Neurocytoskeletal Protective Effect of Melatonin: Importance for Morpho-functional Neuronal Polarization

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Abstract: Neurons have a highly asymmetric shape and they are constituted by two functional domains: the axonal, and the somatodendritic domains. Axons are cellular processes that make contact with target cells to transmit information, while dendrites located in the somatodendritic domain are specialized in the reception of information. During neurodevelopment, neurons acquire the highly morphofunctional polarization through a dynamic cytoskeletal organization. Melatonin, the main indolamine secreted by the pineal gland has two important properties which play a key role in the maintaining of neuron polarization: it is a potent free radical scavenger, and it is a cytoskeletal modulator. Melatonin stimulates cytoskeletal polarization through PKC and ROCK activation by recruiting cells at early stages of neurodevelopment for later differentiation. At later stages, melatonin induces neurite and microtubule enlargement by a calmodulin antagonism. Moreover, melatonin prevents the asymmetric shape lost induced by oxidative stress, a condition present in neuropsychiatric diseases, and abolishes the cytoskeletal damage caused by prolonged treatment with antipsychotics, restoring the morphofunctional polarization. Moreover, in organotypic cultures, melatonin at nanomolar concentrations enhances the number of dendrites and their complexity in hilar neurons of the hippocampus. In addition, melatonin stimulates the formation of new neurons *in vitro* and in a rodent model. In this review we will describe current evidences indicative of the melatonin participation in the neuronal morphofunctional differentiation as a cytoskeletal modulator. Also we will discuss the implications of the loss of neuronal polarization in neuropsychiatric diseases and the potential therapeutic utility of melatonin for the treatment of these illnesses.

Keywords: Cytoskeletal, neurites, growth cones, neurodevelopment, doublecortin, melatonin.

INTRODUCTION

In neurons, the cytoskeleton is composed by actin microfilaments, neurofilaments and microtubules. The neuronal cytoskeleton is essential for the formation of new neurites and synapses, axonal transport, neurotransmitter release, receptor positioning as well as for axonal, dendrite and synaptic structural support. These functions depend on the polarized morphology of neurons that is determined by the cytoskeleton [1,2]. Neurons are highly asymmetric shaped and have two domains: the axonal, and the somatodendritic domain. Axons are cellular processes that make contact with target cells to transmit information, while dendrites located in the somatodendritic domain are specialized in the reception of information. During neurodevelopment, neurons acquire the highly morphofunctional polarization. In this process dynamic cytoskeletal reorganization takes place together with a differential distribution of proteins to either the dendrites or the axons. Thus, asymmetric distribution of microtubule associated proteins, neurotransmitter transporters, ionic channels, protein signaling effectors, and adhesion molecules occurs during neuronal differentiation [3]. By contrast, in pathological conditions, such as those present in neuropsychiatric diseases, neurons lose their asymmetric shape, and therefore their capability to transmit and receive information [4,5].

Melatonin, the main indolamine secreted by the pineal gland has two important properties which play a key role in the maintenance of neuron polarization: its potent free radical scavenger activity, and its cytoskeletal modulator property [6]. In this review we will describe the dynamic cytoskeletal changes that occur during development that culminate in the highly asymmetric cell polarization. In addition, we summarize our findings that support that melatonin acts on the cytoskeletal structure to promote the highly asymmetric polarization in neuroblastoma N1E-115 cells. Also, we will describe the signaling pathways triggered in response to melatonin that participate in cytoskeletal redistribution and polarization. In addition, the loss of neuronal polarization in high oxidative stress that resembles pathological condition and its reestablishment by melatonin will be described. Furthermore, we will describe the polarized structure of the hippocampus to illustrate the importance of the highly asymmetric shape of neurons for cognition, a brain function regulated in the hippocampus. Finally, we will describe recent data on the melatonin effects on neuronal polarization in rat hippocampal organotypic cultures and in mice. Implications of the loss of neuronal polarization in

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1876-5289/10

neuropsychiatric diseases and the potential therapeutic utility of melatonin for the treatment of these illnesses will be discussed.

ASYMMETRIC DISTRIBUTION OF CYTOSKELETAL ELEMENTS IN NEURONS

Axons and dendrites differ substantially in their molecular composition [7]. Various cytoskeletal proteins are found exclusively or preferentially in axons or dendrites. Also, microtubule polarity differs in these structures and the three main cytoskeletal components are differently organized in axons and dendrites. Cytoskeleton in axons is constituted by microtubules arranged in parallel along the axon. Neurofilaments are intercalated with microtubules, given support to the axonal structure and depending on neurofilament number; axonal caliber is determined [8]. In axons, microfilaments are mainly distributed beneath the cell membrane forming the actin microfilament cortex, and are enriched in growth cones that act as guides to enlarge new formed neurites through microtubule polymerization [9]. In axons, the microtubule-associated protein, tau is abundantly distributed to give stabilization to microtubules. Also, it stimulates tubulin polymerization [10]. Evidence on asymmetric tau polarization in neurons was obtained by experiments where inhibition of tau expression, blocked neuronal tau asymmetric distribution concomitant to an axon maturation failure [11]. Moreover, structural asymmetry in neurons is evidenced by the fact that microtubule arrays in axons have uniform polarity with the growing end, the plus end, located at the most distal part of the soma, while in dendrites microtubules have mixed polarity. Dendrites are enriched in the microtubule associated protein 2 (MAP2), and stable tubulin only polypepetide (STOP) which are polarized into dendrites and absent in axons. Both, microtubule associated proteins play a key role in stabilize the structure of dendrites [12]. Microtubules in dendrites are characterized by a uniform distribution and they are equidistantly spaced across all the dendrite showing a lattice-like appearance. Also they have a mixed polarity, in which approximately 50% of minus-ends and 50% of plus-ends distal from soma, in proximal mature dendrites [13]. On the other hand, neurofilaments are more numerous than microtubules and are grouped in branches of 30 neurofilaments in large dendrites giving them a rigidity property [14]. Also, they appear as strands of a rope in longitudinal sections. Microfilaments in dendrites are concentrated underneath the plasmatic membrane at dendritic spines, in pre- and post-synaptic terminals as well as in growth cones compromised to form new dendrites and they are highly dynamic [15].

NEURONAL POLARIZATION OCCURS DURING NEURODEVELOPMENT

One key question in neuronal development is how polarity and the highly asymmetric shape of neurons is established and maintained. Dynamic cytoskeletal rearrangements take place along the entire processes of neurodevelopment which determine the neuronal shape and structural polarization. Neurodevelopment starts with neuritogenesis, which is characterized by spherical shape breakage of immature neurons, and by emerging nascent neurites from the soma, which steadily enlarge to establish the axonal dendrite polarity characteristic of neuronal cells [16]. Neuritogenesis has been described to occur in five stages all characterized by an asymmetric neuronal shape and by a polarized distribution of the three main cytoskeletal components. Stage 1 consists of cellular budding by neuroblast spherical shape break. Stage 2 is characterized by the beginning of neurite development and implicates branching of the main neurite by sprouting of several minor neurites and the formation of a meshwork of microfilaments located in sheet-like extension at cell periphery, called lamellipodium [16]. This structure develops long thin protrusions composed of filamentous actin (F-actin) bundles or filopodia, forming the growth cone located at the tip of neurites. Growth cones move away from the cell body allowing microtubules to form a neurite shaft [17,18]. At stage 3, one of the neurites elongates rapidly to form the axon. The remaining neurites grow at a slower rate to form dendrites (Stage 4). Lastly, they continue to branch dendritic spines, allowing synaptic contacts assemble and the generation of spontaneous electrical activity throughout the neuronal network (Stage 5) [18,19]. Once axons and dendrites are differentiated, cytoskeletal dynamic changes participate in axon and dendrite maintenance.

MELATONIN PROMPTS ASYMMETRIC DISTRIBUTION OF CYTOSKELETAL COMPONENTS DURING NEURODEVELOPMENT

The effects of melatonin on cell polarization during neurodevelopment have been studied mainly in N1E-115 neuroblastoma cells. These cells *in vitro* develop the five stages of neuritogenesis and when they are differentiated exhibit many of the properties of natural nerve cells, including cell morphology, electrical excitability, the expression of nerve specific enzyme activities involved in neurotransmitter synthesis, as well as the expression of neurotransmitter receptors. Moreover, N1E-115 cells show a dynamic cytoskeleton capable of extending processes, several millimeters in length, that contain microtubules [20-22], neurofilaments, and microfilaments. Therefore, we used N1E-115 cells to study the cytoskeletal dynamics in presence of melatonin.

Despite that melatonin effects on molecule polarization was observed in amphibian dermal melanophores three decades ago as pigment aggregation in amphibian melanocytes [23], cytoskeletal asymmetric distribution was demonstrated by studying neurite formation in N1E-115 cells, a process which implicates microtubule enlargement and polarization [24]. In this study it was demonstrated that melatonin increased two-fold the number of cells with neurites with 1 and 100 nM concentration suggesting that melatonin prompts neuronal differentiation at the nocturnal plasma and cerebrospinal fluid concentrations, respectively breaking the neuronal spherical shape at stage 1 of neuritogenesis in Da Silva an Dotti neurodevelopment model [16]. Calmodulin antagonism by melatonin was demonstrated to participate in microtubule enlargement induced by the indole in N1E-115 cells [25]. *In vitro* experiments demonstrated that melatonin blocked the decreased polymerization rate and the total polymerization at equilibrium elicited by calmodulin activated by calcium similarly to the calmodulin antagonist, trifluoperazine and the compound 48/80. These effects were corroborated in cytoskeletons *in situ*, suggesting a melatonin´s membrane-receptor independent mechanism and mediated by direct binding to calmodulin [25].

In addition to the effects of melatonin on microtubule polarization and enlargement in neurites of N1E-115 cells, vimentin intermediate filaments and microfilaments, also are reorganized in presence of melatonin through protein kinase C (PKC) activation [26,27]. A PKC alpha dependent vimentin rearrangement was demonstrated to occur after 30 minutes of melatonin incubation in N1E-115 cytoskeleton *in situ*. In presence of either the phorbol ester or melatonin, vimentin relocalization at the perinuclear area was observed. This effect was blocked by an anti-PKC alpha antibody, while an anti-PKC epsilon antibody was unable to block this melatonin effect on vimentin organization [27]. Thus, because vimentin intermediate filaments act supporting microtubules in nerve cells and they are reorganized during neurite enlargement and maturation in presence of melatonin, data suggest that they participate as support for the asymmetric morphology of N1E-115 cells treated with the indole. In addition to increased vimentin phosphorylation by a PKC stimulated by melatonin, microfilament phenotypes described as typical of stage 2 of neuritogenesis were formed in cultures incubated with melatonin at the nocturnal cerebrospinal fluid concentration. Increases in the number of cells with growth cones, and filopodia, were observed after 3 and 6 hours of melatonin incubation [28].

The mechanisms by which melatonin increases these microfilament phenotypes involves ROCK and PKC activation since the specific inhibitors, Y27632 and bisindolylmaleimide, respectively, blocks the increased formation of growth cones and filopodia as well as neurites. Furthermore, the activity of both enzymes increased in presence of melatonin was inhibited with the specific inhibitors. Thus, melatonin stimulates the neurodevelopment by recruiting cells for later differentiation. This notion is supported since melatonin increases the number of cells with immature neurites, breaking the sphere (stage 1), by increasing the number of cells with growth cones and filopodia (stage 2) and by increasing the number of cells in stage 3 with longer neurites.

MELATONIN INDUCES CELL POLARIZATION IN OXIDATIVE STRESS N1E-115 CELL CULTURE MODELS

In schizophrenia and bipolar disorder, loss of dendrites in the prefrontal cortex as well as reduction in the hippocampus volume associated with loss of gray and white matter has been related to an altered cytoskeletal organization [29]. Moreover, neurotransmission impairment and cognitive dysfunction, together with brain structural abnormalities strongly suggest that the highly morphofunctional asymmetric shape of neurons is lost in neuropsychiatric disorders. Besides, oxidative stress is another pathophysiological alteration present in neuropsychiatric disorders related to the loss of cytoskeletal polarization [30-33]. This notion is supported because in culture cells, oxidative stress causes aberrant organization of cytoskeletal components. High levels of free radicals cause actin depolymerization [34]. Also in N1E-115 cells microfilaments and microtubules collapse in presence of chemical substances that produces high levels of free radicals such as okadaic acid, hydrogen peroxide and the antipsychotics clozapine and haloperidol. Thus, these evidences indicate that oxidative stress causes the loss of neuronal morphology and the highly asymmetric shape [35-37].

Current knowledge indicates that melatonin prevents cytoskeletal damage by reducing oxidative stress and by re-establishing the cytoskeletal organization and therefore the asymmetric shape in damaged neurons [29,37]. Melatonin attenuates cytoskeletal disruption produced by high levels of free radicals generated by hydrogen peroxide, and high doses of the antipsychotics haloperidol and clozapine [29]. N1E-115 cells incubated with either 100 μM hydrogen peroxide, 100 μM haloperidol, or 100 μM clozapine or 50 nM okadaic acid undergo a complete cytoskeletal retraction around the nucleus [29]. By contrast, NIE-115 cells incubated with either of these compounds followed by the nocturnal cerebrospinal fluid concentration of melatonin (100 nM) showed a well preserved cytoskeleton and neuritogenesis [29,37]. Re-establishment by melatonin of neurite formation, microtubule enlargement, and microfilament organization in microspikes and growth cones in cells previously damaged with either haloperidol, clozapine or okadaic acid have been observed[1]. Also, the indole abolished increased lipid peroxidation and apoptosis and tau hyperphosphorylation caused by these compounds. Hyperphosphorylation of the microtubule-associated protein tau causes microtubule de polymerization and the inhibition of axonal transport. Tau protein binds to microtubules for stabilizing their structure. Hyperphosphorylated tau is dissociated from microtubules and is abnormally assembled in paired helicoidal filaments found abundantly in dementia patients. Melatonin abolished increased tau phosphorylation and paired helical filament formation in animal models and in N1E-115 cells produced by wortmannin, isoproterenol, calyculin, haloperidol and okadaic acid. Together, data strongly suggest that melatonin acts as a neurocytoskeletal protector by decreasing tau hyperphoshorylation and preserving the microtubular structure. They also suggest that melatonin may improve cognition by impeding neuronal damage caused by tau hyperphosphorylation. Furthermore, the results indicate that melatonin protects, prevents or re-establish the neurocytoskeletal organization and the morphofunctional asymmetric shape disrupted by compounds that generates high levels of oxidative stress. In support to this notion it has been demonstrated that melatonin diminished the symptoms of tardive diskynesia caused by long-term neuroleptic treatment that has been associated with antipsychotic toxicity and neuronal death. Also toxic effects of clozapine such as agranulocytosis have been associated with high levels of free radicals and cytoskeletal alterations [38]. Thus, melatonin may be useful in the treatment of schizophrenia because it reduces the cytoskeletal and neuronal damage caused by prolonged treatment with antipsychotics. Moreover, cytoskeletal collapse precedes apoptosis [39], melatonin may improve neuronal survival by cytoskeletal stabilization and through maintaining the highly asymmetric shape of neurons (Fig. **1**).

NEURONS IN THE HIPPOCAMPUS ARE POLARIZED AND HAVE A HIGHLY ASYMMETRIC SHAPE

The hippocampus is a brain structure implicated in memory and learning and is deteriorated in neuropsychiatric disorders [40]. The hippocampal formation comprises four cy-

[1] Benítez-King G, Ortiz-López L, Jiménez-Rubio G, Ramírez-Rodríguez G. Haloperidol causes cytoskeletal collapse in N1E-115 cells through Tau hyperphosphorylation induced by oxidative stress: Implications for neurodevelopment. *Submitted*.

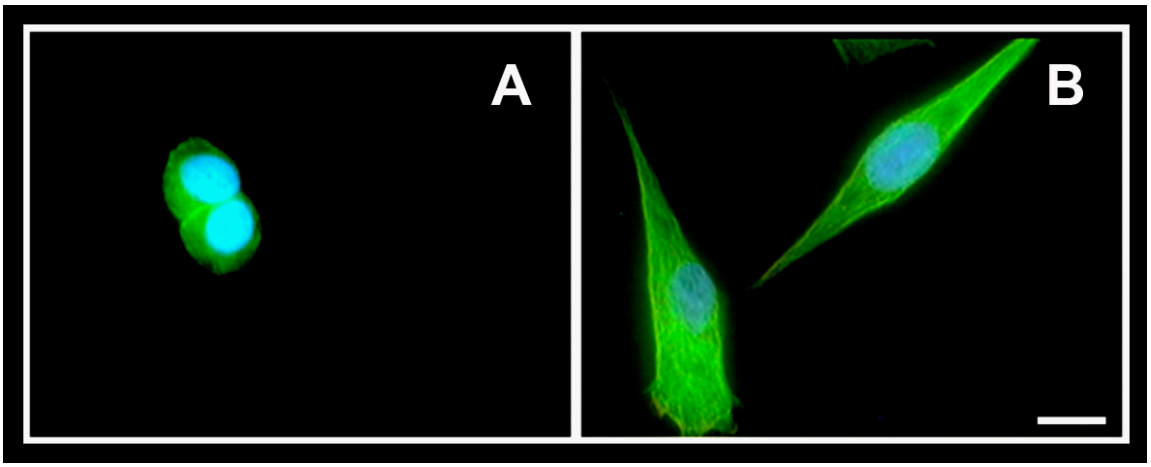

Fig. (1). Cytoskeletal neuroprotective effect of melatonin against the damage caused by hydrogen peroxide. N1E-115 cells were incubated with (**A**) 100μM hydrogen peroxide for an hour causing a collapsed-shape of microtubular network. (**B**) Cells incubated with 100 μM hydrogen peroxide for an hour followed by 3 hours of 100 nM melatonin show that melatonin restores the morphofunctional polarity indicated by the presence of long processes. Bar =50 μm.

toarchitectonically distinct regions: a) dentate gyrus, b) hippocampus, which has three subdivisions named *cornus ammonis* (CA) and are enumerated, CA3, CA2 and CA1, c) subicular complex, in which are included the presubiculum, the parasubiculum and the subiculum and d) enthorrinal cortex [40,41].

Each of the hippocampal regions is linked, one to the next, by unique and largely unidirectional projections formed by asymmetric shaped neurons. This neuronal circuitry is mainly an excitatory pathway, which begins in the enthorrinal cortex where cells of the superficial layers send axonal projections to the dentate gyrus that form part of the perforant pathway. Likewise, the granular cells of the dentate gyrus give rise axons called mossy fibers that connect with pyramidal cells of CA3. Finally, the Shaffer collateral axons are the source of the input to the pyramidal cells of CA1, which in turn projects to the subicular complex and enthorrinal cortex for closing the hippocampal processing loop. We could resume this circuit in the early literature term of "trisynaptic circuit" by emphasizing the first three major links as follows: EC→DG (perforant path), DG→CA3 (mossy fibers), CA3→CA1 (Shaffer collaterals) (Fig. **2**). Due to three-dimensional highly organized laminar distribution of its inputs and outputs, their cytoarchitectonic organizations and their highly polarized neurons, the hippocampus has been ideally suited to use it as a model system for neurobiological research [40,41].

MELATONIN REESTABLISHES AND INDUCES HIPPOCAMPAL CELL POLARIZATION

Hippocampus damage produces alteration in the neuronal polarity and the loss of neurons [42]**.** Considering that melatonin exerts its protective role acting as antiapoptotic substance and for favoring cytoskeletal organization [43], several evidences came out in the last years to show the melatonin effects on neurocytoskeleton and its implications for morphofunctional neuronal polarization in the hippocampus and in other brain regions in physiological and non-physiological conditions [44-48].

Neuronal polarity is important due to axons are cellular processes that make contact with target cells to transmit information, while dendrites located in the somatodendritic domain are specialized in the reception of information. Neuronal morphology is affected when the hippocampus is injured and consequently the loss of polarity affects memory and cognition [48,49]. In pinealectomized rats, supplementation of melatonin prevented the loss of neurons in CA1 and CA3 regions of the hippocampus [49]. Melatonin also reduced the death of pyramidal neurons preventing the impairment of place learning and memory [48]. Moreover, in ischemia the indole affected neuronal morphology in the hippocampus. In this sense, Gonzalez-Burgos and cols; reported that in a global cerebral ischemia model melatonin treatment induced changes in the dendritic spines of pyramidal neurons in CA1 [46,48]. Pyramidal neurons of rats treated with melatonin show a higher proportional density of mushroom spines than that in control rats [46].

An important process modulated by melatonin is apoptosis, also in the hippocampus [45]. It is known that the indole activates the Akt pathway, a canonical survival pathway, preventing the neuronal death in the hippocampus [50]. In 2006, Lee and cols; demonstrated the protective mechanism of melatonin on kainic acid excitotoxicity. Melatonin treatment prevented the death and the cytoarchitectural changes of pyramidal neurons in the CA3 region. This effect was accompanying by an increase in glial cell line-derived neurotrophic factor (GDNF). Thus, melatonin exerts its protective effect through the activation of Akt and through GDNF that will activate neuronal PI3K/Akt pathway [50]. This work suggests that, in addition to the decrease in neuronal death melatonin, also, maintains neuronal cell structure or polarization.

During aging cell structural changes and neuronal death occur possibly by the instability of synaptic connectivity [51]. Chronic melatonin administration to aged rats decreased the loss of MAP2 in the hippocampal CA1 and CA3 regions [52] and a relative increment in MAP2 protein in comparison with control rats was observed. Additionally, in

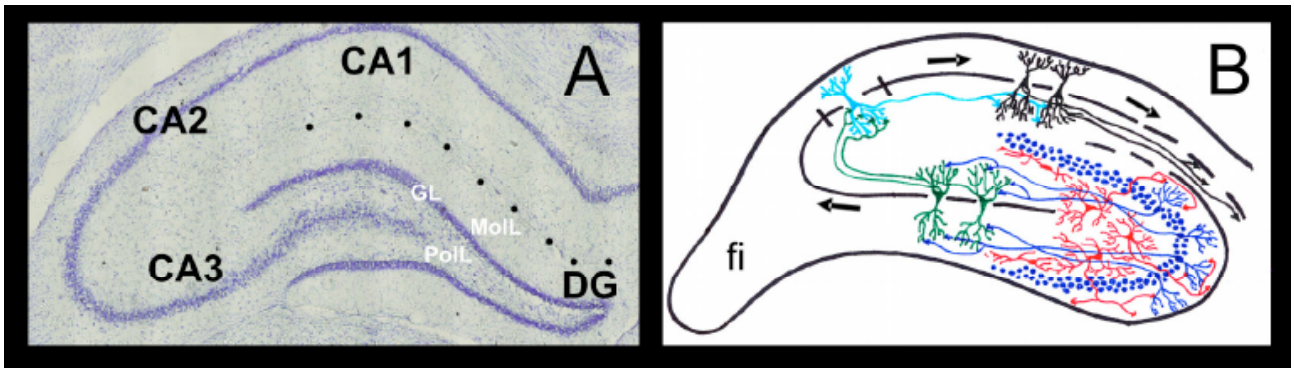

Fig. (2). Hippocampal formation. (**A**) Hippocampal divisions and subdivisions of the dentate gyrus in a Nissl staining micrograph (20x), in which is observed the *cornus ammonis* regions (CA1, CA2 and CA3) and the dentate gyrus (DG) that comprises three layers: molecular layer (MolL), granular layer (GL) and polymorphic layer (PolL). Points highlight the hippocampal fissure. (**B**) A diagram of the hippocampal formation, which points out high polarized neurons of granular layer (granular cells, blue), polymorphic layer (mossy cells and HIPAC cells, red), CA3 (pyramidal cell, green) and region termed fimbria (fi). Arrows indicate trisynaptic circuit unidirectionality.

older rats, chronic melatonin administration decreased the lost of MAP2 [52]. MAP2 is a microtubule stabilizer, then the fact that melatonin increases relative levels of this protein might support that the indole improves dendritic stability during brain aging.

Moreover, during development of the hippocampus, melatonin induces effects at structural cytoskeletal level. In hypoxic animals dendrites are affected by nitric oxide increased. This effect was counteracted by melatonin administration and it was accompanied by a reestablishment of dendrites in the developing hippocampus [53]. Effects of melatonin indicate that modulation of the dendritic spines and dendrite structure might be related to the cytoskeleton arrangements and with the hippocampal development and functionality.

Despite that little is known about hilar neurons, their loss has been described in aging and in some neuropsychiatric diseases as Alzheimer's disease [54], Parkinson's disease [55] and epilepsy [56]. In organotypic hippocampal slices, an *ex vivo* model based on the culture of explants that preserves hippocampal architecture and connectivity [57,58], we found that melatonin increases the number of dendrites as well as the complexity and the length of hilar neurons. Thus, the effects of melatonin on the highly asymmetric shape of neurons are elucidated in this *ex vivo* model.

Besides to the melatonin effects on cytoskeleton reorganization [59] and on dendrite complexity of hilar neurons, we found that melatonin also modulates the new neuron formation in the dentate gyrus of the hippocampus in adult mice [60]. Dendrite organization of new neurons is important for cell survival and integration in the neuronal circuitry [61]. Data of our group show that melatonin also modulates the dendritic morphology of new neurons. Based on the doublecortin expression, chronic melatonin makes more complex the dendritic tree of newborn neurons[2]. This effect suggest that modulation of cytoskeleton components by melatonin might be important for establishing synaptic connectivity of new neurons in the hippocampus of adult mice. Thus, melatonin favors hippocampal functionality by the well established neuronal contacts in this region important for learning and memory.

CONCLUDING REMARKS

Polarity and the highly asymmetrical shape are intrinsic to neuronal function. In neurons, somatodendritic domain receives and decodes incoming information and axonal domain transmits information to target cells. Neuronal polarity is cytoskeleton dependent. Then, cytoskeletal components organization will determine the correct neuronal polarization for establishing synaptic contacts that are necessary for the precise transmission of information in the brain. Neuronal polarization is affected in several neuropsychiatric disorders as depression, schizophrenia and also in Alzheimer's and Parkinson's diseases. Progressive loss of neuronal polarity is a known histopathological event in neuropsychiatric diseases related to oxidative stress. The loss of neuronal polarization would affect cognition. Therefore, the melatonin effects on neuronal polarization are important. This event is characterized by cytoskeletal collapse that underlies the loss of structural polarity and it is known that it precedes neuronal death and the disappearance of synaptic connectivity. Considering that the indole exhibits the properties to modulate cytoskeleton organization and prevents apoptosis, melatonin is important for re-establishing neuronal connectivity in the brain, specifically in areas important for cognition as the hippocampus.

Finally, melatonin is a potent free-radical scavenger that at the same time acts as a cytoskeleton regulator; thus, data discussed here strongly suggest that through its neuroprotective role melatonin could be useful in prevention and alleviation of psychiatry diseases that exhibit synaptic connectivity disruption.

ABBREVIATIONS

PKC = Protein kinase C

ROCK = Rho dependent kinase

[2] Ramírez-Rodríguez G, Ortiz-López L, Domínguez-Alonso A, Benítez-King G. Chronic melatonin modulates dendrite maturation in adult hippocampal neurogenesis. *Submitted*.

CA1	=	*Cornus ammonis* 1
CA2	=	*Cornus ammonis* 2
CA3	=	*Cornus ammonis* 3
EC	=	Enthorrinal cortex
DG	=	Dentate gyrus
STOP	=	Stable tubulin only polypeptide
MAP2	=	Microtubule-associated protein 2
Akt	=	Protein kinase B
GDNF	=	Glial-derived neurotrophic factor

ACKNOWLEDGEMENTS

This work was supported by the National Council of Science and Technology (CONACYT) Grant No. 86863. We thank Leonardo Ortiz-Lopez for preparing figures.

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Received: October 10, 2009 Revised: October 18, 2009 Accepted: October 22, 2009