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Melatonin as a neuronal differentiation factor: therapeutic implications for dementia

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Original article

SUMMARY

Dementias are progressive, neurodegenerative neuropsychiatric illnesses with a high worldwide prevalence. They are ranked among the most important diseases causing disability in the elderly. Within these patients, the Central Nervous System shows anatomic-structural alterations at a cellular and sub-cellular level associated with cognitive deficiencies. In Alzheimer's disease in particular, histopathological markers such as amyloid plaques and neurofibrillar tangles have been characterized. It has been acknowledged that oxidative stress and neuroinflammation take part in the etiology and development of the disease. Neuronal precursors of the human olfactory epithelium have been recently characterized as an experimental model adequate for identifying trait biomarkers and to study the physiopathology of various neuropsychiatric diseases as well as the neurodevelopment process at a cellular, molecular and pharmacological level. This review presents evidence that sustains that melatonin can be used in the treatment of dementias due to its antioxidant capacity, its anti-inflammatory effect, as well as the inhibiting effect on the hyperphosphorilation of *tau* protein and on amyloid plaque formation. Likewise, by stimulating both formation of new neurons as well as neuritogenesis at its early stages and formation of dendrites, melatonin could contribute at counteracting the loss of cognitive functions present in these ailments.

Key words: Melatonin, dementia, biomarkers, neuroephithelium, neuritogenesis.

RESUMEN

Las demencias son enfermedades neuropsiquiátricas, progresivas, neurodegenerativas y con una alta prevalencia a nivel mundial. Ocupan uno de los primeros lugares como enfermedades que causan incapacidad en los adultos mayores. En estos pacientes el Sistema Nervioso Central presenta alteraciones anatómico-estructurales a nivel celular y subcelular que se asocian con deficiencias cognitivas. En particular, en la enfermedad de Alzheimer se han caracterizado marcadores histopatológicos como las placas amiloides y las marañas neurofibrilares. Se sabe que el estrés oxidativo y la neuroinflamación participan en la etiología y el desarrollo de la enfermedad. Recientemente se caracterizó a los precursores neuronales del neuroepitelio olfatorio humano como un modelo experimental adecuado para identificar biomarcadores de rasgo y para estudiar la fisiopatología de diversas enfermedades neuropsiquiátricas, así como el proceso del neurodesarrollo, a nivel celular, molecular y farmacológico. En este trabajo se presenta la evidencia que sustenta que la melatonina puede ser útil en el tratamiento de las demencias, por su capacidad antioxidante, por su efecto anti-inflamatorio, así como por el efecto inhibidor de la hiperfosforilación de la proteína *tau* y de la formación de placas amiloides. Además, al estimular la formación de nuevas neuronas, la neuritogénesis en sus etapas tempranas y la formación de dendritas, la melatonina podría contribuir a contrarrestar la pérdida de las funciones cognitivas que se observa en estos padecimientos.

Palabras clave: Melatonina, demencia, biomarcadores, neuroepitelio, neuritogénesis.

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Dementias are neurodegenerative illnesses suffered by around 30 million people worldwide.[1,2] They rank sixth among disabling mental illnesses, with 4.6 million new cases every year.[3] Alzheimer's disease (AD) is the most common of dementias, affecting mostly the elderly. According to age, prevalence rates vary between 5 and 8% in people 65 and older, 15 to 20% in people 75 and older, and 25 to 50% in people who are 85 and older.[4,5]

Patients suffering AD show cognitive disorders (agnosia), during the sleep-wake cycle and in their ability to perform everyday activities (apraxia), among other symptoms.[4,6] Anatomic-structural, cellular and sub-cellular alterations[7,8] can be observed in the Central Nervous System (CNS). Generalized brain atrophy[9] and gradual loss of gray matter formed by neuronal nuclei and dendrites[10] can be observed, at a macroscopic level, using techniques of tran-

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scranial magnetic resonance and positron emission tomography. Atrophia involves the limbic system (hippocampus, amygdala and parahippocampal gyrus), the brain cortex, the entorhinal cortex, the association cortex and other subcortical regions including the cholinergic system of the basal prosencephalon, the corpus striatum, the thalamus and the cerebellum, as well as the frontal, temporal, parietal and occipital lobes.[9,11] Alterations in brain regions are related to cognitive impairments such as aphasias, caused by damage in Broca's and/or Wernicke's area at the left hemisphere and the arcuate fasciculus connecting both areas; apraxias, caused by injuries in Broca's area and the corpus callosum as well as the parietal-frontal association areas, motor cortex and other areas associated to motion, depending on the kind of apraxia.[9] Besides, patients with AD show reduced neuronal activity in areas of the prefrontal cortex and in all areas of hippocampal formation including the *subiculum* and associated with deficiencies in verbal episodic memory.[9]

One pathological characteristic present in dementias is a decrease in volume of the hippocampus.[11,12] It has been associated with neuronal loss at the *hilus* and in the *cornus amonis* CA1 region, among others.[13,14] It has also been related to a loss of axons and to the decrease in the number of dendrites in the CA1 region,[15-17] as well as a reduction in their extension,[18] in the number of dendritic terminals and in the total length of dendrites in the parahippocampal gyrus.[19] This decrease of the structure of dendrites reduces the amount of spines and as a consequence the assembly of synaptic unions and of new neural circuits participating in neuronal plasticity and synaptic excitability inherent to memory and learning processes.[7,11,15,20,21] Decrease of synaptic contacts in neocortex and in the molecular layer of the dentate gyrus of the hippocampus has been identified in patients suffering AD, as well as loss of afferent pathways from neurons located in the entorhinal cortex.[22-24]

BIOMARKERS OF ALZHEIMER'S KIND DEMENTIA

Along with structural changes at a brain level, several brain trait biological markers have been described regarding patients with an AD diagnosis, oxidative stress among them, and it has been showed that patients with AD have high levels of free radicals in the frontal cortex[25] through brain mapping techniques and transcranial magnetic resonance. The levels of free radicals are also high in the plasma of these patients and their antioxidant capacity is diminished.[26] It has also been described that oxidative stress plays a crucial role in AD since it causes diminishing synaptic densities, reduction of neurotrophic factors such as NGF and BDNF[27] and collapse of the neural cytoskeleton,[28] altogether causing generalized brain atrophy. Free radicals and reactive oxygen and nitrogen species (ROS/NOS) also activate signaling cascades associated to inflammation which, as described below, also plays an important part in the etiology of neurodegenerative ailments.

Difuse or neuritic amyloid plaques, also called senile plaques, have also been considered a biomarker of AD. They have been described in the neocortex and in the hippocampus during early stages of the disease.[29] These plaques are formed by aggregation of beta amyloid peptide (Aβ) at the extracellular matrix of the brain.

Neurofibrillar tangles are another hystopatological marker[9,11] and are considered one of the main *post-mortem* diagnostic criteria for AD. They are formed by aggregates of paired helical filaments (PHF) consisting of hyperphosphorylated *tau* protein. This protein, associated to microtubules, is found in axons and participates in anterograde axonal transport. When it joins microtubules, it stabilizes them and it also acts by promoting the polymerization of tubulin.[30] Excess phosphorylation of *tau* causes dissociation of this protein of microtubules and, as a consequence, depolymerization of tubuline as well as loss of morphofunctional symmetry in neurons,[31] reduction in the number of synaptic contacts and interruption of axoplasmic transport.[32]

It is currently accepted that in the etiology of neurodegenerative illnesses in general and of AD in particular, there is a chronic neuroinflammation component. In this sense it has been showed that microglia and activated astrocytes are accumulated and associated to senile plaques in the brains of these patients, as well as with a greater amount of proinflammatory cytokines, chemokines, complement proteins, reactive oxygen and nitrogen species as well as other inflammation mediators.[33,34] The activation of microglia is produced by the presence of Aβ deposits and is, in principle, a protective response, since it is directed to the elimination of such deposits by means of phagocytosis.[35,36] Under normal conditions there is equilibrium between pro- and anti-inflammatory processes which allows for tissue repair and for the preservation of neural function. In neurodegenerative ailments, this equilibrium is broken and a chronic process is unchained which promotes recruiting and activation of microglia and astrocytes producing various pro-inflammatory mediators. They favor the activation of enzymatic systems associated to inflammation.[33] In response to cellular stress, neurons, astrocytes and the vascular endothelium generate more Aβ, which perpetuates the process.[33,37,38] The presence of these biochemical and cellular factors, together with the depletion of tissue antioxidant mechanisms and the loss of neuroprotective functions of astrocytes, as well as homeostasis of glutamate,[39] contribute to generate a microenvironment where exitotoxicity and neurodegeneration are favored, as well as retraction of synapses with its consequent cognitive deficit and, eventually, neuronal death.

On the other hand, in the last few years it has been also suggested that systemic inflammation plays a crucial role in the pathogenesis of dementias and an association

has been found between high levels of peripheral inflammatory markers such as C-reactive and IL-6 seric proteins and a moderate risk of suffering dementia.[40,41] In spite of the great amount of evidence regarding this topics, cellular and molecular mechanisms implied in chronic neuroinflammation as associated to dementias are complex and have not been completely elucidated. Just as well, therapeutic strategies developed in this area, especially the administering of non-steroideal anti-inflammatory drugs (NSAIDs), have offered partial results in terms of prevention and delay in the progression of the disease; nevertheless, they have not been effective in its advanced stages.[42]

EXPERIMENTAL MODELS USED IN THE STUDY OF DEMENTIAS

Studies have been developed to understand the physiopathology of AD in various animal models as well as in cellular and organotypic cultures.[43,44] Studies in rodents allowed to establish that injuries in the hippocampus produced by chemical or mechanical agents are associated with space memory deficiencies,[45] whereas the organotypic culture model of this brain structure has allowed for the definition of neural connections and of neural nuclei involved in memory and cognition as well as to attain the study of neuroprotective and neuroregenerative drugs potentially useful for the treatment of AD.[46-48] Slices of hippocampus in culture remain viable for several weeks, keeping their tridimensional architecture as well as the integrity and functionality of neural circuitry[49] and pharmacology effects can be topologically evaluated in them, both in adult neurons and in new neurons formed in the dentate gyrus.[47,50-52] In this model, the neurotoxic effects of Aβ have been tested on the aggregation of *tau* protein and the protective effect of melatonin (MEL) on neurotoxicity induced by peptid Aβ25-35.[44]

Apart from these experimental models, in recent years, the olfactory neuroepithelium (ONe) began to be used to study potential molecular markers of AD.[53-55] This epithelium contains the most external neurons of the organism, since they are placed in a peripheral region in contact with the environment and are therefore available to be obtained and isolated.[56-58] The first studies on characterization of biomarkers were made in ONe biopsies obtained *post-mortem*. In one cohort of 79 patients diagnosed with AD an increase was described in the amount of *tau*-FHP protein, of cytoplasmic Aβ aggregates and of alpha synuclein as potential proteic markers of the disease.[59] ONe is a tissue analogous to the neural tube giving origin to CNS and constituted by different types of cells: basal, subtentacular, multipotent, neuronal precursors and sensory neurons.[60,61] The latter are continually regenerating due to the proliferation of multipotent cells which are differentiated in order to become olfactory sensory neurons.[60,61] We have recently developed a method to isolate ONe cells in ambulatory neuropsychiatric patients by means of non invasive exfoliation of the nasal cavity and the implementation of culture conditions for the selection, propagation, differentiation and cryopreservation of neuronal precursors.[62] Neuronal lineage cells cryopreserved in banks which were later defrosted and recultured, kept their electrophysiological and phenotypical properties[62] and were able to develop the arrangements of cytoskeleton characteristic of neuritogenesis.[62] These data suggest that the neural precursors obtained from the nasal cavity of neuropsychiatric patients constitute an adequate experimental model to investigate the physiopathology of AD, the cellular and molecular aspects of neurodevelopment such as the differentiation of neurites in axons and dendrites, as well as various pharmacological actions on this process, and for the characterizations of trait biomarkers.[62]

MELATONIN AS AN ALTERNATIVE IN THE TREATMENT OF DEMENTIAS

During the last few years it has been suggested that MEL (N-acetil-5-methoxytryptamine) can be used for the treatment of AD (review: Rosales et al., 2012).[63] Administering this indoleamine to subjects suffering AD causes improvement in disorders of the circadian rhythms[64-66] as well as diminishing cognitive disfunction.[67] Pre-clinical trials support this concept since MEL acts as a free radical scavenger and thence reduces oxidative stress and apoptosis.[68] Particularly in neurons in the hippocampus and in neurons in culture, this hormone diminishes the levels of lipid peroxidation caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),[69] 6-hydroxydopamine (6-OHDA)[70] and kainic acid.[71] MEL also protects the neuronal cytoskeleton from structural disorganization produced by free radicals.[28] In microglia cultures stimulated with Aβ1-42 peptide, MEL pre-treatment reduces NADPH oxidase activity and assembly and the consequent production of superoxide anion and its derivative ROS.[72]

Okadaic acid (OA) increases oxidative stress[73] and inhibits PP1 and PP2A phosphatases,[74] which increases hyperphosphorilation of *tau* protein, formation of FHP and cytoskeleton retraction around the nucleus.[75] MEL, in concentrations similar to those circulating in plasma and cerebrospinal fluid (1 and 100 nM, respectively) blocks, prevents and reverts the increase in lipid peroxidation and apoptosis induced by OA in N1E-115 cells, as well as cytoskeleton collapse, which remains organized in the cytoplasm and in neurites in the presence of OA and MEL. Similar results were obtained when other oxidizing agents such as hydrogen peroxide and haloperidol.[28,76,77] On the other hand, reduction of circulating levels of MEL produced by surgical removal of the pineal gland (pinealectomy) has been associated with high indices of oxidative stress, decreased memo-

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ry and cytoskeleton disorganization in rat hippocampus.[78-81] Treatment with MEL administered intraperitoneally during a week, reverted these effects and pinealectomized animals injected with MEL showed values similar to those of control group in the amount of microtubules and microphilaments determined in the cytoskeleton-membrane fraction,[81] which suggests that the degree of oxidative stress, cytoskeleton organization and cognition are related.

MELATONIN REDUCES LEVELS OF PHOSPHORILATED *TAU*

Another one of the molecular changes blocked by MEL is the hyperphosphorilation of *tau* protein, associated to oxidative stress. In N1E-115 neuroblastoma cells previously incubated with 50 nM OA, 100 μM hydrogen peroxide or 100 μM haloperidol, MEL (1 or 100 nM) diminishes 100% of the relative amount of phosphorilated *tau* in serine 404.[77,82] Also, in animal AD models, such as 3xTg-AD mice, it has also been proven that MEL diminishes hyperphosphorilated *tau* levels as well as behavioral symptoms similar to those of dementia such as anxiety and loss of exploratory behavior.[83] Analogously, it has been described that there is an increase in the levels of hyperphosphorilated *tau* associated to a cognitive detriment in pinealectomized animals.[80]

MELATONIN STIMULATES EARLY NEURITOGENESIS AND DENDRITOGENESIS

Apart from its antioxidant effect, MEL works as a modulator of cytoskeleton organization and, as a consequence, of morphofunctional polarity development in neurons. This process implies differentiation of two cellular compartments: somatodendritic and axonal domain.[84] In culture, this process is initiated after detaching the neurons from the substrate and reseeding. Round cells develop one or multiple neurites presenting growth cones at their most distal end. Later, one of the neurites elongates and is differentiated from the axon. The remaining short neuritis are differentiated in dendrites and in the end, functional polarization and synapses[84] formation takes place. It has been proven that calmodulin (CaM) kinase II participates in the formation of neuritis and dendrites by means of phosphorilation of MAPs and STOPs (microtubule associated proteins and stable tubule only polypeptides).[85,86] STOPs participate in the development and neuronal differentiation. They are proteins that interact with CaM and which are required in the formation of neurites.[87] They also stabilize microtubules and are concentrated in cold-stable and drug-resistant domains in mature axons.[88] In N1E-115 cells in culture, MEL induces neurite formation and their elongation by means of stimulation of microtubules polymerization and through increase in the organization of actin in growth cones.[89,90] Also, indoleamine stimulates early neuritogenesis by means of the activation of protein kinase C (PKC) and RHO kinase.[90] We have recently showed, in organotypical hippocampus cultures, that MEL increases dendrite formation as well as their elongation and complexity, with an optimal time of six hours in culture, in neurons and interneurons in the *hilus* zone, part of the trisynaptic hippocampal circuit, which plays a key part in the integration of spatial memory.[52,91,92] These effects were concentration-dependent and maximal response was obtained with a 10^{-7} M MEL concentration. CaM kinase II participates in this response since dendrite formation is not stimulated by MEL in presence on the specific inhibitor of this enzyme KN-62 and of the specific inhibitor of PKC, bisindolylmaleimide (non-published data). These results and the fact that indoleamine in cells in culture activates PKC and induces phosphorilation of CaM and its translocation to the cytoskeleton membrane fraction, suggest that this enzyme is down the pathway of PKC in the signaling pathway of MEL.[93,94]

MELATONIN HAS ANTI-INFLAMMATORY EFFECTS ON THE CENTRAL NERVOUS SYSTEM

It is known that the administering of MEL inhibits activation of microglia and production of pro-inflamatory cytokines in models of acute neuroinflammation caused by bacterial infection[95] or by cerebral ischemia[96] in rats. Also, in the model of cerebral infection, treatment with MEL(100 mg/kg) diminishes the number of apoptosic neurons,[95] while in the ischemia-reperfusion model, administering of MEL (5 mg/kg) reduces cerebral infarction and associated neurobehavioral sequelae.[96] At a molecular level, MEL inhibits the expression of chemokines ARNm such as CCL2 (MCP-1), CCL5 y CCL9 (MIP-1γ) induced by LPS in a cellular line of microglia.[97] This effect was mediated by inhibition of transcriptional activity of NF-κB y STAT/GAS.[97] In mouse brain[98] and rat hippocampus[44] organotypical cultures stimulated by Aβ1-40 and Aβ25-35 peptides, respectively, MEL diminishes secretion of pro-inflammatory cytokines and prevents activation of microglia and astrocites induced by exposition to peptide Aβ25-35.[44] Along these effects, MEL inhibits the expression of pro-inflammatory iNOS and COX-2 enzymes, in C6 astrocyte cell line,[99] without inhibiting enzyme COX-1 and it has been suggested that indoleamine might have therapeutic effects similar to those of NSAIDs, but without its negative effects.[100] Reduction in the levels of pro-inflammatory cytokines such as TNF-α and IL-6, enzymes such as iNOS and COX-2 and transcription factors such as NF-κB, and the simultaneous stimulation of antioxidant systems, as those associated to Nrf2 cascade, among others, are considered as part of the moduling mechanisms of melatonin in neuroinflamation.[101,102]

Current evidence indicates that MEL can be useful in AD treatment and in the treatment of general tauopathies on account of its antioxidant capacity, of its sleep-wake rhythm synchronizing effect, of its anti-inflamatory effect as well as of the inhibiting effect of hyperphosphorilation of *tau* and of formation of amyloid plaques. In recent years it has been shown that MEL stimulates the formation of new neurons, neuritogenesis in its early stages as well as dendrite formation. Nonetheless it is not yet known if indoleamine stimulates morphofunctional differentiation in other stages of this process such as the formation of axons, dendritic spines, competent and electrically active synapses.

All this shows that it is of the utmost importance to continue studying neuronal development in the presence of MEL and to define those action mechanisms involved in this process as well as the effects of this indoleamine on the main trait biomarkers of dementias within the stablished neurodegeneration models as well as in the neuronal precursors obtained from the nasal neuroepithelium of neuropsychiatric patients.

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