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Role of MAP1B in axonal retrograde transport of mitochondria

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Synopsis

Microtubule associated proteins MAP1B and Tau are well known for binding to microtubules and to stabilize these microtubule structures. An additional role for microtubule binding proteins (MAPs) has recently emerged where they appear to participate in regulating transport of cargoes on the microtubules found in axons. In this role, tau has been associated with the regulation of anterograde axonal transport. We now report that MAP1B is associated with the regulation of retrograde axonal transport of mitochondria. This finding potentially provides for precise control of axonal transport by MAPs at several levels: controlling the anterograde or retrograde direction of transport depending on the type of MAP involved, controlling the speed of transport and controlling the stability of the microtubule tracks upon which transport occurs.

Introduction

A neuron can be divided into three regions: the cell body (or soma), the axon, and the dendrites. The morphological differentiation from a precursor cell to a neuron containing the three indicated regions is regulated by the cytoskeleton [1]. As a component of the cytoskeleton, the microtubules are necessary for the extension and maintenance of neuritic processes. Underlying these processes are the microtubules, dynamic structures that are stabilized by microtubule associated proteins (MAPs) [2]. Importantly, different MAPs are present in axonal and somatodendritic microtubules. In developing neurons, MAP1B and tau are the main MAPs found in axons, while another MAP, MAP2, is found in dendrites [3].

MAP1B has been shown to be the first MAP which is expressed in developing neurons "in situ" [4]. The expression of MAP1B is downregulated during brain development until it essentially disappears from the axon when synaptic contacts are formed between neurons in the central nervous system [5-8]. Tau expression is more complex where six protein isoforms can be grouped into two classes. One class contains three imperfect repeats composed of a highly conserved tubulin binding motif followed by a less conserved amino acid sequence, while the other class contains an additional fourth repeat. The class with three tubulin binding domain repeats is predominantly expressed in developing brain, whereas the class with four tubulin binding domain repeats is generally expressed in adult brain [9-11].

Axonal microtubules serve as tracks for the bidirectional transport of various organelles including mitochondria and synaptic vesicles between the cell body and the axonal tips. As the tracks, microtubules are intrinsically polar structures with two differentiated ends (a plus end and a minus end) and all axonal microtubules have their plus ends pointing towards the axon terminal [12, 13]. As the motors, two protein superfamilies, the kinesins and the dyneins [14], serve as molecular machines to drive the transport of these organelles along microtubule tracks. Most kinesins move towards the plus end of microtubules and participate in anterograde transport [14]. In contrast, dyneins moves towards the opposite (minus) end and participates in retrograde transport [15].

In addition to stabilization of microtubules, it has recently been suggested that tau protein may also contribute to the regulation of anterograde axonal transport of mitochondria [16-18]. This suggestion opens the idea that MAP1B, as a microtubule associated protein like tau, might also participate in regulation of axonal transport of organelles. We now report our MAP1B studies in axonal transport of mitochondria by using cultured neurons from mice deficient in that protein.

Our results indicate a novel role for MAP1B in the regulation of the retrograde axonal transport of mitochondria.

Experimental

Animals

Wild type R1/NMR1 and C57BL/6 mice were used in this study.

The generation of the *map1b* mutants (R1/NMR1) by gene trapping was reported previously [19]. The generation of the *tau* mutants (C57BL/6) have been previously described [20].

Western-Blot

Protein mixtures were separated on 10% (v/v) acrylamide gel to isolate tau and/or 6% (v/v) acrylamide gel for MAP1B isolation.

Antibodies used were: 125 (MAP1B) (1/20, [21]), 7.51 (Tau) (1/100. a gift from Dr. C.M. Wischik) and tubulin (1/2000, SIGMA).

Primary culture

Hippocampal neurons were cultured as described by Banker and Cowan [22]. Pregnant wild type, tau -/- and map1b -/- mice were killed at gestational day 18 and the embryos removed under sterile conditions.

Dissociated hippocampal neurons from the embryos were plated on glass coverslips coated with 1mg /ml poly-L-lysine and 20 μ g/ml laminin. After incubation for 3 hr. in medium containing 10% horse serum (GIBCO), the cells were transferred to N2 and B27-supplemented medium (GIBCO).

Immunofluorescence

Cells were fixed 24h post-plated with 4% paraformaldehyde and 4% sacharose for 20 min at 37°C and the washed with phosphate buffered saline (PBS). The cells were then permeabilized with 0,2% triton-X-100 in PBS for 5` at room temperature and incubated with a solution containing 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperature (RT, about 20° centigrades). The cells were incubated with the primary antibodies diluted in PBS and 1% BSA for 1 hour at RT, after wards the coverslips were washed three times with PBS. The cells were then exposed to the different antibodies for 1h at RT. Finally, the coverslips were washed 3 times with PBS and mounted with FluorSave.

Analysis of mitochondrial motility.

After 1 day of culture, neurons were incubated with 200nM of Mitotracker Red CM-H2Xros (Invitrogen) and the mitochondria movement was recorded by using a video camera. One image is taken about 30 s. MetaMorph software was used to analyze the data.

Results

Characterization of the mouse lacking MAP1B.

We initially characterized our MAP1B knockout (MAP1B -/- [23]) and tau knockout (tau -/- [20]) mice using Western blot analysis of brain lysates. Specific antibodies directed against tubulin reacted with tubulin proteins in wild-type, MAP1B knockout and tau knockout mice (Figure 1A). Anti-MAP1B antibodies failed to react with any proteins in lysates from MAP1B knockout brains, but did react with MAP1B proteins from the brain lysates of tau knockout and wild-type mice. Anti-tau antibodies failed to reach with any proteins in lysates from tau knockout brains, but did react with tau proteins from the brain lysates of MAP1B knockout and wild-type mice. These results confirm that MAP1B knockouts lacked MAP1B proteins and that tau knockouts lacked tau proteins.

Morphology of hippocampal neurons from wild type and MAP1B deficient mice.

Hippocampal neuronal cultures from E18 embryos were established as previously described by [24]. After 1 day in culture to reach stage II, axonogenesis starts to be observed. When compared to cultures of wild-type mice that express MAP1B proteins, a significant decrease in axonal length was observed in MAP1B -/- neurons that lack the MAP1B protein (Figure 1B, C) while the axon diameter was found to be similar for processes from neurons that either lacked or contained MAP1B proteins (Figure 1C). As a control, the morphology of tau -/- neuronal cultures was examined under the same conditions (Figure 1B, C, D) and as previously reported [20], a decreased axonal length was also observed in tau -/- neuronal cultures. These results suggest that the lack of microtubule associated proteins, either MAP1B or tau, is associated with decreased growth of axonal length in primary neuronal cultures.

To study the microtubule/microtubule associated protein complex as a functional unit, we have investigated the association between mitochondria and this complex. As shown in Figure 2, we find a higher proportion of mitochondria at the distal end of axons than at the ends proximal to the growing axon in our neuronal cultures. While this bias for more mitochondria at the distal

end of axons was maintained in tau -/- neuronal cultures, a different pattern was observed for MAP1B -/- neurons where slightly higher staining of mitochondria was observed in the proximal region of the growing axon. Combined with our previous report [25] that dispersion of Golgi complex was observed in MAP1B -/- neurons, this new result suggests that retrograde axonal transport of mitochondria may be affected in MAP1B -/- neurons.

A faster mitochondrial retrograde transport was found in developing neurons lacking MAP1B.

Video imaging of mitochondria on axons has shown that no movement, anterograde movement, and retrograde movements are permitted. One combination of these movement types is known as saltatory movement that involves frequent stops and starts in either the anterograde or in the retrograde directions [26]. Since tau is known to participate in mitochondrial transport, we also wanted to measure the participation of MAP1B in mitochondrial movement in axons. As shown in the still pictures from time lapse imaging of stained mitochondria in axons, in Figure 3, we compared the velocities of mitochondrial movement in neuronal cultures from wild-type, tau -/-, and MAP1B -/- mice. Figure 3 is an example of that type of analysis. Figure 3A shows axonal retrograde movement of a single mitochondria through the axon of a neuron isolated from the brain of a wild type mouse. Figure 3B indicates the axonal transport found in a neuron from a tau -/- mouse, tested as an additional control. Figure 3C shows the axonal transport in two different neurons lacking MAP1B. These two neurons have a different axonal length. Using these pictures, the velocities of movement of individual mitochondria that did not stop or "pause" were measured and plotted as a histogram in Figure 4A. In comparison to wild-type neurons, the top velocities of mitochondrial movement in the anterograde fashion are enhanced in tau-/- neurons while the top rates of mitochondrial movement in the retrograde fashion are enhanced in Similarly, the average velocities of all mitochondria analyzed show MAP1B-/- neurons. increased anterograde velocities in tau -/- neurons and increased retrograde velocities in MAP1B -/- neurons. Although the fraction of mitochondria moving in the anterograde and the fraction moving in the retrograde directions are similar between all three genotypes (Figure 4B), the mitochondria with the fastest velocities of movement are found in neurons lacking MAP1B or lacking tau. Similarly, the fraction of mitochondria that do not constantly move because they stop or pause, is significantly decreased in neurons lacking MAP1B or lacking tau when compared to wild-type controls (Table 1). These results suggest that microtubule associated proteins are associated with regulating the velocities of mitochondrial movement along the microtubule and that the lack of MAP1B increases velocities in the retrograde direction while the lack of tau increases velocities in the anterograde direction.

For tau -/- mouse, our results support the previous observation indicating that tau mainly acts as an inhibitor of organelle-motor attachment [17]. Thus, in the absence of tau an increase in the number of mitochondria bound to motor proteins will be expected. Our data on "pausing" indicate that it is the case for tau, as expected, but also in the case of MAP1B -/- depletion.

Mitochondrial movement does not depend on axonal length.

We have previously indicated that the average length of the axon in cultured neurons, after a 1 day of culture, is shorter in MAP1B lacking neurons compared to wild type neurons. Thus, we have compared the mitochondrial movement in MAP1B -/- neurons containing axons with a different length. Figure 3C shows that essentially there are not differences in mitochondria velocity in axons with different lengths from MAP1B -/- neurons. Similar figures to those of Table 1 were observed (not shown) for the transport on short and long axons in MAP1B -/- neurons.

Discussion

Brain microtubules contain some associated proteins that contribute to the stabilization of microtubules needed to maintain neuron morphology. In the axon of a developing neuron there are two of these microtubule associated proteins (MAPs), MAP1B and Tau. For Tau protein it has been found that not only that it could stabilize in vitro assembled microtubules, but also that it can compete for microtubule binding with protein motors that are involved in the movement of organelles, like mitochondria, (in non neuron and neuron), mainly by disturbing their anterograde transport [16, 18]. Mitochondria can move in both, anterograde and retrograde directions, in one axon [27]. For the anterograde movement Kinesin-1 family (the nomenclature used is the standardized kinesin nomenclature [28]) have been involved for mitochondria transport [15]. For the retrograde transport probably dynein [29] is involved, since the velocity of mitochondrial retrograde transport is consistent with that of dynein driving movements, but not with alternative motors like kinesin-13 [26]. Mitochondria change movement direction frequently and very fast, suggesting that it could not be easy an exchange from one motor to another for the opposite direction. Axonal transport of MAPs, like tau, has been also recently studied [30, 31].

The proper functioning of neurons depends upon their ability to form synapses and to then supply those structures with the proper organelles, energy sources and molecules that support synaptic transmission. Maintaining the supply of synaptic components requires that they be transported from the cell body to the synapse and back again, through specialized neuritic processes that include axons and dendrites. One of the key features in the formation of these processes is the presence of microtubules, which can provide a structural frame, a motive force, and a track upon which supplies, and additional components can be moved. These inherently dynamic microtubule structures must then be stabilized with the microtubules associated proteins, MAP1B and tau, to provide scaffolds for the maintenance of neuritic process's morphology and for reliable tracks upon which synaptic components can be moved.

While the movement of mitochondria in both anterograde and in retrograde directions could be explained by undirectional movement of kinesins alone or dynein alone bound to the mitochondria and to the microtubule, the frequent and rapid change of directions of movement would imply that an exchange of one motor for another on a rapid time scale would be required. Such an implication would further predict that anterograde mitochondrial movement is regulated independently of retrograde mitochondrial movement although this point is under discussion [32, 33]. However, our work and those of others also shows that MAP1B and tau are bound to the microtubule tracks, and we now show that removal of MAP1B or tau results in increased velocities of retrograde or anterograde movement of mitochondria, respectively. This result suggests that a new mechanism could contribute to the observed movement of mitochondria in axons where MAP1B and/or tau could compete with kinesin and/or dynein motors for binding or positioning on the microtubule tracks. Depending upon the density of MAPs and motors in spatial proximity to one another, rates of movement could be finely regulated and if needed, could be completely stopped, a situation consistent with the observed movement of mitochondria in axons. While the mechanisms underlying a complete change in the vector of direction remains poorly understood, the postulate that MAPs could temporarily stop movement for a long enough pause in time to permit an exchange of motors that drive mitochondrial movement is plausible. Alternatively, if multiple kinesin and dynein motors are attached to a single mitochondria, then the well-positioned presence of selected MAPs may encourage detachment of motors from the microtubule tracks that would permit a net change in the vector of direction.

Thus, we suggest that Tau protein could regulate anterograde transport whereas MAP1B could regulate retrograde transport. However, the motor regulating retrograde transport, dynein, is first anterogradely transported to the axonal ends before it moves to the minus end of microtubules playing the role of the motor for retrograde transport [23]. Thus, a defect in anterograde transport could indirectly affect retrograde transport. This could explain the slight effect found for retrograde transport in Tau -/- neurons.

In the present work we have indicated a possible role for MAP1B in retrograde mitochondria transport. Recently, it has been indicated that C190RF5, a homologue of MAP1B could affect to

mitochondria transport in non neurons cells [34], or that other homologue of MAP1B, futsch protein, leads to axonal transport defects [35]. For the mitochondria retrograde transport, dynein may be involved, and, it has been indicated that MAP1B could interfere with dynein motor functions through its binding to LIS1 [25], or by doing an EB1 like function [36].

Also, in this work, we have found that MAP1B could regulate, in a negative way, the axonal retrograde movement of mitochondria, just the opposite that Tau protein does, that mainly prevents axonal anterograde transport [18, 37].

By taking into account the previous observations, we can suggest that in a developing neuron the anterograde transport of mitochondria is favoured. This is suggested by the following facts: a) the main Tau isoform present, in that tau developing neuron, is tau containing only three binding tubulin sequences (Tau 3R). This Tau isoform will interfere less with axonal transport than that containing four tubulin binding sequences (Tau 4R), b) the retrograde axonal transport will be down-regulated by the presence of MAP1B. A possible relation between the increase in anterograde and the decrease in retrograde transport, with axonogenesis, was previously suggested [38].

Afterwards, once neuron maturation occurs and synapsis takes place, MAP1B is not longer expressed and Tau 4R expression increases [1]. In that situation axonal anterograde transport should slow down (decelerate) whereas retrograde transport should increase. In summary, axonal MAPs like MAP1B and Tau may have not only a structural role like proteins involved in microtubule stabilization, but they appear to have an additional role in regulating mitochondria axonal transport.

Abbreviations: room temperature (RT); microtubule associated protein (MAP)

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	Wild type	Tau -/-	MAP1B -/-
Number of mitochondrial:	20	22	20
anterograde moves	20	32	20
Number of mitochondrial:			- /
retrograde moves	20	28	24
Velocity anterograde (µm/s)	0,60	0,68	0,60
Velocity retrograde (µm/s)	0,43	0,53	0,90
Range velocity (µm/s):			
anterograde	0,2-1,0	0,2-1,2	0,2-1,1
Range velocity (µm/s):			
retrograde	0,2-0,7	0,2-0,9	0,4-1,4
Frequency relative			
anterograde	56% ± 5	$49\% \pm 6$	$51\% \pm 4$
Frequency relative			
retrograde	44% ± 7	51% ± 5	49% ± 5
Pausing	29% ± 5	6% ± 2	2% ± 1

Table 1

Mitochondria transport in wild-type (40 samples), tau -/- (60 samples), and MAP -/- (44 samples) neurons. The data of Figure 4 were taken into account. For the frequency of anterograde and retrograde movements up to 100 hundred mitochondriae, from each sample, were tested, and the variation of the results is shown. Additionally, pausing was measured, in 100 mitochondria from each sample.

Legends to the Figures

Figure 1: A) Western-Blot analyses of brain extracts derived from control (wt), MAP1B deficient (*map1b*) and Tau deficient (*tau*) mice are shown. The samples have been analyzed with antibodies recognizing MAP1B and Tau proteins. For the loading control we have used antibody against tubulin. B) Double immunofluorescence analysed by confocal microscopy with an antibody recognizing specific axonal proteins. Hippocampal neurons from wild-type and *map1b-/-* mice were incubated with Tau1 antibody, and hippocampal neurons from *tau-/-* mice were incubated with SMI31 antibody. All the neurons were stained with an antibody recognizing tubulin protein. C) Axon caliber width (n=10). The range of caliber of hippocampal neurons of wild type mice is between 0,74-0,94µm, for *tau-/-* mice between 0,56-0,72µm and for the *map1b-/-* mice between 0,72-0,89µm D) Axon length in hippocampal neurons from wild-type, *tau-/-* and *map1b-/-* mice is between 7,5-8,1 µm and for the *map1b-/-* mice is between 3,2-4,8 µm.

Figure 2: Hippocampal neurons from wild type, *tau-/-* and *map1b-/-* mice; A) analyzed by double immunofluorescence showing tubulin and MitoTracker distribution. Bars: 50 μ m; B) the immunofluorescence intensity at distances of the cell soma up to 100 μ m (wt) or 75 μ m (*tau-/- and map1b-/-*) are indicated.

Figure 3: The figure shows a retrograde mitochondrial transport in hippocampal neurons from wildtype mice (A), *tau-/-* mice (B) and *map1b-/-* mice (C), in a long axon and in a short axon. The movement of a single mitochondria, at different times, is indicated for each paradigm. In the Figure, only are indicated some photograms of a video recording. By looking at this video it is possible to indicate that we were looking at the same (single) mitochondria.

Figure 4: A. Histograms depict anterograde and retrograde velocity distribution of mitochondrial movement in neurons from wild type, *tau-/-* and *map1b-/-* mice. B. Histograms of the frequency of anterograde and retrograde mitochondrial movements in neurons from wild type, *tau-/-* and *map1b-/-* mice. Different number of mitochondria (40 mitochondria for wild type, 60 for tau -/-, and 44 for MAP1B -/- mice, were analyzed). The data from those different mitochondriae are summarized in Table 1.



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Figure 2



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