that takes place in a time- and chromosome region-specific way to allow or prevent expression of gene groups under circadian control.

The rhythmicity of chromatin remodeling strongly contrasts with long-lasting deviations of chromatin structure as found in tumor cells, being indicative of profound deregulation. The fact that several core and accessory oscillator genes are tumor repressors may be seen as a sign for incompatibility of persistent tumor-specific deviations with a pervasive cyclicity that repeatedly rearranges chromatin structure on a daily basis. Interactions between DNA and histone modification may participate in the chronobiological remodeling processes.

The above-mentioned discovery of HAT activity of the core oscillator protein CLOCK has given rise to studies on rhythmic histone acetylation. In mouse liver, cycles of histone H3 acetylation have been observed in the promoter regions of *Per1*, *Per2* and *Cry1* genes, findings that extended to a participation of p300, another HAT enzyme, which coprecipitated with CLOCK [65]. The rhythms of H3 acetylation were shown to be

in good agreement with those of gene expression. Later, rhythms of H3 acetylation were shown to exist on a broad scale of genes. This was extensively reviewed, showing that circadian oscillations were associated with corresponding changes in H3K9ac and, additionally, H3K27ac and also the trimethylated form, H3K4me3 [66]. In post-mortem human brains, the H3 acetylome revealed a widespread rhythmicity, which was, moreover, reduced in amplitude in AD patients [67]. The aforementioned antagonism between acetylation by CLOCK and deacetylation by SIRT1 has a specific role in cellular oscillators [21,68,69], but also extends to the control of other circadian-driven genes [66]. The dynamic changes in acetylation/deacetylation are not restricted to histones and core oscillator components. Rhythmic changes of this type were also observed in the glucocorticoid receptor [70]. However, it seems important to remain aware of the numerous other players in the field of histone acetylation/deacetylation. Class 2a histone deacetylases (HDACs) and, more specifically, HDAC3 were shown to be involved in the expression of circadian rhythms [71,72]. Additionally, other modifications of histones as well as further regulatory factors have to be considered. Circadian rhythms of histone modification have been observed concerning H3K9ac and H3K27ac, but also in H3K4me1, and H3K36me3 [11,73]. A substantial role of the histone methyltransferase MLL1 in the circadian oscillator has been reported that involves rhythmic H3K4me3 formation [74]. Interestingly, a connection between MLL1 activity and SIRT1 has been detected, since MLL1 was shown to be deacetylated by SIRT1 [75]. On the background of cyclic histone methylation, it would not be surprising that demethylases play an additional, antagonistic role. Infact, the demethylase JARID1a was reported to influence the circadian oscillator via inhibition of HDAC1, which leads to enhanced CLOCK and BMAL1 expression [76], another cross-connection between demethylation of a regulatory compound and histone acetylation.

Alterations of protein modifications in the circadian oscillator system can be also associated with melatonin, in multiple ways. On the one hand, circadian changes can be expected to influence melatonin levels, which are known to occur in terms of progressive reductions during aging and in various senescence-related pathologies [77,78] and which frequently develop in parallel to flattening of circadian amplitudes [24]. On the other hand, melatonin is capable of modulating other rhythms, generated in both central and peripheral oscillators, in terms of both phase resetting and increasing amplitudes [24,25]. Moreover, these effects of melatonin do apparently not consist of direct up- or downregulations of oscillator components via the usual melatonergic signaling pathways, but rather seem to involve other epigenetic factors, in particular, SIRT1 [11,24], which is known to enhance circadian amplitudes [21-24]. Moreover, numerous accessory oscillator components have been shown to be influenced by melatonin and may contribute to chronobiological modulation [79]. With regard to SIRT1, it is of utmost importance to distinguish between nontumor and tumor cells, as summarized elsewhere [11,24]. Especially in aging, melatonin has been repeatedly shown to upregulate SIRT1, which is otherwise typically decreased at advanced age, whereas SIRT1 is increased in various tumor cells to supranormal levels that are substantially reduced by melatonin, effects that are accompanied by inhibition of tumor cell proliferation and, sometimes, by tumor-specific apoptosis.

Other studies have less focused on the circadian connection, but have been conducted under neurobiological or protective aspects. In response to melatonin, area-specific changes in histone modification were described, in particular, increased acetylation of histones H3 and H4 in the hippocampus, and of H4 in the striatum, but no such changes were detected in midbrain and cerebellum [80]. An increase in histone H3 acetylation was observed in the neural stem cell line C17.2, at melatonin concentrations of 0.1 and 1 nM [81]. In addition, rises in mRNA expression of HDAC3, HDAC5 and HDAC7 were described, which remained, however, relatively moderate and were interpreted as a compensatory feedback to melatonin-induced hyperacetylation.

Another link between melatonin and histone modification seems to be mediated by Nrf2 (nuclear factor erythroid 2-related factor 2). Numerous publications have demonstrated the upregulation of Nrf2 and activation of its downstream pathways by melatonin [15,82-87], with an additional contribution by SIRT1 [87]. The role of Nrf2 is of utmost interest to epigenetics. On the one hand, epigenetic mechanisms at various levels (DNA methylation/demethylation, histone acetylation/deacetylation, histone methylation/demethylation, microRNAs) are involved in the regulation of Nrf2 and its negative regulator kelch-like ECH-associated protein 1 (Keap1), whereas, on the other hand, Nrf2 likely exerts epigenetic effects, e.g., by promoting site-specific histone acetylation and/or inhibition of histone deacetylation [88,89]. As numerous pathways are converging at Nrf2, many details still remain to be clarified. An aspect of particular relevance to aging as well as to chronic age-related diseases concerns Nrf2-mediated protection against oxidative stress, which is typically associated with senescence [15,88]. These findings are well in accordance with similar effects observed with melatonin [52,90-93]. An additional complication results from the negative relationship between Nrf2 and NF-κB, which is correspondingly modulated by melatonin in terms of reducing NF-κB activity [15]. In the future, the signaling network of melatonin, Nrf2 and NF-κB and the numerous converging pathways will have to be analyzed in many more details.

Noncoding RNAs, exosomes, and ncRNA Links between Epigenetic Mechanisms

The discovery of long and short noncoding RNAs (ncRNAs) has opened a new field of epigenetic research

and shown that countless functions are modulated by these RNA species. These actions include cross-connections between the levels of epigenetic modifications concerning DNA, histones and regulatory players such as transcription factors and readers of modified sites. In particular, numerous ncRNAs display circadian rhythms in different tissues, especially SCN, retina, brain and liver and, sometimes, profoundly influence circadian oscillator components, as recently summarized [11]. As reported there in detail, numerous lncRNAs (long noncoding RNAs) including lincRNAs (long intergenic noncoding RNAs), imprinted ncRNAs, asRNAs (antisense RNAs), snoRNAs (small nucleolar RNAs) and countless miRNA (microRNAs) exhibited circadian oscillations. A case of special interest concerned the rhythm of antisense RNA of the *Per2* gene (as *Per2* RNA) [73]. The precise actions of the asRNAs remain to be further elucidated. This statement is valid for many other, especially the longer, ncRNAs. The relatively poorly understood snoRNAs may be also of higher relevance than previously believed. Knockout of the *Snord116* locus caused changes in the expression of over 6000 genes in the brain at zeitgeber time (ZT) 6 hours and over 3000 genes at ZT 16, perhaps, partially due to the observed altered expression of *Clock*, *Cry1*, *Cry2*, *Per1*, and *Per2* [94].

More details are known on miRNAs, which have been shown to change the expression and phasing of oscillator components or to be driven by them [11]. For instance, knockdown of *miR-219* was shown to lengthen the circadian period, whereas a *Cry1*/*Cry2* double knockout abolished the *miR-219* rhythm [95]. More information of actions of miRNAs on circadian rhythms has accumulated during the last years [96-101], but the complete details would exceed the scope of this article. However, a specific point should be addressed because of its fundamental importance. MicroRNAs were not only detected in cells, but also in exosomes. In fact, *miR-152* and *miR-494* have been shown to exhibit circadian rhythms in the serum [102]. This finding indicates that that the exchange of circadian information between cells and within the multioscillator system is not restricted to hormones and neurotransmitters, but also involves microRNAs released as exosomes into the blood and, presumably, other body fluids.

Compared to the amply documented cycles and actions of miRNAs in the field of circadian rhythms, the respective information on melatonin is relatively smaller and, in particular, not really coherent because of contextual differences. A few data are available on the role of microRNAs in the pineal gland. *Aanat* mRNA was shown to be targeted by *miR-483* [103] and *miR-325-3p* [104], but these downregulations were only obtained in a neonatal context. Members of the *miR-183-96-182* cluster, otherwise known as regulating factors in the mammalian SCN [11], were expressed in zebrafish pineals [105]. Importantly, *miR-183* did not only influence the circadian oscillator, but additionally targeted *Aanat2* mRNA.

Information on relationships between melatonin and miRNAs mainly concerned either protective effects of the pineal hormone, in different systems, or oncostatic actions. In a rat scopolamine toxicity model of AD-like memory losses, an increase of *miR-124* was observed that was reversed by melatonin, along with correction of the targeted *Egr1* mRNA [106]. Lipopolysaccharide (LPS)-induced neonatal brain inflammation in rats caused changes in *miR-34a*, *miR-146a*, and *miR-126*, along with reduction of SIRT1 expression, effects that were reversed by melatonin [107]. Premature senescence of cardiac progenitor cells by H_2O_2 was prevented by melatonin by maintaining the expression levels of lncRNA H19 and its derivative *miR-675* [108]. In a murine model of alcoholic liver disease, protection by melatonin could be related to the downregulation of *Btg2* (B-cell translocation gene 2) and *Yy1* (yin yang 1) by enhancing *miR-497* expression [109].

In the context of oncostatic actions, a recent study reported downregulation of *miR-155* by melatonin in several human glioma cell lines, along with reduced c-MYB (myeloblastosis proto-oncogene) expression, proliferation and migration [110]. An earlier, more detailed study in MCF-7 breast cancer cells revealed changes in miRNA expression by 1 nM or 100 nM melatonin. Twelve miRNAs were significantly upregulated (*miR-7-1*, *miR-140-5p*, *miR-148b*, *miR-151-3p*, *miR-362-3p*, *miR-374b*, *miR-497*, *miR-505*,*miR-509p*, *miR-658*, *miR-769-5p*, *miR-1977*) and 10 downregulated (*miR-30e*, *miR-222*, *miR-223*, *miR-324p*, miR-519e, *miR-574-5p*, *miR-670*, *miR-1207-3p*, *miR-1244*, *miR-1257*). The analysis of 5'-utr sequences showed that the 22 miRNAs might be able to target 2029 mRNAs [111].

Conclusion

Melatonin secretion by the pineal gland is known to decline by age and, even more profoundly, in various age-related pathologies, in particular, neurodegenerative diseases, and in the complex of metabolic syndrome and type 2 diabetes [51,77-79]. Therefore, it will be of utmost importance to study in detail and on a broader scale the changes that can be induced by melatonin in aging mammals, as far as possible, including the human. However, researchers have to be aware that findings obtained in nocturnally active rodents are not necessarily translatable to the diurnally active human [24,112-114]. Moreover, it is important to strictly discriminate between effects obtained in nontumor and in tumor cells, in which completely opposite actions of melatonin have been observed, notably, concerning the aging suppressor and accessory oscillator component SIRT1 [11,24,52,114]. The connection between melatonin and SIRT1 [24,26] also sheds light on the roles of melatonin in the circadian system and its aging-depending deterioration. On the one hand, the pineal gland is steered by the circadian master clock, SCN, but on the other hand, melatonin feeds back to the SCN and also influences pe-

ripheral oscillators [25]. Therefore, it will be necessary to dissect direct and oscillator-mediated indirect effects of melatonin. This will require analyses of melatonin-induced changes in oscillator components, at the levels of DNA methylation/demethylation, histone modification and contributions by miRNAs, perhaps also other ncRNAs.

The majority of aging-associated changes in both melatonin levels and the circadian system cannot be explained by mutation-based genetic alterations. In part, they may be attributed to progressing degenerative processes [58], but there seems to be a considerable and, to date, frequently underrated contribution of epigenetic alterations. The demonstrated possibility of re-initiating rhythms that had disappeared in the course of aging [42] clearly shows that these reversible changes were of epigenetic rather than degenerative nature. Age-dependent epigenetic modulation of oscillator components [11,43] also indicate a substantial gerontological relevance of these mechanisms.

Contrary to its circadian role, melatonin may exert similar effects in both nocturnal rodents and humans in the field of antioxidative protection, especially when studied at high pharmacological doses. Its antioxidant actions, originally discovered by its radical scavenging properties [115,116], are meanwhile known to comprise numerous additional effects including the avoidance of free-radical formation and the detoxification of reactive intermediates by upregulating antioxidant enzymes [58,117,118] as well as contributions of protective melatonin metabolites [119-121]. Again, numerous epigenetic effects are highly likely to exist in the field of antioxidative actions of melatonin, although the actual knowledge is largely confined to the induction of antioxidant enzymes, the regulation of cyclooxygenase 2 and inducible NO synthase [14,15] as well as some other anti-inflammatory actions [11,52].

The antioxidant effects of melatonin also concern two areas of particular importance to gerontology, namely, mitochondrial function and immunosenescence. Mitochondrial dysfunction and the development of a proinflammatory phenotype represent major sources of free radical generation and cause an age-related, persistent low-grade oxidative stress, in which melatonin has been shown to be beneficial [52,92,93,122,123]. Low chronic oxidative stress was also shown to affect telomere length, especially via formation of 8-oxodG in the telomeric sequence TTAGGG [92,124,125]. The documented prevention of 8-oxodG formation by melatonin [59,60] can be assumed to attenuate this epigenetic effect on telomeres. Whether melatonin also influences the alternative lengthening of telomeres (ALT), which has been shown to be influenced by oxidative stress, remains to be studied. A further aspect to be followed more in detail will be the assumed beneficial role of melatonin in the senescence-associated secretory phenotype (SASP), which fuels low-grade inflammation by secretion of proinflammatory cytokines. In this field, the protective role of melatonin seems to be related to the inhibition of iNOS expression and additional anti-inflammatory effects [52,92]. Again, as SASP is based on the upregulation of previously silent genes, epigenetics can be concluded to play a major role in this cellular alteration. The direct evidence for this assumption is still restricted to a few details concerning NF-κB [126] and the involvement of MLL1 [127].

Under practical aspects of application in the human, potentially beneficial actions of melatonin will have to be studied more in detail [128,129] and this should also consider its role in epigenetic regulation mechanisms. A particular problem is related to the inevitably lower mechanistic orientation of clinical work and the difficulties of bench-to-bed translation. Besides controlled clinical studies, melatonin is also used by countless subjects without prescription, mostly in the second half of life, for purposes of promoting sleep or improving the antioxidative protection system. This is frequently done in the absence of any awareness of doses required for a specific application, of circadian effects and of changes in the immune system. The possibility that undesired effects occur concerning sleep fragmentation, phase shifting into wrong directions [130], and proinflammatory actions, especially in autoimmune diseases [114], is mostly not perceived prior to such a self-treatment. Moreover, the possibility of reduced melatonergic efficacy by melatonin receptor desensitization is usually not considered, something that has, at least, been observed preclinically [131]. The reduction of melatonergic signaling, especially in circadian phases when melatonin levels should be high, can be assumed to also result in epigenetic effects, similar to those observed after LAN [35,37]. However, this would require confirmation under realistic conditions. The participation of epigenetic processes in alterations of the circadian system have been matter of this article, and their role in autoimmune diseases is an emerging topic [132-134]. With regard to effects of melatonin in both the circadian and the immunological fields, epigenetic effects of exogenous melatonin are highly likely. Although this possibility has been addressed in the context of LAN [135], the direct evidence in humans is actually insufficient for any robust judgment.

The future demands formelatonin research in the gerontological field concern a more systematic investigation of epigenetic processes in aging animals and, as far as possible, humans. Such studies should not so much focus on pharmacological challenges by toxins, endotoxemia and excitatory agents that secondarily cause microglia activation, since these treatments usually require counteractions by high supraphysiological doses of melatonin and, therefore, poorly reflect the pathophysiology of an aging organism. Therefore, it seems to be more important to investigate physiological

effects of melatonin just on the basis of aging, but under consideration of possible chronobiological actions of melatonin. The discrimination of oscillator-mediated and direct effects of melatonin may become a major challenge in such experiments, but the outcome will be of high value in either case. It is a remarkable fact that much more is known about epigenetics in the functioning of circadian oscillators and rhythms of epigenetic factors than in the field of melatonin, although the pineal hormone is a chronobiotic. Therefore, this gap has to be filled in the next future. Moreover, studies on gerontological effects of melatonin should not, again and again, refer to factors that have been multiply studied before, such as NF-κB or Nrf2, but systematically consider the various levels of epigenetic actions, i.e., DNA and histone modification including the discrimination of writers, readers and erasers, as well as the modulation of these processes by the countless ncRNAs. In particular, the number of known miRNAs is steadily increasing, indicates roles in numerous cellular processes and may affect, at least, one third of the genome, perhaps more. It seems highly likely that melatonin, representing an orchestrating, pleiotropic regulator, controls numerous miRNAs. Understanding these effects will be a requirement for interpreting changes, when melatonin is declining because of age and age-related diseases.

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Conflicts of Interest

The author reports no conflict of interest.

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