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Melatonin Beyond Its Classical Functions

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Abstract: The perception of melatonin as a mediator of darkness, formed in a circadian fashion, circulating in subnanomolar concentrations, and removed as 6-sulfatoxymelatonin, reflects only a sector within a spectrum of actions. This ubiquitous compound present in bacteria and eucaryotes is exceptionally pleiotropic, in terms of binding proteins, receptor distribution, G protein coupling, electron-exchange reactions, and secondary effects by metabolites, such as 5-methoxytryptamine and methoxylated kynuramines. Membrane receptors are located, e.g., in the vertebrate suprachiasmatic nucleus, pars tuberalis, brain, vasculature, and leukocytes. Binding proteins include quinone reductase 2, ROR/RZR transcription factors, calmodulin, calreticulin, nuclear and mitochondrial proteins. Actions *via* hormonal subsystems, growth factors, neurotransmission and immune system lead to further secondary effects. Single-electron transfer reactions are basis of radical scavenging, non-enzymatic metabolism and interactions with electron transport systems. The metabolite, N^1-acetyl-5-methoxykynuramine, is a potent inhibitor of prostaglandin synthesis and of neuronal NO synthase, an NO scavenger and a mitochondrial modulator.

Keywords: Aging, antioxidants, kynuramines, melatonin, neuroprotection, signal transduction.

INTRODUCTION

The indoleamine melatonin is perceived within the community mostly in a very limited context, in the role of a chronobiotic, which exhibits a circadian rhythm, is released at night from the pineal gland, transmits – as a troll among hormones – the signal darkness to other organs, in particular, to the suprachiasmatic nucleus (SCN) and, in seasonal breeders, to the eminentia mediana and pituitary. As a hormone, it is present in the circulation in usually subnanomolar concentrations. Considerable progress has been made concerning the regulation of its biosynthesis, its actions *via* membrane receptors and its chronobiological functions.

However, the exclusive consideration of this classical role is insufficient. Melatonin is a ubiquitous compound which has been found in any taxa studied so-far, including bacteria, eucaryotic unicellulars, macroalgae, plants, fungi as well as invertebrate and vertebrate animals [1-4]. In some of these organisms, the amounts of melatonin are remarkably high and exceed by orders of magnitude that what one is accustomed to find in vertebrates. Dinoflagellates exhibit basal levels between 20 nM and 10 μM, but concentrations can rise dramatically in response to temperature changes and may transiently approach the millimolar range [2,5,6]. In yeast, melatonin levels depend on the availability of its precursor, tryptophan, and may rise to 40 μM [2,7]. Very high concentrations were detected in some vascular plants and, after such findings had been made especially in medicinal plants [3,8,9], lists of 'record holders' were repeatedly published [4,10-14]. Since melatonin levels are highly variable among plant species, plant organs, fruits and seeds, the role of melatonin has to be diverse in these organisms [4]. Even within the vertebrate body, melatonin concentrations can profoundly differ from those in the pineal gland and the circulation. Numerous extrapineal sites of melatonin synthesis exist, and in some of them, quantities or concentrations considerably exceed those in pineal and blood plasma [14-16]. In extrapineal sites, with exception of the retina and, where present, the parietal organ, circadian rhythms may exhibit low amplitudes or even be virtually absent, and the transmission of dark signals seems to be rather unlikely in organs like bone marrow or gastrointestinal tract. Again, the conclusion has to be that melatonin plays a number of different roles.

The multiplicity of melatonin's effects is remarkable, already within a vertebrate. Major areas of actions are schematically depicted in Fig. (**1**). These areas should not be seen as separate entities, but are rather overlapping in many details. The chronobiotic function of melatonin can influence each of them, but not necessarily at any site.

MELATONIN AND ITS METABOLITES IN UNICELLS

Melatonin has been demonstrated and measured in bacteria (*Rhodospirillum*, *Erythrobacter* and *Escherichia coli*), euglenoids, trypanosomids, ciliates, numerous dinoflagellates, and several chlorophyceans [2,17]. Though representing a secondary unicell, yeast (*Saccharomyces*) shall also be mentioned here [7]. In the majority of species, reliable determinations have been made, using safe extractions, calculations of recovery, and independent procedures, such as RIA and HPLC with electrochemical detection [18,19]. In addition to melatonin, its metabolites 5-methoxytryptamine (5-MT) and 5-methoxytryptophol (5-ML) have been measured in dinoflagellates, in *Euglena*, in the ciliate *Tetrahymena*, and in yeast [2]. In a marine, bioluminescent dinoflagellate species, *Lingulodinium polyedrum* (syn. *Gonyaulax polyedra*), the formation of 5-methoxyindole-3-acetic acid (5-

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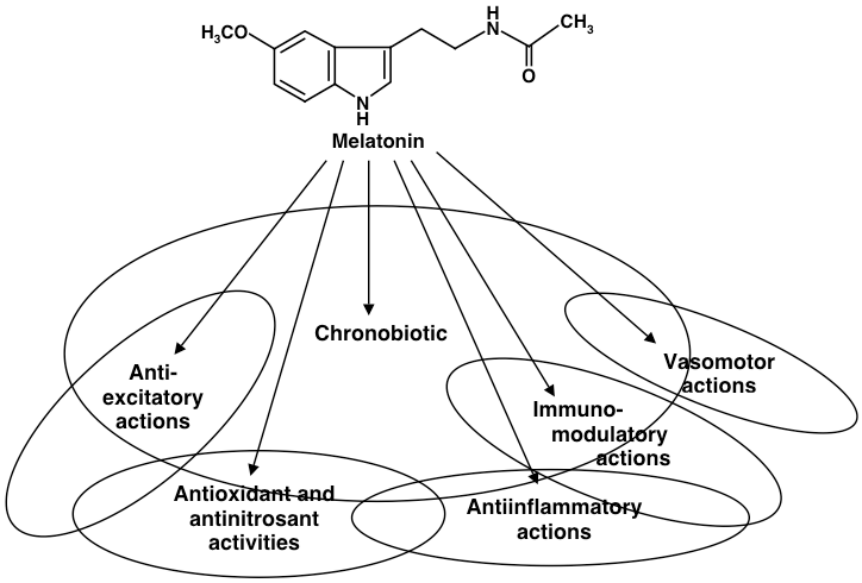

Fig. (1). The fields of melatonin's major actions. The chronobiotic actions are overlapping with all other functions, however, to a varying extent, as indicated by the graphic representation. Additional overlaps exist between some of the other areas of action. Interdependence and independence are depicted. For specific details see current text.

MIAA) [20-22], N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) [22-24] and pinoline [25] was also investigated.

Robust circadian rhythms of melatonin were discovered especially in *L. polyedrum* [18] and *Euglena gracilis* [2]. The rhythm in *L. polyedrum* persisted in constant darkness (DD) [23] and, contrary to the situation in vertebrates, also in constant light (LL) [26]. The latter finding is in accordance with strong rises observed in *L. polyedrum* when cells were subjected in LL to a decrease in temperature [5,6]. Differences in melatonin levels between light- and dark-exposed cells were also reported for the photosynthetic bacterium *Erythrobacter longus* [27] and, perhaps rhythmic, time patterns for chlorophyceans like *Dunaliella* and *Chlamydomonas* species, but details demonstrating a truly circadian periodicity have not been provided [2]. No rhythmicity was detected in yeast [3,7], a finding which may be related to the absence of regulatory flanking regions in the arylalkylamine *N*-acetyltransferase (AA-NAT) homolog [28]. In the ciliate *Tetrahymena*, melatonin was reported to be decreased by light, however, only gradually [29]. The most thoroughly studied rhythm, in *L. polyedrum*, is characterized by a steep rise directly after onset of darkness. In the course of the night, the melatonin level declines to almost basal values, largely because of deacetylation to 5-MT by an aryl acylamidase (AAA), which peaks, in coincidence with its product, 5-MT, during the last hours of dark phase [26,30,31].

Where studied in detail, i.e., in *Lingulodinium* and in *Saccharomyces*, the pathway of melatonin biosynthesis is identical with that known from vertebrates, in a prevailing sequence of tryptophan → 5-hydroxytryptophan → serotonin → *N*-acetylserotonin → melatonin. Especially in *Saccharomyces*, a certain fraction of externally given 5-MT was reacetylated to melatonin, whereas exogenous melatonin was largely converted to 5-MT [7]. These findings are in good agreement with the usually observed relative substrate affinities of AA-NAT for serotonin and 5-MT, and also with those determined in *L. polyedrum* [32-34].

Contrary to vertebrates, which eliminate the circulating melatonin to a great deal by predominantly hepatic P_{450} monooxygenases (CYP1A2, CYP1A1, CYP1B1) and subsequent conjugation with sulfate, such a pathway designed for excretion would not be meaningful in aquatic unicells, which can easily release undesired compounds to the environment. Consequently, the main catabolic pathway is, in organisms like *Lingulodinium* and *Saccharomyces*, that one starting with deacetylation. In both species, 5-MT is further oxidized by a monoamine oxidase (MAO), to give 5-methoxyindole-3-acetaldehyde, which can be further converted by alcohol dehydrogenase to 5-ML, a compound found in these and other unicells. However, in *Lingulodinium*, the predominant fate of 5-methoxyindole-3-acetaldehyde is that of oxidation by aldehyde dehydrogenase to 5-MIAA, which is released from the cells, does practically not re-enter them from the relatively alkaline seawater and does not exhibit any demonstrable effect in this species [20-22]. 5-MT is also the likely source of pinoline found in *L. polyedrum*, as it is usually the case in other systems, including the mammalian and avian brain, retina and pineal [35-37]. Reactions of melatonin with free oxygen radicals, as they occur in any aerobic organism, and which are likely with this indoleamine owing to its remarkably high reactivity to radicals [38-40], are also possible in any of the unicells mentioned. A main product deriving from melatonin by radical-mediated or enzymatic pyrrole-ring cleavage, AFMK, was found in *Lingulodinium*, in particular as a consequence of photocatalytic reactions promoted by compounds present in the dinoflagellate [22-24].

A few responses and actions of melatonin have been identified in unicells. *Tetrahymena* was reported to increase and release melatonin in response to exogenously given small amount of this indoleamine, an effect interpreted as kind of an imprinting [41]. In *Paramecium tetraurelia*, melatonin increased the clonal life span [42]. One of the earliest effects reported for melatonin was detected in another ciliate, *Stentor coeruleus*, in which it interfered with the microtubu-

lar arrangement in the cytostome field [43], an effect reminiscent of similar findings in plants and mammalian cells [4,44]. In the dinoflagellate *Pyrocystis acuta*, melatonin induced phase shifts in the circadian rhythm of spontaneous bioluminescence, and the phase response curve exhibited an advance part more pronounced than the delay part [2]. These results were obtained at concentrations of 100 μM, levels which are, however, attained in dinoflagellates [2,5,6]. In *Lingulodinium polyedrum*, melatonin did not show substantial phase shifts when cells were kept in constant dim light, but it moderately increased a phase advance induced by a light pulse at CT 15 (circadian time 15 h) [45]. However, in experiments not designated to phase shifting but rather to antagonize oxidative stress, moderate phase delays were repeatedly observed in DD [46]. Similar delays were observed in another member of this genus, *L. spiniferum* [2].

A chronobiological role of melatonin as a control factor rather than resetting agent seems to exist in the regulation of enzymes which oscillate in a circadian fashion. In *L. polyedrum*, melatonin downregulates two diurnally peaking enzymes, which oscillate with high-amplitude, tryptophan hydroxylase [30] and superoxide dismutase [46]. Another enzyme with a circadian maximum at onset of photophase, glutathione S-transferase, is moderately suppressed [46]. The findings on the two antioxidant enzymes profoundly differ from the situation in vertebrates, in which such components of the antioxidative protection system are usually upregulated by melatonin [47-49].

Another strong effect of high biological relevance for *L. polyedrum* is the upregulation of AAA [30]. The circadian melatonin peak after onset of darkness is followed with a certain delay by a maximum of AAA, which is causative for a corresponding peak in 5-MT, as already mentioned. Progressive rises in the amplitude of the AAA rhythm were observed when cells were subjected to a temperature step from 20 to 15°C [50], a treatment also leading to dramatic rises in melatonin levels, by orders of magnitude [5,6]. As a consequence of these increases in both melatonin and its catabolizing enzyme, AAA, strong elevations of 5-MT are observed [1,4-6].

The findings mentioned have considerable implications for the physiology and ecology of such dinoflagellates. Melatonin has been demonstrated to be not only a mediator of photoperiodic information, but also of temperature signals. This was also observed in *Alexandrium lusitanicum* and, however, as a response to high temperatures, in a tropical strain of *Amphidinium carterae* [4]. The extent of these temperature effects, as observed in dinoflagellates, exceeds by far moderate influences of temperature in any other organism studied.

Melatonin has turned out to generate high quantities of another bioactive molecule, 5-MT. In the dinoflagellates, 5-MT is a signal molecule that is, under certain aspects, much more potent than the parent compound. Its primary action consists in the opening of proton channels in the membrane of acidic vacuoles [1,4,23,33,34]. Depending on the degree of stimulation by different 5-MT concentrations, this can have two consequences. At moderate concentrations, the intracellular, conducted proton potentials moving along the membrane of the acidic vacuoles of bioluminescent species initiate light emission by the entrance of protons into the microsources (scintillons), where they induce conformational changes in the luciferin-binding protein leading to luciferin release. By virtue of this mechanism, 5-MT is, contrary to melatonin, a very potent stimulator of bioluminescence in *L. polyedrum* [23,33,51,52] and in several other species, too [2]. When 5-MT formation was blocked by inhibition of tryptophan hydroxylase or by oxidant-mediated destruction of methoxyindoles, the circadian peak of spontaneous bioluminescence was suppressed. This suppression was reverted by any metabolite leading to 5-MT, but not by other methoxyindoles, such as 5-ML [31,52-57]. Therefore, the glow peak, which coincides with the 5-MT maximum [26,31], was concluded to require the presence of physiologically elevated 5-MT levels.

Even higher 5-MT concentrations were obtained in *Lingulodinium* after a temperature drop leading to high melatonin and AAA induction. In this case, the massive proton transfer caused a cytoplasmic acidification, which was not seen with melatonin [23]. Similar acidifications were observed in other dinoflagellate species, too [2]. The decrease in cytosolic pH induced the formation of asexual cysts, a dormant state capable of surviving adverse conditions [1,4,23,33,34,58-60]. This effect was clearly attributable to proton transfer, since it was mimicked by any other means leading to cytoplasmic acidification, including protonophores, mechanical stress, low external pH and other acidifying agent such as epinephrine, another stimulator of bioluminescence [23,33,60-62]. Re-alkalinizations allowed cells to excyst again. Encystment in response to 5-MT was demonstrable in numerous dinoflagellates, whereas, in several other species not forming typical pellicle cysts, immobilization of still viable cells was observed [2].

ROLES FOR MELATONIN IN PLANTS

The presence of melatonin has now been shown in numerous plants and was repeatedly reviewed [1-4,8-14,63,64]. Despite the high number of demonstrations, the knowledge on physiological functions has remained remarkably scarce, mainly because investigators were less interested in plant physiology, but rather in finding nutritional or medicinal sources of the indoleamine many beneficial actions are ascribed to. A chronobiological role had been sought more or less in vain [4]. In a suitable test organism, *Chenopodium rubrum*, a nocturnally peaking circadian rhythm was described [65], but an involvement in photoperiodic short-day responses remained uncertain [4,64]. Likewise, no short-day responses were detected in some lemnaceans and in *Kalanchoë tubiflora* [4]. Rhythms reported for some other plants such as tomatoes were not confirmed, but rather changes during fruit ripening [66]. A well pronounced rhythm was described in the water hyacinth, *Eichhornia crassipes* [67]. In this species, the maximum was attained at the end of photophase, and the periodicity of melatonin was accompanied by another robust rhythm of the metabolite AFMK.

An auxin-like growth stimulation was found in both a dicot, *Lupinus* [68], and in monocots. Growth stimulation in coleoptiles of several poaceans was associated with another auxin-like action, growth inhibition of roots [69]. Whether these effects are physiologically relevant and whether melatonin metabolites may be involved, is an intriguing question

to be answered in the future. The presence of particularly high melatonin levels in fruits and especially oily seeds indicated a possible role in differentiation processes and maintenance of dormancy, perhaps, in combination with antioxidative protection in dry seeds, in which enzymatic mechanisms cannot work [4,70].

Radical reactions of melatonin are, of course, possible in any aerobic organism, the question is, however, that of rates and quantities. In *Eichhornia*, particularly high levels of AFMK were detected, which attained a maximum at the end of photophase, i.e., a time at which light-induced damage to photosystems and to auxiliary regenerating mechanisms are highest and, thus, oxidant formation [4]. This may indicate progressive oxidation of melatonin in the course of the light phase by free radicals, singlet oxygen, photocatalytically active compounds, peroxidases and/or other hemoproteins. Such melatonin-consuming reactions may, on the other hand, be regarded as contributions to photoprotection. A possible role in photoprotection had been assumed to be one of melatonin's functions in animal tissues, such as organs rich in photocatalytically active porphyrins like the rodent Harderian gland, in photoreceptors of some molluscs and crustaceans, and in several macro- and microalgae, too [44,71]. This may be similarly relevant to higher plants [4], as indicated by several observations: (i) light-dependent turnover of melatonin, (ii) UV-induced rises, and (iii) considerably higher melatonin contents found in plants exposed to high natural radiation, e.g., at alpine, mediterranean and subtropical sites, compared to same species in other locations or in greenhouses [4,12].

TISSUE MELATONIN IN VERTEBRATES

With regard to actions beyond non-classical functions, extrapineal sites of melatonin formation in the vertebrate body deserve particular attention. Like the pineal gland, some of them also represent extrusions of the intermediate brain, such as the parietal organ of reptiles [72] and the retina of most vertebrates [73-84]. These organs exhibit robust, nocturnally peaking high-amplitude rhythms in melatonin, comparable to that found in the pineal gland. Insofar, they may appear as a variation of the same theme, but, except for retinas of amphibia and some other non-mammalian vertebrates, they contribute only poorly to the circulating hormone. Moreover, the presence [85], rhythmicity and release [86] of melatonin were shown in the rat hypothalamus, another area of the intermediate brain, but site-specific formation remains to be demonstrated.

The poor release from the mammalian retina clearly shows that melatonin serves, in this organ, other functions and is not or poorly acting as a hormone. In avian and mammalian retinas, melatonin strongly downregulates dopamine formation and release [77,80,81]. Contrary to the predominantly noradrenergic upregulation of melatonin biosynthesis in the mammalian pineal, retinal stimulation is mediated by a GABAergic mechanism, involving mainly $GABA_A$, but also partially $GABA_B$ receptors [81,87]. Melatonin and dopamine are obviously inversely correlated, because light, in turn, depresses retinal melatonin *via* dopamine D_1 or D_4 receptors [88-90]. In species in which melatonin is poorly released from the retina, it cannot be metabolized in the same way as the circulating hormone. In fact, is it largely converted to 5-MT, a finding which has even been made in amphibia, which liberate substantial amounts of melatonin [79,91-93]. Deacetylation by a specific melatonin deacetylase, as in the retina, but also by AAAs or an AAA-like activity of eserine-sensitive acetylcholinesterase are also relevant for the brain [16]. 5-MT, as a bioactive compound, exerts various effects in the central nervous system, as summarized in ref. [16], but the physiological relevance of respective pharmacological experiments is not always easy to judge. In cultured retinal cells, 5-MT was reported to prevent the forskolin-induced rise in cyclic AMP, in an action independent of melatonin receptors [94], a finding of still uncertain meaning.

Other areas in the central nervous system have also been suspected to be sources of melatonin [16]. This may be seen in relation to AA-NAT expression in various brain areas, which is, however, not necessarily associated with melatonin biosynthesis, because of separate central nervous actions of NAS and uncertainties concerning the presence of hydroxyindole *O*-methyltransferase, which may, however, be locally replaced by other methyltransferases [16]. Nevertheless, relatively high concentrations of about 0.7 μM were reported for the whole mouse brain [95]. On the other hand, high concentrations of melatonin also enter the CNS from the pineal gland *via* the pineal recess [96,97].

Melatonin synthesis was also described for the membranous cochlea, and smaller amounts of the indoleamine were found in the cochlear nerve of guinea pigs [98]. Temporal variations have been described, but data were not sufficient for describing a robust rhythm. The importance may be sought in protective actions within this highly vulnerable organ.

Several extrapineal sites of melatonin biosynthesis are devoid of robust circadian rhythmicity. In some of them, this has not been thoroughly studied yet. The rodent Harderian gland exhibits only weak diurnal changes, which may be almost absent. Usually, only a small, transient drop after onset of light is observed [99-103], which may not be of circadian nature. From these findings, the fundamental conclusion can be drawn that mammalian tissue melatonin is not necessarily associated with darkness. One could even suspect that light entering the Harderian gland might, by photocatalytic conversion to AFMK, create a signaling molecule perhaps indicative for the photophase. Variations in Harderian melatonin, as observed within sexual or seasonal cycles [99,101-103], do not indicate transmission of dark signals, but are rather a consequence of regulation by gonadosteroids, which strongly influence this gland [99,101,104-107].

The gastrointestinal tract is another, important site of melatonin formation, which has been reviewed several times [14,108-113]. The indoleamine is synthesized in the enterochromaffin cells, but circulating melatonin can be also taken up from the blood. Owing to its size, the entire gastrointestinal tract contains about 400 – 500 times more of melatonin than the pineal gland [110,111]. The uptake of circulating melatonin has been demonstrated by elevating plasma melatonin during daytime to nighttime levels [114,115]. A substantial fraction was released unmetabolized from the gut to the lumen. In other studies, enterohepatic cycling of melatonin was demonstrated and, consequently, high quantities of

the indoleamine were detected in the bile fluid [113,116,117]. As melatonin can enter the gut by reuptake from the lumen, its resorption from the food is not surprising. It is possible to elevate diurnal blood plasma levels by feeding vegetables rich in melatonin [118], whereas decreases were observed after melatonin-depleted food [119]. Whether intestinal bacteria also contribute to the melatonin content of the gut is a question that has been addressed [3], but is not yet solved.

Circadian rhythms have been described for gastrointestinal melatonin, but this should not be implicated in the transmission of dark signals. The amplitudes remain much lower than those in the pineal gland or the retina and usually attain maximum/minimum ratios of only 2:1 or lower, and may sometimes remain undetectable [14,110,111]. In some avian and mammalian species, the circadian peak of duodenal melatonin appeared earlier than that of the pineal gland [85,120,121], a finding requiring further elucidation. This is possibly indicative of a relative independence of the gut from the pineal.

In the absence of a specific stimulus, gastrointestinal melatonin is poorly entering the circulation. However, it can be released into the blood, in response to food intake [14,110,111] and, in particular, to high tryptophan [113,122]. The tryptophan-induced rise in plasma melatonin is higher than the nocturnal circadian peak originating from the pineal gland. The dramatic but transient melatonin surge from the gastrointestinal tract was reported to be unaffected by pinealectomy, but strongly diminished by partial portal ligature [122]. The physiological meaning of the postprandial melatonin release is poorly understood, but it should not be primarily a chronobiotic one. During daytime, melatonin released from the gut is almost ineffective because it appears mostly in the silent zone of the circadian phase-response curve [14]. Apart from the postprandial liberation to the blood, gastrointestinal melatonin seems to act largely through paracrine and luminal routes. It was shown to participate in the regulation of gastrointestinal motility [110,111]. Melatonin receptors have been identified in this organ [113]. Pharmacological experiments have also demonstrated cytoprotective, antioxidant and antiinflammatory actions as well as melatonin's efficacy in supporting wound healing and antagonizing esophagitis, gastritis, peptic ulcer, pancreatitis and colitis [113].

An organ that has been debated as another site of melatonin biosynthesis is the skin. The expression of enzymes required for melatonin formation has been demonstrated, such as tryptophan hydroxylase, AA-NAT and, as alternate enzymes, arylamine *N*-acetyltransferase (NAT) subforms, and HIOMT [123-128]. A major difficulty in judging the relevance of cutaneous melatonin results from discrepancies between levels measured in skin biopsies and in cultured keratinocytes. Earlier attempts of quantifying this indoleamine in skin samples by extraction and liquid chromatography techniques failed to demonstrate the presence of melatonin [124,129], but this may have been a matter of safe extraction under avoidance of oxidative destruction, a problem that is present with various non-classical sources of melatonin [19]. On the other hand, cultured HaCaT keratinocytes were shown to contain remarkable amounts of about 30 μM melatonin (under consideration of an erratum) [130]. It seems rather unlikely – but is not impossible – that this entirely deviating result is only a consequence of a dysregulation in the cultured, immortalized cells.

Melatonin is believed to act as a photoprotective agent in the skin [126,130-132]. In HaCaT keratinocytes, oxidative metabolites, such as 6-hydroxy-, 2-hydroxy- and 4-hydroxymelatonin and AFMK were also detected, and exposure to UVB caused rises in 2-hydroxymelatonin and AFMK [130]. It attenuated UV-induced apoptoses [131] and supported the maintenance of the mitochondrial membrane potential [132]. On the other hand, melatonin was reported to exert oncostatic effects in human melanoma cell lines [133]. Applicability in practice as an anti-cancer drug remains to be studied.

Several further tissues and cells have been reported to synthesize melatonin, such as the human ovary [134], bone marrow [135], in which the indoleamine was assumed to modulate hematopoiesis [136], various types of leukocytes [137-140], platelets [141], and even erythrocytes [142]. The role of the indoleamine in bone marrow, leukocytes and presumably also other cells relevant to the immune system seems to be of high relevance, as discussed in the section on multiple lines of defense. The formation in platelets and erythrocytes should not be overrated and may only reflect unavoidable side reactions taking place when platelet-derived serotonin comes into contact with unspecific *N*-acetyl- and *O*-methyltransferases. Elevated concentrations of melatonin were detected in various other tissues, too [140,143,144], but the alternative of biosynthesis *vs*. accumulation by uptake from the circulation remains to be clarified.

This overview on the presence, formation and functions of tissue melatonin shall be completed by addressing a few aspects of fundamental relevance. As briefly mentioned with reference to the mammalian retina, a poor release of melatonin to the circulation has two consequences. First, the indoleamine has to be metabolized differently from the circulating hormone, and routes of deacetylation to 5-MT or of pyrrole-ring cleavage to AFMK may be more important than hydroxylation or demethylation by CYPs. Second, the maintenance of elevated concentrations in a synthesizing but poorly secreting tissue requires an explanation. Because if its amphiphilicity, melatonin is usually believed to cross any membrane and, therefore, to reach any compartment or body fluid. In fact, externally administered melatonin is found soon in many places, but it does not equally distribute within the body [114,115]. Some organ systems such as the gut resorb relatively more melatonin than others. This observation may reflect the capacity of cells to maintain high concentrations by synthesizing but poorly releasing melatonin. If the assumption of passive diffusion through membranes is correct, the answer can be only sought in the presence of intracellular binding sites, which should be distinct from receptors but efficiently sequester the indoleamine. Results indicating melatonin retention were to date only obtained in the dinoflagellate *Lingulodinium polyedrum* [21,143]. Externally given melatonin accumulated intracellularly, for a couple of hours, to manyfold higher levels. Although the extracellular concentration did not substantially change because of a much

larger medium volume compared to cell volume (about 2000:1), the accumulation ceased by time and was partially reverted. No indication existed for an active transport mechanism. In mammalian tissues, high amounts of melatonin were sometimes found in nuclei and mitochondria. Nuclear non-receptor binding proteins [144] may contribute to sequestration, for quantitative reasons [145].

DIVERSITY OF ACTIONS BY DISTRIBUTION OF MEMBRANE RECEPTORS AND G PROTEINS

Numerous melatonin bindings sites have been detected in CNS areas and many peripheral organs by means of the ligand [^{125}I]iodomelatonin (summarized in ref. [44]). This reflects one aspect of the remarkable pleiotropy of this molecule. More specifically, the G protein-coupled membrane receptors MT_1 and MT_2, identified and analyzed in the pioneering work by SM Reppert and colleagues [146-153], are found in vertebrate tissues or cells. These include, e.g., retina, various brain areas, the hypothalamic median eminence, pituitary, choroid plexus, cerebral and peripheral vasculature, reproductive organs, adrenal cortex and several leukocytes [140,153-161]. In non-mammalian vertebrates, the receptor Mel_{1c} is present. For reasons of nomenclature, this receptor cannot be classified within the MT-terminology, and is not identical with a binding protein formerly called MT_3.

Instead of listing all places in which MT_1 and MT_2 have been demonstrated, we shall focus on divergencies of actions mediated by these receptors. The classical, chronobiotic effects of melatonin, especially exerted in the circadian pacemaker, the SCN, involve signaling *via* the G_i protein, leading in the case of MT_1 mainly to acute suppression of neuronal firing, in that of MT_2 predominantly to circadian phase shifts [15,150-153,162-164]. Since both actions are mediated by the same G protein and a decrease in cAMP, it is not surprising that these receptors can partially substitute for each other, as can be also seen in Sibirian hamster species, which do not possess the MT_2 receptor, but respond to melatonin by phase shifts [165]. However, similar or synergistic actions by the two receptors are by no means universal, but rather depend on the relative expression of alternately coupling G proteins as well on the effector proteins controlled by the various α subforms or by βγ. Therefore, effects mediated by MT_1 or MT_2 can be expected to be not only cell-specific, but also divergent if not antagonistic. In fact, such a complexity is observed. Co-activation or alternate activation of G_o or G_q has been repeatedly demonstrated [153,154,157,166-169]. In some cases, including studies in transfected cells, G_z or G_{16} were reported to differentially couple to melatonin receptors [157,170,171]. Contrary to decreases in cAMP caused by G_i proteins, rises in cAMP were also described, e.g., for the *Xenopus* Mel_{1c} receptor when acting *via* α_z coupling to adenylyl cyclase type II [172]. G_i-dependent mechanisms may not only affect cAMP levels, but also modulate, in some cells, K^+ conductance, and, *via* βγ, stimulate phospholipase Cβ (PLCβ), which may also occur with G_o [157]. PLCβ activation seems to be a more general phenomenon of either parallel or alternate melatonin signaling present in various target tissues, including the SCN [173]. Both pertussis toxin-sensitive (G_i/G_o) and -insensitive (e.g., G_q) G proteins can be involved [157,174-177]. The consequences of PLCβ activation are highly divergent, cell type-specific, and reach from stimulation of PKC subforms, CaM kinases, opening of Ca^{2+}-activated K^+ channels to modulation of numerous other protein kinases, including the MAP and jun terminal kinase pathways. An extreme example for divergent actions mediated by MT_1 and MT_2 has been described for the vasomotor control by melatonin. While MT_1 leads to a pertussis toxin-sensitive vasoconstriction *via* opening of BK_{Ca} channels, MT_2 causes vasodilation [16, 153,178-181].

NUMEROUS OTHER BINDING SITES

The existence and physiological relevance of other binding sites has been vividly debated for quite some time. Meanwhile, their presence is beyond doubt. However, not all of them can be classified as receptors, especially when signaling mechanisms have not been demonstrated. One of these binding sites was originally named MT_3 and believed to represent another membrane receptor. However, the protein is, in fact, the mainly cytosolic enzyme quinone reductase 2 (= QR2 = NRH:quinone oxidoreductase 2 = NQO2; NRH = dihydronicotinamide riboside) [182-188]. QR2 is expressed in several tissues, including the brain [183,189,190]. It may be of interest that some of its polymorphic subforms have been related to Parkinson's disease [191], but, in mechanistic terms, the meaning of this finding is unclear. With regard to the activity of a quinone-reducing enzyme, a relationship to redox processes and detoxification seems likely, at first glance. However, the situation is not that simple. Disruption of the QR2 gene leads to bone marrow myeloid hyperplasia [192], indicating a function beyond detoxification of xenobiotics. Although a function in ubiquinone reduction was assumed, the precise role of this enzyme is not understood, especially with regard to the meaning of melatonin binding. Recently, melatonin has been suggested to act as a cosubstrate serving as a hydrogen/electron donor to other redox cofactors such as FAD [193], an assumption which seems possible with regard to melatonin's redox properties, but which would need direct experimental support. Perhaps, this discussion should not be exclusively focussed on melatonin, because *N*-acetylserotonin, which is formed in many brain areas presumably without further *O*-methylation, has a similar affinity to QR2 [16]. Even without a convincingly demonstrated physiological role, QR2 may gain pharmacological relevance, since it is assumed to be a molecular target of resveratrol and of anti-malarian drugs such as chloroquin or paraquine [186].

Several other binding proteins are related to calcium signaling and distribution. One of them is calmodulin (CaM), which acts as a subunit and regulator of several protein kinases and other enzymes. Its affinity to melatonin is sufficient for mediating effects at elevated physiological concentrations, as attained in some tissues [194-197]. This may be also relevant for counteracting hyperphosphorylation-related cytoskeletal changes in neurodegenerative diseases [198]. Two enzymes of high physiological significance have been shown to be inhibited by melatonin *via* binding to CaM, namely, CaM kinase II [196] and neuronal NO synthase [44,199]. In addition, melatonin causes PKCα-dependent phosphorylation of calmodulin [200], presumably involving membrane receptors, an effect that reinforces and perpetuates CaM-dependent suppressions. Modulation of CaM and protein kinases mentioned are involved in cytoskeletal

[195,197,198] effects, which extend from neuroblastoma cells to ciliates and plants [1,44,195,198]. Melatonin has been shown to act as a ligand of another important Ca^{2+}-binding protein, calreticulin [144]. Additionally, a nuclear protein with high homology to calreticulin was discovered in that study, and another binding protein that was structurally different.

Another category of nuclear binding proteins can be classified as receptors in the proper sense. Although their affinity is lower than that of the membrane receptors, they may be relevant with regard to elevated melatonin concentrations in some tissues as well as to autocrine and paracrine effects. These proteins are the transcription factors RORα1, RORα2 and RZRβ, which belong to the retinoic acid receptor superfamily [201-204]. Additional splice variants exist, but their relationship to melatonin is less clear. RORα1 and RORα2 seem to be involved in immunomodulation, in addition to the membrane receptors. RZRβ is especially expressed in the central nervous system, including the pineal gland [15,16,140]. RORα was also assumed to participate in upregulations of antioxidant enzymes by melatonin [204].

A further melatonin binding site, with a dissociation constant of 150 pM and a total number of specific binding sites of 30 fmol/mg, has been detected in rat brain mitochondria [16]. Mitochondrial effects of melatonin on electron leakage from the respiratory chain indicate that this protein may be localized at the amphipathic ramp of complex I. Another mitochondrial action was only observed at elevated concentrations, namely a direct inhibition of the opening of the mitochondrial permeability transition pore [205], a finding that would imply another, low-affinity mitochondrial binding site.

DIVERSITY OF METABOLISM AND ACTIONS OF METABOLITES

As outlined in preceding sections, the metabolism of melatonin is taxon- and organ-dependent and not restricted to 6-hydroxylation. Deacetylation to 5-MT or demethylation to *N*-acetylserotonin have been mentioned above. Effects of these bioactive compounds in vertebrates have been reviewed elsewhere [16]. Several other indolic compounds can derive from 5-MT, and some of them are biologically active, but, perhaps, only in a pharmacological or pathophysiological context. *N*,*N*-dimethyl-5-methoxytryptamine, formed from 5-MT or by *O*-methylation of bufotenin, is as an endogenous hallucinogen [16]. 5-ML, which can be formed from either 5-MT or 5-hydroxytryptophol, was shown in some species to vary in a diurnal and seasonal fashion, and to exert some antigonadal effects summarized elsewhere [25], which may, however, be inferior to those of melatonin. In one case, 5-ML was reported to be more efficient than melatonin, namely, in decreasing basal body temperature, an action shared with 5-hydroxytryptophol and *N*-acetylserotonin [206]. This potentially important finding has never been followed up. An almost forgotten melatonin analog, *O*-acetyl-5-methoxytryptophol, which can be formed from 5-ML and is present, e.g., in the pineal gland, was shown to inhibit nicotinic and muscarinic acetylcholine receptors and to decrease pituitary prolactin and LH [16].

Pinoline, a tricyclic compound belonging to the β-carboline family, is formed from 5-MT under uptake of a C-atom [35-37]. It is a natural metabolite, but a certain fraction is formed artificially during extraction, so that physiological levels remain uncertain. In the CNS, pinoline binding sites have been demonstrated, which may be identical with the imipramine binding site [207-210]. It does not only interfere with serotonin uptake, but also acts as a MAO A inhibitor [211,212], thereby potentiating the enhancement of extracellular serotonin levels. The interference with extracellular serotonin availability can explain the neuro- and psychotropic effects of this compound, but this may be mainly a matter of pharmacology or, perhaps, of pathophysiology.

Another tricyclic metabolite is cyclic 3-hydroxymelatonin (c3OHM), a compound formed by sequential interaction of melatonin with two free radicals, under physiological conditions, two hydroxyl radicals [213-215]. After melatonin administration, it can be detected in the rodent urine [216], and it is strongly elevated after exposure to ionizing radiation [213]. With the exception of radical scavenging, no other effect of c3OHM is known to date.

Entirely different compounds, the 5-methoxylated kynuramines and their derivatives, are produced from melatonin upon cleavage of the pyrrole ring. The primary kynuramine metabolite, AFMK, is formed by numerous reactions ranging from enzymatic catalysis, by indoleamine 2,3-dioxygenase or by myeloperoxidase, to pseudoenzymatic mechanisms involving, e.g., hypervalent oxyferryl-hemoglobin or hemin, to various photochemical and radical reactions [49, 217,218]. AFMK can be either formed (i) directly from melatonin, e.g., by the enzymes mentioned, by pseudoenzymatic reactions, by singlet oxygen, or (ii) by combination of an intermediate melatonyl radical with a superoxide anion, or (iii) from c3OHM, and, perhaps, also (iv) by 3-hydroxylation of 2-hydroxymelatonin. The broad spectrum of reactions leading to the same product, AFMK, is exceptional. Moreover, this kynuramine is frequently the major product formed in various oxidation systems, especially when they are not designed to generate a single radical species, but take account of the physiological prevalence of superoxide anions [49,219], which can serve as terminators of radical reaction chains [25,49]. In quantitative terms, AFMK formation has been underrated for a long time, partially because of the lack of sufficiently sensitive assays and availability problems. Although plasma and urinary amounts remained low in respective studies, even after oral administration of melatonin [216,220,221], one should not judge from these findings on the relevance of the pathway, which seems to be much more important in non-hepatic tissues. It would be a misconception to argue on the basis of circulating melatonin levels, because much higher amounts of the indoleamine are found outside the blood, quantities which are not generally entering the blood and are not preferentially hydroxlated. AFMK was demonstrable in the retina and in the lateral brain ventricle [221]. It also appeared in the blood after intraperitoneal melatonin administration [221]. Moreover, it was detected as a major brain metabolite exceeding by far the hydroxylated indolic metabolites [222]. Also with regard to its formation by myeloperoxidase, the AFMK route was assumed to contribute to about one third of total melatonin catabolism [223]. Furthermore, the high amounts of AFMK found in HaCaT keratinocytes [130] and in the plant *Eichhornia* [67] seem to shed light on the biological relevance of this compound.

Attempts of identifying actions of AFMK were of limited success, when effects on the reproductive system [224,225] and affinities to benzodiazepine [226,227] and melatonin receptors [228,229] were investigated. However, an interesting chronobiological effect of AFMK was described, which was never followed up later in other systems, namely, an acceleration of the reentrainment of the melatonin rhythm in rats [230]. More recently, the life cycles of malaria parasites (*Plasmodium chabaudi* and *P. falciparum*) were shown to be synchronized by AFMK in the upper nanomolar range, an effect associated with rises in cytosolic calcium [231]. However, this action was blocked by the MT_1/MT_2 antagonist luzindole [231], a finding raising the question of a direct or indirect interference of AFMK with melatonin receptors, despite the low affinities determined for direct binding of the kynuramine to the mammalian receptors.

Although cyclic voltammetry demonstrates a preference of AFMK for two-electron transfer reactions [217], so that the molecule is only a moderate radical scavenger [218,232], is has been used successfully for antagonizing oxidative damage and stress, however, at pharmacological concentrations. AFMK protected DNA from oxidative damage by hydroxyl radicals generated in a chromium(III)-based Fenton-analog reaction [233], or by a δ-aminolevulinic acid/Fe^{2+} system [234], but it remained less efficient than melatonin. AFMK reacts with this highly aggressive radical species, but rather moderately or even poorly with other radicals [218,232]. Nevertheless, protective effects by AFMK were observed in living cells, such as inhibition of toxicity by glutamate, H_2O_2, or amyloid $\beta_{25\text{-}35}$ peptide in hippocampal neurons [217], and, in mice exposed to X-rays, in which damage to DNA, proteins and lipids was attenuated [235]. AFMK was also reported to be a more efficient inhibitor of lipopolysaccharide (LPS)-induced formation of TNF-α and IL-8 in neutrophils, compared to melatonin [236], an effect which cannot be explained by affinity to free radicals released in response to LPS.

The discrepancy between moderate radical scavenging and potent protection may be explained in two different ways. Either the effects are caused by – to date unknown – mechanisms modulating antioxidative enzymes or other protective proteins, or they imply the participation of less inert metabolites. In fact, the most frequently investigated product, N^1-acetyl-5-methoxykynuramine (AMK), is a much more potent radical scavenger [218,232] and otherwise biologically active substance. AMK is formed by deformylation of AFMK, e.g., by arylamine formamidase (= kynurenine formamidase) [14,25,49,218] or by hemoperoxidase ("catalase") [49,237] (Fig. **2**). Recently another, photochemical mechanism by UV light has been described [238]. AMK formation may not be the exclusive route of AFMK metabolism, since oxidation reactions by free radicals led to a couple of C2-substituted 3-indolinones, which represent a novel class of oxidation products [239].

AMK is presumbly not just an end product, as previously believed, because it readily interacts with reactive oxygen species [49,218,232], carbonate radicals [218,232], reactive nitrogen species, such as •NO and nitroxyl (HNO) [240-242], and is, therefore, rapidly consumed. Among the products, the stable, nitrosated derivative 3-acetamidomethyl-6-methoxycinnolinone (AMMC), formed by interaction with •NO or HNO [240-242] (Fig. **3**), may be of particular interest, because other members of the cinnoline family have been used as medicaments or investigative drugs with antiallergic, antitumor, anxiolytic or other neurotropic properties [240].

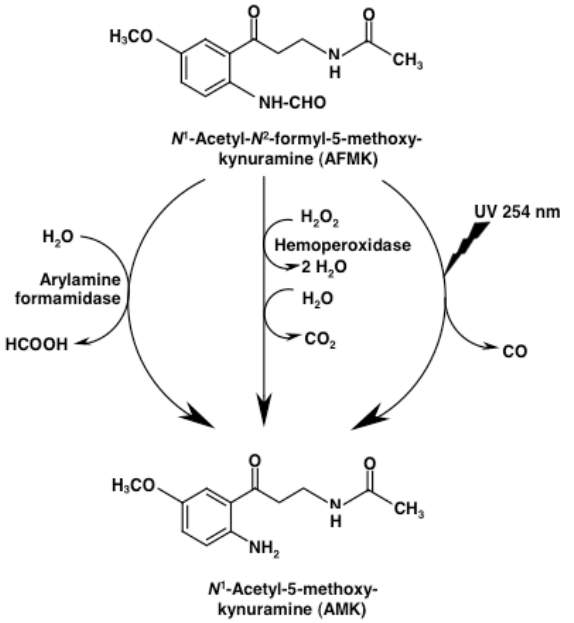

Fig. (2). Three pathways leading from the melatonin metabolite AFMK to the deformylated product AMK. For further details see refs. [49] and [238].

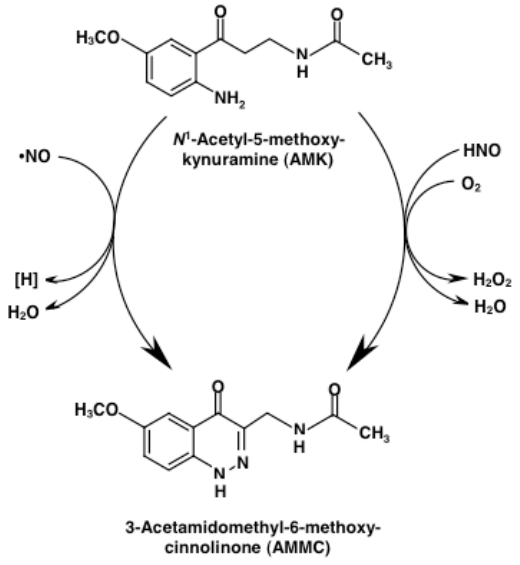

Fig. (3). Two pathways of AMK nitrosation, by the NO radical or by nitroxyl (HNO), lead to the same cinnolinone, AMMC. For further details see refs. [240-242]. The reaction with HNO includes two alternate possibilities differing in the sequence of consecutive steps [242]. A third pathway initiated by interaction of AMK with the nitrosonium cation (NO^+) has been chemically identified [242], but is not depicted because it may be physiologically irrelevant with regard to the extremely short half-life of NO^+ in aqueous solution at neutral pH.

AMK itself also displays several potentially important pharmacological properties. It was reported to be a cyclooxygenase inhibitor by far more potent than acetylsalicylic acid (aspirin) [243]. More recently, AMK was shown to specifically downregulate, at pharmacological concentrations, cyclooxygenase-2 expression in macrophages, but not that of cyclooxygenase-1 [244]. Effects observed in the nanomolar range concerning mitochondrial electron transport will be discussed next. Another action on neuronal NO synthase was also detected at very low concentrations [245]. This enzyme was demonstrably inhibited already at 10^{-11} M.

With regard to the biological activities of its metabolites, melatonin appears as a compound which does not exclusively display effects mediated by its own signaling mechanisms, but additionally represents a prodrug causing various secondary effects *via* its products [14]. Concerning AFMK and AMK, this should be seen in the context of the whole kynuramine family, an own class of biogenic amines, whose other members can be unsubstituted or hydroxylated at C-5, and methylated or dimethylated at N-1. Several effects of kynuramines have been summarized elsewhere [246].

THE MULTIPLE LINES OF DEFENSE

Protective effects have been ascribed to melatonin and some of its metabolites in numerous publications. However, many investigators solely focus on antioxidative actions such as radical scavenging to explain these actions. In fact, protection comprises several other mechanisms, which involve prevention of radical formation and different lines of defense (Fig. **1**). Oxidative stress, which may be suppressed by pharmacological doses of melatonin, is frequently associated with other dysfunctions as a consequence and not the cause of disorders. In such cases, reversal of oxidative stress requires interference with the primary cause.

Already the chronobiotic actions of melatonin may contribute to the suppression of oxidative damage. Dysphased circadian rhythms and problems of coordination and coupling of oscillators are a potential source of oxidative stress. Enhanced oxidative damage to proteins and lipids was observed in clock mutants of *Drosophila* and Syrian hamster and is likely to occur upon repetitive phase shifting [247]. This view may be also in line with the observation that short-period mutations in clock genes can lead, in mice, to a cancer-prone phenotype [247-249]. Adjustment and appropriate coordination of rhythms may, thus, attenuate oxidative stress and damage to macromolecules. A second preventive action of melatonin to be discussed below in detail is that of antiexcitatory actions of melatonin in the CNS [49,247]. Antiexcitatory and antiexcitotoxic effects prevent excitation-dependent calcium overload, secondary rises in radical formation, and antagonize mitochodrial dysfunction and cell death. Safeguarding of mitochondrial electron flux, attenuation of electron leakage and diminution of Ca^{2+}-dependent •NO formation [29,247] should be regarded as an important strategy for preventing mitochondrial dysfunction, as will be discussed in the subsequent section.

Another line of defense concerns the immune system. Since this aspect has been repeatedly reviewed in the last years [15,140,250-256], only a general outline will be given. Melatonin is clearly an immunomodulatory agent, which not only acts on several types of leukocytes, but is also formed by some of them, such as monocytes, eosinophiles, mast cells and NK cells. Several leukocyte-derived cell lines were also shown to synthesize the indoleamine. Certain amounts found in thymocytes and epithelial cells [140] may be formed there or alternately taken up from the circulation. Various forms of leukocytes are activated by melatonin, in particular, T, B and NK lymphocytes, monocytes and splenocytes. These processes are associated with modulations of cytokine release and comprise stimulations of IL-2, IL-6 and IL-12 production, whereas levels of IFNγ or TNFα were sometimes found to be decreased, but in other cases increased [140,253,257]. In the context of antiinflammatory aspects of immunomodulation, an inhibition of the PGE_2-induced suppression of IL-2 formation may be of particular interest [139,153,258]. Immunomodulation seems to be the result of concerted actions not only involving different cell types and their respective cytokines, but also different melatonin receptors. MT_1 was found to mediate effects concerning IL-2, but the role of this receptor may extend to other functions, because the presence of this receptor was shown in numerous leukocyte subtypes [140,153,158,251]. Signaling *via* MT_2 is involved in splenocyte proliferation and antagonizes leukocyte rolling [153,259]. In avian splenocytes, growth stimulation is mediated *via* the Mel_{1c} receptor [260]. Leukotriene B_4-induced endothelial leukocyte adhesion was ascribed to "MT_3" [259], but caution seems due because of the absence of signaling pathways known for QR2. ROR and RZR subforms are also expressed in various leukocytes and related cells such as splenocytes, thymocytes and Jurkat cells, and some of melatonin's immunological actions are assumed to be mediated by these transcription factors [202,250,251,260]. The coordinated actions of membrane and nuclear receptors in the immune system appear as an intriguing and potentially important field.

An aspect bridging immunological and antioxidant effects concerns the antiinflammatory actions of melatonin and its metabolite AMK. This is not limited to the suppression of proinflammatory prostaglandins, but extends to inhibition of •NO formation, scavenging of •NO and attenuation of secondary oxidant, nitrosating and nitrating effects by this gaseous messenger and its metabolites, in particular, peroxynitrite [49]. Protective antiiflammatory actions involving •NO metabolism have been reported to be efficient even in animals models of severe sepsis, such as cecal ligation and puncture [261-264].

In addition to the above-mentioned avoidance of radical formation, detoxification of free radicals and other oxidants may be regarded as another line of defense. The mechanisms involved are, again, multiple. At the level of gene expression, upregulations of antioxidative enzymes have been repeatedly described, in particular, of glutathione peroxidase [47-49,265-275], glutathione reductase [49,269,272,276], in some tissues, Cu,Zn- and/or Mn-superoxide dismutases [47-49,271,272,273,275,277-280], catalase [48,49,275,281], and supporting enzymes that increase the availability of reduced glutathione, such as glucose-6-phosphate dehydrogenase [47,49,269] and γ-glutamylcysteine synthase [47,49]. Prooxidant enzymes were shown to be downregulated, in particular, 5- and 12-lipoxygenases [47,49,282-284] and NO synthases [44,47,49,270,285-293]. The effects on 5-

lipoxygenase were attributed to an ROR/RZR binding site in the promotor [202], and similar assumptions have been made for antioxidative enzymes [204]. However, the involvement of membrane receptors in the control of antioxidative enzymes was reported in other cases [294,295]. This issue urgently deserves clarification. Some further contributions to the defense in favor of oxidatively, nitrosatively or otherwise challenged cells seem to be related to signaling pathways and transcription factors. In murine macrophages, C6 glioma cells and skeletal muscle, downregulation of inducible NO synthase and, in glioma cells, cyclooxygenase-2, was shown to be associated with prevention of NFκB activation [287,296,297]. Similar findings were obtained in the suppression of renal inflammation [298]. Inhibition of NFκB activation by melatonin was also observed in various models of oxidative stress [299-301], including Aβ peptide toxicity [302] and brain trauma [303]. This antagonism to NFκB seems to be a general phenomenon, although it is not clear in each of these studies whether its activation is the result of intracellular rises in oxidants or signaling by cytokines, and at which level melatonin interferes. Melatonin-sensitive signaling cascades have not been studied extensively, and although the involvement of Akt [304], FAS ligand expression and c-Jun phosphorylation [305] have been occasionally shown, a coherent picture cannot be drawn yet, especially as these finding were made in contextual situations as different as inflammation, apoptosis and growth control.

Downregulation of steroid and related receptors by melatonin have been reported for several organs. Interference with the glucocorticoid receptor was decribed in thymus [306-308], brain (hippocampus, hypothalamus, cerebellum) [309-312], pituitary and liver [309,313], for thymus and cerebellum especially in terms of prevention of apoptosis. Similar downregulations were described for estrogen receptor ERα and retinoic acid receptor RARα [314]. While the interference with these steroid and retinoic acid receptors is beyond doubt, the suppression mechanisms by which melatonin acts would require more experimental clarification. While some evidence was presented for the involvement of membrane receptors [310,314,315], an additional MT_1- and RORα-independent mechanism was reported for thymocytes, which showed an inhibition of nuclear translocation of the glucocorticoid receptor [308]. Authors concluded on interference with Hsp90. This effect is not necessarily a direct one, but might have been mediated by immunophilins, as frequently observed in glucocorticoid resistance [316,317].

The last line of defense, that of direct radical scavenging, should be relevant only in the presence of high melatonin concentrations, for reasons of stoichiometry with radicals generated and proportions to other, usually more abundant antioxidants. This has been the case in numerous studies performed with pharmacological doses, but may be likewise valid for cells producing or storing elevated amounts of melatonin. This can be relevant for tissues like the rodent Harderian gland, for plants rich in melatonin as well as their seeds, and for some dinoflagellates. In *Lingulodinium*, elevated but physiologically possible concentrations of melatonin protected, without upregulation of antioxidative enzymes, against lethal oxidative stress by H_2O_2 [46] and sublethal stress by paraquat [31,318,319].

Radical scavenging by melatonin has been reviewed several times [1,14,40,44,47,49,218,237,269,270,272,289], so that this will not be repeated here in any detail. As will be found there, melatonin scavenges with high efficacy hydroxyl radicals (•OH), some kinds of peroxyl radicals (•OOR), carbonate radicals (CO_3•$^-$), various radicals deriving from excited states of photocatalysts, other organic and some xenobiotic radicals. Superoxide anions (O_2•$^-$) are only scavenged at reasonable rates in the presence of enzymatic or pseudoenzymatic catalysts, or by melatonin's reactive radical intermediates, the melatonyl cation or neutral radicals. Melatonin can also interact with •NO and •NO_2, whereas the frequently mentioned scavenging of peroxynitrite (which is highly reactive but not a radical) cannot be easily distinguished from the scavenging of peroxynitrite-derived radicals, which also lead to melatonin nitration. Although other indoles were sometimes reported to be similarly efficient as melatonin, this has to be seen with caution. It is particularly important that reactive intermediates from melatonin are capable of terminating radical reaction chains [49,219], thereby forming AFMK or c3OHM. Other indoles may scavenge free radicals at high rates, but the reaction chains are often not terminated so that the balance is a prooxidant one. Apparent rates of scavenging may also depend on the oxidation system. If this is designed in a way not easily allowing termination, e.g., in a chemist's attempt of studying the influence of a single radical species – which is highly unbiological – a prooxidant indole may appear as the "better" scavenger. If one considers, however, the physiological prevalence of superoxide anions, which terminate the radical chains, melatonin turns out to be superior to its structural analogs, in terms of antioxidative protection [320]. The oxidation chemistry of melatonin is clearly unique and not only dependent in the indole moiety, but strongly determined by both the 5-methoxy and the *N*-acetyl residues. While the effect of the methoxy group is not surprising, that of the acetyl group is estonishingly important, as demonstrated by conversion rates [320] and the formation of c3OHM [213]. With regard to the distance of this group from the aromate and to the possibility of forming a third ring, we assume the existence of a hydrogen bond between the acetyl-O and the pyrrole-N, which should also influence the pK of the melatonyl cation radical formed by single-electron donation. The cation radical is usually assumed to have a pK of about 4 and, thus, to readily form the neutral radical at physiological pH [215], but this conclusion is based on measurements with other indoles not forming such a bridge.

Although melatonin can eliminate in a scavenger cascade up to 10 free radicals [239], it does not do this in any situation. In the presence of an enzymatic (indoleamine 2,3-dioxygenase) or pseudoenzymatic catalyst (hemin), just a single O_2•$^-$ molecule will be scavenged. Except for cells rich in melatonin, radical scavenging by this indoleamine may not suffice for quantitative elimination of oxidants, but still yield significant amounts of bioactive metabolites [49,247].

ELECTRON TRANSFER REACTIONS AND THE ROLE IN MITOCHONDRIA

Radical scavenging, as outlined above, reflects a property which should be of particular importance for melatonin's actions in mitochodria. As demonstrated by cyclic voltam-

metry, melatonin displays a pronounced preference for single-electron transfer reactions [40]. What is otherwise seen in respective experiments as electron exchange with a free radical, may be something of more profound biological relevance in this organelle, namely, a basis for interactions with components of the electron transport chain. In this context, the pronounced amphiphilicity of melatonin may be decisive. This allows the molecule to enter the mitochondrion, and also to interact with components in an amphiphilic or even hydrophobic environment. The same is valid for its metabolite AMK. Moreover, melatonin has been shown to reduce cytochrome c [321] and cytochrome oxidase [322]. In both cases, AFMK was formed under the experimental conditions, and the action of cytochrome oxidase was interpreted as an atypical kind of peroxidase reaction, but, with regard to the preference for single-electron exchange, the initial step would be that of a one-electron donation, regardless of whether pyrrole-ring cleavage takes place in secondary reactions. However, the donation of electrons to the electron transport chain may, under physiological conditions, lead to other reactions, too, provided that the resonance-stabilized radical intermediates of melatonin are sufficiently stable. It was, therefore, assumed that melatonyl radicals might take up electrons again from iron-sulfur cluster N2 of complex I, which represents a bottleneck of electron flux and from where electrons are easily leaking out [247]. This may be similarly possible with an AMK-derived cation radical, so that both melatonin and AMK might create an electron shuttle bridging between the site of electron overflow and downstream sites of electron acceptance, thereby attenuating electron leakage to oxygen and formation of superoxide-derived oxidants in the mitochondrion [14,49,247,323]. Whether the action at N2 is sufficiently described by this model, remains to be analyzed in detail. Other data describing a mitochondrial high-affinity melatonin binding site [16] may indicate that the indoleamine exerts a regulatory function at the amphipathic ramp of complex I. Unlike other bioenergetic agents that modulate electron flux by substrate-specific stabilization of N2, such as theanine, melatonin appears to exert a ligand-specific stabilization. An interaction of melatonin with complex I, thereby supporting electron flux, was also observed in studies using MPTP/MPP^+ [324,325].

A support of mitochondrial functions by melatonin has been repeatedly described in various models. In those based on intraorganellar changes, most of the data were obtained for submitochondrial particles. Such data do not reflect *in vivo* flux rates, but rather flux capacities. Nevertheless, a support of complex I and complex IV activities as well as rises in ATP formation were repeatedly observed, already at near-physiological concentrations [326-331]. The same was found with AMK in the nanomolar range [330]. Effects on activities of submitochondrial particles may either reflect enhanced stabilization of its components, by maintenance of active conformations or prevention of oxidative destruction, or enhanced *de novo* synthesis. The expression of subunits 1 – 3 of complex IV was, in fact, shown to be upregulated by melatonin [330]. Since eucaryotic gene expression requires time, but as mitochondrial effects are also seen in short-term experiments, this finding indicates another area of complexity of melatonin's actions. In fact, the situation is even more complicated insofar as melatonin also influences mitochondrial glutathione [331] and •NO formation and metabolism [261-264], factors which additionally modify the level of intramitochondrial oxidants and integrity of the components of the electron transport chain. The relevance of the interaction with such oxidants and prooxidants within the mitochondrion is supported by findings in septic mice, in which melatonin attenuated mitochondrial NO synthase activities and enhanced, at the same time, electron flux and ATP formation [261-264,332]. The interrelation between melatonin and changes in mitochondrial function are, moreover, of considerable gerontological interest, as will be discussed in a following section.

The preeminent role of mitochondria in the induction of apoptosis has been frequently studied and repeatedly reviewed [49,331,333,334]. Prevention of apoptosis was described many times, but the reasons for this may be diverse and depend on the mode of action of the respective inducers. In many cases, pharmacological doses of melatonin will have suppressed cell death by reducing damage by oxidants. In other cases, downregulation of •NO formation or of signaling molecules such as NFκB may have been decisive. However, protection from apoptosis by agents interfering with the respiratory chain was achieved by melatonin, as summarized elsewhere [49]. In the future, it will be of particular importance to judge the significance of melatonin's direct effect on the mitochondrial permeability transitition pore [205] and the contribution of this effect to rescuing of cells in the respective models.

In summary, the mitochondrial actions of melatonin seem to profoundly exceed antioxidant effects and reflect bioenergetic control at key steps of electron and proton flux.

ANTIEXCITOTOXICITY AND ANTICONVULSANT EFFECTS

In the context of its chronobiotic actions, melatonin is involved in sleep initiation and phasing [15,335-337]. These effects, which are mediated through mechanisms involving the SCN and the hypothalamic sleep switch, are working in humans and diurnally active vertebrates, but not in the nocturnal animals, in which sleep is associated with photophase. However, sedating effects by melatonin are also found in nocturnally active species, such as rodents [338-341]. They have to be, therefore, different from the chronobiotic actions, although they are also subject to circadian variations. These findings extend to other, related influences of antiexcitatory/antiexcitotoxic, anticonvulsant, anxiolytic, antihyperalgesic and antinociceptive nature [15,339,340,342-346], which go beyond the chronobiotic and sleep-promoting activities. Melatonin-induced decreases in locomotor activity have been even found in *Caenorhabditis elegans* [347], an organism that does not exhibit a robust melatonin rhythm, but rather temporal fluctuations which are largely explained by melatonin uptake from its food, *Escherichia coli* [3]. Melatonin's sedating capacity may, thus, appear as an ancient property. However, detailed analyses in vertebrates reveal another remarkable complexity of antiexcitatory mechanisms, which involve modulations of signaling by GABA and glutamate receptors [346,348], secondary effects by decreases of cytosolic Ca^{2+} *via* $GABA_c$ [349] or metabotropic $mGlu_3$ receptors [350], interference with neuronal NO synthase, by melatonin or its metabolite AMK

[15,44,49,199,245,247,285,286], changes in K^+ currents [351], and potentiation of strychnine-sensitive glycine-induced currents [352]. Although the relative contributions of these mechanisms should be different in the various forms of antiexcitatory actions and also depend on CNS regions, the attenuation of neuronal excitation seems to be a general theme. These antiexcitatory and, moreover, antiexcitotoxic effects are not only important in terms of seizure prevention and treatment, but also one basis, amongst others, of radical avoidance and antiapoptotic activities [49].

It may be also noted that melatonin has been successfully applied in a couple of otherwise intractable cases of children with subtypes of epilepsy and West syndrome [353-355]. In a sense, this treatment may have been even a causal one, since very low baseline melatonin levels were found in some intractable patients with temporal lobe epilepsy [356]. In these subjects, strong rises in melatonin were observed following seizures [356], so that a relationship between melatonin dysregulation and overexcitation should be worth of further investigation.

SECONDARY EFFECTS BY INFLUENCES ON OTHER HORMONAL AND NEURONAL SUBSYSTEMS

In its classical role as a hormone, melatonin is known to influence several important hormone systems, thereby initiating a host of secondary effects. In the context of seasonality, this has been extensively studied for the hypothalamic-pituitary-gonadal axis. In other species undergoing a seasonal fattening cycle, similar observations have been made for the thyroid-directed axis. In terms of circadian regulation, melatonin participates in the phasing of high-amplitude hormonal rhythms, for which glucocorticoids give the most impressive example. These actions beyond the scope of this review extend, however, to other functions, in particular concerning the immune system, which is also subject to seasonal variations and in which melatonin interacts with thyroid hormones, glucocorticoids and, sometimes, gonadosteroids [357-362]. Melatonin influences further hormonal systems, too, in a presumably circadian fashion. MT_1 receptors were recently demonstrated in both rat [363] and human [364] pancreatic islet cells. In the material from human islets, MT_1 was expressed only in α cells, melatonin did not decrease cAMP, but stimulated glucagon secretion in a $Ca^{2+}{}_i$-dependent manner, presumably another case of alternate G protein coupling and regulation of PLCβ. Signaling *via* G_q, PLC and Ca^{2+} is in accordance with another study [365]. Secondarily, the paracrine glucagon release stimulated insulin secretion, an otherwise well-known effect. The clinical relevance of interactions between melatonin and pancreatic hormones has been emphasized [365,366].

Another area of high interest is the interaction of melatonin with molecules participating in neuronal communication. This has been addressed above for some neurotransmitter systems and for •NO, but likewise applies to neurotrophic factors. Colocalization of MT_1 and expression of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) was demonstrated in glioma cells [367] and, more importantly, in neural and glial progenitor cells [368]. Moreover, melatonin was shown to stimulate the expression of these neurotrophic factors in stem cells [369]. Such findings may turn out to be of great value for maintenance and recovery of brain functions.

We are not aiming here to discuss the interactions of melatonin with any hormone, growth factor or other signaling molecule. However, it seems necessary to direct the investigators‘ attention to the numerous secondary effects melatonin exerts by modulating other communication systems within an organism and to the consequences thereof.

MELATONIN, AGING AND NEURODEGENERATIVE DISORDERS

The multiple protective actions of melatonin have been a reason for considering this indoleamine as a health-promoting and potentially life-extending agent. While there can be no doubt about the beneficial effects concerning neuroprotection [36,198,270,323,370], the issue of life extension is a very particular one and should not be simply subsumed under the keyword of “anti-aging“ with its, sometimes, non-scientific meaning. It is important to first perceive that a prolongation of lifetime, if it is observed, can have different causes and that the various model organisms and strains of them do not tell identical stories. If, e.g., a particular inbred mouse strain develops by age certain tumors from which the majority of animals die, melatonin’s chemopreventive action rather than antagonization of aging in the proper sense is responsible for the effect observed. This has, in fact, been demonstrated in laboratory mice [371-373]. Moreover, the outcome of a melatonin treatment may be different in nocturnal and diurnal organisms, and the ability or non-ability to grow throughout life makes another difference. Life extension (mean and maximum life span) by the considerable amount of, at least 50%, has been described in a gerontological model organism, the rotifer *Philodina acuticornis* [373,374]. Of course, this finding cannot be directly transmitted to vertebrate animals or to humans. Although several early studies in mammals may have suffered from methodological problems, and although some more recent evidence may still be circumstantial, anybody who has treated rodents with melatonin for extended periods of time will have observed the “Methusala syndrome“ [374], i.e., the healthy condition of old melatonin-treated animals concerning mobility, glossy fur, absence of skin inflammations and low osteoporosis. Usually these animals die without a prolonged phase in poor health state.

A beneficial role in aging animals requires explanations, and, with regard to the extreme pleiotropy of melatonin, the answers are by far not self-evident or banal. Several consequences result from the various protective actions, and the respective areas are, again, overlapping and influencing each other (Fig. **4**). As a result, they can finally contribute to healthy aging. Melatonin may already support good health conditions by influencing the circadian master clock, the SCN. Some indications for this may be found in a study on the aging degu, *Octodon degus*, a rodent from Chile, in which melatonin not only enhanced amplitude and activity/rest ratio of temperature and locomotor activity rhythms, but also led to a persistence of this effect after cessation of treatment [375]. This would require substantiation, also because some effect was already seen with the vehicle (0.01% ethanol in drinking water). Nevertheless, this possibility of a chronobiologically based amelioration is not at all absurd.

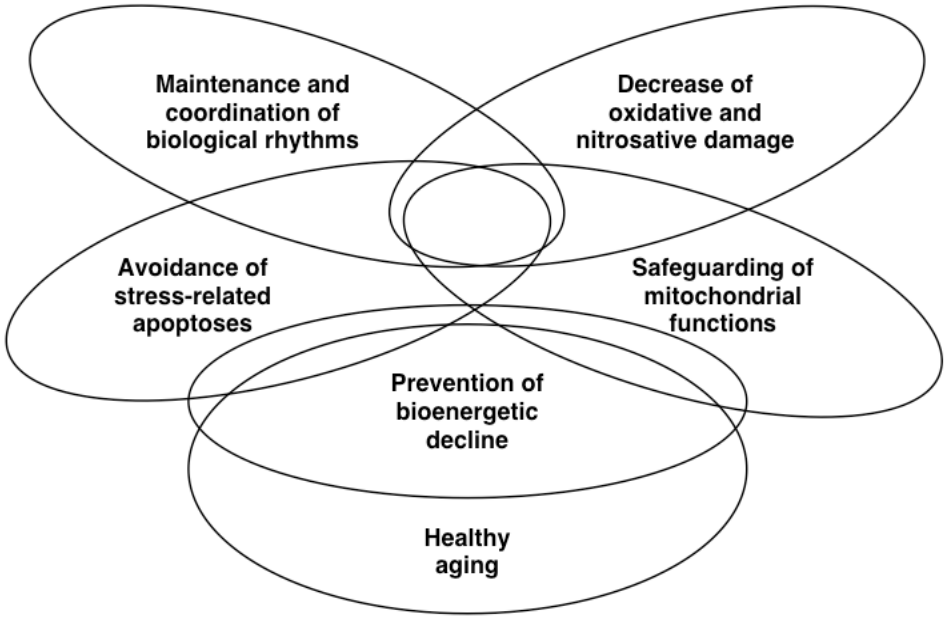

Fig. (4). The multiple consequences of melatonin's actions. Interrelations and mutual influences are indicated by the different degree of overlap, as depicted. The upper four areas comprise primary actions and are interrelated, as indicated by their specific intersections. Cell survival and mitochondrial function are basis for preventing bioenergetic decline, a universal feature of senescence.

Age-dependent declines in rhythmic functions [376] not only enhance morbidity and oxidative stress [247], but have been shown to be reverted by SCN transplantations, which also caused a certain extent of rejuvenilization [377].

Other actions of melatonin may contribute to an improved health state, too. Age-related inflammatory processes as well as susceptibility to inflammatory stimuli were demonstrated to be antagonized by melatonin in aged and, especially, senescence-accelerated rodents [378,379]. These finding may also extend to melatonin's immunomodulatory role in a broader sense. Amelioriations by attenuating oxidative and nitrosative stress have been repeatedly discussed [289,380,381]. More specifically, normalizations of mitochondrial functions including electron flux and ATP formation have been observed in old and senescence-accelerated mice [327-329,382], effects which may have considerably decreased oxidant formation [49,247,383].

We do not want to advocate here a premature use of melatonin in aging humans, without knowing many more details required for a safe application, especially in diseased subjects. However, very high doses (suppositories, ca. 300 mg/day) over a year have been applied without complications in ALS patients [370]. The use of melatonin by elderly persons is, at least, worth of further, detailed investigation. This can be done, of course, only on the basis of controlled production and storage of melatonin preparations. Possibly impure pills sold over the counter and stored by the user under inappropriate conditions leading to chemical decay are unsuitable and potentially harmful.

CONCLUSION

The main message of this article can be summarized by three terms: ubiquity, pleiotropy and complexity. Ubiquity, which includes numerous, if not all, taxa of living beings, already implies diversity of actions, and only a small number of organisms ouside the vertebrates has been investigated in details of physiological relevance. Future investigators should not only intend to re-discover effects already known from mammals or birds, but orient themselves at the physiology and ecology of the respective organism.

Pleiotropy has multiple levels and facets. This includes the role as a pineal hormone, but the effects are not limited to sites as SCN, median eminence and pituitary, but extend to numerous organs or tissues. In this chronobiological function, melatonin exerts effects *via* other hormonal subsystems, growth factors, neurotransmitters and the immune network, which lead to further secondary effects. One can truly state that melatonin, in this role, orchestrates numerous rhythmic functions of the body, many of which have not been considered in classical investigations. The situation may be entirely different in extrapineal organs which also synthesize melatonin. Since the extrapineal amounts of this indoleamine exceed by far that in the pineal gland and the circulation, the relevance of tissue melatonin demands further research. Although considerable differences in temporal dynamics and metabolism have been demonstrated, important issues such as synthesis, turnover and retention have not been fully addressed yet.

Complexity starts with the different binding sites, some of which are receptors of varying affinity, and continues with cell-specific coupling to G proteins and other downstream effects. Moreover, melatonin is the parent compound of several bioactive metabolites, whose contribution to the spectrum of melatonin's actions deserves further clarification.

Temporal orchestration, pleiotropy and complexity are fascinating phenomena. On the other hand, they complicate investigations in intact organisms and may be regarded as a caveat concerning interpretations and undesired side effects, in both physiological experiments and pharmacological use.

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