Melatonin-mitochondria Interplay in Health and Disease

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Abstract: Although two main hypotheses of mitochondrial origin have been proposed, i.e., the autogenous and the endosymbiotic, only the second is being seriously considered currently. The 'hydrogen hypothesis' invokes metabolic symbiosis as the driving force for a symbiotic association between an anaerobic, strictly hydrogen-dependent (the host) and an eubacterium (the symbiont) that was able to respire, but which generated molecular hydrogen as an end product of anaerobic metabolism. The resulting proto-eukaryotic cell would have acquired the essentials of eukaryotic energy metabolism, evolving not only aerobic respiration, but also the physiological cost of the oxygen consumption, i.e., generation of reactive oxygen species (ROS) and the associated oxidative damage. This is not the only price to pay for respiring oxygen: mitochondria possess nitric oxide (NO•) for regulatory purposes but, in some instances it may react with superoxide anion radical to produce the toxic reactive nitrogen species (RNS), i.e. peroxynitrite anion, and the subsequent nitrosative damage. New mitochondria contain their own genome with a modified genetic code that is highly conserved among mammals. The transcription of certain mitochondrial genes may depend on the redox potential of the mitochondrial membrane. Mitochondria are related to the life and death of cells. They are involved in energy production and conservation, having an uncoupling mechanism to produce heat instead of ATP, but they are also involved in programmed cell death. Increasing evidence suggest the participation of mitochondria in neurodegenerative and neuromuscular diseases involving alterations in both nuclear (nDNA) and mitochondrial (mtDNA) DNA. Melatonin is a known powerful antioxidant and anti-inflammatory and increasing experimental and clinical evidence shows its beneficial effects against oxidative/nitrosative stress status, including that involving mitochondrial dysfunction. This review summarizes the data and mechanisms of action of melatonin in relation to mitochondrial pathologies.

Keywords: Oxidative stress, nitric oxide, mitochondrial diseases, melatonin therapy.

INTRODUCTION

Mitochondrial Functions

Mitochondria are specialized in the rapid oxidation of NADH and FADH produced during glycolysis, Krebs cycle and β-oxidation of fatty acids by the transfer electrons from these precursors to oxygen. The electron transport chain (ETC) is a system of oxido-reductan protein complexes (complexes I, II, III and IV) and two electron carriers (coenzyme Q and cytochrome c) in the inner mitochondrial membrane. According to the chemiosmotic hypothesis, C-I, C-III and C-IV pump protons yielding a proton gradient along the mitochondrial inner membrane. This proton gradient is a source of free energy that is dissipated when protons enter the inner mitochondrial membrane *via* the ATP synthase [1]. During this process, ADP is phosphorylated to ATP. Mitochondrial DNA codifies several components of the respiratory complexes: 7 of C-I; cyt b corresponding to a cofactor of C-III; 3 of the C-IV and 2 of the ATP synthase [2]. In aerobic cells, oxidative phosphorylation (OXPHOS) is responsible for production of 90-95% of the total amount of ATP, and more than 90% of the respiratory phosphorylation is catalyzed by ATP synthase, an enzyme converting the respiratory chain-produced electrochemical proton potential difference ($\Delta\mu_H^+$) into ATP [3]. The respiration-produced $\Delta\mu_H^+$ can be utilized by mitochondria not only to form ATP but also to support some other energy-consuming processes including transport of certain solutes from the cytosol to the matrix. Mitochondria are also of central importance for physiological Ca^{2+} handling, acting as a reservoir for Ca^{2+}. Mitochondrial Ca^{2+} regulates the activity of mitochondrial dehydrogenases as well as nucleic acid and protein synthesis [4]. Several factors have been proposed to regulate respiration including ATP (respiratory control), Ca^{2+} and proton leak [5].

Dissipation of energy as heat to maintain body temperature at a level higher than in the environment is another important function of mitochondria. The mechanism is referred to as thermoregulating uncoupling of respiration and phosphorylation. Uncoupling results in dissipation of the respiratory chain-produced $\Delta\mu_H^+$ due to increased proton conductance of the inner membrane. Thus, energy released by respiration is dissipated immediately as heat without formation and hydrolysis of ATP. Non-esterified fatty acids proved to be compounds mediating the thermoregulatory uncoupling. They operate as protonophorous uncouplers with the help of special uncoupling proteins (UCPs) [6]. It is known that in

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the rat, T_3 influences in rat the expression of nine nuclear-encoded respiratory genes, regulates mitochondrial RNA synthesis through both the activation of a mitochondrial transcription factor A (TFAM) and the specific mitochondrial T_3 receptors, and stimulates the expression of the mRNA for UCP2 and UCP3 [7-9].

UCPs are proton transporters across the inner mitochondrial membrane, driven only by the membrane potential [10]. One of the proposed mechanisms for proton transport involves fatty acids, which provide one or more carboxyl groups along the translocation channel, and deliver their protons to an acceptor group (carboxyl groups of the other fatty acid), which in turn delivers protons into the matrix. The UCP family, including UCP1, UCP2, UCP3, UCP4 and UCP5 form a subfamily within the gene family of mitochondrial anion carriers [11]. While UCP1 is known to play an important role in regulating heat production during cold exposure, possible roles for other UCPs are yet controversial and they may include: regulation of ATP synthesis; control of reactive oxygen species (ROS) production by mitochondria; control of adaptative thermogenesis in response to cold and diet, and regulation of fatty acid oxidation [12, 13]. These UCPs probably do not transport protons except in the presence of specific activators [14]. Fatty acids, ROS and free-radical-derived alkenals are activators of proton transport through UCPs, whereas purine nucleotides are inhibitory [12, 13]. A failure to control ROS damage can cause the collapse of multiple vital functions, including mitochondrial energy conservation, culminating in loss of membrane integrity and cell death by necrosis and/or apoptosis [6]. The UCP-dependent uncoupling mitochondrial depolarization reduces ROS production and thus inhibits the permeability transition pore (PTP) preventing the proapoptotic cascade. It was suggested that decreased superoxide anion radical (O_2^{-}•) production occurs because uncoupling increases the rate of electron transport, diminishing the probability that electrons will escape from the respiratory chain and interact with molecular oxygen. Other functions, including UCP2 and UCP3 mediation of ROS signaling and insulin secretion and transport or export of fatty acids and fatty acid peroxides, as well as a direct interaction between UCP2 with the antiapoptotic bcl-2 family, also have been proposed [6, 14].

Mitochondrial DNA

Mitochondrial DNA consists of a closed-circular, doubled-stranded DNA molecule of about 16.6 kbp. Most information is encoded on the heavy (H) strand, with genes for two rRNAs, 14 tRNAs, and 12 polypeptides. The light (L) strand codes for 8 tRNA, and a single polypeptide. All 13 polypeptides are constituents of the enzyme complexes of the ETC. The genes lack introns and, except for one regulation region, intergenetic sequences are absent or limited to a few bases. Replication and transcription in mitochondria depend upon trans-acting nuclear-encoded factors [2, 15].

Transcription of mtDNA is controlled by a human dissociable transcription factor (TFAM) acting in concert with the mitochondrial RNA polymerase and a factor mediating attenuation of transcription (MTERF). As no intron sequences are present in vertebrate mtDNA, and intergenetic sequences are minimal. Processing of the long polycistronic H- and L-strand messengers is thought to be a relatively simple process requiring only a few enzymes. Genes for tRNAs flank the two rRNAs genes and nearly every protein gene, suggesting that the secondary structure of the tRNA sequences provide the punctuation marks in the reading of the mtDNA information. One initiation factor (mtIF-2) and three mitochondrial elongation factors (mtEFs) have been identified and participate in the polypeptidic chain elongation. Mammalian mtDNA replication is a slow and unidirectional process. DNA polymerase γ (POLG) is the only DNA polymerase present in mitochondria, and it is necessary for mtDNA synthesis. In addition to its role in transcription, TFAM appears to have a function in maintenance of mtDNA [2, 16]. Because the mitochondrial genes encode only a few proteins, almost all of the mitochondrial proteins must be imported into the mitochondria after the proteins are synthesized by cytoplasmic free ribosomes as preproteins [17]. These usually have 20 amino acid N-terminal extensions (presequences), which can direct the preproteins to the mitochondria [18]. Cytoplasmic import factors deliver the preproteins to the outer surface of the mitochondria; then import systems of the outer membrane (Tom, translocase of the outer membrane) and the inner membrane (Tim, translocase of the inner membrane) transport the preproteins to their final destinations [19, 20]. Fundamental mechanisms of mitochondrial protein import seem to be conserved from eukaryotes to mammals.

Mitochondria and Apoptosis

Mitochondria exert a central role in eukaryote life and death [21-23]. Apoptosis, or programmed cell death, is a genetically controlled pathway that can eliminate unwanted cells. Apoptosis may be initiated for homeostatic regulation, aging, and to eliminate potentially tumorigenic cells. Apoptosis is now recognized as an essential aspect of development [24]. Mitochondria promote the release of proapoptotic factors including cytochrome *c* and other "death factors" in the intermembrane space [25], and activate the apoptotic cascade leading to cell death [26]. Under some conditions, Ca^{2+} overload leads to mitochondrial swelling, loss of respiratory control, collapse of $\Delta\psi_m$, and release of matrix Ca^{2+} caused by a permeabilization of the mitochondrial inner membrane (PTP) to molecules up to 1.5 kDa. Structurally, the PTP is formed by the adenine nucleotide translocase (ANT), and electrophysiological studies also have shown the interaction of PTP with the membrane porins and with the mitochondrial benzodiazepine receptor [4, 27].

The PTP can switch from low- to high-conductance states. The conformational switch appears to be dependent on the saturation of the internal Ca^{2+} binding of the channel. The low-conductance state of PTP may be responsible for mitochondrial volume homeostasis, and contributes to a significant part of the final cytosolic Ca^{2+} signalling [27]. Thus, under its low-conductance conformation, the PTP does not impair mitochondrial functions and is operated by changes in matrix pH accompanying mitochondrial Ca^{2+} uptake. The high-conductance state of the PTP involves a massive opening of many such pores activated by the cooperative binding of two Ca^{2+} ions to its matrix domain, with a molecular cut-off of 1.5 kDa. This induces, *in vitro* al least, a complete

collapse of the proton gradient, allowing for the efflux of a variety of other ions, and of small molecules such as pyrimidic and adenylic nucleotides, and promotes the diffusion of components from the incubation medium into the matrix, e.g. sucrose. The high-conductance state of PTP is highly regulated, and exhibits the features of a Ca^{2+}-, voltage- and pH-gated channel [27], modulated by the redox and phosphate potentials. Opening of the PTP appears to be regulated by direct binding of a mitochondrial cycloplilin (cyclophilin D) to its matrix domain, accounting for the inhibitory effect of Cyclosphorin A (CsA). In this context, two processes take place: the permeabilization of the outer mitochondrial membrane and PTP opening. The first event releases cytochrome c to cytosol, whereas PTP causes $\Delta\psi_m$ collapse [28], considered the point of no return, i.e., the point at which apoptosis can no longer be reversed [29].

The PTP may be also regulated by the ROS leaking from the ETC. The shift from a low to a high-conductance state is promoted by the oxidation of NADPH by oxidative stress. This impairs the antioxidant function of glutathione (GSH) [30]. The participation of ROS in the opening PTP is clear, since PTP opening does not occur in the absence of molecular oxygen [30]. The PTP possesses at least two redox-sensitive sites that both increase the probability of opening after oxidation: the S-site, a dithiol in apparent redox equilibrium with matrix GSH, and the P-site, in apparent redox equilibrium with the pyridine nucleotides [31]. Glutathione disulfide (GSSG) is probably the immediate oxidant of the S-site and many pore inducers such as hydrogen peroxyde (H_2O_2) appear to affect the pore through changes at the level of GSH rather than the direct oxidation of the S-site. In turn, oxidation of the P-site by oxidized pyridine nucleotides can induce PTP under conditions where the GSH pool is maintained in a fully reduced state. Under conditions of oxidative stress, the mitochondrial levels of GSH and reduced pyridine nucleotides are connected through energy-linked transhydrogenase and glutathione reductase (GRd) and thus it is difficult for these compound to independently modulate the S- and the P-site *in vivo* [31].

Apoptosis may have evolved in glycolyzing host cells to punish respiring guests if they formed excessive ROS [3]. In fact, the protomitochondria brought respiration to the partnership and with it the power to kill the new cell through the production of ROS [32]. It is obvious that in modern organisms, the functions of apoptosis (at mitoptosis) are not restricted by elimination of the ROS-overproducing mitochondria and cells. However, apoptotic stimuli are processed inside the cell in such a way that as increases in intramitochondrial (intracellular) levels of ROS are initiated. The production of $O_2^{-}\bullet$ and H_2O_2 by the ETC is the inevitable side effect of the ETC induced by one or two electron reduction of O_2 [3]. In some instances, the production of ROS increases and may induce PTP opening. Increased PTP in mitochondria cannot survive due to the collapse of $\Delta\psi_m$, since PTP permits the efflux of molecules up to 1.5 kDa; the high molecular mass compounds in the matrix exert an osmotic effect and water enters the matrix causing its swelling. As a result, mitochondrial cristae straighten and the outer membrane is broken since it is much smaller that the inner. The lost of the outer membrane integrity means that all the intermembrane proteins are released into the cytosol including some involved in apoptosis, e.g. cyt c, apoptosis-inducing factor (AIF) and some procaspases [3, 29, 33]. Cyt c and AIF form a complex with the cytosolic Apaf-1 and ATP. The complex hydrolyzes inactive procaspase 9 to active caspase 9, which in turn hydrolyzes procaspase 3 to caspase 3. Caspase 3 attacks some other key proteins resulting in controlled cell death [34]. In the interplay bewteen life and death there are many other families of proteins. Among them, the Bcl-2 family of proteins (Bcl-2, Bcl-x_L, Mcl-1,…) inhibit apoptosis by preventing the mitochondrial release of the intermembrane proteins [34, 35], whereas Bax, Bcl-x_S, Bad, Bak,… promote apoptosis [36]. Once the process gets past the mitochondria, the anti-apoptotic proteins have no effect [26].

Mitochondrial Pathologies

The functional activity of mitochondria depends on a precise cross-talk between two different genetic systems, i.e., nuclear and mitochondrial genomes [37]. A series of mechanisms controlling the mitochondrial genetic system and intergenomic communications have been recently summarized [38]. Thus, defects of mitochondrial metabolism may be associated with mutations of mtDNA or nDNA. Abnormalities of mitochondrial metabolism causing human disease have been recognized for more than 30 years. They encompass defects of fatty acid oxidation, Krebs cycle enzymes and the OXPHOS system. Some of these pathologies are not a primary cause of an alteration in mitochondrial metabolism, but it is altered changed of these. Alterations in energy production accompany abnormal hormonal changes (hyper and hypothyroidism-induced changes in UCPs expression altering $\Delta\psi_m$) [3, 9, 39], or as a consequence of ischemia/reperfusion, excitotoxicity, or sepsis. In all of these conditions, there is an increase in ROS production and an alteration in mitochondrial function that may lead to cell death [40, 41]. Moreover, as mtDNA encodes proteins of the OXPHOS, such mutations frequently result in a deficiency in one or more constituents of these enzymes complexes. A recently-described group of alterations involves mitochondrial transmembrane carrier deficiencies that constitute the mitochondriopathies [42]. In addition to increasing the quantity of ROS which DNA, they produce an elevation in poly-(ADP-ribose) synthetase (PARS) to repair the damaged DNA. This enzyme ADP-rybosylates proteins depleting the intracellular concentration of its substrate, NAD^+, slowing the ETC and ATP production [43, 44]. Primary OXPHOS defects [45] are caused by mutations of mtDNA or nDNA genes encoding subunits of the ETC complexes, including mutations affecting mitochondrial targeting of protein, i.e., the N-terminus sequence. A defect in the importance of the Rieske iron-sulphur center has been postulated. Other alterations of mtDNA occur, including changes in tRNA and protein encoding genes of the ETC complexes, and cyt b. In turn, OXPHOS deficiencies may result in increased ROS [45]. Secondary OXPHOS deficiencies are induced by both genetic and environmental factors. Alteration in mtDNA transcription, translation and replication are also included [45]. Endogenous and exogenous toxins may impair OXPHOS. Aging itself may be a result of ROS production and mitochondrial damage. Toxins such as MPTP cause neurodegenera-

tive diseases, especially Parkinsonism, and demonstrate the involvement of ROS in this pathology [45, 46].

Neuromuscular Disorders

Nuclear mutations can affect genes encoding enzymatic or structural mitochondrial proteins, assembly factors, translocases, intergenomic signalling mitochondrial protein importation, the composition of the lipid bilayer of the inner mitochondrial membrane, and mitochondrial dynamics. mtDNA mutations fall in three main categories: sporadic rearrangements (deletions/duplications), maternally inherited rearrangements (duplications), and maternally-inherited point mutations.

Disorders Due to Defects of mtDNA

A large number of diseases are related to alterations in mtDNA. Most mutations result in well-defined syndromes althought clinical overlap often takes place in mtDNA-related disorders. Mitochondrial genetic differs from mendelian genetic in several ways: i) polyplasmy, which means that each cell contain hundreds or thousands of mtDNA copies; ii) heteroplasmy, which means that harmful mutations of mtDNA usual affect some but not all mtDNAs; iii) threshold effect, since a minimal critical number of mutant mtDNAs (tipically 80-90%) is required to cause mitochondrial dysfunction and to express clinical symptoms; iv) mitotic segregation, since at cell division the proportion of mutant mtDNA in daughter cells may vary, thus explaining how the clinical phenotype in patients with mtDNA-related disorders may vary over the years; and v) maternal inheritance, since all mtDNA derives from oocyte. The mtDNA alterations include:

Sporadic Rearrangements of mtDNA (Single Deletions or Duplications)

There are three main clinical syndromes: i) Kearns-Sayre syndrome, a subtype of progressive external ophthalmoplegia (PEO) with early onset (before 20 years), limb weakness and fatigue; ii) Pearson syndrome, manifested in infancy as a severe hematopoietic disorder with sideroblastic anemia and exocrine pancreas dysfunction; and iii) sporadic PEO with ragged-red fibers (RRF) [47, 48].

Maternally Inherited Rearrangements of mtDNA

Although there is no evidence that single mtDNA deletions are inherited, there are a few disorders in which duplications/deletions are maternally transmitted. These conditions are usually associated with diabetes and myopathy [47].

mtDNA Point Mutations

About 200 mtDNA point mutations have been associated with human diseases. The most frequents diseases caused by mtDNA mutations are: i) myoclonus epilepsy with RRF, characterized by myoclonus, generalized seizures, cerebellar ataxia, and myopathy. Muscle biopsy shows RRF, which are typically COX negative; ii) mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), characterized by convulsive episodes with hemiparesia or hemianopia, before 40 years and often in childhood. Common features include generalized seizures, migraine-like headache, vomiting and dementia. MELAS is caused by a mutation in the $tRNA^{Leu(UUR)}$, causing a decreased in mitochondrial protein synthesis; iii) myoclonic epilepsy with ragged red fibers (MERRF) showing myopathy and myoclonus in association with generalized seizures. MERRF is caused by a mutation in $tRNA^{Lys}$, causing a reduction in mitochondrial protein synthesis; iv) neuropathy, ataxia, retinitis pigmentosa (NARP) consisting in a multisystem disorder of young adults comprising neuropathy, ataxia, seizures, dementia and retinitis pigmentosa. NARP results from a mutation in ATPase 6, causing a defect in CV; v) maternally inherited Leigh syndrome (MILS), a more severe syndrome than NARP, manifested in infancy with developmental delay, hypotonia, seizures, pyramidal signs, ataxia, retinitis pigmentosa, and with the neurological features of LS. MILS is also caused by a mutation in ATPase 6, causing a defect in CV; vi) Leber hereditary optic neuropathy (LHON) which causes loss of vision in young adults, and it is due to mutations in ND1, ND4 and ND6; vii) Myopathy, multisystemic diseases or another phenotypes which can be caused by mutations in other tRNAs, ND1, ND4, cyt b or COXIII [47, 48]. In mtDNA-related mitochondrial encephalomyopathies, cells die because the lack of an adequate energy supply and the decrease in $\Delta\psi_m$ that triggers PTP and apoptosis, although small proportions of non-mutant genomes seem to be sufficient to protect tissue from defects of the ETC. In neurons, the incapacity to maintain adequate ATP levels would lead to a partial neuronal depolarization and excitotoxicity, and muscle cells seem to die mainly by apoptosis [47, 49].

Disorders Due to Defects of nDNA

Defects of Genes Encoding Enzymatic/Structural Proteins and Assembly Factors

Defects in CI and CII have been associated with mutations in nDNA genes encoding subunits of these complexes. These mutations cause autosomal recessive forms of Leigh syndrome, a devastating encephalopathy with characteristically symmetric lesions of the basal ganglia and the brainstem. Other mendelian diseases due to mitochondrial respiratory chain defects include primary ubiquinone (CoQ_{10}) deficiency, an autosomal recesive syndrome with a clinical spectrum that encompasses five major phenotypes [50]. Although it was first described 30 years ago, the first molecular defects were identified only recently. Specifically, the first mutations in two genes encoding the first two CoQ_{10} biosynthetic enzymes (*PDSS2* subunit of COQ1, and *COQ2*) were identified in infants or children with encephalomyopathy or Leigh syndrome and nephrotic syndrome [51, 52]. Later, mutations in *PDSS1* and other in *COQ2* were indentified in infants or children with encephalomyopathy and/or nephrotic syndrome [53, 54]. Recently, a mutation in *COQ9*, a CoQ_{10} biosynthetic gene with unknown function, was identified in a child with intractable seizures, global developmental delay, hypertrophic cardiomyopathy and renal tubular dysfunction. Nevertheless, the consequences of primary CoQ_{10} deficiency include a severe bioenergetic defect or a combination of mild bioenergetic defect and oxidative stress, which seems to be dependent of the CoQ_{10} levels [55]. Other syndromes have also been associated with a secondary CoQ_{10} deficiency. These include autosomal recessive cerebellar ataxia of unknown etiology in children and caused by mutations in *ADCK3* in adults [50, 56], the syndrome of ataxia and ocu-

lomotor apraxia (AOA1) caused by mutations in the aprataxin gene (*APTX*) [57], and a predominantly myopathic form of glutaric aciduria type II (GAII) caused by mutations in the electron transfer flavoprotein dehydrogenase gene (*ETFDH*) [58]. The usual treatment of CoQ_{10} deficient patients is the oral supplementation with CoQ_{10}. However, the cause of the lack of positive response in some patients requires investigation [50, 52, 54, 59].

Mitochondria respiratory chain complexes need other factors for proper assembly and function. That is the case with SURF1, SCO2, SCO1, COX10, and COX15, which are required in the assembly of CIV. Mutations in some of the genes encoding these factors have been associated with COX-deficient Leigh syndrome or other multisystemic fatal infantile disorders, in which encephalopathy is accompanied by cardiomyopathy (*SCO2*, *COX15*), nephropathy (*COX10*), or hepatopathy (*SCO1*) [47]. Mutations in assembly factors of CIII and CV have been also identified [47].

Disorders Due to Defects of Intergenomic Communication

In this group, the primary genetic modification is in nDNA genes (encoding proteins involved in replication, maintenance and translation of mtDNA) but the consequence of this mutation is a quantitative or qualitative abnormality of mtDNA. A quantitative mtDNA alteration is a partial or severe mtDNA depletion which frequently causes myopathy or hepatopathy, but the central nervous system and kidney may be also affected. Mutations in different genes have been identified as a cause of mtDNA depletion including *POLG*, *SUCLA2* (encoding the β subunit of succinyl-CoA synthetase), *MPV17* (encoding a protein with unkown function) and genes encoding enzymes involved in the mitochondrial nucleotide metabolism, *DGUOK*, *TK2* and *RRM2B* [47].

mtDNA alterations are often represented by multiple mtDNA deletions, which are frequently manifested by PEO associated in some cases to exercise intolerance, hearing loss and psychosis. Mutations in *ANT1*, *PEO1* and *POLG* have been associated with PEO and multiple mtDNA deletions.

A particular disease in this category is MNGIE (mitochondrial neurogastrointestinal encephalomyopathy) since a combination of mtDNA depletion, multiple mtDNA deletions and point mutations are found in many organs of MNGIE patients [60, 61]. The primary cause of the disease is a mutation in *TYMP* gene [62], which encodes thymidine phosphorylase (TP), and enzyme that catalyzes the phosphorilysis of thymidine and deoxyuridine. A defect in the function of TP causes an accumulation of thymidine and deoxyuridine provoking an unbalance in the mitochondria deoxynucleotides pool, which in turns causes mtDNA alterations [63]. Defects of mtDNA translation also cause combined respiratory chain complex deficiencies and can be caused by mutations in several genes [47].

Disorders Due to Defects of Mitochondrial Protein Importation

Transporting proteins to mitochondria is accomplished through targeting signals localized at the N-terminus of polypeptides. Some alterations involving this disorder have been described, although considering the complexity of the multistep process involving protein import, it seems likely more defects will be uncovered [47].

Disorders Due to Alterations in the Lipid Bilayer of the Inner Mitochondrial Membrane

Cardiolipin is a phosphatidylglycerol enriched molecule in mitochondrial inner membrane, where it is intimately associated to ETC. Mutations in the tafazzin gene (*TAZ*) causes Barth syndrome, which is associated to alterations in the composition and concentration of cardiolipin, leading to an abnormal mitochondrial architecture and function [47].

Disorders Due to Abnormalities in Mitochondrial Dynamics

Mitochondria are dynamic organelles and form tubular networks that may favor the delivery of organelles to areas of high energy demands. Then, defects in proteins responsible of mitochondrial motility-fusion-fission cause another group of mitochondrial disorders. At least mutations in 13 different genes, i.e. *OPA1*, *MFN2* and *GDAP1*, have been associated to defects in mitochondrial dynamics.

Neurodegenerative Pathologies

Neurodegenerative diseases of different ethiologies may share mitochondrial dysfunction as a final common pathway. Recent studies using cybrid cell lines certainly support this posibility. Parkinson's disease (PD) is characterized by bradykinesia, rigidity and tremor. Mitochondrial involvement in PD is suggested by deficiencies of C-I in substantia nigra [64], with a parallel reduction in GSH levels, suggesting the existence of oxidative stress. In platelets of PD patients C-I is also decreased, and in some cases is accompanied by C-II, C-III and C-IV deficiencies. Studies with cybrids have shown that alterations in C-I is due to a defect in the mtDNA [64]. This defect is accompanied by an alteration in the expression of C-IV activity and a reduced $\Delta\psi_m$, which lowers the apoptotic threshold. Mitochondrial involvement in the pathology of PD has been genetically supported by the finding of *POLG* mutations in early-onset Parkinsonism in different families [65, 66]. In some cases, the *POLG* mutations were accompanied by mtDNA deletions, ragged-red and cytochrome c oxidase-negative fibers and low activities of mitochondrial complexes containing mitochondrial DNA-encoded subunits [65, 66]. On the contrary, a recent review of the evidence for primary mtDNA mutations in PD led to the conclusion that there is no convincing proof for a primary role of mtDNA mutations in this neurodegenerative disorder [67]. However, a series of nuclear genes (PARK1, PARK2,…PARK8) are recognized to be associated with the familial form of PD. In addition to the genetic origin, evidence has accumulated that an interaction of environmental toxins with the products of these genes may create oxidative damage and mitochondrial dysfunction leading to cell death [68]. These environmental toxins influence PD, as shown by the C-I inhibitory effects of MPTP, rotenone and paraquat. The C-I inhibition is prevented by free radical scavengers indicating oxidative damage to C-I. Moreover, MPTP also stimulates NMDA-dependent nNOS activity thereby increasing NO• production [69], and decreasing the content of mtDNA [70].

Huntington's disease (HD) is a neurodegenerative disorder characterized by ataxia, chorea and dementia. It is known to be caused by an alteration in a gene for nDNA encoding huntingtin, a widely expressed protein of unknown function but associated with inappropriate apoptosis. The pathology of HD involves mainly the GABA-containing neurons of the caudate nucleus [64]. There is a mtDNA deletion (mtDNA4977) in HD patients which is especially common in the frontal and temporal lobes of the cerebral cortex, although its significance is unclear [71]. Excitotoxicity has been suggested to play an important role in this disease. This includes activation of NMDA-dependent neuronal nitric oxide synthase (nNOS) and NO• production. NO• and particularly peroxynitrite ($ONOO^-$) mediate the oxidative damage. There are also deficiencies in the activity of C-II, C-III and C-IV in caudate and in a lesser extend in putamen in HD. Aconitase, an iron-sulphur-containing enzyme is particularly susceptible to inhibition by O_2^-• and NO•/$ONOO^-$, as are C-II and C-III, which are FeS-containing enzymes [64, 72]. The subsequent oxidative damage to proteins, lipids and mtDNA reduces $s\Delta\psi_m$ and induces apoptosis.

Hereditary spastic paraparesis (HSP) is another hereditary disease involving a nDNA mutation. It may be present in children or adults. A new gene defect has been recently described encoding paraplegin which contains an N-terminus sequence and is imported into mitochondria. Muscle biopsies show mitochondrial alterations including cytochrome oxidase negative fibers.

Wilson's disease, usually present in children and adolescence, is accompanied by liver failure with movement disorders (dystonia, parkinsonism), and is caused by a mutation in the gene encoding a mitochondrial P-type ATPase, leading to copper accumulation and ROS generation [64, 72].

Friedreich's ataxia (FA) is an adolescent autosomal disease with progressive ataxia, dysarthria, skeletal deformations, hyporeflexia, pyramidal features and cardiomyopathy. Pathology includes distal axonopathy affecting the large sensory axons of the dorsal root ganglia and the spinocerebellar and pyramidal tracts in the cord with loss of neuronal perikarya. The genetic defect results in a deficiency of frataxin protein, the function of which is not known. Since it has an N-terminus sequence and is associated with mitochondrial membranes, a role in mitochondrial physiology was proposed. There are several deficiencies of complexes I-III and in the Krebs cycle enzyme aconitase. There is a parallel increase in the mitochondrial iron levels and, *via* the Fenton reaction, oxidative damage to mtDNA may also occur [64, 72].

Alzheimer's disease (AD), is associated with a decrease mRNA expression of mtDNA encoding cytochrome oxidase (COX) subunit II, although it has been proposed that other nDNA-encoded COX subunits may be also altered [73]. A recent review found little evidence in support a role of mtDNA mutations in the development of AD (Howell, 2005). Also, β-amyloid peptide generates ROS in a metal-catalyzed reaction inducing neuronal cell death in a ROS-mediated process resulting in damage to neuronal membrane lipids, proteins and nucleic acids. This suggests that the use of antioxidants such as vitamin E, melatonin or estrogens may be beneficial in AD [72, 74].

Epilepsy may involve mitochondrial dysfunction which may contribute to neuronal damage during seizures, as in the case of myoclonic epilepsy and generalized tonic-clonic seizures. The OXPHOS defects, reduced ATP production, free radical generation and altered Ca^{2+} handling may all contribute to neuronal damage and epileptogenesis [72].

Mitochondria and Aging

Two schemes have been proposed as genetic models of aging. One is that aging is a genetically programmed event. Specific aging genes, functioning as hierarchical clocks, might exist to cause aging and death of the individual. The alternative, but not a mutually exclusive view, is that environmental insults and/or endogenous ROS and reactive nitrogen species (RNS) may cause genetic damage and mutations [75]. The proposal that free radicals, produced by normal aerobic metabolism, cause, at subcellular locations, random tissue damage that impairs cellular function and proliferative capacity was proposed as a cause of aging by Harman in 1956 [76]. Aging is then the result of the failure of various defense and repair mechanisms which normally counteract the radical-induced damage [75]. However, no single cause for aging should be considered as being exclusively responsible. Rather normal aging is probably the sum of multiple genetic and environmental factors.

The mitochondrial theory of aging states that the persistent accumulation of impaired mitochondria is the driving force of the aging process [77-79]. This theory is continuously gaining new experimental support. The existence of age-related mtDNA deletions and their relation to oxidative stress further support this hypothesis. The mtDNA inherited variability could play a role in successful aging and longevity in humans [80], whereas continuous damage to mtDNA leads to a bioenergetic crisis. It has been demonstrated that the levels of mitochondrial transcripts in *Drosophila* during aging are significantly reduced, which means that the ability of mtDNA to perform transcriptional activity decreases [81]. However, an increase in mtDNA damage in response to oxidative stress in human cells has been reported [82]. Experimental accumulations of mtDNA deletions and point mutations have been observed in several species including mice and humans, and are correlated with a significantly reduced life span [83, 84]. Moreover, POLG deficient mice accumulate high levels of mtDNA mutations resulting in a premature aging phenotype [85, 86]. Additionally, some genes encoded by nDNA involved in aging have been identified [75].

There is increasing consensus that ROS and RNS are a major cause of aging [87]. Aging is accompanied by structural changes in mitochondria including their reduction in number and increase in size, and a decrease in C-IV and C-V activities. Senescence-accelerated mice (SAMP8) show a reduction in mitochondrial respiratory chain activities resulting in a loss of ATP levels [88, 89]. This mitochondrial malfunction seems to be a consequence of the mitochondrial oxidative damage accumulated during aging [88, 89], as well as the existence of an inflammatory process during aging with the subsequent production of RNS [90]. These changes may impair energy-dependent neurotransmission, contributing to senescence-related decline in memory and other brain functions that are apparent in this mouse [91, 92]. The muta-

tion rate of mtDNA is much higher than that of nDNA because expression of the entire genome is essential for the maintenance of mitochondrial bioenergetic function, while only about 7% of the nuclear genome is expresses during cell differentiation [92]. Moreover, three factors make mtDNA particularly vulnerable to ROS/RNS: the mtDNA is located close to the inner membrane, just near from the generation of both ROS and RNS; mtDNA is not extensively condensed and protected by histones; and the mtDNA repair is limited [93].

Oxidative injury is not limited to mtDNA but also to mitochondrial membranes. This may lead to a progressive lipid peroxidation (LPO) and cross linking damage, with concomitant changes in the respiration rate, ATP synthesis, membrane fluidity and permeability, Ca^{2+} homeostasis and apoptosis. The free radical theory of aging provides a rationale for intervention by means of antioxidant administration [94, 95]. In fact, mitochondrial aging may be due to chronic oxidative stress, and the O_2^{-}• generated by mitochondria leads to the formation of other ROS/RNS [88-90, 96, 97]. Collectively, oxidants reduce GSH availability thereby producing oxidative damage to mtDNA, lipids and proteins, which is manifested as mitochondrial aging and, in turn, cell and organism aging [88].

Mitochondria Repair Mechanisms

The increase in mitochondrial mass and mtDNA content are early molecular events in human cells in response to oxidative stress [82]. Most of the oxygen taken up by human cells is reduced to water *via* the action of mitochondrial C-IV by the addition of 4 electrons to each O_2 molecule. The intermediate steps of oxygen reduction are the formation of O_2^{-}•, H_2O_2 and hydroxyl radical (HO•), corresponding to a reduction by one, two and three electrons, respectively. Additionally, NO• and its metabolite $ONOO^{-}$ are other RNS produced in the mitochondria. Mitochondrial DNA is not protected by histones and lies in close proximity to the free radical-producing ETC. Mutations in mtDNA or nDNA genes encoding mitochondrial proteins and/or changes in ROS production may induce mitochondrial damage which, as noted above, is the basis for aging and several diseases including neurodegenerative diseases and mitochondriopathies.

A large number of DNA base modifications caused by oxidative stress have been detected. One of the most widely studied is 8-hydroxydeoxyguanosine (8-oxo-dG). This mutagenic lesion also accumulates with age. Mitochondria defend against oxidative stress using two main mechanisms: eliminating ROS (antioxidants and scavengers) and repairing the damaged molecules. The former include SOD which actively dismutates O_2^{-}• to H_2O_2; the latter agent is then transformed to water by glutathione peroxidase (GPx). In this process, GSH is oxidized to GSSG and the enzyme GRd restores GSH levels. The glutathione recycling system is highly active in mitochondria; these organelles do not synthesize GSH and are devoid of another antioxidative enzyme, catalase. Thus, mitochondria mainly depend on their own GSH pool, although they can also import GSH from the cytosol [98]. Thus, under physiological conditions, equilibrium between the mechanisms generating and those scavenging superoxide and other ROS should be in equilibrium. In the case of mitochondrial respiratory chain deficiencies, however, an overexpression of antioxidative enzymes occurs [98].

Endogenous metabolic processes generate ROS yielding oxidized bases that are removed from the DNA mainly by the base excision repair (BER) pathway. Adducts due to UV exposure are removed by a nucleotide excision repair (NER) pathway [99]. Mitochondria are able to carry out BER. The first repair enzyme detected was uracil DNA glycosylase. Homologs to the yeast repair enzymes, OGG1, which excises 8-oxo-dG from DNA, have been found in mouse and human mitochondria. The formamidopyridine DNA glycosylase, an enzyme that detects 8-oxo-dG, has been reported in rat hepatic mitochondria. Removal of 4-nitroquinoline lesions from mtDNA, which is normally accomplished by NER pathways, has been proposal. However, NER as it exists in the nucleus, does not exist in mitochondria, and thus, the role of NER protein in mitochondrial repair remains unclear [99]. It has been reported that the endonucleolytic activity of the enzyme that specifically cleaves 8-oxo-dG oligonucleotides is higher in 12 and 23-months old than in 6-months old rats. Thus, the mitochondrial capacity to repair 8-oxo-dG seems to increase with age [99].

Mitochondria and Stem Cells

There are three types of stem cells: the pluripotent embryonic stem cells (ESC) that have the potential to differentiate into any cell type in the organism; the multipotent cells derived from adult tissue including umbilical cord blood and amniotic fluid, which can differentiate into a limited number of cells types of their own lineage, e.g., mesoderm only, and precursor cells, which are adult stem cells committed to differentiation. While most stem cell studies have focused on the activity of the nuclear genome, characteristics of the mitochondrial genome have been largely ignored.

Some authors have hypothesized that stem cell competence may be verified using functional mitochondrial characteristics [100]. Differentiation of mouse and human ESC results in changes in mitochondrial structure, morphology and pattern of cytoplasmic localization. Mitochondria in stem cells tend to localize perinuclearly [100]. Moreover, ESC have relatively few mitochondria with poorly developed cristae [101, 102], and restricted oxidative capacity. As cells are allowed to differentiate, the number of mtDNA copies increase and these differentiated cells contain increased numbers of mitochondria with distinct cristae, dense matrices and high membrane potentials. These features suggest the initiation of metabolic activity through OXPHOS [103]. Because ESC display low oxygen consumption and thus, poor OXPHOS, an elevation in ATP content per cell may therefore reflect a loss of stemness and the subsequent onset of differentiation [100, 102]. Therefore, preservation of immature mitochondria with a perinuclear arrangement, reduced expression of OXPHOS enzymes and low metabolic activity in ESC has led to the suggestion that these mitochondrial properties might be important for the maintenance of pluripotency and should be considered as another ESC marker. Departures from this profile indicate that cells are differentiating or perhaps becoming senescent.

The increase in mitochondrial mass is accompanied by elevated ATP production and, thus, by a greater generation of ROS. Undoubtedly, the intracellular levels of ROS are higher in differentiated than in undifferentiated ESC, due to the increase in OXPHOS metabolism in the former [104]. An increase in ROS levels might have a role in cell signaling and regulation of proliferation and differentiation. Exposure to low levels of ROS has been reported to enhance ESC differentiation whereas continuous exposure to high levels of ROS results in inhibition of differentiation [104]. Therefore, differentiating cells probably activate effective antioxidant systems, including catalase, GPx and others. In summary, successful differentiation of embryonic cells *in vivo* or ESC *in vitro* involves initiation of mtDNA transcription and replication, an increase in the number of mitochondria, and regulation of the enzymes required for aerobic metabolism in order to fulfill the elevated ATP requirements of fully differentiated cells.

ESC from embryos created by *in vitro* fertilization procedures have been reported to exhibit various forms of mtDNA mutations, and it is not known whether metabolic functions of ESC are affected by mtDNA mutation or mitochondrial deletions [105]. Considering that mutations in mtDNA have been linked to a wide range of disorders including diabetes, cardiovascular disease, neurodegenerative and neuromuscular diseases, and cancer [106, 107], therapeutic defective cell replacement could lead to the development of these diseases. Thus, mtDNA anomalies could have widespread implications for biomedical applications of stem cells as well as for studies on their behavior *in vitro* [100].

Melatonin and Mitochondrial Pathology

In the last 25 years, an increasing amount of evidence supports new roles and mechanisms of action of melatonin. The actions of melatonin depend on receptor- and non-receptor-mediated processes, the latter accounting for the antioxidant properties of melatonin [108]. Receptor-mediated events for melatonin involve both membrane and nuclear receptors [109-112], and the existence of a membrane-nuclear signaling pathway has been proposed [113]. Some of the protective effects of melatonin on the cell seem to be mediated by genomic regulation, and some genes, including 5-lipoxygenase gene in human B lymphocyte, reportedly regulated by melatonin [114]. In addition, the expression of some genes, mainly related to the cell redox state and inflammatory status including GPx, GRd, SOD, inducible nitric oxide synthase (iNOS) and cytokines are also under genomic regulation by melatonin [115-119]. In addition, the specific binding of melatonin to Ca^{2+}-calmodulin (Ca-CaM) appears to regulate some CaCaM-dependent enzymes such as nNOS [120-122]. The discovery of the mitochondrion being a target for melatonin action opens new perspectives to understand the mechanism of action of melatonin, and may help to explain the antiapoptotic and thermogenic effects of the indoleamine [123-126].

In a number of experimental and clinical situations a beneficial effect of melatonin has been reported in those pathologies involving mitochondria dysfunction especially in the cases of secondary cause of the disease, including ROS-induced DNA damage, excitotoxicity and neurodegenerative diseases such as PD, AD and epilepsy, sepsis and aging [89, 127-133]. Melatonin´s ability to counteract excitotoxicity and ROS-induced DNA damage has been described under a variety of different experimental paradigms. Melatonin prevents DNA damage in human blood cells exposed to ionizing radiation, and reduces genetic damage to lymphocytes which were exposed to ionizing radiation after their removal from the individual who consumed melatonin [134]. Oxidation of guanine bases in DNA from rat liver induced by whole body ionizing radiation was prevented by melatonin administration [135]. Furthermore, the DNA damage caused by the chemical carcinogen safrole or by chromium is reduced by melatonin [136, 137]. Using the comet assay, it was shown that treatment with melatonin reduced neural DNA fragmentation by exposure of rats to extremely low frequency magnetic fields [138]. Melatonin counteracts paraquat-induced genotoxicity in mice [139], as well as ferric nitrilotriacetate-induced DNA damage and H_2O_2-induced DNA damage in U-937 cells [140, 141]. The protective effects of melatonin against DNA damage was estimated by measuring the 8-oxo-dG levels in the brain of kainic acid-treated rats [142].

When DNA repair mechanisms are induced, the activation of the nuclear enzyme PARS triggers an energy-consuming repair cycle reducing cellular NAD^+ levels. This also occurs in rats treated with zymosan, a non-bacterial agent which causes cellular injury by inducing the production of $ONOO^-$ and consequent PARS activation. In this situation, there is also an inhibition of mitochondrial respiration due to $ONOO^-$. The administration of melatonin protects cellular energy depletion and prevents the occurrence of DNA damage [143]. Renal and hepatic DNA damage induced by the carcinogen δ-aminolevulinic acid was assessed by measuring the levels of 8-oxo-dG, which were reduced by melatonin [144, 145]. Rat lung and spleen production of 8-oxo-dG induced by δ-aminolevulinic acid are also lowered by melatonin [146].

Particularly interesting findings were described when the effect of melatonin on mitochondrial membrane fluidity was tested. Mitochondrial membrane fluidity decreased after the animals were treated with δ-aminolevulinic acid with these changes being reversed by melatonin co-treatment [144]. However, no changes in mitochondrial membrane lipid peroxidation (LPO) levels were reported and thus, the effects of melatonin on mitochondrial membrane fluidity may be independent of its ability to counteract lipid damage [144, 145]. The effects of melatonin in maintaining optimal membrane fluidity in mitochondrial membranes may depend on its ability to localize in the membrane itself, in a superficial position in lipid bilayers near the polar heads of membrane phospholipids [147]. In this position melatonin would be near of the mitochondrial proteins which then would be protected from ROS. It should be note that δ-aminolevulinic acid damage to mitochondria results in the disruption of the $\Delta\psi_m$ and enhanced membrane permeability [146, 148] leading to reduction in ATP, PTP opening and apoptosis. Thus, melatonin may protect protein complexes in the inner mitochondrial membrane and thereby improve ETC.

A series of experiments have provided strong evidence for the anti-excitotoxic properties of melatonin both *in vivo* and *in vitro*. Anti-convulsant activity of melatonin was ini-

tially showed to be related to its effects on both brain GABA-benzodiazepine receptor complex and Na^+, K^+-ATPase [149-153]. However, due to the inhibitory effect of melatonin on the NOS/NO system, and effect of the indoleamine on glutamate-induced excitotoxicity was soon proposed. A melatonin deficiency is associated with increased brain damage after stroke or excitotoxic seizures in rats [154], and an anticonvulsant activity of melatonin against seizures induced by a series of drugs in mice was reported [155]. Melatonin protects cultured cerebellar neurons from kainate excitotoxicity [156]. Quinolinic acid, a neuroactive metabolite of tryptophan implicated in some neurodegenerative diseases [157], induces neuronal degeneration when injected into animals, an effect counteracted by melatonin administration [158].

Electrophysiological experiments document the antagonism of melatonin at the level of the NMDA receptor which is involved in excitotoxicity [159-162]. The effect of melatonin was specific, dose-dependent and was independent of melatonin receptors. Thus, an intracellular action of melatonin in inhibiting the NMDA-dependent excitotoxic events was further demonstrated with synthetic kynurenamines supporting an inhibition of the NOS/NO• system, the main mediator of glutamate-dependent excitotoxicity [121, 163, 164]. The effects of melatonin against brain excitotoxicity were the basis for the clinical use of melatonin in infantile seizures [165, 166]. Melatonin also protects against excitotoxicity by reducing the autoxidation of dopamine (DA) which occurs in some degenerative diseases as PD [167]. These effects were demonstrated in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP)-induced PD in mice [168, 169]. The ability of melatonin to reduce DA autoxidation was tested elsewhere and it showed a greater potency than other antioxidants including vitamin E and C, and that of L-deprenyl, a monoamine oxidase (MAO) B inhibitor which also has antioxidant properties [167]. The protective effects of melatonin in mouse models of PD include specific actions of the indoleamine on the mitochondria from s. nigra and striatum. Melatonin administration normalized complex I activity and oxidative status in mitochondria from these nuclei. Looking for the targets of melatonin action, it was recently shown that melatonin reduced the activity of the mitochondrial iNOS (i-mtNOS), thus decreasing mitochondrial $NO^{\cdot}$ levels, preventing the respiratory inhibition produced by NO• at the level of complex IV [170].

The neuroprotective effects of melatonin were also tested against neurodegenerative manifestations in AD [171]. When neuroblastoma cells were incubated with β-amyloid (βA), more than 80% of the neurons died due to apoptosis, but the presence of melatonin reduced cellular death and DNA damage in a dose-related manner [172]. In human platelets, melatonin also protected against βA-induced damage [173, 174]. The protective properties of melatonin were extensively tested in models of aging, which involves pervasive cell damage. In different models of aging and age-related diseases including cancer and cataracts, melatonin administration has been shown to be protective. The fact that melatonin decreases with age was then suggest as one of the causes of aging in mammals [128, 175-181].

The benefit effects of melatonin on mitochondrial function have also been tested in sepsis, an acute inflammatory process. In sepsis models induced by LPS in rats or by cecal ligation and puncture (CLP) in mice, melatonin reduces the overexpression of iNOS/i-mtNOS, decreases mitochondria ROS/RNS production, induces the activity of the GSH cycle, increases mitochondria respiratory chain activities, restores ATP synthesis and increases animal survival [133, 182-184]. Melatonin improves the clinical outcome of septic newborns [185].

The importance of melatonin as antioxidant depends on several characteristics: it is both lipophilic and hydrophilic, and it passes all bio-barriers with ease. It is available to all tissues and cells, where it scavenges free radicals [186-188]. Melatonin distributes in all subcellular compartments, being especially high in the nucleus and mitochondria. This means that melatonin is available at the sites in which free radicals are being maximally generated, thus decreasing the potential damage [189, 190]. Melatonin, identified by Lerner as a product of the mammal pineal gland [144], is also found in several tissues including the retina, cells of the immune system, gut, bone marrow, skin and its appendages and in the human ovary and testes [191-199]. It seems that these tissues may produce the melatonin required for antioxidant regulation [200] since this melatonin does not enter the circulation. Also, most of these tissues have much higher levels of melatonin than concentrations in blood. Levels of melatonin 2-3 orders of magnitude higher than maximal blood melatonin concentration are present in bile [201]. Another fluid that contains very high levels of melatonin is the cerebrospinal fluid (CSF) [202].

It was reported that expression of genes of the key enzymes for melatonin synthesis, N-acetyl-transferase (NAT) and hydroxyindol-O-methyl-transferase (HIOMT), are found in many the organs [203]. Thus, these organs may synthetized their own melatonin and do not depend totally that delivered by circulation to provide this indolamine. This suggests that each organ may in part produce the melatonin that it needs independently of its circulating levels. Thus, the concept of what constitutes a physiological level of melatonin is changing, and physiological levels must be defined based on specific fluid and subcellular organelles [204].

Melatonin and Apoptosis

The observation that melatonin influences apoptotic cell death is a documented regulatory effect of melatonin on cell survival. The possibility that the antioxidant properties of melatonin account its inhibitory effect on apoptosis was investigated *in vivo* and *in vitro* by measuring DNA fragmentation. These experiments showed that melatonin administration counteracts apoptosis in rat thymus. In cultured thymocytes, 1 nM of melatonin decreases cell death by 20% [205]. It was suggest that melatonin down-regulate the glucocorticoid receptor in thymocytes, which may explain its antiapoptotic effect in the thymus [206]. In primary cultures of cerebellar granule neurons, melatonin protects them from singlet oxygen-induced apoptosis [207]. Melatonin also inhibited pre-B-cell apoptosis during lymphopoiesis in mouse bone marrow; this has implications for neoplasia since boosting the formed B cells would have effects on humoral immunity

[208]. Melatonin was also shown to protect bovine cerebral endothelial cells from hyperoxia-induced DNA damage and apoptotic death [209].

Since apoptosis is a possible mechanism involved in neuronal death documented in several neurodegenerative diseases including PD, AD and epilepsy, it would be expected that melatonin may exert antiapoptotic effects in these diseases. In fact, in neuroblastoma cells exposed to the Alzheimer β-amyloid peptide, melatonin prevented cell death [172]. Melatonin also prevents apoptosis induced by MPTP in mouse [169] and by 6-hydroxydopamine in PC12 cells [210]; these findings could be of potential clinical importance in the treatment of PD. Melatonin also abrogated cell death induced by cysteamine pretreatment of the PC12 cells; cystamine treatment involves mitochondrial iron sequestration [211]. The age-associated accumulation of redox-active iron in subcortical astrocytes may facilitate the bioactivation of DA to neurotoxic free radical intermediates and thereby predispose the nervous system to PD and other neurodegenerative diseases. Melatonin counteracts very efficiently DA autoxidation by reducing iron-dependent ROS production by mitochondria [167]. In rats injected with kainic acid to produce excitotoxicity-induced apoptotic cell death, melatonin significantly attenuated apoptosis, an effect linked to the reduction in oxidative damage and an increased GSH content [212]. In a spontaneous, age-induced model of apoptosis using cerebellar granule cells, it was shown that melatonin and Ca^{2+}-channel blockers such as amlodipine, inhibited spontaneous apoptosis [213]. This antagonism between melatonin and Ca^{2+}-channels was also demonstrated in electrophysiological and binding experiments [162]. Striatal neurons growing in low density culture in serum-free medium and in the absence of glia die within 3 days by apoptosis. The presence of melatonin rescues striatal neurons from impending cell death, which may have important consequences in neurodegenerative diseases involving nigrostriatal pathway as in PD [214].

The relation of melatonin with cell death was tested in several cell cancer models. In an ovarian carcinoma cell line it was found that melatonin exerts an oncostatic action linked to a nuclear effect of the indoleamine, since the melatonin nuclear receptor agonist CGP 52608 caused a similar effect [215]. Interestingly, melatonin seems to enhance apoptosis in carcinoma cells, as has been demonstrated with Ehrlich ascites carcinoma cells. In this case, changes in GSH were not detected during the proapoptotic effects of melatonin [216]. Similarly, in colon mucosa and colon tumors induced by 1,2-dimethylhydrazine in rats, melatonin behaves as a potent stimulator of apoptosis [217]. It was recently shown that melatonin also inhibited the LOOH-triggers cell death, in a similar manner to that of CsA, an inhibitor of the permeability transition pore [218]. An intriguing result was found with U-937 cells. While melatonin counteracted the H_2O_2-induced DNA damage in U-937 cells [141], other authors were unable to confirm the antiapoptotic role of melatonin against 7-ketocholesterol-induced apoptosis in the same cell type, although melatonin prevented O_2^{-}• generation by mitochondria [219]. In general, it seems that the antioxidant and to some extend the GSH-enhancing effects of melatonin may account for melatonin´s antiapoptotic activity in non-cancerous cells.

Melatonin Actions on Mitochondria

Three main considerations suggest a role for melatonin in mitochondrial homeostasis. First, the mitochondrion is the organelle with the highest ROS/RNS production into the cell, and melatonin is a powerful scavenger of ROS and RNS. Second, mitochondria depend on the GSH uptake from cytosol, although they have GPx and GRd to maintain the GSH redox cycling; melatonin improves the GSH redox cycling and increases GSH content by stimulating its synthesis in the cytosol. Third, melatonin exerts important antiapoptotic effects (Fig. **1**) in normal cells and most of the apoptotic signals originate from the mitochondria [125].

The relationships between melatonin and mitochondria have been known for several years, but to date the existence of a specific role of the indoleamine on mitochondrial homeostasis remain enigmatic. Following the lines of evidence that an aerobic organism entered into an anaerobic one, the subsequent symbiosis had beneficial consequences for the two organisms [220]. However, the anaerobic one had an unexpected problem, i.e., oxygen is highly toxic and oxidizes many molecules of the host. The hybrid organisms had to acquire new antioxidant mechanisms not only to preserve themselves from oxygen toxicity but also to preserve the enzymatic machinery required to produce ATP highly efficiently. Additionally when ROS produced by the guest were excessive, the host organism evolved a trigger to initiate mitoptotic signals to eliminate the damaging symbiotic organelle [3]. Since melatonin was present in the invading unicellular organisms [221], the question arises as to what was the function of melatonin in the mitochondria of multicellular organisms?

Chronic melatonin administration increases the number and size of mitochondria in the pineal and in ependymal epithelium of the choroid plexus [222, 223]. Binding experiments with ^{125}Iodomelatonin also revealed a high percentage of specific binding sites in the mitochondrial fraction of the pigeon brain and in the spleen of guinea pigs [224, 225]. In the hamster hypothalamus, higher binding of ^{125}Iodomelatonin was recorded in the mitochondrial pellet than in the nuclear pellet [226]. Soon thereafter, it was shown that melatonin influenced of on mitochondrial activity throughout the circannual cycle [227]. Milczarek [228] showed that melatonin inhibit NADPH-dependent lipid peroxidation in human placental mitochondria. Melatonin protects fetal rat brain against oxidative mitochondrial damage [229]. Finally, a protective effect for melatonin against the MPP+-induced inhibition of C-I of ETC was also shown [230].

The ability of melatonin to influence mitochondrial homeostasis was initially tested *in vivo*. In this study it was shown that melatonin to normal rats significantly increased the activity of the complex C-I and C-IV of the mitochondrial ETC measured in mitochondria obtained from brain and liver, whereas C-II and C-III were unaffected [231]. Melatonin also counteracted ruthenium red-induced inhibition of the C-I and C-IV in brain and liver mitochondria when melatonin was given simultaneously with ruthenium red [231].

To further test the antioxidant ability of melatonin against mitochondrial oxidative stress, *in vitro* experiments using isolated mitochondria prepared from rat brain and liver

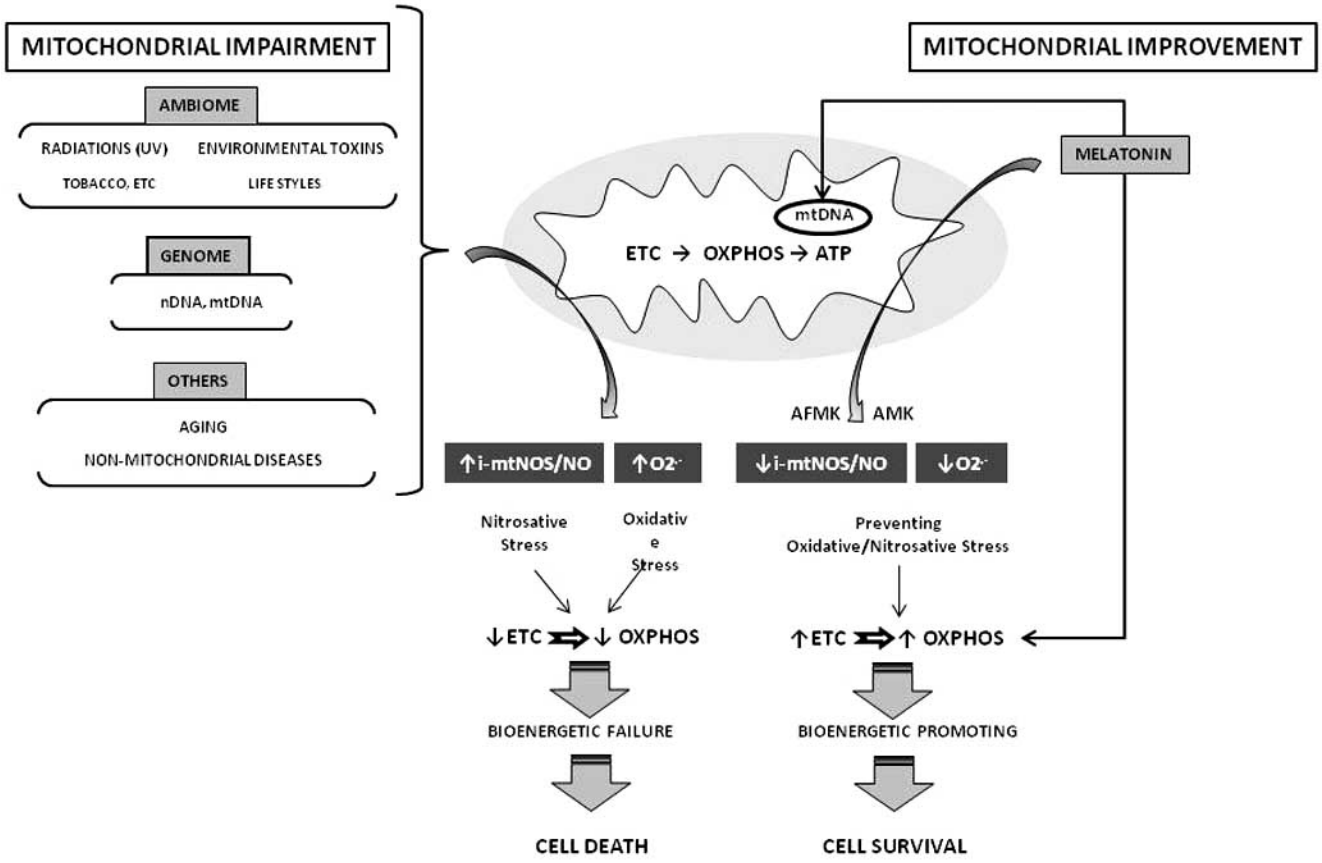

Fig. (1). Schematic representation of the effects of melatonin on the mitochondrion. Mitochondrial impairment may depend on a series events, including environmental factors, mtDNA- and nDNA-dependent mitochondrial diseases, and non-mitochondrial diseases. The term ambiome has been defined as the set of environmental factors (radiations, toxins, life styles, tobacco, etc.) that, with the genome and proteome, influence normal human development but they can also determine disease (Mora and Sanguinetti, 2004 [255]). The mechanisms involved in the ETC failure mainly depend on the generation of ROS and RNS in the mitochondria. Oxidative stress and ATP depletion favored opening of the mitochondrial permeability transition pore, which in turn induces mitochondrial swelling and the release of proapoptotic factors. These proapoptotic factors activate a caspase cascade in the cytosol leading to cell death. Melatonin scavenges ROS and RNS, thus avoiding free radical attack against mtDNA and the complexes of the ETC. Moreover, melatonin improves the activity of the ETC complexes and ATP synthesis. Finally, melatonin increases transcriptional activity of the mtDNA, improving mitochondrial physiology. As a consequence of the effects of melatonin, the mitochondrion recovers to a almost normal function avoiding PTP induction and apoptosis.

were performed. Oxidative stress was induced by incubation of these mitochondria with t-butyl hydroperoxide (t-BHP), which oxidizes pyridine nucleotides, depletes the mitochondrial GSH pool and inhibis both GPx and GRd activities [232]. In this situation, 100 nM melatonin counteracted these effects, by restoring basal levels of GSH and the normal activities of both GPx and GRd. N-acetyl cysteine (NAC) and vitamins E and C were unable to exert any significant effect on t-BHP-induced oxidative stress in mitochondria despite the high doses of these compounds that were used [233]. Interestingly, melatonin increased the activity of the C-I and C-IV in a dose-dependent manner, the effect being significant at 1 nM melatonin [233]. Melatonin was also able to counteract the cyanide-induced inhibition of the C-IV, restoring the levels of cyt $a+a_3$. Moreover, melatonin increased the activity of isolated C-I by blue native polyacrylamide gel electrophoresis (PAGE). The effects of melatonin are of likely physiological significance since the indoleamine increased the ETC activity coupled to OXPHOS, which was reflected in an elevated ATP synthesis, either in normal mitochondria or in mitochondria depleted of ATP by cyanide incubation [234, 235].

Recently, an study performed *in vitro* with normal mitochondria addressed some of the mechanisms involved in the actions of melatonin on this organelle [235]. Using high-resolution respirometry, rat liver mitochondria were analyzed for oxygen consumption, ROS generation, membrane potential, and ETC activity. Melatonin decreased oxygen consumption in the presence of ADP in a concentration-dependent manner; it reduced $\Delta\psi_m$ and, consequently inhibited the production of $O_2^{-}\bullet$ and H_2O_2. At the same time, melatonin maintained the RCR and the efficiency of oxidative phosphorylation and ATP synthesis while increasing the activity of the respiratory complexes (mainly complexes I, III, and IV). Kinetic experiments showed that mitochondria take up melatonin in a time- and concentration-dependent manner and thus, the effects of melatonin on this organelle were due to its presence within the mitochondria [235].

These data support the hypothesis that melatonin participates in the physiological regulation of mitochondrial homeostasis.

Together, the data summarized herein suggest a direct effect of melatonin on mitochondrial energy metabolism (Fig. **1**), providing a new homeostatic mechanism regulating mitochondrial function [125, 181, 231, 233]. First, melatonin scavenges H_2O_2 [187] the most abundant ROS produced into the mitochondria from O_2^{-}•. This reduces the loss of the intramitochondrial GSH pool and lowers mitochondrial damage [144, 145]. This effect is also supported by the observation that melatonin increases mitochondrial membrane fluidity thereby at least partially protecting against protein oxidative damage. Due to the high content of proteins in the inner mitochondrial membrane, this effect of melatonin may also account for its ability to improve ETC activity. Second, improving mitochondrial respiration and ATP synthesis increases the rate of electron transport across the ETC and reduces ROS production. Due to the high redox potential of melatonin (-0.94 V) [188], this molecule may donate an electron to the C-I of the ETC. Thus, melatonin improves ETC and reduces mitochondrial oxidative damage. These effects reflect the ability of melatonin to lower the harmful reduction in $\Delta\psi_m$ that may trigger PTP opening and the apoptotic cascade. Other important consequence of the effects of melatonin on mitochondria is its role in thermogenesis [124]. The effects of melatonin are similar to those derived from the mild uncoupling exerted by UCPs: reduced ROS production without affecting ATP production. Thus, melatonin reduces heat production by mitochondria and induces a more efficient use of substrates in terms of ATP production.

An important question becomes apparent from these data. If melatonin improves OXPHOS and ATP synthesis, does melatonin exert some effect on mtDNA transcriptional and/or translational activity? It was shown in tumor cell studies that melatonin exerts an oncostatic effect unrelated to nascent DNA synthesis [236]. However, when melatonin was added to cultured J774 macrophages, the indoleamine reduced the suppression of mitochondrial respiration and inhibited the development of DNA single strand breakage in response to peroxynitrite [237]. In other set of experiments, it was shown that melatonin administration prevents oxidative degradation of mtDNA and reduction of mtDNA transcripts in several tissues including liver, heart, skeletal muscle and brain [238, 239]. In addition, a direct effect of melatonin on mitochondrial genome expression in brown adipocytes of the Siberian hamster was documented [240].

Because of these findings, we performed a series of experiments to analyze the possible effects of melatonin of the expression of the mtDNA encoded polypeptide subunits of the C-IV under both *in vivo* and *in vitro* conditions. Starting from the mtDNA-encoded subunits I, II and III of the C-IV, a quantitative analysis of the mRNAs of these subunits by means of quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed [241]. Rats were intraperitoneally injected with melatonin (10 mg/kg body weight) or vehicle and sacrificed at different times after treatment to obtain the livers used for the determinations. The results showed a significant increase in the expression of the mRNAs for the three subunits tested. The increases in the mRNAs content were time-dependent, peaking at 90 min after melatonin administration. This time-dependence of melatonin action agrees very well with the time-dependent changes in complex IV activity after melatonin injection to rats reported elsewhere [231]. Thus, it seems that mtDNA transcriptional activity and complex IV enzyme activity are two melatonin-related events. To further analyze the effects of melatonin, another set of rats was intraperitoneally injected with melatonin (10 mg/kg) or vehicle for 10 days and then sacrificed and their livers immediately used for mitochondria preparation. Fresh mitochondria were incubated as described [242], and incorporation of labelled UTP to mRNA was analyzed. The results showed that the animals treated with melatonin have lower mRNA levels than the controls, an effect partially counteracted in pinealectomized animals. Based on the effect of melatonin on ATP production [125], and the effect of ATP on mitochondrial mRNA synthesis [242], 10-days of melatonin treatment in rats may increase significantly the ATP production that in turn inhibits mRNA synthesis.

Melatonin, Mitochondria, and Stem Cells

Whereas a role of mitochondria in stem cell proliferation and/or differentiation begins to have experimental support, the role of melatonin remains unclear. One can presume that, in view of the specific and significant effects of melatonin on mitochondrial physiology, the indoleamine may also affect mitochondrial physiology in stem cells. It was recently reported that melatonin modulates the proliferative and differentiative ability of the neural stem cells (NSC) from fetal mouse brain in a concentration and exposure-timing dependent manner [243]. Pharmacological concentrations of melatonin (1-100 μM) applied during the proliferation period, the proliferation diminished. Interesting, neural differentiation of these cells increased without affecting astroglial differentiation. Other data point towards a net hippocampal neurogenesis in adult mice by melatonin [244]. The effects of melatonin on neural proliferation and differentiation might be partly resulting from melatonin´s activity in mitochondria and thus, additional studies are required to uncover underlying melatonin´s actions on NSC.

CONCLUDING REMARKS

The observations described herein suggest that melatonin acts as a uncoupling agent in mitochondria to reduce oxygen consumption, ROS generation, and heat production, while maintaining or even increasing ATP production, and increasing mtDNA expression. These effects may be the basis for the presumed anti-aging properties of melatonin. The physiological reduction of melatonin levels with age presumably promotes an increase of mitochondrial generation of ROS that impairs mitochondrial metabolism, favoring apoptosis. Additionally, the age-dependent reduction in mtDNA transcriptional activity may also partially depend on the reduction of melatonin levels with age. On the other hand, the protective properties of melatonin in many degenerative and inflammatory disorders, which exhibit mitochondrial alterations, may be also related to melatonin´s homeostatic role in mitochondria. Considering these observations, melatonin may be useful for the treatment of some mitochondrial dysfunctions involving mtDNA damage and/or other mitochon-

driopathies. The mitochondria are now considered a potentially important target for drug delivery, and strategies to prevent mitochondrial damage or to manipulate mitochondrial function may provide new therapies for these disorders [245]. Moreover, the application of antioxidant therapy in oxidative stress-related diseases is now acquiring increasing clinical interest [246, 247]. However, antioxidants such as vitamins E and C, which are used in megadoses to exert their antioxidant effects, and resveratrol, may produce pro-oxidant and genotoxic effects [248-250]. Moreover, the effects of melatonin on NSC proliferation and/or differentiation open as a promising new field in the homeostatic roles of the indoleamine. On the basis of data summarized in this report (Fig. **1**), melatonin becomes an interesting pharmacological tool in mitochondrial-related diseases since it easily reaches the mitochondria, it regulates the mitochondrial redox status and mtDNA transcriptional ability, and it is metabolized to other compounds with strong antioxidant ability [251-253]. Finally, the lack of significant toxic effects of melatonin treatment at both physiological and pharmacological doses [165, 254] allows for a wide margin of safety in clinical trials.

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ABBREVIATIONS

8-oxo-dG	=	8-hydroxydeoxyguanosine
AD	=	Alzheimer's disease
AIF	=	Mitochondrial apoptosis-inducing factor
ANT	=	Adenine nucleotide translocase
BER	=	Base excision repair mechanism
CaCaM	=	Calcium-camodulin complex
C-I, C-II, C-II, C-IV	=	Complex I, II, II and IV of the ETC
CLP	=	Cecal ligation and punture
CoQ10	=	Ubiquinone
COX	=	Cytochrome oxidase (complex IV of the ETC)
CsA	=	Cyclosphorin A
Cyt	=	Cytochrome
DA	=	Dopamine
$\Delta\mu_H^+$	=	Mitochondrial electrochemical proton potential
$\Delta\psi_m$	=	Mitochondrial membrane potential
ESC	=	Embryonic stem cells
ETC	=	Mitochondrial electron transport chain
FA	=	Friedreich's ataxia
GPx	=	Glutathione peroxidase
GRd	=	Glutathione reductase
GSH	=	Glutathione
GSSG	=	Glutathione disulfide
H_2O_2	=	Hydrogen peroxide
HD	=	Huntington's disease
HIOMT	=	Hydroxyindol-O-methyl-transferase
HO•	=	Hydroxyl radical
HSP	=	Hereditary spastic paraparesis
iNOS	=	Inducible nitric oxide synthase
LHON	=	Leber hereditary optic neuropathy
LPO	=	Lipid peroxidation
MELAS	=	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MERRF	=	Myoclonic epilepsy with ragged red fibers
MILS	=	Maternally inherited Leigh syndrome
MNGIE	=	Mitochondrial neurogastrointestinal encephalomyopathy
MPTP	=	1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine
mtDNA	=	Mitochondrial DNA
mtEF	=	Mitochondrial elongation factor
mtIF-2	=	Mitochondrial translation initiation factor 2
MTERF	=	Mitochondrial transcription termination factor
NAC	=	N-acetyl cystein
NAT	=	N-acetyl-transferase
nDNA	=	Nuclear DNA
NER	=	Nucleotide excision repair mechanism
NMDA	=	N-methyl-D-aspartate
nNOS	=	Neuronal nitric oxide synthase
$NO^!$	=	Nitric oxide
O_2^{-}•	=	Superoxide anion
$ONOO^-$	=	Peroxynitrite
OXPHOS	=	Oxidative phosphorilation
PAGE	=	Polyacrylamide gel electrophoresis
PARS	=	Poly-(ADP-ribose) synthetase
PD	=	Parkinson's disease

PEO	=	Progressive external ophthalmoplegia
POLG	=	DNA polymerase γ
PTP	=	Mitochondrial permeability transition pore
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
RRF	=	Ragged-red fibers
RT-PCR	=	Reverse transcription polymerase chain reaction
SOD	=	Superoxide dismutase
T_3	=	Triiodo tyronine
t-BPH	=	t-butyl hydroperoxide
TFAM	=	Transcription factor A, mitochondrial
Tim	=	Translocase of the inner mitochondrial membrane
Tom	=	Translocase of the outer mitochondrial membrane
UCP	=	Uncoupling proteins
VDAC	=	Voltage-dependent anion channel

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