Review

Melatonin and retinoid orphan receptors: Demand for new interpretations after their exclusion as nuclear melatonin receptors

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ABSTRACT

The demonstrated incapability of the retinoic acid receptor-related orphan receptor-α (RORα) to bind melatonin inevitably requires consequences for interpreting numerous reports on actions of this protein as far as it was believed to be a nuclear melatonin receptor. While the synthetic compound CGP 52608 is, in fact, a ligand of RORα, effects obtained with this molecule can no longer be attributed to melatonin. Moreover, the sometimes assumed interplay between melatonin membrane receptors and RORα as nuclear receptors has to be dropped. Conclusions on melatonin's actions via RORα that were based on a lack of demonstrable involvement of membrane receptors appear to have been precocious. Nevertheless, findings on melatonin uptake into the nucleus may still be taken as a hint for nuclear melatonin receptors, but this would require thorough characterization. Although RORα does not bind melatonin, it is interrelated to the latter in regulatory terms by involvement of cellular circadian oscillators. A mode of action seems to be the upregulation of sirtuin-1 by melatonin, deacetylation of poly ADP ribose polymerase-γ coactivator-1α (PGC-1α) by sirtuin-1, and facilitation of RORα binding to its response element by deacetylated PGC-1α, a route that had been shown to exist in circadian oscillators, thereby enhancing their amplitude.

Keywords: Circadian, Melatonin, Nuclear Receptors, Retinoid receptors, Sirtuin-1.

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1. INTRODUCTION

The early reports on nuclear melatonin receptors [1-6] promised to open a new area of research, which was expected to expand our knowledge of melatonin signaling beyond the pioneering and highly fruitful work on G protein-coupled melatonin receptors [7-10]. The assumed properties as nuclear melatonin receptors were especially attributed to the ROR/RZR (retinoic acid receptor-related orphan receptor/retinoid Z receptor) subfamily of retinoid receptors. Till date, the term RZR is still found in melatonin literature, although this protein is meanwhile classified as an ROR subform (RZRβ = RORβ; human gene ID: 6096). However, the validity of the reports on melatonin binding by RORs was vividly debated from the beginning, but the doubts were poorly expressed in the literature and remained largely restricted to reviewer's comments concerning the lack of

confirmation in leading groups. Part of the problem might have been that the key conclusions, as proposed in papers of just a single group [1-6], were not corrected earlier in the literature by other laboratories. In 1997, the claim of melatonin binding to RORs was retracted [11]. However, the idea of RORs as nuclear melatonin receptors was continuously proposed after that date, even by members of the group who had retracted the original article [5,6]. An early review indicated a need for thoroughly investigating the "still debatable questions whether and under which situations melatonin does serve as a physiological modulator of the activities of these receptors" [12]. Nevertheless, the number of publications on RORs as melatonin receptors rose steadily and this role was regarded by many researchers as a matter of fact. The countless reports interpreting melatonin effects by actions via RORs led to the consequence that this aspect had to be incorporated in many review papers, including those of mine, because the large body of published results could not be ignored. The skepticism concerning these proteins as mediators of melatonin's actions could, for a while, only be expressed by stating that the problem was not yet settled and that the role of RORs in melatonin signaling had to be analyzed in-depth with regard to the circadian oscillators [13]. This connection to the circadian system was forwarded because RORα, the subform that has been most frequently claimed to be a nuclear melatonin receptor, acts as a component of circadian oscillators by binding to RORE (retinoid orphan receptor response element) sequences in the control regions of *Bmal*, *Clock* and *Npas2* genes. Therefore, a chronobiotic like melatonin, which modulates circadian oscillators, might have indirect effects on the oscillator machinery that change RORα activity and expression. The role of the oscillator in RORα expression is evident from the fact that its gene is E-box-driven via CLOCK/BMAL1 binding [14,15], as known for other oscillator components, such as *Per* and *Cry* genes, as well as various circadian-controlled genes (CCGs). Concerning the upregulation of *Per*, *Cry* and *Npas2* by RORα, a mode of action that comprises a contribution by melatonin has become more likely with regard to the involvement of sirtuin-1 (SIRT1) in RORα binding to RORE [16,17], as will be discussed below. In other words, effects of melatonin that are associated with RORs may be explained by indirect actions via SIRT1 and the circadian oscillator.

The doubts concerning melatonin binding to RORs were strengthened by the fact that identified natural and synthetic ligands are typically lipids including steroids that are structurally highly different from melatonin [18,19]. More recently, it was directly shown in a screening of ligands that RORα does not bind melatonin, or related compounds such as 5-methoxytryptamine or 5-methoxytryptophol and the metabolite AFMK (N^1-acetyl-N^2-formyl-5methoxykynuramine) [20,21]. Admittedly, several subforms of RORα exist that have not all separately investigated concerning melatonin binding, and RORβ (= RZRβ) has also not been tested. However, RORβ is only expressed in some tissues such as brain, pineal gland, retina and spleen, contrary to the ubiquitously expressed RORα splice variants [22]. However, most of the studies that had investigated melatonin effects were related to RORα or, specifically, its abundant splice variant RORα1. In particular, RORα was intensely investigated in immune cells. In total, the majority of reports on melatonin binding to RORs can be dropped, at least what the most highly expressed RORα subforms is concerned.

The lack of melatonin binding does not at all mean that the respective results reported on cell biological changes under the influence of melatonin have generally lost their value, especially when inhibitions of MT_1/MT_2 signaling failed to suppress the effects. Of course, the reasons for a lack of suppression have to be excluded, such as too low doses of the inhibitor relative to that of melatonin. Of value remain certainly data on changes in the expression of RORs in response to melatonin. However, what is now needed in this case is thinking about alternate interpretations.

2. EFFECTS OF CGP 52608, PERHAPS VALUABLE DATA, BUT WITHOUT DIRECT RELATIONSHIP TO MELATONIN

The report that the synthetic thiazolidine drug CGP 52608 is a ligand of RORα [2] may be taken as valid finding, as long as the opposite has not been demonstrated, although the same publication also claimed melatonin to act via RORα. Again, the conclusion is widely based on a single publication [2]. Several other reports considering CGP 52608 as an RORα ligand, without specific reference to melatonin [23-25], may be compatible with this assumption, especially, as effects of the thiazolidine dione were shown to enhance RORα-dependent transcriptional activity [25], but direct binding assays were not repeated. Confirmation by an independent study may be required. The more recent report that excluded binding of melatonin and its metabolites to RORα [20] focused on natural ligands and, therefore, did not test CGP 52608 or other thiazolidine diones. As long as this point has not been definitely clarified, it may be even conceivable that this compound acts independently of ROR binding, although it may influence RORs indirectly.

The publications that studied effects of CGP 52608 in the context of melatonin were based on the assumption that melatonin really binds with reasonable affinity to RORα and that the synthetic ligand mimics melatonin effects via this nuclear receptor, a precocious conclusion and fundamental misconception. The possibility that CGP 52608 acts in different ways was underrated. One of the early studies on this compound discussed this ligand in the context of thiazolidine diones as antiarthritic drugs [26]. However, this does not perfectly meet the properties of melatonin in this disease. Although melatonin does display anti-inflammatory properties under certain conditions [27,28], it can behave in a proinflammatory way especially in arthritis [28-30].

The belief that CGP 52608 mimics melatonin effects represents an overinterpretation that has led to many questionable conclusions. This was especially problematic when this drug was applied to organisms in which its specificity had not been tested or presence and properties of RORs not sufficiently documented, such as plants. Although plants are known to produce numerous compounds that bind to mammalian RORs, they presumably do not possess these proteins. Although some genes with limited homology to mammalian nuclear receptors have been detected in plants, the retinoid receptors seem to have evolved in early metazoans [31]. Even if RORs were present in plants, eventual actions of CGP 52608 could have only been successfully studied if its binding to such RORs had been confirmed. Therefore, the expectancy that CGP 52608 might mimic melatonin effects in a plant like *Chenopodium rubrum* was a bit audacious. In fact, the findings obtained in this species with this drug may not allow conclusions. When given, in experiments on photoperiodism, late in a night of long duration, flowering was partially suppressed by CGP 52608, and, by the way, also by melatonin [32]. However, partial suppression of a physiological function in plants by long exposure to darkness in combination with a drug of unclear action may simply reflect weakening of the organism. Another case of application of CGP 52608 to organisms devoid of knowledge on RORs concerns dinoflagellates [33]. Induction of asexual cysts by this compound may have been caused by melatonin-independent actions, in particular, as melatonin exerts this effect by conversion to the direct cyst inducer, 5-methoxytryptamine [34,35], a metabolite that is readily formed from melatonin [36] and acts, in the encystment response, at much lower concentrations than melatonin [37].

Sometimes but not generally, melatonin and CGP 52608 exerted similar effects, e.g., in the suppression of cancer cell proliferation [23,24,38-45]. However, some authors cautiously discussed the possibility of different mechanisms of action that may lead to an apparently same result [38]. Occasionally, the antiproliferative effect of melatonin could not be related to MT_1/MT_2 actions, but, as effects with CGP 52608 were observed, involvement of the putative nuclear melatonin receptor was assumed [45]. However, the criterion that opposed the involvement of the membrane

receptors concerned extremely high concentrations of melatonin that were required (above 1 mM) and the lack of inhibition by luzindole or 4-P-PDOT at these high melatonin levels [45]. CGP 52608 required similarly high concentrations. As all agents including the presumed ROR ligand were only effective far beyond receptor saturation levels, it seems that conclusions on receptor types should have been rather avoided. Moreover, the high levels required in that study strongly contrast with other findings on antiproliferative actions by CGP 52608, which were pronounced at 1 – 100 nM [23,24,42] or 1 µM [43]. In some reports, the inhibition that was already detectable at 1 or 10 nM became stronger at 100 nM and even more at 1 µM [23,24]. If the increase towards micromolar concentrations is interpreted in terms of receptor binding, this would imply that saturation would require by orders of magnitude higher concentrations than those for MT_1/MT_2. The alternative would be that CGP 52608 exerts different, indirect effects that remain to be identified. If both melatonin and CGP 52608 are considered as antiproliferative agents in several cancer cell lines, at reasonable concentrations, it would be necessary to identify converging effects that control the cell cycle. From our actual point of view, it should be underlined that melatonin and RORα share the property of acting at circadian oscillators [13,17,46,47], as will be discussed below in more detail. This possibility might resolve some contrasting findings.

However, different results on cell proliferation were also obtained with melatonin and CGP 52608, in some other cases. In uveal melanoma cells, melatonin reduced cell growth via MT_1/MT_2 signaling, but CGP 52608 remained ineffective [48]. In rat epididymal epithelial cells, melatonin stimulated proliferation, whereas CGP 52608 turned out to be inhibitory [49]. In the breast cancer cell line MCF-7, proliferation was inhibited by either melatonin or CGP 52608, but their kinetics differed profoundly [50]. Moreover, melatonin and the nonselective melatonergic agonist AMMTC were shown to reduce the transcription of a RORE-luciferase reporter construct. This contrasts with the finding that suppression of MT_1 signaling by luzindole or *MT_1* antisense RNA downregulates RORα expression in peripheral mononuclear blood cells and Jurkat cells [51].

In one study on CGP 52608, a suppression of 5-lipoxygenase was interpreted to be responsible for its antiproliferative action [52]. This effect was attributed to a RORE sequence in the 5-lipoxygenase control region that specifically binds the splice variant RORα1. One might have expected that binding to RORE would activate the gene, as known, e.g., from the circadian oscillator genes *Bmal1*, *Clock* and *Npas2*. In the DU 245 prostate cancer cells studied, this led instead to a suppression of an increase in 5-lipoxygenase expression induced by medium change. However, this effect was observed at the mRNA level only after 36 - 60 h of exposure to CGP 52608 and at protein level not before 96 h [52]. The long duration required would need an explanation and the exclusion of secondary effects. At first glance, these findings seem to be in line with earlier reports on CGP 52608-induced suppression of 5-lipoxygenase [3,4], which had been repeatedly regarded as a contribution to antioxidative protection [22]. However, another study in the promonocytic cell line U937 stated that melatonin did not downregulate this enzyme, but that 5-lipoxygenase rather promoted ROS formation [53], another case of contrasting effects by melatonin and CGP 52608.

3. A LOOK AT RORS IN MELATONIN'S IMMUNOLOGICAL EFFECTS

Apart from the differences that became apparent in U937 cells, various reports have dealt with presumed nuclear melatonin receptors in the immunological field. Several otherwise important studies came to the conclusion that melatonin acts via RORs, in particular RORα1, in upregulating proinflammatory cytokines, such as IL-2 and IL-6 [54-56]. This conclusion seemed to be supported by a report that 2-[^{125}I]-iodomelatonin was not only bound to purified rat thymus and spleen nuclei, with Kd values in the upper picomolar range, but was also displaced by CGP 52608 [57].

Nevertheless, this RORα ligand remained without effect in another IL-2-related context. Prostaglandin E_2 (PGE_2) is known to inhibit IL-2 production in human lymphocytes via cAMP. This effect was reduced by melatonin via MT_1 signaling, expectably by $G\alpha_i$ protein-mediated inhibition of adenylyl cyclase, but not by CGP 52608, which devoid of this type of signaling [58]. At that time, the conclusion was that leukocytes that respond to melatonin are modulated by both membrane and nuclear receptors. In fact, immune cells usually express MT_1, some also MT_2 receptors, and also consistently RORα [59]. The presence of these receptors remains a matter of fact, but RORα can longer be regarded as a nuclear melatonin receptor, although its expression may be influenced by melatonin.

4. THE MELATONIN – SIRT1 – CIRCADIAN OSCILLATOR CONNECTION

The relationship between melatonin and SIRT1 was first discussed in the context of cancer, since melatonin strongly suppressed SIRT1 in tumor cells, such as human prostate cancer cell lines and murine prostate adenocarcinoma [60] as well as human breast cancer cell lines [61,62]. Interestingly, a relationship between melatonin and RORα became apparent in this context [61], which was reminiscent of earlier findings in MCF-7 breast cancer cells [50]. Melatonin was shown to downregulate RORα, with the consequence of reduced expression of the oscillator gene *Bmal1* [61]. The same should be assumed for the *Clock* gene, which is similarly regulated via a RORE sequence to which RORα binds as a transcription activating ligand. Moreover, the respective cancer cells were shown to not express the other important oscillator gene, *Per2*, under basal conditions [61]. This repression is plausible because *Per2* as well as some other oscillator genes display tumor suppressor properties and, therefore, have to be epigenetically silenced in cancer cells [13,63]. These findings indicated that the circadian oscillators of cancer cells are dysregulated.

In fact, the relationship between melatonin and SIRT1 turned out to be profoundly different in nontumor cells. Especially, in the context of aging, which is associated with reduced secretion of melatonin and reduced expression of SIRT1, exogenous melatonin enhanced SIRT1 levels, as recently summarized [17]. On this basis, it was concluded that aging oscillators differ from the dysregulated and largely dysfunctional oscillator machineries of tumor cells [17,63]. Moreover, recent data indicate that melatonin can act via SIRT1 as a downstream factor, because several melatonin effects were suppressed by sirtuin inhibitors such as sirtinol and EX527 or *Sirt1* siRNA [64-73]. Therefore, SIRT1 was recently classified as a partial mediator of melatonin's actions[74, 75]. This would also be consistent with a considerable functional overlap between melatonin and SIRT1, as evident from literature recently summarized [75]. Although the upregulation of SIRT1 by melatonin and the inhibition of melatonin effects by blocking SIRT1 has meanwhile been documented in a number of cases, the complete sequence from MT_1 or MT_2 activation to SIRT1 effects has not yet been elaborated step by step. Insofar, this gap remains to be filled before the melatonin-SIRT1 relationship may exceed the quality of a hypothesis.

This relationship gains importance with regard to RORα, as this transcription factor as well as SIRT1 is interacting with circadian oscillators. SIRT1 activity depends on the oscillator-driven expression of nicotinamide phosphoribosyltransferase (NAMPT), which results in a cycle of NAD^+ concentration [76-78]. NAD^+ levels determine the activities of the various sirtuins [78,79]. Moreover, SIRT1 enhances circadian amplitudes in both central and peripheral oscillators. This effect has been explained by two mechanisms. The first one is based on interactions of SIRT1 with E-box-binding proteins at the control regions of *Per* and *Cry* genes [77,78,80]. As the *RORα* gene also contains E-boxes [14,15], a direct effect of SIRT1 on the expression of this gene is possible, in addition to indirect actions by amplitude enhancement via other oscillator components. The second mechanism concerns the activation of RORE-controlled genes such as *Bmal1* and *Clock*

[16]. This has been demonstrated in the in the central master clock, SCN, and discussed with regard to its relevance in aging and the possibility of preventing declining amplitudes in senescence by upregulation of SIRT1 [16]. The mechanism consists of deacetylation of PGC-1α (poly ADP ribose polymerase-γ coactivator-1α) by SIRT1. The deacetylated form of PGC-1α binds to RORα, thereby facilitating the activation at the RORE sequences in the control regions of *Bmal1* and *Clock* genes. These findings indicate that SIRT1 can influence the expression, the cyclicity and the transcription-regulating activity of RORα. If SIRT1 is also considered as a partial downstream factor of melatonin [74,75], this would mean that melatonin may influence RORα expression, circadian amplitudes and circadian gene expression, including clock genes and E-box or RORE containing circadian-controlled genes (CCGs) via SIRT1. Secondarily, the influences on the circadian clocks, which also alter NAMPT expression and the NAD^+ cycle, will presumably have additional effects on the constitutively chromatin-associated SIRT6 and, thereby, modulate the daily chromatin remodeling and associated gene expression of those CCGs that are not directly driven by E-box- or RORE-dependent mechanisms.

Although CGP 52608 has been reported to be a ligand of RORα, no data are available for an eventual interaction of this compound with SIRT1 signaling. Another open point of SIRT1 signaling concerns the relationship to melatonin in experiments in which the latter has been applied in highly supraphysiological concentrations that clearly exceed receptor saturation. In these cases, in which high melatonin was especially given to counteract strong toxicological insults, effects cannot be explained by receptor-mediated signal transduction pathways, but may be related to other properties of melatonin such as radical scavenging or mitochondrial protection, in which some actions require elevated concentrations [81,82].

In summary, the known role of RORα in circadian oscillators, in conjunction with the described signaling route of melatonin via SIRT1 and its effects on RORα expression and transcriptional activity, offers possibilities of newly interpreting findings that had related melatonin to changes and actions of RORα. The new interpretations do not require a physical interaction between melatonin and RORα. Moreover, the involvement of circadian oscillators strongly suggests a more systematic consideration of the temporal dynamics of melatonin effects on RORα and its downstream actions. With regard to tumor cells, the dysregulation of their oscillators has to be taken into account. This may also help to explain divergent effects concerning up- or downregulation of RORα and RORα-dependent functions.

5. WHAT CAN BE CONCLUDED FROM CHANGES IN ROR AND SIRT EXPRESSION?

The participation of both RORα and SIRT1 in the cellular circadian oscillator mechanisms as well as the rhythmicity of melatonin secretion indicate that alterations observed in experimental studies should consider the possibility of changes in the extent of observed effects within the circadian cycle, according to the phase of treatment. It is fundamental chronobiological knowledge that the apparently same treatment can lead to strongly divergent results when applied in different circadian phases. This does not only concern phase shifting according to the phase response curve, but also the susceptibility to drugs and endocrine factors [83,84]. As both RORα and SIRT1 are under circadian control via E-boxes, any experimentally induced change should vary within the circadian cycle, at least under conditions of properly operating oscillators. Moreover, exogenous melatonin may influence these parameters differently, especially because of rhythms in MT_1 and MT_2 expression. This rhythmicity has been demonstrated in various tissues, e.g., SCN [85] and other hypothalamic nuclei [86], adrenal gland [87], and liver [88]. In the latter study, it was also shown that pinealectomy blunted these rhythms, whereas RORα expression was increased [88].

Another important point that is frequently overlooked concerns the difference between expression and activity. Although some investigators obviously infer that a change in expression, at least at the protein level, would correspond to similar changes in the activity of an enzyme, this is, from a fundamental point of view, a misconception. In the case of RORα, its activity can influenced by the acetylation/deacetylation balance of its interaction partner PGC-1α, which in turn is controlled by SIRT1 [16]. The difference between expression and activity is even more evident in sirtuins, because their activities are not determined by their protein concentrations, but rather by the NAD^+ level [76-80]. The contrast between these parameters became especially obvious in a study on ovarian cancer on the effect of BRCA1 (breast cancer 1, early onset) on SIRT1 [89]. Inactivation of BRCA1 caused a reduction of SIRT1 expression, but, surprisingly, an increase of NAD^+ concentration and, therefore, enhanced SIRT1 activity. Conversely, BRCA1 overexpression led to increased SIRT1 expression, but to decreases in NAD^+ levels and SIRT1 activity. It remains to be clarified to what extent this negative correlation reflects NAD^+ consumption by active deacetylases and/or by co-regulated poly(ADP-ribose) polymerase 1 (PARP1), which shares properties with SIRT1 in terms of E-box-related regulation of oscillator genes and various other functions [90-92] and which has been shown to deplete NAD^+ levels upon overactivation [93]. Alternately, effects via the dysregulated circadian oscillators of the tumor cells may be taken into consideration. On the other hand, under conditions of aging, when SIRT1 expression has declined and may have become rate-limiting, a – usually moderate – upregulation of SIRT1 may correlate with an increased SIRT1 activity, especially if effects are shown to be suppressed by sirtuin inhibitors. In any case, studies on SIRT1 expression should be accompanied by activity measurements or, at least, by controlling the effects using SIRT inhibitors or *Sirt1* siRNA. Investigators should be aware of the role of SIRT1 activity for the biological actions of RORα.

6. HOW DOES MELATONIN UPREGULATE SIRT1?

Assuming that several – or many? – effects of melatonin are transmitted by SIRT1 leads to the important question on the mechanism of SIRT1 upregulation. To date, this can be only insufficiently answered. A possibility for approaching this problem may be to seek for control elements in the *Sirt1* promoter and to analyze whether melatonin might be able to regulate the respective binding proteins. The E-box in the *Sirt1* promoter would only be of relevance if melatonin acts on circadian oscillators independently of SIRT1, but not if SIRT1 mediates the melatonin effect to the oscillator, e.g., via PGC-1α deacetylation and facilitation of RORα binding to ROREs in oscillator genes. However, no such mechanism is known that explains a direct amplitude effect by melatonin. There are only a few publications on other response elements in the *Sirt1* promoter, which may be candidates for melatonin's actions. Two mechanisms have been related to the antioxidant properties of SIRT1, which are of interest with regard to melatonin's spectrum of actions. One of them starts with the phosphorylation of ERK5 (extracellular-signal-related kinase-5; also known as big ERK, BERK). The activated ERK5 phosphorylates the myocyte enhancer factor-2 (MEF2), which binds as pMEF2 to the *Sirt1* promoter and stimulates transcription [94]. In another study conducted in the context of bone metabolism and osteoblastogenesis, melatonin was shown to activate via MT_2 not only ERK1/2, as known since long, but additionally ERK5 [95]. Therefore, a mechanistic connection between melatonin and SIRT1 upregulation may exist. However, for reasons of caution, one should be aware of the possible contextual limitations of these studies. Especially, it would be important to look for similar upregulations of ERK5 and MEF2 in other cells, to see whether this is a more general route.

The second mechanism that has related SIRT1 upregulation to oxidative stress concerns two nCaRE sequences (negative calcium responsive elements) in the *Sirt1* promoter. These elements form a cross-like double hairpin structure, which serves as a binding site for APE1 (apurinic/apyrimidinic endonuclease 1). Upon oxidative damage to the DNA in the hairloop (presence of 8-oxoG), base excision repair (BER) enzymes including APE1 are recruited to the damaged site and form a loop that further recruits RNA polymerase II to the promoter at a site close to the transcriptional start, a position that allows gene expression [96]. For the moment, there is not good reason to assume that this mechanism is stimulated by melatonin, but it rather seems to be an autonomous response to DNA damage.

It will be of importance to discriminate in the future between these two mechanisms and to identify an eventual participation of melatonin in SIRT1 upregulation. Of course, these examples only reflect the actual state of knowledge and other mechanisms may be discovered. This may even include processes mediated by noncoding RNAs, such as miRNAs, lncRNAs, eRNAs (enhancer RNAs) or asRNAs (antisense RNAs) [97]. In the case of SIRT1 expression, an *asSirt1* was shown to enhance posttranscriptional SIRT1 expression by eliminating the *Sirt1*-mRNA targeting *miR-34a* [98]. In any case, moderate enhancement of SIRT1 levels which remain much below those in cancer cells will be of interest for the understanding of melatonin's actions, for circadian regulation and the role of RORα.

7. MELATONIN AND THE NUCLEUS

There can be no doubt that melatonin is able to enter the nucleus. The capability of melatonin to protect DNA in the nucleus from oxidative damage [99,100] can be hardly explained on an exclusive extranuclear elimination of free radicals, which are mostly not far-reaching enough for a cytosolic-nuclear transgression without reacting with other compounds. In earlier literature, melatonin was repeatedly reported to be present and to accumulate in the nucleus [101-104]. Two studies using 2-[^{125}I]-iodomelatonin communicated the presence of nuclear high-affinity binding sites [57,104]. However, when melatonin was infused during the day to reach nocturnal blood levels, no particular accumulation was detected in the nucleus, contrary to increased levels in mitochondria [105,106]. Leaving apart the eventual technical differences and methodological problems concerning the exact determination of nuclear melatonin concentrations, the impression remains that melatonin may attach to nuclear binding sites. With regard to the absence of melatonin binding to RORα [20,21], the nature of such sites would require clarification. The reports on high-affinity binding may raise the question of whether membrane-bound receptors such as MT_1 might be located at the nuclear envelope. This may not appear as unlikely as previously thought, when signaling of G protein-coupled receptors was believed to be only associated with the plasma membrane. The recent demonstration of MT_1 in the outer mitochondrial membrane [107] shows that this receptor is present in intracellular membranes and, at least, in the case of mitochondria, functional. Thus, the presence of MT_1 in both membranes of the nuclear envelope cannot be excluded for the moment. These membranes are interconnected at the nuclear pores and additional connections to the ER membranes exist. It is still an open question whether other binding sites of sufficient affinity and abundance exist in the nucleoplasm or in association with chromatin. The number of melatonin-binding proteins may be larger than usually believed. The presence of binding sites that are different from G protein-coupled receptors and other frequently discussed proteins such as calmodulin and quinone reductase 2 (= QR2 = NRH:quinone oxidoreductase 2 = NQO2) has received some support. This was assumed in a study on melatonin effects on the NMDA receptor, in which its redox site was discussed with regard to possible melatonin binding [108]. In another investigation, melatonin binding to calreticulin was reported [109]. In the same study, two

other, functionally not yet characterized nuclear binding proteins were mentioned, one of them with homology to calreticulin. These examples have not been mentioned to advocate anything concerning specific proteins that have been discussed as putative melatonin binding sites. In all these cases, the experimental basis is not sufficiently broad for substantial conclusions. However, these examples may illustrate that the question of nuclear binding sites is not yet settled, and a new race for their identification may commence. Moreover, a binding site, if existing at all, may not possess the quality of a receptor, which would require the demonstration of a signaling pathway. Alternately, a binding site may serve sequestration of melatonin.

8. CONCLUSION

The elimination of RORs, in particular RORα1, from previous concepts of melatonin's cellular actions leads to the necessity of explaining a number of reported effects that had been ascribed to these proteins:

(1) A frequently made observation concerns effects of RORα activation, e.g., by CGP 52608, or overexpression. The interpretation that this mimics melatonin effects has to be dropped, even in cases in which melatonin exerts same or similar effects. To understand such actions that are shared with melatonin, they should be tried to explain on the basis of mechanisms that are influenced by both melatonin and RORα. A machinery that is modulated by either regulator would be the cellular circadian oscillator. If similar effects are obtained, a route by which melatonin might act on RORα could consist in the upregulation of SIRT1 expression, deacetylation of PGC-1α and facilitation of RORα binding to RORE, as outlined above.

However, several cases exist in which the actions of melatonin and CGP 52608 turned out to be incongruent, as indicated in this article. The reasons remain to be identified. They might include additional actions of the one or the other compound. Especially the routes of primary melatonergic signaling via cAMP decrease or ERK1/2 upregulation, with numerous secondary effects on metabolic regulators, may lead to changes that are beyond the spectrum of ROR actions. If actions via a circadian oscillator appear to be likely, the effects may be strongly phase-specific. Differences in the duration of action, e.g., because of dosages applied, might already lead to divergent results, if they cover different phases of the oscillator. The use of extremely high concentrations of agents may also result in poorly interpretable data.

(2) In other cases, modulation of RORα by melatonin was assumed, or an interplay of membrane receptors and RORα. Again, the most promising approach for newly interpreting such data may be found in the consideration of actions via circadian oscillators.

(3) Some conclusions on the involvement of RORs in melatonin's actions were only based on the absence of demonstrable effects via the membrane receptors, e.g., because of lack of inhibition by luzindole or other melatonergic antagonists. First, the conclusion on an involvement of RORs represents a relatively weak argument, since the identification as a receptor had been missing. Moreover, studies with receptor antagonists have to consider their distribution kinetics and affinities relative to those of melatonin or other agonists, to avoid misinterpretations because of poor antagonist levels.

(4) Loading of melatonin to the nucleus or its constituents should be experimentally revisited. Binding to the nuclear envelope has to be distinguished from association with intranuclear proteins. If the latter can be confirmed, the hard work of identifying their nature will be inevitable to arrive at convincing interpretations.

As a bottom line, investigators are encouraged to re-analyze the findings that were originally ascribed to RORs in their erroneously assumed role as nuclear melatonin receptors. Such re-investigations should consider the newly discovered pathways of secondary signaling by

melatonin, such as actions via sirtuins, under consideration of the necessary distinction between expression and NAD^+-dependent activity.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

REFERENCES

1. Becker-André M, Wiesenberg I, Schaeren-Wiemers N, André E, Missbach M, Saurat JH, Carlberg C (1994) Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. *J. Biol Chem* **269**: 28531-28534.
2. Wiesenberg I, Missbach M, Kahlen JP, Schräder M, Carlberg C (1995)Transcriptional activation of the nuclear receptor RZRα by the pineal gland hormone melatonin and identification of CGP 52608 as a synthetic ligand. *Nucleic. Acids Res.* **23**: 327-333.
3. Steinhilber D, Brungs M, Werz O, Wiesenberg I, Danielsson C, Kahlen JP, Nayeri S, Schräder M, Carlberg, C (1995) The nuclear receptor for melatonin represses 5-lipoxygenase gene expression in human B lymphocytes. *J. Biol. Chem.* **270**: 7037-7040.
4. Carlberg C, Wiesenberg I (1995) The orphan receptor family RZR/ROR, melatonin and 5-lipoxygenase: an unexpected relationship. *J. Pineal Res.* **18**: 171-178.
5. Wiesenberg I, Missbach M, Carlberg C (1998) The potential role of the transcription factor RZR/ROR as a mediator of nuclear melatonin signaling. *Restor. Neurol. Neurosci.* **12**: 143-150.
6. Carlberg C (2000) Gene regulation by melatonin. *Ann. NY. Acad. Sci.* **917:** 387-396.
7. Reppert SM, Weaver DR, Ebisawa T (1994) Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron* **13**: 1177-1185.
8. Reppert SM, Godson C, Mahle CD, Weaver DR, Slaugenhaupt SA, Gusella JF (1995) Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. *Proc. Natl. Acad. Sci. USA* **92**: 8734-8738.
9. Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VK, Reppert SM (1997) Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron* **19**: 91-102.
10. Jin X, von Gall C, Pieschl RL, Gribkoff VK, Stehle JH, Reppert SM, Weaver DR (2003) Targeted disruption of the mouse Mel1b melatonin receptor. *Mol. Cell Bio.l* **23**: 1054-1060.
11. Becker-André M, Schaeren-Wiemers N, André E, Wiesenberg I, Missbach M, Saurat JH, Carlberg C (1997) Erratum (Correction and Addition) to: Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. *J. Biol. Chem.* **272**: 16707.
12. Smirnov AN (2001) Nuclear melatonin receptors. *Biochemistry* (*Mosc*) **66**: 19-26.
13. Hardeland R, Madrid JA, Tan D-X, Reiter RJ (2012) Melatonin, the circadian multioscillator system and health: the need for detailed analyses of peripheral melatonin signaling. *J. Pineal Res.* **52**: 139-166.

14. Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, FitzGerald GA, Kay SA, Hogenesch JB (2004) A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron* 43: 527-537.
15. Zhang EE, Kay SA (2010) Clocks not winding down: unravelling circadian networks. *Nat. Rev. Mol. Cell Biol.* **11**: 764-776.
16. Chang H-C, Guarente L (2013) SIRT1 mediates central circadian control in the SCN by a mechanism that decays with aging. *Cell* **153**: 1448-1460.
17. Hardeland R (2017) Melatonin and the pathologies of weakened or dysregulated circadian oscillators. *J. Pineal Res.* **62**: e12377; DOI: 10.1111/jpi.12377.
18. Bitsch F, Aichholz R, Kallen J, Geisse S, Fournier B, Schlaeppi JM (2003) Identification of natural ligands of retinoic acid receptor-related orphan receptor alpha ligand-binding domain expressed in Sf9 cells – a mass spectrometry approach. *Anal. Biochem.* **323**: 139-149.
19. Solt LA, Burris TP (2012) Action of RORs and their ligands in (patho)physiology. *Trends Endocrinol. Metab.* **23**: 619-627.
20. Slominski AT, Kim TK, Takeda Y, Janjetovic Z, Brozyna AA, Skobowiat C, Wang J, Postlethwaite A, Li W, Tuckey RC, Jetten AM (2014) RORα and RORγ are expressed in human skin and serve as receptors for endogenously produced noncalcemic 20-hydroxy- and 20,23-dihydroxyvitamin D. *FASEB J.* **28**: 2775-2789.
21. Slominski AT, Zmijewski MA, Jetten AM (2016) RORα is not a receptor for melatonin (response to DOI 10.1002/bies.201600018). *Bioessays* **38**: 1193-1194.
22. Hardeland R, Cardinali DP, Srinivasan V, Spence DW, Brown GM, Pandi-Perumal SR (2011) Melatonin – A pleiotropic, orchestrating regulator molecule. *Prog. Neurobiol.* **93**: 350-384.
23. Moretti RM, Marelli MM, Motta M, Polizzi D, Monestiroli S, Pratesi G, Limonta P (2001) Activation of the orphan nuclear receptor RORalpha induces growth arrest in androgen-independent DU 145 prostate cancer cells. *Prostate* **46**: 327-335.
24. Moretti RM, Montagnani Marelli M, Motta M, Limonta P (2001) Oncostatic activity of a thiazolidinedione derivative on human androgen-dependent prostate cancer cells. *Int. J. Cancer* **92**: 733-737.
25. Park Y, Hong S, Lee M, Jung H, Cho WJ, Kim EJ, Son HY, Lee MO, Park HG (2012) N-methylthioureas as new agonists of retinoic acid receptor-related orphan receptor. *Arch. Pharm. Res.* **35**: 1393-1401.
26. Missbach M, Jagher B, Sigg I, Nayeri S, Carlberg C, Wiesenberg I (1996) Thiazolidine diones, specific ligands of the nuclear receptor retinoid Z receptor/retinoid acid receptor-related orphan receptor alpha with potent antiarthritic activity. *J. Biol. Chem.* **271**: 13515-13522.
27. Carrillo-Vico A, Lardone PJ, Álvarez-Sánchez N, Rodríguez-Rodríguez A, Guerrero JM (2013) Melatonin: buffering the immune system. *Int. J. Mol. Sci.*. **14**: 8638-8683.
28. Hardeland R (2016) Opposite effects of melatonin in different systems and under different conditions. *Curr. Top. Biochem. Res.* **17**: 57-69.
29. Maestroni GJM, Cardinali DP, Esquifino AI, Pandi-Perumal SR (2005) Does melatonin play a disease-promoting role in rheumatoid arthritis? *J. Neuroimmunol.* **158**: 106-111.
30. Maestroni GJM, Otsa K, Cutolo M (2008) Melatonin treatment does not improve rheumatoid arthritis. *Br. J. Clin. Pharmacol.*. **65**: 797-798.
31. Owen GI, Zelent A (2000) Origins and evolutionary diversification of the nuclear receptor superfamily. *Cell. Mol. Life Sci...* **57**: 809-827.

32. Kolář J, Macháčková I, Johnson CH (1999) Effects of a melatonin analogue CGP 52608 in the photoperiodic flower induction in a short-day plant, *Chenopodium rubrum*. *Biol. Rhythm Res.*. **30**: 243.
33. Tsim ST, Wong JT, Wong YH (1996) CGP 52608-induced cyst formation in dinoflagellates: possible involvement of a nuclear receptor for melatonin. *J. Pineal Res.* **21**: 101-107.
34. Hardeland R (1999) Melatonin and 5-methoxytryptamine in non-metazoans. *Reprod. Nutr. Dev*. **39**: 399-408.
35. Hardeland R, Pandi-Perumal SR, Poeggeler B (2007) Melatonin in plants – Focus on a vertebrate night hormone with cytoprotective properties. *Funct. Plant Sci. Biotechnol.*. **1**: 32-45.
36. Fuhrberg B, Hardeland R, Poeggeler B, Behrmann G (1997) Dramatic rises of melatonin and 5-methoxytryptamine in *Gonyaulax* exposed to decreased temperature. *Biol. Rhythm Res*. **28**: 144-150.
37. Balzer I, Hardeland R (1991) Photoperiodism and effects of indoleamines in a unicellular alga, *Gonyaulax polyedra*. *Science* **253**: 795-797.
38. Karasek M, Pawlikowski M (1999) Antiproliferative effects of melatonin and CGP 52608. *Biol. Signals Recept.* **8**: 75-78.
39. Petranka J, Baldwin W, Biermann J, Jayadev S, Barrett JC, Murphy E (1999)The oncostatic action of melatonin in an ovarian carcinoma cell line. *J. Pineal Res*. **26**: 129-136.
40. Pawlikowski M, Kunert-Radek J, Winczyk K, Melen-Mucha G, Gruszka A, Karasek M (1999) The antiproliferative effects of melatonin on experimental pituitary and colonic tumors. Possible involvement of the putative nuclear binding site? *Adv. Exp. Med. Biol*. **460**: 369-372.
41. Moretti RM, Montagnani Marelli M, Motta M, Limonta P (2002) Role of the orphan nuclear receptor RORα in the control of the metastatic behavior of androgen-independent prostate cancer cells. *Oncol. Rep.* **9**: 1139-1143.
42. Karasek M, Gruszka A, Lawnicka H, Kunert-Radek J, Pawlikowski M (2003) Melatonin inhibits growth of diethylstilbestrol-induced prolactin-secreting pituitary tumor in vitro: possible involvement of nuclear RZR/ROR receptors. *J. Pineal Res*. **34**: 294-296.
43. Herrera F, Mayo JC, Martín V, Sainz RM, Antolin I, Rodriguez C (2004) Cytotoxicity and oncostatic activity of the thiazolidinedione derivative CGP 52608 on central nervous system cancer cells. *Cancer Lett*. **211**: 47-55.
44. Winczyk K, Lawnicka H, Pawlikowski M, Kunert-Radek J, Karasek M (2006) Growth-inhibitory action of melatonin and thiazolidinedione derivative CGP 52608 on murine 16/C breast cancer cells. *Neuro. Endocrinol. Lett*. **27**: 351-354.
45. García-Navarro A, González-Puga C, Escames G, López LC, López A, López-Cantarero M, Camacho E, Espinosa A, Gallo MA, Acuña-Castroviejo D (2007) Cellular mechanisms involved in the melatonin inhibition of HT-29 human colon cancer cell proliferation in culture. *J. Pineal Res*. **43**: 195-205.
46. Hardeland R (2018) Brain inflammaging: roles of melatonin, circadian clocks and sirtuins. *J. Clin. Cell Immunol*. **9**: 543; DOI: 10.4172/2155-9899.1000543.
47. Hardeland R (2018) Recent findings in melatonin research and their relevance to the CNS. *Cent Nerv Syst Agents Med. Chem*. **18**: 102-114.
48. Roberts JE, Wiechmann AF, Hu DN (2000) Melatonin receptors in human uveal melanocytes and melanoma cells. *J. Pineal Res*. **28**: 165-171.
49. Li L, Wong JT, Pang SF, Shiu SY (1999) Melatonin-induced stimulation of rat corpus epididymal epithelial cell proliferation. *Life Sci*. **65**: 1067-1076.

50. Ram PT, Dai J, Yuan L, Dong C, Kiefer TL, Lai L, Hill SM (2002) Involvement of the mt1 melatonin receptor in human breast cancer. *Cancer Lett*. **179**: 141-150.
51. Lardone PJ, Carrillo-Vico A, Molinero P, Rubio A, Guerrero JM (2009) A novel interplay between membrane and nuclear melatonin receptors in human lymphocytes: significance in IL-2 production. *Cell Mol. Life Sci*. **66**: 516-525.
52. Moretti RM, Montagnani Marelli M, Sala A, Motta M, Limonta P (2004) Activation of the orphan nuclear receptor RORα counteracts the proliferative effect of fatty acids on prostate cancer cells: crucial role of 5-lipoxygenase. *Int. J. Cancer* **112**: 87-93.
53. Radogna F, Sestili P, Martinelli C, Paolillo M, Paternoster L, Albertini MC, Accorsi A, Gualandi G, Ghibelli L (2009) Lipoxygenase-mediated pro-radical effect of melatonin via stimulation of arachidonic acid metabolism. *Toxicol. Appl. Pharmacol*. **238**: 170-177.
54. Garcia-Mauriño S, Gonzalez-Haba MG, Calvo JR, Rafii-El-Idrissi M, Sanchez-Margalet V, Goberna R, Guerrero JM (1997) Melatonin enhances IL-2, IL-6, and IFN-gamma production by human circulating $CD4^+$ cells: a possible nuclear receptor-mediated mechanism involving T helper type 1 lymphocytes and monocytes. *J. Immunol*. **159**: 574-581.
55. Garcia-Mauriño S, Gonzalez-Haba MG, Calvo JR, Goberna R, Guerrero JM (1998) Involvement of nuclear binding sites for melatonin in the regulation of IL-2 and IL-6 production by human blood mononuclear cells. *J. Neuroimmunol*. **92**: 76-84.
56. García-Mauriño S, Pozo D, Calvo JR, Guerrero JM (2000) Correlation between nuclear melatonin receptor expression and enhanced cytokine production in human lymphocytic and monocytic cell lines. *J. Pineal Res*. **29**: 129-137.
57. Rafii-El-Idrissi M, Calvo JR, Harmouch A, García-Mauriño S, Guerrero JM (1998) Specific binding of melatonin by purified cell nuclei from spleen and thymus of the rat. *J. Neuroimmunol*. **86**: 190-197.
58. Carrillo-Vico A, García-Mauriño S, Calvo JR, Guerrero JM (2003) Melatonin counteracts the inhibitory effect of PGE2 on IL-2 production in human lymphocytes via its mt1 membrane receptor. *FASEB J*. **17**: 755-757.
59. Carrillo-Vico A, García-Pergañeda A, Naji L, Calvo JR, Romero MP, Guerrero JM (2003) Expression of membrane and nuclear melatonin receptor mRNA and protein in the mouse immune system. *Cell Mol. Life Sci*. **60**: 2272-2278.
60. Jung-Hynes B, Schmit TL, Reagan-Shaw SR, Siddiqui IA, Mukhtar H, Ahmad N (2011) Melatonin, a novel Sirt1 inhibitor, imparts proliferative effects against prostate cancer cells in vitro culture and in vivo in TRAMP model. *J. Pineal Res*. **50**: 140-149.
61. Hill SM, Frasch T, Xiang S, Duplessis T, Mao L (2009) Molecular mechanisms of melatonin anticancer effects. *Integr. Cancer Ther*. **8**: 337-346.
62. Proietti S, Cucina A, Dobrowolny G, D'Anselmi F, Dinicola S, Masiello MG, Pasqualato A, Palombo A, Morini V, Reiter RJ, Bizzarri M (2014) Melatonin down-regulates MDM2 gene expression and enhances p53 acetylation in MCF-7 cells. *J. Pineal Res*. **57**: 120-129.
63. Hardeland R (2014) Melatonin, noncoding RNAs, messenger RNA stability and epigenetics — evidence, hints, gaps and perspectives. *Int. J. Mol. Sci*. **15**: 18221-18252.
64. Cristòfol R, Porquet D, Corpas R, Coto-Montes A, Serret J, Camins A, Pallàs M, Sanfeliu C (2012) Neurons from senescence-accelerated SAMP8 mice are protected against frailty by the sirtuin 1 promoting agents melatonin and resveratrol. *J. Pineal Res*. **52**: 271-281.
65. Yu L, Sun Y, Cheng L, Jin Z, Yang Y, Zhai M, Pei H, Wang X, Zhang H, Meng Q, Zhang Y, Yu S, Duan W (2014) Melatonin receptor-mediated protection against myocardial ischemia/reperfusion injury: role of SIRT1. *J. Pineal Res*. **57**: 228-238.
66. Yu L, Liang H, Dong X, Zhao G, Jin Z, Zhai M, Yang Y, Chen W, Liu J, Yi W, Yang J, Yi D, Duan W, Yu S (2015) Reduced silent information regulator 1 signaling exacerbates

myocardial ischemia-reperfusion injury in type 2 diabetic rats and the protective effect of melatonin. *J. Pineal Res*. **59**: 376-390.
67. Yang Y, Jiang S, Dong Y, Fan C, Zhao L, Yang X, Li J, Di S, Yue L, Liang G, Reiter RJ, Qu Y (2015) Melatonin prevents cell death and mitochondrial dysfunction via a SIRT1-dependent mechanism during ischemic-stroke in mice. *J. Pineal Res.* **58**: 61-70.
68. Zhao L, An R, Yang Y, Yang X, Liu H, Yue L, Li X, Lin Y, Reiter RJ, Qu Y (2015) Melatonin alleviates brain injury in mice subjected to cecal ligation and puncture via attenuating inflammation, apoptosis, and oxidative stress: the role of SIRT1 signaling. *J. Pineal Res.* **59**: 230-239.
69. Han D, Huang W, Li X, Gao L, Su T, Li X, Ma S, Liu T, Li C, Chen J, Gao E, Cao F (2016) Melatonin facilitates adipose-derived mesenchymal stem cells to repair the murine infarcted heart via the SIRT1 signaling pathway. *J. Pineal Res*. **60**: 178–192.
70. Bai XZ, He T, Gao JX, Liu Y, Liu JQ, Han SC, Li Y, Shi JH, Han JT, Tao K, Xie ST, Wang HT, Hu DH (2016) Melatonin prevents acute kidney injury in severely burned rats via the activation of SIRT1. *Sci. Rep.* **6**: 32199; DOI: 10.1038/srep32199.
71. Yang W, Kang X, Qin N, Li F, Jin X, Ma Z, Qian Z, Wu S (2017) Melatonin protects chondrocytes from impairment induced by glucocorticoids via NAD^+-dependent SIRT1. *Steroids* **126**: 24-29.
72. Shah SA, Khan M, Jo MH, Jo MG, Amin FU, Kim MO (2017) Melatonin stimulates the SIRT1/Nrf2 signaling pathway counteracting lipopolysaccharide (LPS)-induced oxidative stress to rescue postnatal rat brain. *CNS. Neurosci. Ther.* **23**: 33-44.
73. Peng Z, Zhang W, Qiao J, He B (2018) Melatonin attenuates airway inflammation via SIRT1 dependent inhibition of NLRP3 inflammasome and IL-1β in rats with COPD. *Int. Immunopharmacol.* **62**: 23-28.
74. Hardeland R (2018) Extended signaling by melatonin. *Cell Cell. Life Sci. J.* **3**: 000123.
75. Hardeland R (2018) Melatonin and inflammation—Story of a double-edged blade. *J. Pineal Res*. **65:** e12525. DOI: 10.1111/jpi.12525.
76. Nakahata Y, Sahar S, Astarita G, Kaluzova M, Sassone-Corsi P (2009) Circadian control of the NAD^+ salvage pathway by CLOCK-SIRT1. *Science* **324**: 654-657.
77. Bellet MM, Orozco-Solis R, Sahar S, Eckel-Mahan K, Sassone-Corsi P (2011) The time of metabolism: NAD^+, SIRT1, and the circadian clock. *Cold Spring Harb Symp Quant Biol.* **76**: 31-38.
78. Sahar S, Sassone-Corsi P (2013) The epigenetic language of circadian clocks. *Handb. Exp. Pharmacol.* **217**: 29-44.
79. Masri S (2015) Sirtuin-dependent clock control: New advances in metabolism, aging and cancer. *Curr. Opin. Clin. Nutr. Metab. Care* **18**: 521-527.
80. Grimaldi B, Nakahata Y, Kaluzova M, Masubuchi S, Sassone-Corsi P (2009) Chromatin remodeling, metabolism and circadian clocks: the interplay of CLOCK and SIRT1. *Int. J. Biochem. Cell Biol.* **41**: 81-86.
81. Andrabi SA, Sayeed I, Siemen D, Wolf G, Horn TF (2004) Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism responsible for anti-apoptotic effects of melatonin. *FASEB J.* **18**: 869-871.
82. Hardeland R (2017) Melatonin and the electron transport chain. *Cell Mol. Life Sci.* **74**: 3883-3896.
83. Lemmer B, Labrecque G (1987) Chronopharmacology and chronotherapeutics: definitions and concepts. *Chronobiol. Int.* **4**: 319-329.
84. Reinberg AE (1992) Concepts in chronopharmacology. *Annu. Rev. Pharmacol. Toxicol.* **32**: 51-66.

85. Masana MI, Benloucif S, Dubocovich ML (2000) Circadian rhythm of mt1 melatonin receptor expression in the suprachiasmatic nucleus of the C3H/HeN mouse. *J. Pineal Res.* **28**: 185-192.
86. Pinato L, Ramos D, Hataka A, Rossignoli PS, Granado MD Junior, Mazzetto MC, Campos LMG (2017) Day/night expression of MT_1 and MT_2 receptors in hypothalamic nuclei of the primate Sapajus apella. *J. Chem. Neuroanat.* **81**: 10-17.
87. Richter HG, Torres-Farfan C, Garcia-Sesnich J, Abarzua-Catalan L, Henriquez MG, Alvarez-Felmer M, Gaete F, Rehren GE, Seron-Ferre M (2008) Rhythmic expression of functional MT1 melatonin receptors in the rat adrenal gland. *Endocrinology* **149**: 995-1003.
88. Venegas C, García JA, Doerrier C, Volt H, Escames G, López LC, Reiter RJ, Acuña-Castroviejo D (2013) Analysis of the daily changes of melatonin receptors in the rat liver. *J. Pineal Res.* **54**: 313-321.
89. Li D, Bi FF, Chen NN, Cao JM, Sun WP, Zhou YM, Li CY, Yang Q (2014) A novel crosstalk between BRCA1 and sirtuin 1 in ovarian cancer. *Sci. Rep.* **4**: 6666. DOI: 10.1038/srep06666.
90. Aguilar-Arnal L, Sassone-Corsi P (2013) The circadian epigenome: how metabolism talks to chromatin remodeling. *Curr. Opin. Cell Biol.* **25**: 170-176.
91. Luna A, Aladjem MI, Kohn KW (2013) SIRT1/PARP1 crosstalk: connecting DNA damage and metabolism. *Genome Integr*. **4**: 6; DOI: 10.1186/2041-9414-4-6.
92. Mendelsohn AR, Larrick JW (2017) The NAD+/PARP1/SIRT1 axis in aging. *Rejuvenation Res*. **20**: 244-247.
93. Wang S, Yang X, Lin Y, Qiu X, Li H, Zhao X, Cao L, Liu X, Pang Y, Wang X, Chi Z (2013) Cellular NAD depletion and decline of SIRT1 activity play critical roles in PARP-1-mediated acute epileptic neuronal death in vitro. *Brain Res.* **1535**: 14-23.
94. Lopez-Royuela N, Rathore MG, Allende-Vega N, Annicotte JS, Fajas L, Ramachandran B, Gulick T, Villalba M (2014) Extracellular-signal-regulated kinase 5 modulates the antioxidant response by transcriptionally controlling Sirtuin 1 expression in leukemic cells. *Int. J. Biochem. Cell Biol.* **53**: 253-261.
95. Maria S, Samsonraj RM, Munmun F, Glas J, Silvestros M, Kotlarczyk MP, Rylands R, Dudakovic A, van Wijnen AJ, Enderby LT, Lassila H, Dodda B, Davis VL, Balk J, Burow M, Bunnell BA, Witt-Enderby PA (2018) Biological effects of melatonin on osteoblast/osteoclast cocultures, bone, and quality of life: Implications of a role for MT2 melatonin receptors, MEK1/2, and MEK5 in melatonin-mediated osteoblastogenesis. *J. Pineal Res.* **64**: e12465.; DOI: 10.1111/jpi.12465.
96. Antoniali G1, Lirussi L, D'Ambrosio C, Dal Piaz F, Vascotto C, Casarano E, Marasco D, Scaloni A, Fogolari F, Tell G (2014) SIRT1 gene expression upon genotoxic damage is regulated by APE1 through nCaRE-promoter elements. *Mol. Biol. Cell* **25**: 532-547.
97. Hardeland R (2018) Hardeland, R. (2018): On the relationships between lncRNAs and other orchestrating regulators: Role of the circadian system. *Epigenomes* 2: 9. DOI: 10.3390/epigenomes2020009.
98. Wang GQ, Wang Y, Xiong Y, Chen XC, Ma ML, Cai R, Gao Y, Sun YM, Yang GS, Pang WJ (2016) Sirt1 AS lncRNA interacts with its mRNA to inhibit muscle formation by attenuating function of miR-34a. *Sci. Rep.* **6**: 21865. DOI: 10.1038/srep21865.
99. Tan D, Reiter RJ, Chen LD, Poeggeler B, Manchester LC, Barlow-Walden LR (1994) Both physiological and pharmacological levels of melatonin reduce DNA adduct formation induced by the carcinogen safrole. *Carcinogenesis* **15**: 215-218.
100. Karbownik M, Tan D-X, Reiter RJ (2000) Melatonin reduces the oxidation of nuclear DNA and membrane lipids induced by the carcinogen δ-aminolevulinic acid. *Int. J. Cancer* **88**: 7-11.

101. Menendez-Pelaez A, Reiter RJ (1993) Distribution of melatonin in mammalian tissues: the relative importance of nuclear versus cytosolic localization. *J. Pineal Res.* **15**: 59-69.
102. Acuña-Castroviejo D, Pablos MI, Menendez-Pelaez A, Reiter RJ (1993) Melatonin receptors in purified cell nuclei of liver. *Res. Commun. Chem. Pathol. Pharmacol.* **82**: 253-256.
103. Menendez-Pelaez A, Poeggeler B, Reiter RJ, Barlow-Walden L, Pablos MI, Tan D-X (1993) Nuclear localization of melatonin in different mammalian tissues: immunocytochemical and radioimmunoassay evidence. *J. Cell Biochem.* **53**: 373-382.
104. Acuña-Castroviejo D, Reiter RJ, Menéndez-Peláez A, Pablos MI, Burgos A (1994) Characterization of high-affinity melatonin binding sites in purified cell nuclei of rat liver. *J. Pineal Res.* **16**: 100-112.
105. Messner M, Hardeland R, Rodenbeck A, Huether G (1998) Tissue retention and subcellular distribution of continuously infused melatonin in rats under near physiological conditions. *J. Pineal Res.* **25**: 251-259.
106. Messner M, Hardeland R, Rodenbeck A, Huether G. (1999) Effect of continuous melatonin infusions on steady-state plasma melatonin levels, metabolic fate and tissue retention in rats under near physiological conditions. *Adv. Exp. Med. Biol.* **467**: 303-313.
107. Suofu Y, Li W, Jean-Alphonse FG, Jia J, Khattar NK, Li J, Baranov SV, Leronni D, Mihalik AC, He Y, Cecon E, Wehbi VL, Kim J, Heath BE, Baranova OV, Wang X, Gable MJ, Kretz ES, Di Benedetto G, Lezon TR, Ferrando LM, Larkin TM, Sullivan M, Yablonska S, Wang J, Minnigh MB, Guillaumet G, Suzenet F, Richardson RM, Poloyac SM, Stolz DB, Jockers R, Witt-Enderby PA, Carlisle DL, Vilardaga JP, Friedlander RM (2017) Dual role of mitochondria in producing melatonin and driving GPCR signaling to block cytochrome c release. *Proc. Natl. Acad.. Sci USA* **114**: E7997-E8006.
108. Escames G, León J, López LC, Acuña-Castroviejo D (2004) Mechanisms of *N*-methyl-D-aspartate receptor inhibition by melatonin in the rat striatum. *J. Neuroendocrinol.* **16**: 929-935.
109. Macías M, Escames G, Leon J, Coto A, Sbihi Y, Osuna A, Acuña-Castroviejo D (2003) Calreticulin — melatonin. An unexpected relationship. *Eur. J. Biochem.* **270**: 832-840.