Melatonin, Mitochondrial Electron Flux and Leakage: Recent Findings and Resolution of Contradictory Results

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Abstract

Melatonin’s functions exceed by far the role of a hormone that mediates the signal darkness. In extrapineal tissues, it is produced in considerably higher quantities than in the pineal gland, and its local metabolism can be considerably different from the hepatic first-pass 6-hydroxylation/conjugation pathway of the circulating hormone. Tissue melatonin can be converted to various bioactive compounds, among which \(N^1\)-acetyl-5-methoxykynuramine (= AMK) is of particular interest in the context of mitochondrial function. Both melatonin and AMK act in multiple ways on mitochondria. Oxidative and nitrosative damage is prevented by upregulating antioxidant enzymes, decreasing NO synthase activities, and scavenging of reactive oxygen and nitrogen species. AMK very efficiently reacts with NO congeners to form a stable product, 3-acetamidomethyl-6-methoxy-cinnolinone (= AMMC). Decreases in NO levels attenuate peroxynitrite formation and damage by peroxynitrite-derived radicals as well as the direct inhibition of electron flux by NO. Under conditions of inflammation and sepsis, activities of Complexes I and IV, sometimes also Complex III, maintenance of the mitochondrial membrane potential and ATP formation can be substantially supported by melatonin or AMK. Melatonin exerts antiapoptotic actions by upregulating antiapoptotic Bcl proteins, preventing Bax translocation, and directly inhibiting the mitochondrial permeability transition pore, at low affinity (\(K_i = 0.8 \mu M\)). Melatonin interacts, at high affinity (\(K_i = 150 \text{ pM}\)), with a binding site at the amphipathic ramp of Complex I, thereby presumably modulating electron flux. In aging and, especially, senescence-accelerated mice, enhanced electron leakage is reduced by melatonin. Depending on conditions, e.g., state 3 or state 4 respiration,
melatonin may either increase or decrease electron transport. This is not necessarily contradictory, but may be explained by either removing secondary bottlenecks of electron flux or by regulating the primary bottleneck at Complex I. Additional sirtuin-mediated actions of melatonin may favor cellular metabolism via mitochondrial growth stimulation.

**Keywords:** Aging, Electron leakage, Electron transport chain, Inflammation, Melatonin, N\(^1\)-Acetyl-5-methoxykynuramine, Nitric oxide

### Introduction

A relationship between melatonin and mitochondria may not be immediately obvious to everybody. At first glance, melatonin may only appear as the pineal hormone which mediates the signal “darkness”, but, in fact, it has numerous additional functions [39,48,132]. It is produced in various extrapineal sites [39,48], attains extrapineal quantities exceeding by orders of magnitude those found in pineal gland and circulation [10,39,44,48], and exhibits numerous protective actions [37, 44,48,65,100,110,114], including antioxidative and antinitrosative defense as well as interference with stress- or hormone-induced apoptosis [100,107,123,124]. Mitochondria, sites of respiration, are involved in the intrinsic apoptotic pathways, represent a major source of oxidants as a consequence of electron leakage, and have become of particular interest with regard the numerous mitochondrial diseases meanwhile identified [19,64,67,84,104]. Thus, a deeper view reveals numerous relationships between melatonin and mitochondria (Table 1), which have meanwhile received substantial support by experimental data. This review will summarize such findings, but also intends to resolve some problems which have arisen by observations of either enhancements or attenuations of electron flux under the influence of melatonin. As will be shown, these findings are not really contradictory, as it might appear at first glance, but rather have to be attributed to different situations. In the end, both effects will turn out to be beneficial and reveal that melatonin facilitates adaptation to different challenges.

### Table 1. Overview of the mitochondrial actions of melatonin and AMK

<table>
<thead>
<tr>
<th>Action</th>
<th>Area of relevance</th>
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<tr>
<td>Protection of ETC proteins from oxidative damage by upregulation of antioxidant enzymes and radical scavenging by melatonin and AMK</td>
<td>Avoidance of oxidative stress</td>
</tr>
<tr>
<td>Antioxidative protection of lipids, in particular, cardiolipin, by melatonin and, perhaps, AMK</td>
<td>Maintenance of favorable lipid environment for Complexes III and IV</td>
</tr>
<tr>
<td>Downregulation of iNOS by melatonin and AMK, inhibition of nNOS by AMK, scavenging of NO congener by AMK</td>
<td>Inflammation, sepsis, and, perhaps, neurodegeneration</td>
</tr>
<tr>
<td>Direct and indirect support of activities of Complex I,</td>
<td>Inflammation, sepsis</td>
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Complex IV, and, perhaps, also Complex III

Binding of melatonin to amphipathic ramp of Complex I

<table>
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<tr>
<th>Reduction of electron throughput and leakage</th>
<th>Control of electron flux rate</th>
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<tr>
<td>Upregulation of antiapoptotic Bcl proteins, e.g. Bcl-2, and prevention of Bax translocation</td>
<td>Aging</td>
</tr>
<tr>
<td>Direct inhibition of permeability transition pore</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Sirtuin upregulation, with possible consequences for mitochondrial growth</td>
<td>Aging</td>
</tr>
</tbody>
</table>

For references see current text; acronyms: AMK = N\(^1\)-Acetyl-5-methoxykynuramine; ETC = electron transport chain; iNOS = inducible nitric oxide synthase; nNOS = neuronal nitric oxide synthase

Both Melatonin and Its Metabolite AMK are Relevant to Mitochondria

As shown in Table 1, not only melatonin, but also its metabolite N\(^1\)-acetyl-5-methoxykynuramine (= AMK) exerts mitochondrial effects. AMK derives from the parent compound via the pyrrole ring cleave pathway (Fig. 1). This metabolic route is particularly relevant in non-hepatic tissues [38,39,44,46,48], especially in the central nervous system [46,56], and in myeloperoxidase-expressing leukocytes [28,52,141]. The primary metabolite, N\(^1\)-acetyl-N\(^2\)-formyl-5-methoxykynuramine (= AFMK), is formed by numerous enzymatic, pseudoenzymatic, photocatalytic and radical reactions [38,52]. Since mitochondria represent, in an average animal cell, a major source of reactive oxygen species, it may be of particular importance that AFMK is a typical melatonin product formed by interaction with free radicals.
Fig. 1. Overview of the kynuric pathway of melatonin metabolism, including reactions of AMK with reactive nitrogen species. For details see refs. [41,52]. [37,38,49,51,106,130]. AFMK is easily deformylated by arylamine formamidase [37,38,44,48,51], hemoperoxidase [37,38,44,133], or by UV of short wave-length [48,126].

Melatonin has become known as a remarkably potent scavenger of various free radicals and also as an efficient regulator of antioxidant and prooxidant
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enzymes [34,37,44,49,51,110,114,122,129,133,134]. AFMK is less reactive, because of its preference for two-electron transfer reactions, but was also shown to display cell-protective properties [37,131]. However, the deformylated secondary product AMK is, again, a potent scavenger of hydroxyl, peroxy, carbonate and some heteroaromatic radicals [52,115,135]. Additionally, AMK turned out to efficiently scavenge reactive nitrogen species, including all three NO congeners [34,38,41,42], effects which may be of particular importance for mitochondrial function, e.g., in situations of inflammation and sepsis. In this regard, AMK differs from melatonin by forming a stable product, 3-acetamidomethyl-6-methoxycinnolinone (= AMMC), which does not re-donate NO like nitrosomelatonin and other N-nitrosated aromates [41,42]. Moreover, AMK has been shown to be a high-affinity inhibitor of neuronal NO synthase [71], and is discussed as a regulator of inducible NO synthase in certain models, in addition to other antiinflammatory actions [45,62,83]. Its relevance to mitochondrial function has been repeatedly discussed [1,37,43,128].

Protection from Oxidative and Nitrosative Stress, Including Inflammation and Sepsis

By virtue of its multiple antioxidative actions, which include upregulations of glutathione peroxidase, glutathione reductase, γ-glutamylcysteine synthase, sometimes also catalase, mitochondrial Mn- and cytosolic Cu,Zn-superoxide dismutases, and glucose-6-phosphate dehydrogenase as a source of reducing equivalents in support of glutathione reduction (summarized in refs. [37,44,108,109,111,114]), melatonin protects mitochondrial DNA, proteins and lipids, and also preserves mitochondrial ultrastructure, as shown in numerous studies using different models of challenge by oxidotoxins, inflammation, ischemia-reperfusion, and UV exposure [2,22,58,59,70,80,86,95,99,101,114,138,140]. A mitochondrial peculiarity of these studies concerns the protection of cardiolipin from peroxidation, since this molecule is required for maintaining a suitable lipid environment of Complexes III and IV [80,101,102], and is, therefore, important for avoiding secondary bottlenecks in the electron transport chain (ETC), which would have consequences for eventual electron backflow and leakage, and thus, additional oxidant formation.

Oxidative stress can also lead to direct impairment of electron flux through the ETC, e.g., by protein carbonylation, ubiquinone oxidation, interaction with sulphhydryl groups and protein-bound iron. In fact, corresponding observations, including protection and restoration of ETC function by melatonin, have been made with excitotoxins, such as kainic acid, and neurotoxins more directly affecting mitochondria, such as 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (= MPTP)/1-methyl-4-phenylpyridinium (= MPP⁺), which also cause oxidative stress and are indirectly or directly associated with ETC dysfunction [14,16,17,63,90,136,142,143].

Other causes of ETC impairment result from reactions of NO and its reactive metabolites. Electron leakage from the ETC to molecular oxygen leads to the superoxide anion (O₂•⁻), which combines at substantial rates with the NO
radical (•NO) to form peroxynitrite (ONOO\(^-\)), because of similar affinities of O\(_2\)\(^•\) to superoxide dismutases and to •NO. The highly diffusible •NO, whether produced in the cytosol or by mitochondrially targeted NO synthases in the matrix, can contribute to mitochondrial peroxynitrite formation. Peroxynitrite, apart from being highly reactive itself, combines with either a proton or CO\(_2\), the latter being highly abundant in the matrix, and these adducts decompose to radical pairs, •NO\(_2\) and •OH, or •NO\(_2\) and CO\(_3\)• (the carbonate radical), respectively. The irreversible mitochondrial damage typically seen in septic shock is currently believed to be mainly caused by chemical reactions of peroxynitrite and its reactive products [3,7,24]. Melatonin scavenges especially •OH, ••NO, perhaps also peroxynitrite (which is difficult to discriminate from actions of its decomposition products) [5,37,70,111-113,126,144,145] and, with particular relevance to high mitochondrial CO\(_2\) concentrations, CO\(_3\)• [5,49,144,145]. However, no stable nitrated products are formed from melatonin [144], and N-nitrosomelatonin easily decomposes [6], so that reactive nitrogen species are kept in the system when metastable intermediates transiently appear from interactions with •NO or peroxynitrite. However, melatonin additionally downregulates NO synthase subforms, thereby also attenuating peroxynitrite formation [24,37,111,112,114,128]. As briefly mentioned in the previous section, AMK is likewise capable of efficiently scavenging •OH [37,52,115], •NO – as well as its congeners NO\(^+\) and HNO\(^-\) [34, 38,41,42] – (Fig. 1), and CO\(_3\)• [34,38,52]. Contrary to melatonin, not only a stable NO adduct, AMMC, is formed [41,42], but AMK is also stably nitrated by the •NO\(_2\)/CO\(_3\)• radical pair deriving from the peroxynitrite-CO\(_2\) adduct [34,38]. Therefore, the reactive nitrogen species scavenged are eliminated from the system. Moreover, AMK is a potent inhibitor of, at least, neuronal NO synthase [71], so that the metabolite can contribute to melatonin’s suppressive actions on NO and peroxynitrite formation.

With regard to ETC function, the inhibition of NO production has different aspects. First, reduced formation of reactive nitrogen species diminishes both oxidative and nitrosative damage to ETC components, which would include protein damage by tyrosine nitration, nitrosothiol formation and transnitrosation reactions [9,18]. Complex I seems to be particularly vulnerable to S-nitrosation, and this modification was found to result in increased superoxide anion formation as a consequence of enhanced electron leakage [18]. The second aspect concerns the inhibition of ETC components by the •NO molecule, which can interact with cytochrome hemes as well as with protein sulfhydryls. Therefore, high •NO levels can substantially suppress electron flux through the ETC. These effects are, in principle, reversible [23], so that basal or slightly enhanced concentrations of •NO may only moderately reduce flux rates, whereas high levels, as occurring under conditions of septic shock, with a contribution of irreversible secondary changes by peroxynitrite and its metabolites can fully suppress respiration [23].

Measurements of mitochondrial functions in models of inflammation and, even more, of septic shock, reflecting a most dramatic suppression of electron flux, proton translocation, decline of mitochondrial membrane potential (ΔΨ\(_m\)) and ATP formation, impressively demonstrate the beneficial effects of melatonin, given in pharmacological doses. In a murine model of septic shock using cecal ligation and puncture, several studies in skeletal muscle [26,77], heart [25], and diaphragm [77,78] showed unanimously ETC failure including declining ATP
synthesis, which were associated with oxidative damage to mitochondria. All changes were widely reverted by melatonin. The involvement of NO was clearly demonstrated, since dysfunction and all other deleterious changes were absent in knockout mice deficient in inducible nitric oxide synthase (iNOS). Moreover, rises in mitochondrial iNOS activity and nitrite levels in wildtype mice were directly demonstrated, which were, correspondingly, absent in the knockouts [78]. A mitochondrially targeted iNOS subform was identified and shown to be decisive [24-26,77-79]. The suppression of NO formation by melatonin was associated with elevated activities of ETC complexes, improved ATP formation, maintenance of the mitochondrial membrane potential and reduced formation of superoxide and hydrogen peroxide [79].

In this context, it should be noted that melatonin additionally downregulates expression and activity of neuronal NOS (nNOS) [12,13,72,111,137], so that similar protective effects as observed with iNOS-dependent mitochondrial damage may be exerted in the central nervous system, especially in situations of excitotoxicity, associated with elevated Ca\(^{2+}\) influx and nNOS activation. The highly efficient inhibition of nNOS by the brain metabolite AMK, demonstrable already at \(10^{-11}\) M [71], may contribute to mitochondrial protection.

Melatonin, Mitochondria and Longevity

Counteracting severe conditions like septic shock requires high pharmacological doses. However, effects of melatonin are also demonstrable at low, near-physiological concentrations In isolated mitochondria challenged by 100 \(\mu\)M \(t\)-butylhydroperoxide, melatonin was shown to normalize the glutathione redox equilibrium (GSH/GSSG) and to prevent oxidative inactivation of glutathione peroxidase and glutathione reductase are already between 1 and 100 nM [81]. Notably, these effects were not observed with other antioxidants, such as N-acetylcysteine, ascorbate or Trolox, the more water-soluble tocopherol analog, except for a normalization of the GSH/GSSG ratio at elevated Trolox concentrations of 1 mM. For stoichiometrical reasons, these effects cannot be easily explained on the basis of radical scavenging, and also the inefficiency of the other antioxidants would speak against such an interpretation. This argument remains valid, even if a scavenger cascade is considered, which does exist in the case of melatonin. In conjunction with its radical-scavenging metabolites, a total of up to 10 free radicals can be scavenged per melatonin molecule [122]. However, this number is not always reached, depending on the reaction partners, especially in the presence of chain-terminating radicals. Alternately, repetitive actions of a single melatonin molecule (or kynuric metabolite) may be discussed, which would imply organic redox cycling involving the scavenger and its organic radical formed by interaction with another radical. However, no direct evidence is available for such an effect at the mitochondrial level, and only indirect hints from \textit{in vitro} studies exist, showing potentiating effects of melatonin in combination with other antioxidants [105,130]. Nanomolar concentrations of melatonin were, moreover, sufficient for stimulating Complex I and IV activities and ATP synthesis in submitochondrial particles [1,82], and a similar efficacy was reported for AMK [1]. Although isolated mitochondria and submitochondrial particles may tell stories different...
from the *in vivo* situation, a physiological role of melatonin seems to be possible or even likely.

One of the most exciting aspects of both mitochondrial function and melatonin is their role in aging and age-related diseases. As melatonin concentrations are usually declining throughout life, and since beneficial effects of the indole-amine supporting a healthy state in mice have be reported (for review see ref. [103]), a role in safeguarding mitochondrial electron flux and avoidance of electron leakage seems worth of future attention. Anyway, the role of mitochondria in aging has been discussed since long in several variations, including the aspects of radical formation and damage as well as respiratory capacity [32,33,53,54,87].

From the viewpoint of longevity, the role of electron flux capacity is not immediately clear. Studies on electron flux rates in ETC mutants of *Caenorhabditis elegans* have led, at first glance, to entirely divergent results concerning life span. A mutation in a Complex I subunit, *gas-1*, and also another one in the SDHC subunit of Complex II, *mev-1*, caused reduced rates of oxidative phosphorylation and a shortened live span. However, the *clk-1* mutation, which affects formation of the *C. elegans*-specific coenzyme Q9 (CoQ9) and causes accumulation of other ubiquinone analogs (demethoxy-CoQ9 and rhodoquinone9) [61], reduced oxidative phosphorylation was instead associated with increased longevity [60]. Thus, electron flux is not *per se* an indicator of longevity. The *clk-1* mutation may only reflect some general slowing down of temporal processes, in the absence of demonstrable changes in radical formation and damage [8], an effect which can extend life span especially in an ectothermic organism like *C. elegans*. Combinations of mutations predicted to have reduced Complex I activity exhibited unexpectedly long life-spans [60]. On the other hand, the suppression of *gas-1* normalized the rate of oxidative phosphorylation, reduced oxidative damage to mitochondrial proteins and prolonged life [60]. Collectively, these findings may appear to be puzzling, but they are nevertheless informative. First, one can state that decreases in electron flux can, in principle, be beneficial, and this conclusion may, e.g., also apply to moderate reductions by NO. However, the decisive question is that of the relationship between the sites of electron flux reduction and the respective consequences for electron backflow and leakage, as will be discussed in the next section. Therefore, the answer appears to be a matter of the position of the respective bottlenecks in the ETC.

Studies on the aging process have not only been conducted in wild-type, but also senescence-accelerated animals, such as SAMP8 mice, which can be compared with the normally aging SAMR1 mice sharing the same genetic background. Many investigations on the effects of melatonin in SAMP8 are only indirectly related to mitochondria and conducted in models of inflammation, induced or age-dependent oxidative damage, but include the prevention of apoptosis [11,15,35,36,92,120]. Other studies demonstrate influences of melatonin on respiratory functions [94,96-98,118,119,121]. Despite some differences with regard to tissue (liver, cerebral cortex, heart, diaphragm), mode of melatonin administration via drinking water or by i.p. injection, age (oldest animals tested 10 or 12 months), and some other variations in detail, the results collectively show the same changes at advanced age, which were largely reverted by melatonin: SAMP8 animals exhibited reductions in the respiratory control index (RCI), in
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state 3 respiration, in dinitrophenol-uncoupled respiration, which reflects to some extent the respiratory capacity, and also in the ADP/oxygen ratio, in the ATP level, in conjunction with decreases in the activities of Complexes I and IV.

These findings should not be interpreted in a simplistic way. In one study [98], state 4 respiration was found to be increased in aged SAMP8. This would be in agreement with earlier results from aging wild-type mice showing rises instead of declines in Complex IV activity [127]. However, the respiratory effects were associated with decreases in the GSH/GSSG ratio and in glutathione peroxidase activity, along with rises in lipid peroxidation and protein carbonyl. Again, these changes were widely reverted by melatonin. Another complication results from findings in middle-aged rats, as observed in isolated mitochondria, which were either treated with melatonin in vitro or obtained from animals receiving the hormone via the drinking water [116,117]. In these mitochondria, state 4 respiration remained unaffected, whereas state 3 respiration was reduced by melatonin. The results were interpreted in terms of avoidance of excess electron flux, which would, at the same time, attenuate electron leakage and, thus, oxidant formation and damage. Recently, isolated rat liver mitochondria were shown to respond dose-dependently to melatonin by decreasing oxygen consumption, lowering ΔΨₘ and, consequently, attenuating the formation of O₂•⁻ and its metabolite H₂O₂ [79]. At the same time, activities of Complexes I, III, and IV as well as ATP synthesis were found to be increased. These findings, which may appear, at first glance, contradictory, may be interpreted in terms of higher ETC efficiency, thus, avoiding energy losses by electron leakage. This may also indicate that melatonin helps to prevent secondary bottlenecks in the ETC, which can cause electron backflow. In this context, one should recall that electron flux is not a steady, continuous process with only slow temporal variations, but rather highly dynamic with pulse-like characteristics, which generates flashes of O₂•⁻ formation reflecting bursts of electron overflow [139].

The important message from these results is that rises in activities of ECT complexes, which anyway represent flux capacities of isolated submitochondrial particles rather than real in vivo flux rates, can be enhanced in the presence of moderately reduced electron flow and proton pumping. This may be particularly important for understanding age-related changes and effects of melatonin in aging animals. What may appear as a contradiction, may be resolved on this basis. Although aging animals, including SAMP8, may exhibit under certain conditions decreases in ETC complex activities – which are reverted by melatonin –, other conditions can lead to rises in electron flux and even ΔΨₘ, which may be attenuated by melatonin. In a specific tissue, mitochondria differently located within a cell may even vary with regard to aging. This is particularly valid for cardiomyocytes concerning the subsarcolemmal and interfibrillary mitochondria. While the subsarcolemmal subpopulation does not show profound signs of dysfunction during aging, the interfibrillary mitochondria exhibit decreased Complex III and IV activities and increased electron leakage, especially from the Qo site of Complex III, but antioxidative protection especially of cardiolipin can largely restore interfibrillary mitochondrial function [27,57,73-75,88,89].

Therefore, the divergent changes observed show that, in one case, an age-dependent reduction of ETC complex activities may reflect damage to components of the respiratory chain, but a rise can indicate dysfunction as well. Both
conditions may lead to enhanced electron leakage and damage. In a comparative study on activities of the ETC complexes in different aging mouse tissues, the particular imbalance of flux capacities between the complexes became evident [66]. This should create secondary bottlenecks, electron back- and overflow.

Whatever ETC modulation by melatonin may directly cause, the reduction of mitochondrial oxidative and nitrosative stress is collectively and unanimously evident from all pertinent studies. Insofar, it may be not surprising that the indoleamine was shown to increase half-life and maximal life span in SAMP8 mice [121]. However, at the present state of our knowledge, additional indirect mitochondrial effects of melatonin cannot be ruled out, especially in relation to actions of aging-suppressor genes. With regard to the relationships between mitochondria and sirtuins, whose subforms are either targeted to mitochondria or implicated in mitochondrial growth, we had recently suggested to experimentally follow the possible connection between melatonin and sirtuins [50]. This would be especially of interest because of sirtuin-mediated effects of the redox state on mitochondrial growth [33], and the highly evident redox-related improvements by melatonin. In fact, a recent study has reported upregulations of sirtuin 1 expression in SAMP8 mice by melatonin [36]. Sirtuin 1 shares regulatory endpoints with another aging suppressor, klotho, by controlling FoxO transcription factors [76]. Mitochondria-specific antioxidant actions of klotho [55,125] and some additional overlap with melatonin signaling pathways beyond cAMP downregulation [40] exist, so that relationships between the indoleamine and klotho may be also of future interest.

**Melatonin’s Mitochondrial Sites of Action**

Direct influences of melatonin on electron flux, proton pumping and adjustment of $\Delta \Psi_m$, along with reductions of electron leakage are imaginable in two ways. (i) Melatonin or, alternately, one of its redox-active metabolites such as AMK [37,43,47], may interact with the ETC by electron exchange, thereby modulating net electron flux, enabling a re-cycling of electrons and thereby bridging, in terms of an electron shuttle, between components of the ETC [37,43,47]. The other possibility (ii) would be a direct interaction of the indoleamine with a regulatory high-affinity binding site. Until recently, only a low-affinity binding site ($K_i = 0.8 \mu M$) associated with inhibition of the mitochondrial permeability transition pore was known [4]. This may explain protective, antiapoptotic effects of melatonin at elevated local concentrations of the indoleamine. In fact, mitochondrial accumulation of melatonin has been described [79,85]. However, this effect would not explain the regulation of electron flux through the ETC. Meanwhile, a high-affinity binding site has been identified, whose experimental details are not yet published, although the relevant findings and conclusions have already been cited [39,46,48,50]. For this binding site ($K_d = 150 \text{ pM}$), a total number of 30 fmol/mg protein has been determined in rat brain mitochondria. Displacement studies with the ligands capsaicin and dopamine, which are specific for the subunit carrying the iron-sulfur cluster N2, indicated a localization at the amphipathic ramp of Complex I. These findings offer new possibilities for understanding direct regulatory actions of melatonin at the mitochondrial level, and they indicate a control of
electron flux by the indoleamine at the first control point of the ETC at its entrance, being at the same time the primary bottleneck.

Usually, electron flux can be limited especially at Complexes I and IV [69]. The first one represents the entrance bottleneck, which may be circumvented by feeding electrons via Complex II, whereas limitations at Complex IV physiologically depend on oxygen availability. Regulation at the entrance to a pathway should be regarded as a common means for controlling metabolic throughput. In this regard, the observation that melatonin curtails state 3 respiration [116,117] might be interpreted in terms of a melatonin-dependent reduction of electron uptake at Complex I, which would reduce electron leakage and, thus, radical formation. However, melatonin may not just act as a usual negative ligand, but might exerts its effects depending on the conformation of Complex I components, thus allowing rises in electron flux when the proton-moving force is momentarily reduced and $\Delta \Psi_m$ is declining too much for sufficiently maintaining ATP production. Self-regulatory, conformation-based mechanisms should be built-in in the ETC, already with regard to variations in oxygen availability, and independently from melatonin, but they may be controlled by the indoleamine.

The second bottleneck at Complex IV becomes relevant already under physiological conditions, because of the frequently occurring limitations in oxygen supply. Reduced oxygen availability is particularly important in the context of aging and degenerative disorders, and includes impaired vascular function. Partial or almost complete blockade at Complex IV causes retardation of electron transport, eventually backward electron flow and increases in electron leakage, which may occur at Complexes I and III (Fig. 2). Leakage at Complex III may be particularly important in Complex IV dysfunction, whether caused by lack of oxygen or by damage to Complex IV subunits or to cardiolipin, which is also required for its normal function, as mentioned above. Moreover, interruption of the intramonomer electron transfer between the two hemes at the Qo site in Complex III has been identified as another cause of electron dissipation [31], in conjunction with damage to Complex III subunits or, again, to cardiolipin.

Upon additional feed-in via Complex II, as in many experimental systems using isolated mitochondria, backward electron flow is possible at Complex I, so that its by-passing can be only favorable if Complex IV is not rate-limiting (Fig. 2). In this case, the iron-sulfur cluster N2 is the major site of electron leakage. While cysteines in the respiratory chain are anyway prone to oxidative damage [91], N2 at the amphipathic ramp of Complex I is particularly vulnerable to reactive oxygen species, because this peptide arm containing both hydrophilic and lipophilic domains extrudes to the matrix and is, therefore, exposed to water, oxygen and reactive intermediates. Damage at this site not only reduces electron throughput, membrane potential and ATP production, but can also become, via electron dissipation, the starting point of a vicious cycle, which, moreover, may ultimately limit life span [21].

The particularly vulnerable Complex I is prone to damage by oxidants as well as nitrosants, and the resulting protein modifications lead to enhanced electron dissipation to oxygen. Anyway, Complex I is a major site of electron leakage especially to the matrix side [29,30,68,69,93]. Enhanced superoxide formation by electron leakage was particularly observed as a result of S-nitrosation at Complex I [18]. Additionally, the relationship between proton pumping and
electron dissipation at Complex I deserves particular attention. Inhibition of proton pumping by Complex I enhanced superoxide formation, but ETC uncoupling reduced electron leakage [20,21]. These findings clearly support the idea that interruption or momentary retardation of proton and/or electron flux enhance electron leakage, whereas acceleration as caused by uncoupling leads to suction of electrons and, consequently, reduction of superoxide formation. Regulation of electron flux at Complex I may, therefore, be crucial to radical avoidance, and the observed binding of melatonin at the amphipathic ramp could be of high relevance.

Fig. 2. Electron leakage and backflow in different situations of damage to ETC complexes. Abbreviations: Com = complex; CoQ = coenzyme Q; Cyt C = cytochrome C.
Conclusion

The findings summarized here clearly demonstrate that melatonin is a regulator of mitochondrial function, capable of improving electron flux, avoiding electron dissipation and attenuating damage by free radicals. To understand melatonin’s mitochondrial role, it is, however, necessary to interpret the results in a contextual and non-simplistic way. One can neither say that enhancement of reduction of electron flux is per se beneficial or detrimental. In a blockade of electron flux by NO and peroxynitrite, as occurring under conditions of inflammation or, in the extreme, sepsis, the support of electron flux and ATP formation is, without any doubt, beneficial. However, this context has not to be confused with processes occurring during normal aging, including poor coupling between ETC complexes, or limited oxygen supply because of vascular impairments. In these cases, the decisive question is not that of whether the electron flux is moderately enhanced or decreased, but rather that of where the electron are directed to. A rise in electron flux, when occurring in conjunction with a secondary bottleneck, e.g. at Complex IV, or poor coupling between ETC components, will be certainly detrimental. However, in the absence of a secondary bottleneck, the support of electron flux may improve energy supply and energy-dependent cellular functions. Interpretations of the observed positive effects of melatonin on longevity, as correlated with changes of mitochondrial dynamics, have to consider this duality.

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