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Biogenic amines in the reduction of oxidative stress: Melatonin and its metabolites

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Abstract

N-acetyl-5-methoxytryptamine (melatonin) is an endogenous indoleamine produced by all vertebrate organisms. Its production in the pineal gland has been extensively investigated but other organs also synthesize this important amine. Melatonin's functions in organisms are diverse. The actions considered in the current review relate to its ability to function in the reduction of oxidative stress, i.e., molecular damage produced by reactive oxygen and reactive nitrogen species. Numerous publications have now shown that not only is melatonin itself an efficient scavenger of free radicals and related reactants, but so are its by-products cyclic 3-hydroxymelatonin, N1-acetyl-N2-formyl-5-methoxykynuramine, and others. These derivatives are produced sequentially when each functions in the capacity of a free radical scavenger. These successive reactions are referred to as the antioxidant cascade of melatonin. That melatonin has this function within cells has been observed in studies employing time lapse conventional, confocal and multiphoton fluorescent microscopy coupled with the use of appropriate mitochondrial-targeted fluorescent probes. The benefits of melatonin and its metabolites have been described in the brain where they are found to be protective in models of Parkinson's disease, Alzheimer's disease and spinal cord injury. The reader is reminded, however, that data not covered in this review has documented beneficial actions of these amines in every organ where they have been tested. The outlook for the use of melatonin in clinical trials looks encouraging given its low toxicity and high efficacy.

REVIEW

INTRODUCTION

The brain is highly susceptible to damage by free radicals and related molecules (Reiter, 1995). Reasons for this high vulnerability include its, i) high utilization of molecular oxygen (O_2), ii) elevated content of ascorbic acid (which can become a potent prooxidant in the presence of free iron), iii) highly elevated concentrations of readily oxidizible polyunsaturated fatty acids, iv) low levels of antioxidative enzymes, especially catalase, and v) the poor accessibility of some antioxidants, e.g., vitamin E, to the brain.

Free radicals are molecules or portions of molecules that possess an unpaired electron in their valence orbital (Halliwell and Gutteridge, 1989). As a consequence of this deficient electron, radicals often abstract or donate an electron from/to an adjacent molecule thereby damaging it. The molecular mutilation that occurs as a consequence of these interactions is referred to as oxidative stress or oxidative damage. Every organ in the body, and especially the brain, continually sustain free radical damage. The altered molecules accumulate over the lifetime of the organism. Damaged molecules function less efficiently and eventually contribute to aging and diseases.

Figure 1 illustrates some of the damaging agents that originate from O_2. The superoxide anion radical ($O_2^{\cdot -}$), a product that has only weak oxidizing potential, is formed by the single electron reduction of O_2. It is readily dismutated (by ezymes present in mitochondria and in the cytosol, the superoxide dismutases) to a non-radical species, hydrogen peroxide (H_2O_2). In the presence of a transition metal, most frequently iron (Fe^{2+}), it is converted via the Fenton reaction, to the devastatingly reactive hydroxyl radical (·OH). The ·OH is highly destructive and damages any molecule in the vicinity of where it is produced. Since the damage inflicted is especially at the location where the ·OH is generated, it is referred to as on-site damage. Collectively, radicals and radical products that are oxygen based are referred to as reactive oxygen species (ROS).

Another highly reactive species is the peroxynitrite anion ($ONOO^-$) which is abundantly formed by the coupling of the $O_2^{\cdot -}$ with nitric oxide (NO·) (Stankovska *et al.*, 2006). Both these agents are generated in high quantities in the central nervous system (CNS) so $ONOO^-$ production is a major hazard to neurons and glia (Huier & Padmaja*, 1993;* Radi *et al.*, 2001). Some of the toxicity of the $ONOO^-$ may also be a result of its deterioration into the ·OH. Nitrogen based radicals and associated reactants are identified as reactive nitrogen species (RNS).

Fortunately the brain, as well as other organs, are endowed with means of combating the destruction meted out by free radicals and related species. All cells contain molecules that are capable of neutralizing reactive species before they inflict any damage; these are referred to as free radical scavengers. Additionally, cells are equipped with a series of antioxidative enzymes which metabolize toxic reactants to less reactive or totally innocuous molecules. These antioxidative enzymes include the superoxide dismutases (SODs), the glutathione peroxidases (GPx) and glutathione reductase (GRd). Free radical scavengers and antioxidative enzymes work in concert to reduce molecular damage resulting from ROS/RNS. Despite the complexity of the antioxidative defense system, some reactants always escape scavenging and molecular damage is inevitable.

MELATONIN AND ITS METABOLITES AS ANTIOXIDANTS

Melatonin is an indoleamine synthesized in the vertebrate pineal gland from tryptophan (Reiter, 1991a;1991b) Its production in this organ is regulated by the prevailing light:dark environment (Reiter, 1986) with the neural connections between the eyes and the pineal gland involving the retinal ganglion cells, the biological clock [the suprachiasmatic nuclei (SCN) of the anterior hypothalamus], descending neurons that synapse on cells of the intermediolateral cell column of the upper thoracic cord, and the peripheral sympathetic nervous system. Within the pineal, norepinephrine released from postganglionic sympathetic fibers, induces a series of molecular events that culminate in the conversion of serotonin to melatonin (Reiter, 1991a). Melatonin production within the pineal gland is confined to the dark phase, i.e. night, of the light:dark cycle. The superimposion of light at night (Erren *et al.*, 2008) as well as possibly some non-visible electromagnetic radiation (Lerchl *et al.*, 1990; Reiter, 1993) suppresses melatonin synthesis. Light inhibition of nighttime melatonin production is becoming an increasingly major issue given the rampant light pollution that is a consequence of the widespread use of artificial light at night. The inhibition of melatonin by nocturnal light has already been identified as potentially causative in several diseases (Erren *et al.*, 2008). The diseases that may be a consequence of the nighttime suppression of melatonin by excessive nocturnal light are theorized to be a consequence, in part, of the loss of melatonin's oncostatic actions, its free radical scavenging and antioxidant activities.

Investigations in the early 1990s yielded data that suggested that melatonin may be a free radical scavenger (Chen *et al.,* 1993; Tan *et al.*, 1993a). This highly novel concept was tested by Tan *et al.* (1993b) and the data, in fact, revealed that melatonin is a highly efficient scavenger of the destructive ·OH. In the initial publication, the authors documented that melatonin quenched the electron spin resonance spectrum of the adduct formed by the spin trapping agent, dimethypyroline-N-oxide and the ·OH in a pure chemical system. Thus, a least *in vitro*, melatonin neutralized the ·OH. Additionally, Tan and colleagues (1993b) surmised that melatonin scavengers two ·OH to form cyclic 3-hydroxymelatonin (c3-0HM), a product that was identified by carbon- and proton-nuclear magnetic resonance. Furthermore, they identified c3-OHM in the urine of both humans and rats proving that, *in vivo* as well, melatonin scavenges the ·OH resulting in the formation of c3-OHM, which is then excreted in the urine. Furthermore, this group reported that the injection of exogenous melatonin into rats and then subjecting them to whole body ionizing radiation (which produces massive numbers of free radicals) led to highly elevated urinary excretion of c3-OHM. This suggested that melatonin would be a potent protector of molecular damage resulting from radiati-

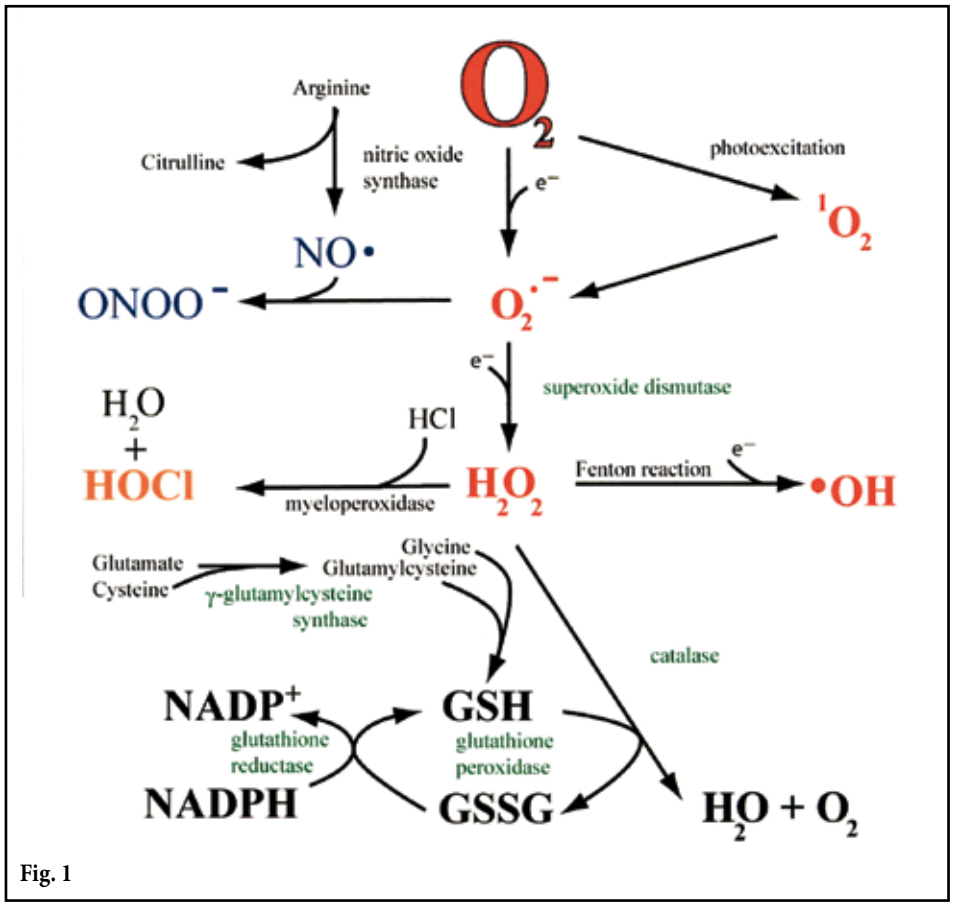

Fig. 1

Fig. 1 (above). Oxygen and nitrogen based reactants that derive from oxygen (O_2). Oxygen based radicals include $O_2^{\cdot -}$ and ·OH., $O_2^{\cdot -}$ also couples with NO· to form ONOO–, a nitrogen based reactant. The most toxic and highly damaging products are the ·OH and $ONOO^-$.

on exposure; subsequent studies have documented that melatonin and its metabolites are in fact powerful radioprotective agents (Vijayalaxmi *et al.*, 2004: Karslioglu *et al.*, 2005; Manda *et al.*, 2007).

Unexpectedly, c3-OHM, like its parent molecule melatonin, also interacts with reactive oxygen species and neutralizes them (Tan *et al.*, 2007). In the process N1-formyl-N2-phenyl-5-methoxykynuramine (AFMK) is generated. AFMK is also capable of functioning in the detoxification of radicals and their derivatives (Tan *et al.*, 2000). In doing so AFMK generates the product, N1-acetyl-5-methoxykynuramine (AMK) (Hardeland *et al.*, 2005; Hardeland *et al.*, 2007). This molecule, like its precursors is also believed to be a scavenger. Each of these metabolites are excreted in the urine (Ma *et al.*, 2006).

This remarkable cascade of reactions whereby melatonin as well as its initial, secondary, tertiary and quaternary derivatives are all potent scavengers is referred to as melatonin's antioxidative cascade (Tan *et al.*, 2002, 2007). In this series of sequential reactions it has been estimated that melatonin is responsible for the scavenging of ten or more reactive damaging agents (Tan *et al.*, 2007). This is unique among free radical scavengers where typically a single antioxidant molecule neutralizes a single radical. This cascade of reactions is illustrated in Figure 2. The detoxification of radical and radical products by melatonin and its derivatives are receptor-independent actions and only require that the scavenger be at the site where the radical product is generated. This is essential since highly reactive agents such as the ·OH and the $ONOO^-$ mediate damage in the immediate vicinity of where they are produced, i.e., the damage is site specific.

Melatonin also has receptor-mediated actions which adds to the capability of this molecule in eradicating radicals and reducing oxidative stress (Reiter *et al.*, 2000; Rodriguez *et al.*, 2004, Tomas-Zapico & Coto-Montes, 2005). Thus, melatonin stimulates a number of antioxidative enzymes which metabolize reactive products to innocuous agents. The enzymes whose activities have been shown to be promoted by melatonin include both Cu/Zn and MnSOD, GPx and GRd (Pablos *et al.*, 1995; Barlow-Walden *et al.*, 1995; Rodriguez *et al.*, 2004). The SOD catalyzes the enzymatic removal of $O_2^{\cdot -}$ from the intracellular environment by dismutating it to H_2O_2 (Reiter *et al.*, 2000). Once H_2O_2 is formed it can be metabolized to water and O_2 by GPx; glutathione (GSH) in this reaction is reduced to its oxidized form (GSSG); this disulfide is converted back

to its reduced GSH by GRd. The cycling of glutathione from GSH→GSSG→GSH is an important function of melatonin. The effects of melatonin on the activities of the antioxidative enzymes are likely receptor-mediated and involve receptors on the plasma membrane and also presumably receptors/binding sites in the nucleus (Tomas-Zapico & Coto-Montes, 2005).

Besides recycling GSH, melatonin influences its synthesis as well. There are two reports documenting that melatonin stimulates the rate limiting enzyme in GSH production, gamma-glutamylcysteine synthase (Urata *et al.,* 1999; Winiarska *et al*., 2006). Since GSH is also an important intracellular antioxidant, stimulation of its production by melatonin would obviously improve the overall ability of the indoleamine to quell oxidative destruction intracellularly.

MELATONIN ACTIONS AT THE MITOCHONDRIAL LEVEL

Interest within recent years has focused on potential mitochondrial actions of melatonin since these organelles are major sites of free radical generation. As electrons pass through the complexes of the mitochondrial respiratory chain, some of them are fumbled and reduce adjacent O_2 molecules giving rise to the $O_2^{\cdot -}$. There is experimental evidence which shows that melatonin acts at the level of Complex I and Complex IV of the respiratory chain (Martin *et al*., 2002; Okatani *et al*., 2003; Leon *et al*., 2005). An action of melatonin at these sites is believed to improve the efficiency of electron flow and reduce the number of electrons that run amuck thereby reducing free radical generation. This action of melatonin is referred to as radical avoidance (Hardeland, 2005) and could be a very significant means by which melatonin restricts oxidative damage.

That melatonin works at the mitochondrial level to scavenge and/or reduce the formation of free radicals has been elegantly visualized in a series of investigations by Jou *et al* (2004; 2005; 2007). With the aid of time-lapse conventional, confocal and multiphoton fluorescent imaging microscopy coupled with non-invasive mitochondria-targeted fluorescent probes, this group successively observed changes in free radical generation in cells with and without melatonin treatment. In these *in vitro* studies, Jou *et al* (2004; 2005) observed that the addition of H_2O_2 to the culture medium in which astrocytes were growing caused a time-related progressive increase in free radical fluorescence in the mitochondria. The elevated free radical generation was followed by membrane blebbing and mitochondrial swelling. Additionally, opening of mitochondrial transition pores, release of cytochrome c, activation of caspase-3, and DNA laddering which led to apoptotic death of the cells was observed. When melatonin was added to the culture medium concurrently with H_2O_2, each of the changes induced by H_2O_2 alone was prevented and apoptosis of the cells was averted.

While the actions of melatonin as a free radical scavenger are unequivocal and of utmost importance, the most recent study by this group (Jou *et al.,* 2007), documented that melatonin scavenges radicals not only in the mitochondria but in the cytosol and nuclei of cells as well. These relationships were investigated using cybrids (cells that have a large-scale deletion of 4977 base pairs of mtDNA); this is referred to as the common deletion. The loss of this mtDNA damages the respiratory chain at four polypeptides of Complex I, one peptide of Complex IV and two peptides of Complex V. This deletion markedly augments mitochondrial ROS formation and enhances the frequency of apoptosis. In what are referred to as W and D cybrid cells, the addition of H_2O_2 to the medium in which these cells were grown caused massive rises in mitochondrial (mROS), cytosolic (cROS) and nuclear (nROS) reactive oxygen species. Even in these cells suffering with a common mitochondrial deletion and extremely high free radical generation, melatonin reduced ROS fluorescence in the mitochondria, cytosol and nuclei (Fig. 3).

PROTECTION OF THE CNS BY MELATONIN

As mentioned in the Introduction, the brain and spinal cord are especially vulnerable to free radical attack and over the course of a lifetime they sustain massive oxidative abuse. A plethora of neurodegenerative diseases have, as part of their basis, accumulated molecular damage and cellular loss due to apoptosis resulting from the persistent bludgeoning by free radicals and related reactants. Models of several of these have been examined to determine whether melatonin has any efficacy in reducing their severity.

1-methyl-4-phenyl-1,2,3,6-tetrahyro-pyridine (MPTP) is a toxin known to cause Parkinson's disease (PD) in humans. MPTP readily crosses the blood-brain barrier, is taken up by astrocytes where it is metabolized to the 1-methyl-4-phenyl pyridinium (MPP^+) by monoamine oxidase B, after which it is released and taken into dopaminergic cells via the dopamine transporter. Inside the dopaminergic neurons it concentrates in the mitochondria and poisons Complex I leading to excessive free radical production and apoptosis of the cells. After loss of the dopaminergic neurons, which are concentrated in the substantia nigra, the signs of PD appear.

Given that MPTP kills neurons via free radical-mediated mechanisms, melatonin would be expected to reduce the cellular death and defer signs of Parkinsonism. When tested in cultured dopamine-containing neurons (Bao *et al*., 2005; Mayo *et al*., 2005) or *in vivo* (Acuna-Castroviejo *et al.*, 1998; Antolin *et al*., 2002), melatonin was capable of attenuating the loss of tyrosine hydroxylase (the rate limiting enzyme in dopamine synthesis), the reduction of dopaminegic cell dead in

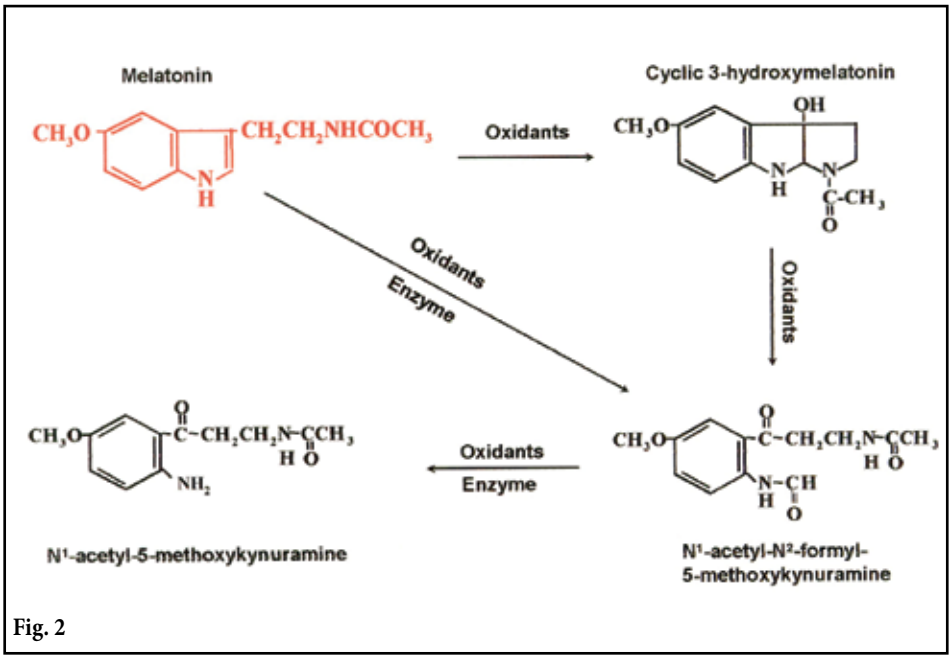

Fig. 2

Fig. 2 (right, above). Products that are formed when melatonin and its metabolites detoxify free radicals. Melatonin scavenges the ·OH to generate cyclic 3-hydroxymelatonin; this latter molecule also functions as a scavenger and produces N1-acetyl-N2-formyl-5-methyoxykynuramine (AFMK). AFMK scavenges radicals to generate N1-acetyl-5-methoxykynuramine (AMF). Melatonin can also be converted to AMFK directly either enzymatically or via radical scavenging. This sequence of reactions is referred to as melatonin's antioxidative cascade (Tan *et al.*, 2002).

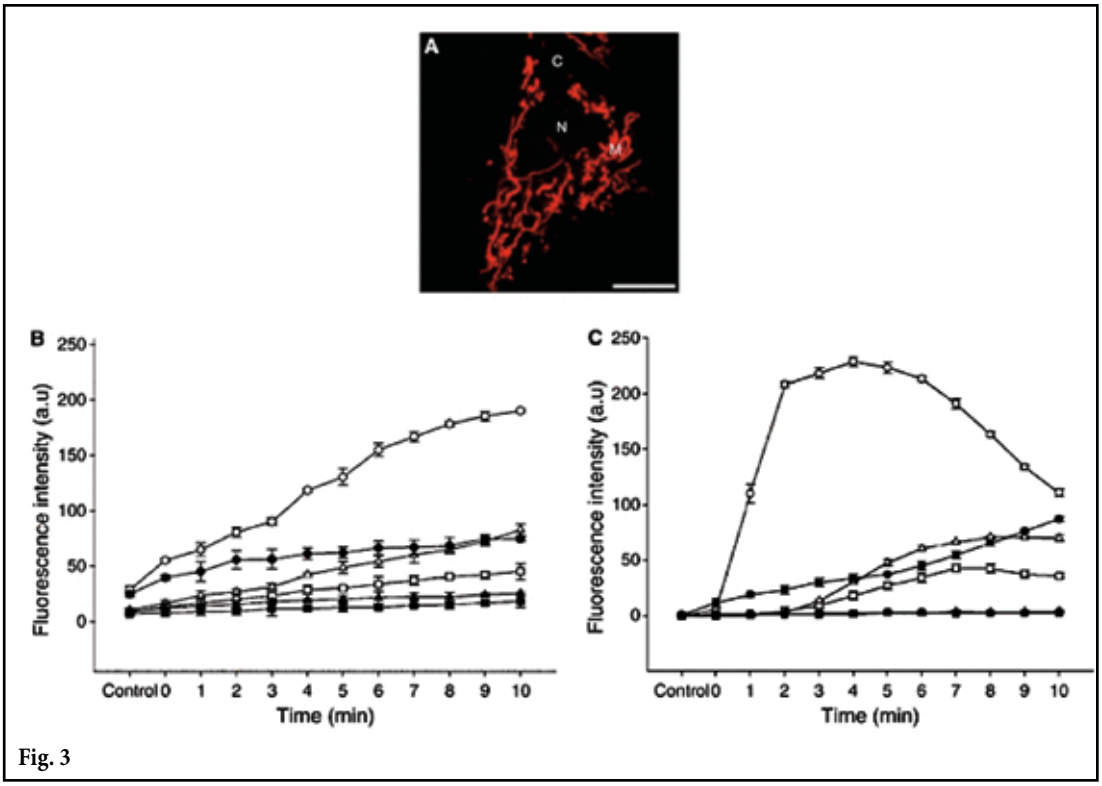

Fig. 3

Fig. 3 (right, below). Subcellular quantification of free radical generation in mitochondria (m), nuclei (n) and cytosol of two cell types (summarized in panels B and C) that have a deletion of 4977 base pairs of their mitochondrial DNA. This defect is referred to as the common deletion and causes the cells to generate augmented numbers of free radicals. Panels B and C show that in both cell types melatonin reduced free radical production (fluorescence intensity) in the mitochondria, nuclei and cytosol documenting that melatonin scavenges radicals in each of these subcellular compartments. Mitochondria (circles); nuclei (squares); cytosol (triangles); Hollow symbols = no melatonin; solid symbols = with melatonin. From Jou *et al.*, 2007).

the substantia nigra and the development of Parkinson-like signs.

In other models of PD including that caused by rotenone (Coulom and Birman; Saravanan *et al.*, 2004, 2007), 6-hydroxydopamine (Mayo *et al.*, 1998) and glutamate excitotoxicity (Herrera *et al.*, 2001; Cheng *et al.*, 2008), melatonin has proven effective in reducing molecular changes and apoptosis. In each of these cases, it was presumed that the antioxidative actions prevented or limited the severity of experimental Parkinsonism.

Major signs of Alzheimer's disease (AD) include the development of β-amyloid-based senile plaques in the vicinity of neurons and the generation of intracellular neurofibrillary tangles. Both malformations are related to free radicals. β-amyloid deposits generate ROS that lead to the oxidation of essential macromolecules in neurons eventually causing them to undergo apoptosis. Neurofibrillary tangles are formed when the cytoskeletal protein, tau, is phosphorylated. The tangles also induce free radical generation that compromises the function of neurons.

The toxicity of amyloid-β has been repeatedly shown to be reduced by melatonin (Pappolla *et al.*, 2000). Similarly, the hyperphorylation of tau is ameliorated when melatonin is present (Yin *et al.*, 2006); melatonin arrests tau metabolism by inhibiting one of the enzymes that causes its phosphorylation, i.e., glycogen synthase kinase-3 (Deng *et al.*, 2005). Additionally, in mice transfected with the human amyloid precursor protein gene, melatonin limits the accumulation of amyloid-β in the brain and forestalls the death of the animals (Matsubara *et al.*, 2003). In humans, melatonin has been shown to reduce mild cognitive impairment in elderly subjects (Curio *et al.*, 2007).

Traumatic brain and spinal cord injury (SCI) are common conditions that jeopardize the quality of life of individuals. A review by Maldonado and colleagues (2007) summarized the high efficacy of melatonin in reducing neural destruction and malfunction associated with blunt craniocerebral trauma. Likewise in crush injury to the spinal cord, melatonin treatment improves the morphological damage as well as the biochemical and molecular biological changes that result from experimental induction of SCI (Samantaray *et al.*, 2008; Nesic *et al.,* 2008). In the study of Samantaray *et al.* (2008), the activity of the neutral peptidase, calpain, was monitored along with many other features after SCI. Associated with the morphological damage, calpain was highly activated. When melatonin was administered, however, restoration of lower calpain levels was achieved and all other parameters of damage were improved as well. Nesic *et al.* (2008) likewise found melatonin reduced neural destruction and allodynia (pain due to mechanical stimuli) after SCI. The improvement in allodynia was presumably related to melatonin's ability to reduce aquaporin 1 (Nesic *et al.,* 2008) in the superficial layers of the dorsal sensory horn of the thoracic cord which was elevated as a consequence of contusion of the cord.

These are only a few examples in which melatonin and/or its metabolites have been shown to be protective of neurons and glia during experimental conditions that were designed to simulate disease processes or damage to the human CNS. Virtually without exception, melatonin either prevented tissue destruction and loss, or improved outcome. While the high efficacy of melatonin and its metabolites as ROS/RNS scavengers has been assumed to be the beneficial feature in these situations, additionally the stimulation of antioxidative enzymes and radical avoidance may have also been involved in improving morphology and restoring function. Certainly, melatonin's ability to easily traverse the blood-brain barrier to enter the brain is also consistent with its highly protective actions in this tissue (Menendez-Palaez *et al.*, 1993).

CONCLUDING REMARKS

The data are unequivocal that melatonin and its metabolites are capable of reducing, via several different means, free radical damage in the CNS. These actions include direct detoxification of radicals and radical products, promotion of the activities of antioxidative enzymes, reducing the formation of radicals (radical avoidance) and enhancing the production of glutathione, an important intracellular antioxidant. In many experimental models, melatonin has proven to be a more effective protector against free radical damage than are the classic antioxidants (Martin *et al.,* 2000; Fischer *et al.,* 2002; 2008).

While this paper briefly reviews a few situations in which melatonin protected the brain from oxidative abuse, the literature is far more extensive on each of these topics than illustrated here. Moreover, herein only melatonin and its relation to PD, AD and SCI was considered. Other CNS diseases/conditions have been shown to be improved by melatonin and/or its by-products.

This article may leave the impression that melatonin's beneficial actions exclusively or primarily relate to the CNS. While its functions in the brain are of potential monumental importance, this indoleamine is equally as active in other organs where it also molecularly preserves the functional integrity of cells. There is also an endless list of studies where melatonin has already been found to be protective (Reiter, 2000; Reiter *et al.*, 2001; 2002; Allegra *et al.*, 2003; Fischer *et al.*, 2008; Peschke, 2008; Tengattini *et al.*, 2008) or in situations where it would likely avert free radical damage (Bruekerova *et al.*, 2006; Mach *et a.l*, 2006; Navarova *et al.*, 2006; Ujhazy *et al.*, 2006). It is anticipated that the literature related to this subject will continue to experience the explosive growth it has witnessed over the last decade. Furthermore, in addition to these basic research investigations it is almost assured that clinical trials with melatonin will continue and expand in scope (Gitto *et al* 2001; Simko and Paulis, 2007; Dominguez-Rodriguez *et al.*, 2008; Wasdell *et al.*, 2008; Wirtz *et al.*, 2008).

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