Rescue of neurons from undergoing hallmark tau-induced Alzheimer’s disease cell pathologies by the antimitotic drug paclitaxel

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Through the use of live confocal imaging, electron microscopy, and the novel cell biological platform of cultured Aplysia neurons we show that unfolding of the hallmark cell pathologies induced by mutant-human-tau (mt-human-tau) expression is rescued by 10 nM paclitaxel. At this concentration paclitaxel prevents mt-human-tau-induced swelling of axonal segments, translocation of tau and microtubules (MT) to submembrane domains, reduction in the number of MTs along the axon, reversal of the MT polar orientation, impaired organelle transport, accumulation of macro-autophagosomes and lysosomes, compromised neurite morphology and degeneration. Unexpectedly, higher paclitaxel concentrations (100 nM) do not prevent these events from occurring and in fact facilitate them. We conclude that antimitotic MT-stabilizing reagents have the potential to serve as drugs to prevent or slow down the unfolding of tauopathies.

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Introduction

Intensive research on the cellular and molecular basis of tauopathy led to the documentation of critical steps in the cascade leading to tau-induced neurodegeneration. Initially, excess tau binds to microtubules (MTs) and competes with MT-kinesin binding sites, thereby down-regulating anterograde axoplasmic transport (Seitz et al., 2002). Tau is then over-phosphorylated and disengages from the MTs (Baas and Qiang, 2005), thereby increasing the susceptibility of MTs to the constitutively active MT-severing proteins, such as katanin and spastin (Ahmad et al., 2005), thereby increasing the susceptibility of MTs to the constitutively active MT-severing proteins, such as katanin and spastin (Ahmad et al., 2005; Baas and Qiang, 2005; Billingsley and Kincaid, 1997; Evans et al., 2005; McNally et al., 2002; Quarmby, 2000). As a consequence, the MTs undergo accelerated fragmentation and become even more susceptible to depolymerization (Qiang et al., 2006). Such alterations in MTs track integrity lead to reduced organelle transport and subsequently to axonal degeneration (Mandelkow and Mandelkow, 1998).

Tau-mediated neurodegeneration was also attributed to other mechanisms including gain of toxic functions (Ding and Johnson, 2008), and mechanical interference with axoplasmic transport (Ballatore et al., 2007).

This cascade of events indicates that MT depolymerization is a critical common downstream event leading to impaired axoplasmic transport and neurodegeneration (Bramlett et al., 1993; Drechsel et al., 1992; Lee et al., 2001; Morfini et al., 2002). Lee et al. (1994) hypothesized that antimitotic, MT-stabilizing reagents may impede depolymerization of MTs and thereby rescue the axoplasmic transport and the well being of the neurons. However, only few studies have been published in support of this hypothesis (Michaelis et al., 2005; Michaelis et al., 1998). Whereas these studies strengthen the concept that a MT-stabilizing reagent may provide some protection in both tau- and Aβ-induced neurodegeneration, a detailed cell biological analysis of the effects of antimitotic drugs on tau-induced neuropathology is not available.

In a recent series of studies we developed the use of cultured Aplysia neurons as a platform to study the cell biology of tauopathy. In these studies we demonstrated that the expression of wild type or mutant-human-tau (Shemesh et al., 2008) in cultured Aplysia neurons leads to the development of pathological alterations similar to those reported in studies of mammalian neurons and other invertebrate models. These include swelling of axonal segments (Stokin et al., 2005), translocation of tau to submembrane domains (Brandt et al., 1995), reduction in the number of MTs along the axon (Cowan et al., 2010a; Cowan et al., 2010b), reversal of their polar orientation (Shemesh et al., 2008; Shemesh and Spira, 2010a), impaired organelle transport (Cowan et al., 2010b; Folwell et al., 2010; Stamer et al., 2002), dramatic accumulation of macro-autophagosomes (Nixon et al., 2008), compromised neurite morphology (Kraemer et al., 2003) and degeneration (Wittmann et al., 2001).

A technical advantage of cultured Aplysia neurons for the study of tauopathy at the cell biological level is the accessibility of the system for long-term, live imaging of a variety of molecular probes, and quantification of the subcellular pathological processes. Using this platform and molecular probes that enable long-term live imaging of...
the subcellular structures and processes, the current study examined the effect of paclitaxel on the kinetics of tau-induced neuropathology.

We report that application of 10 nM paclitaxel prior to the onset of mt-human-tau-induced pathological processes rescues the neurons from undergoing many of the pathological alterations described above. Higher concentrations of paclitaxel (100 nM) do not prevent the unfolding of the pathological processes. Addition of paclitaxel after the onset of the cell pathology does not reverse the situation. We conclude that MT-stabilizing drugs could be used to rescue neurons from undergoing hallmark tau-induced structural pathologies but only within a narrow concentrations range.

Materials and methods

Cell culture

Neurons B1 and B2 from the buccal ganglia of Aplysia californica (Aplysia Resources Facility, Miami, Florida) were isolated and maintained in culture as described previously (Schacher and Proshansky, 1983; Spira et al., 1996). After isolation the neurons were plated in a glass-bottomed dish coated with poly-L-lysine (Sigma) containing culture medium. In the present study we refer to buccal neurons 1 and 2 collectively as B neurons.

Culture medium

The culture medium consisted of 10% filtered hemolymph, obtained from Aplysia fasciata collected in the Mediterranean, diluted in modified Leibovitz’s L 15 Medium as previously described (Spira et al., 1996).

Chemicals and pharmacological reagents

Paclitaxel (Sigma-Aldrich) was stored in a stock solution of DMSO at a concentration of 10 nM, diluted to a concentration of 10 μM in artificial sea water (ASW) and further diluted to a working concentration of 100 or 10 nM at the culture dish. DMSO concentration never exceeded 0.001% in the culture dish. Following preliminary testings, we limited the current study to 10 and 100 nM paclitaxel as at 1000 nM paclitaxel, the EB3-GFP comet tails (a molecular tool to follow MT polar orientations, see below) completely disappeared. Thus, disabling the use of GFP-EB3 as a tool to map the polar directionality was done by image processing in MATLAB, which follows: Consecutive images within a time-lapse series were colored as a function of time. Immobile objects appear white, reflecting the summation of the rainbow.

Electron microscopy

Electron microscopy was performed as previously described by our laboratory (Spira et al., 2003).

mRNA preparation and injection

mRNAs were transcribed in vitro using the recombinant transcription system, as described by our laboratory (Sahly et al., 2003). GFP-tagged EB3 (Nakagawa et al., 2000; Stepanova et al., 2003), double mutant- human-tau containing both missense mutations P301S and K257T (Shemesh et al., 2008) and human LC3-GFP (provided by Zvulun Elazar, Weizmann Institute) were all cloned in pCS2+ expression vector as previously described (Sahly et al., 2003).

The transcribed mRNAs were pressure-injected into the cell body cytoplasm of cultured neurons 4–24 h after plating, as described earlier by our laboratory (Sahly et al., 2003). The reason for the large window of time given for mt-human-tau mRNA microinjection after plating was the variability in time of isolated neurons to adhere to the culture dish. Some batches of neurons adhered faster than the others. This did not affect the nature and dynamics of cell pathology unfolding. The neurons that expressed mt-human-tau were assigned to the different experimental groups (control, tau, tau + 10 nM paclitaxel, tau + 100 nM paclitaxel) randomly.

Confocal microscope imaging

The systems used for confocal imaging included a Nikon C1 confocal system mounted on a Nikon TE-2000 Eclipse microscope system with a Nikon plan-Apo chromat 60× 1.4 NA oil objective. This system was equipped with 3 lasers: blue diode (405 nm), argon (488 nm) and green HeNe (543 nm). Images were collected and processed using EZ-C1 software at 20–24 °C.

GFP-tagged proteins were excited at 488 nm; the emitted fluorescence was collected using a 515/30 nm filter, SR101 and mCherry-tagged proteins were excited with the 543 nm laser, and the emission was collected through a 605/75 nm filter. Lysotracker was excited at 488 nm and the emission was collected through a 605/75 nm filter.

Color coding of SR101 time-lapse sequences was achieved as follows: Consecutive images within a time-lapse series were colored from blue to red. The individual images were then projected to produce a single image in which a moving organelle changes its color as a function of time. Immobile objects appear white, reflecting the summation of the rainbow.

Image analysis

Images were analyzed offline using NIH ImageJ software (Bethesda, MD), MATLAB (MathWorks, Natick, Massachusetts) and Laserpix (Media Cybernetics, L.P.). The figures were prepared using Photoshop (Adobe Systems) and FreeHand software (Macromedia, San Francisco, CA). For further explanation regarding image analysis see Shemesh et al. (2008) and Shemesh and Spira (2010b).

Quantification of the EB3-GFP comet tail length, velocity and directionality

Quantification of single EB3-GFP comet tail length, velocity and directionality was done by image processing in MATLAB, which included the following main steps: (a) an area of interest (AOI) was defined. (b) Frames were converted into binary images, such that EB3 labeled fluorescent “comet tails” were separated from the background. (c) Comet tail length was assessed. (d) The directionality of a single “comet tail” was defined by comparing its position in two consecutive frames, providing a movement vector of individual comet tails. (e) An angle histogram of 100 comet tails within the AOI was produced. (f) Based on the image acquisition rate and the movement vector of each comet tail, EB3-GFP propagation velocity was calculated.

To simplify the presentation and discussion of the MT polar orientations within an axon we subdivided the detailed angle histograms (Fig. 2A, C, E, and G) to three categories as follows: (a) anterograde direction—MTs that point their plus ends away from the cell body, within a section confined between 300° and 60°; (b) retrograde direction—MTs that point their plus ends towards the cell body, within a section confined between 120° and 240°; (c) lateral direction—microtubules that point...
their plus ends perpendicular to the anterograde or retrograde directions and are confined within 60° to 120° and 240° to 300° (Fig. 2B, D, F, and H). For further explanation regarding analysis of EB3 comet tails see Shemesh et al. (2008) and Shemesh and Spira (2010b).

Weighted vector of EB3-GFP comets

To simplify the presentation of the polar orientation of an entire MT population within the main axon we developed a quantitative parameter in the form of a weighted vector. The equivalent length of the weighted vector represents the overall polar orientations of the EB3-GFP comet tails within a standard AOI of 75 × 20 μm. To calculate the weighted vector, the propagation angle of 50 randomly selected comet tails from a standard AOI was determined. All 50 individual EB3-GFP comet tails were assigned an equal vector size of 1 (in arbitrary units [AU]). The x and y components of the 50 vectors were extracted and a weighed vector whose size represents the overall movement of these EB3-GFP comet tails in the axon was calculated.

LC3-GFP/Lysotracker colocalization analysis

For this analysis we used images of LC3-GFP and Lysotracker-labeled organelles taken simultaneously. We created scatter plots between the green image (LC3-GFP) and the red image (Lysotracker). In the scatter plot (fluorogram), the intensity of a pixel in the green image is given in the x coordinate and the intensity of the same pixel in the red image is given in the y coordinate. The scatter plot depicts the distribution of green to red intensities for all the pixels in the images. Hence, in the case of colocalization, the grey levels for the labeled organelles taken simultaneously. We created scatter plots (fluorograms). The intensity of a pixel in the green image (LC3-GFP) and the red image (Lysotracker). (A) A Control neuron characterized by a smooth cylindrical axon. (B) A mt-human-tau expressing neuron which developed axonal swellings. (C) mt-human-tau expressing neuron incubated with 10 nM paclitaxel maintains the normal smooth cylindrical morphology of control axons. (D) mt-human-tau expressing neuron incubated with 100 nM paclitaxel developed an axonal swelling. Scale bar: 100 μm.

Fig. 1. Morphological alterations induced by tau are prevented by 10 nM but not by 100 nM paclitaxel incubation. Neurons were imaged 4 days after mt-human-tau mRNA microinjection using a differential interference contrast microscope. (A) A Control neuron characterized by a smooth cylindrical axon. (B) A mt-human-tau expressing neuron which developed axonal swellings. (C) mt-human-tau expressing neuron incubated with 10 nM paclitaxel maintains the normal smooth cylindrical morphology of control axons. (D) mt-human-tau expressing neuron incubated with 100 nM paclitaxel developed an axonal swelling. Scale bar: 100 μm.

Results

To examine the effects of paclitaxel on tau-induced pathological processes we studied 4 experimental groups. All neurons were microinjected with mRNA encoding the plus end binding protein EB3-GFP to enable live imaging of the MT polar orientation and their spatial distribution (Erez et al., 2007; Erez and Spira, 2008; Kamber et al., 2009; Shemesh et al., 2008; Shemesh and Spira, 2010a; Shemesh and Spira, 2010b): (a) a control group expressing only EB3-GFP (n = 21), (b) a mt-human-tau expressing group (n = 9). (c) As group b, but 1–2 h after the microinjection of mt-human-tau-meruculan mRNA, paclitaxel at a final concentration of 10 nM was added to the culture solution (n = 27). Group (d) as group (c), but with 100 nM paclitaxel (n = 14).

Analysis of the morphological phenotype (by light microscopy) generated under these experimental conditions revealed that in the control group (group a) and the group expressing mt-human-tau and incubated in 10 nM paclitaxel (group c) 95.23% and 88.88% of the neurons (respectively) maintained normal morphology of smooth cylindrically shaped axon for at least 7 days (Fig. 1 A and C). Furthermore, 85% of the control neurons (group a) and 89.47% of group c (expressing mt-human-tau and incubated in 10 nM paclitaxel) revealed normal MT polar orientations (Fig. 2) and normal EB3-GFP spatial distribution (normal EB3-GFP comet tail polar orientation is defined as a situation wherein >60% of the GFP-comets point their plus end anterogradely, and normal spatial distribution is defined as a situation wherein the MT are homogeneously distributed in the axons volume, see Figs. 2 and 3). In contrast, in the mt-human-tau expressing neurons (group b) and the group that expressed mt-human-tau and incubated in 100 nM paclitaxel (group d) the percentage of neurons that underwent pathological changes including swelling of axonal segments and MT polar reorientations was significantly larger. 57.14% of the neurons in group (b) and 46.66% of the neurons in group (d) revealed morphological alterations in the form of swellings along the axons (for example, see Fig. 1). In contrast to group (a) and (c), in which >85% of the neurons revealed normal MT polar orientations, the majority of the neurons of groups (b) and (d) revealed pathological MT polar orientations (66.67% of the neurons in group (b) and 78.57% in group (d) revealed distorted MT polar orientations, Fig. 2C, D and G, H). Statistical analysis revealed no significant change in MT polar orientations over time (48 h and 120 h post plating) in group a (control) and group c (that expresses mt-human-tau in the presence of 10 nM paclitaxel); Chi-square test; group a, χ² = 0.305; df = 2, p = 0.858; group c, χ² = 0.081, df = 2, p = 0.960). In contrast, the MT polar orientations of group b (expressing mt-human-tau) and group d (expressing mt-human-tau in the presence of 100 nM paclitaxel) were significantly altered with time (Chi-square test; group b, χ² = 19.368; df = 2, p < 0.001; group d, χ² = 6.365, df = 2, p = 0.05).

Rescue of the EB3-GFP comet number by paclitaxel

Consistent with earlier results from our laboratory the number of EB3-GFP comet tails per 5 μm, we used the t-test for unequal distribution. To compare EB3 numbers, lengths, velocities and equivalent vector lengths we used paired t-test. For the sector analysis we performed a Chi-square test.

Statistical analysis

To determine the significance of alteration in number of MTs per 5 μm, we used the t-test for unequal distribution. To compare EB3 numbers, lengths, velocities and equivalent vector lengths we used paired t-test. For the sector analysis we performed a Chi-square test.
Fig. 2. Paclitaxel (10 nM) prevents mt-human-tau-induced alterations in MT polar orientation Quantitative analysis of the EB3-GFP “comet tails” polar orientations in control neurons (A and B), in neurons expressing mt-human-tau (C and D), and in neurons expressing mt-human-tau and bathed in 10 nM (E and F) or 100 nM (G and H) paclitaxel for 96 h. The EB3 comet tail propagation angles were measured in relation to the long axis of the axon (defined as 0°). (A and B) Control neurons. Neurons were cultured for 4–24 h. Then the neurons were injected with EB3-GFP mRNA, and imaged 48 and 120 h after plating. The angles of all the EB3-GFP comet tails within a standard area of interest (AOI) are shown in the angle histogram (A). For the sake of convenience the angle histogram was subdivided to three sectors: anterograde, retrograde and perpendicular (for details see Materials and methods section). The percentage of EB3-GFP comet tails pointing the plus end into each sector is shown in (B). (C and D) Five neurons injected with EB3-GFP mRNA, and imaged 48 and 120 h after plating. The angles of all the EB3-GFP comet tails within a standard area of interest (AOI) are shown in the angle histogram (C). For the sake of convenience the angle histogram was subdivided to three sectors: anterograde, retrograde and perpendicular (for details see Materials and methods section). The percentage of EB3-GFP comet tails pointing the plus end into each sector is shown in (D). (E and F) Five neurons injected with EB3-GFP and cerulean-tau mRNA. The shown results were collected at the same time points as the control neurons (A and B). (G and H) Five neurons injected with EB3-GFP and cerulean-tau mRNA and grown in the presence of 10 nM paclitaxel. The images were collected at the same time points as the control neurons (A and B).
mt-human-tau mRNA microinjection (Table 1). Four days later the number of EB3-GFP comet tails was further reduced by 54.88% (Table 1).

In the group expressing mt-human-tau and incubated in 100 nM paclitaxel (group d) there was a robust and statistically significant decrease of 20.68% in comet tail length was detected in tau expressing neurons incubated in 100 nM paclitaxel (Table 1, n = 5 neurons for each group).

Taken together, the above results indicate that 10 nM paclitaxel prevented the unfolding of the mt-human-tau-induced MT pathologies, while higher concentration of paclitaxel (100 nM) was ineffective in preventing the formation of axonal swellings, reorientation of the MTs plus ends, reduction in the number of EB3-DFF comet tails and reduction in their length.

Rescue of the axonal ultrastructure by paclitaxel: An electron microscopic examination

The use of plus end tracking protein family exclusively labels dynamically polymerizing MTs, excluding stable or depolymerizing MTs (Jaworski et al., 2008; Perez et al., 1999). Thus, mapping of MT polar orientation by live imaging of plus end tracking proteins provides incomplete information in that it excludes non-polymerizing MTs (Shemesh and Spira, 2010a). To provide a comprehensive view of the effects of paclitaxel on MT distribution we complemented the above described confocal imaging (Figs. 1 and 2 and Table 1) by electron microscopic observations. In addition, an earlier study revealed that reminiscent to mammalian AD brains, the density of lysosomes and autophagosomes along axons of cultured Aplysia neurons expressing mt-human-tau is dramatically increased (Shemesh and Spira, 2010a). Thus, we examined here whether paclitaxel prevents the development of these neuronal cell pathologies as well.

To that end, the very same neurons that were used for EB3-GFP imaging were chemically fixed and processed for TEM observations (Figs. 3–5).

The axon of control neurons maintained the cylindrical shape of the main axon for a number of weeks. The MTs and neurofilaments in these neurons (group a) are typically aligned in parallel to the long axis of the axon and the MTs are evenly distributed across the axon (Shemesh and Spira, 2010a; Spira et al., 2003). Typically, axons of control neurons do not accumulate autophagosomes and lysosomes (Fig. 3).

In mt-human-tau expressing neurons (group b), the axons are characterized by a significant increase in their macro-autophagosomes, lysosomes, and swollen mitochondrial content (Fig. 4). Typically the axon of mt-human-tau expressing neurons generated a number of swellings in which MT swirls were documented (Shemesh and Spira, 2010a). The distribution of the MTs within the axoplasm is also altered in respect to the control neurons. Whereas the number of MT profiles is elevated in the axonal cortex it is reduced in the axon-core (Fig. 4). The overall averaged number of MTs along 5 μm axonal cortex (from the membrane to the axonal core) was calculated for both group a and group b. We found that in mt-human-tau expressing axon (along its cylindrical parts not including the MT swirls) the average number of MTs was significantly decreased to 61.10% (t-test for unequal variances, α = 0.001, p = 2.79e−17).

The axons of most neurons (92.85%, n = 21) grown in the presence of 10 nM paclitaxel alone (Fig. 5A–D) maintain their cylindrical shape. No change in MT distribution and densities was recorded for the first 48 h. However the density of MTs increased to 212% after 96 h in 10 nM paclitaxel (t-test for unequal variances, α = 0.001, p = 6.87e−23, Fig. 5D). It should be noted that occasionally, along the axons of
neurons incubated in 10 nM paclitaxel, aggregates of organelles are seen.

Neurons grown in the presence of 100 nM paclitaxel alone generated within 24–96 h several bulges along the axon (Fig. S1). The MT density in the axon (both in the swollen areas and in cylindrical compartments) was greatly attenuated (to 27% of control). Examination of neurons incubated in 100 nM paclitaxel for 5, 24, and 48 h revealed that at 24 h of incubation, the number of MTs is elevated in the axonal cortex (to 118.63%), and reduced in the core of the axon. Unexpectedly, 48 h following 100 nM paclitaxel application the number of MTs is elevated (including autophagosomes and lysosomes) due to impaired axonal transport and distribution pattern of these 3 transport markers (Figs. S2 and S3). In contrast, in neurons expressing mt-human-tau (group d) in culture medium containing 100 nM paclitaxel. The MT density in the axon (both in the swollen areas and in cylindrical compartments) is significantly reduced to 67.89% of the control (Fig. 5E in the axonal cortex (Fig. S1). The overall reduction in the density of MTs in neurons expressing mt-human-tau is probably due to impaired axoplasmic transport (Fig. 7 and Fig. S3) and prevented the accumulation of autophagosomes (Kamber et al., 2009), mitochondrial transport by microinjection of human 3-oxoacyl-CoA thiolease (hOXACt) fused to GFP (Zhang et al., 1998), lysosomal transport by the use of Lysotracker (Shemesh and Spira, 2010b) and autophagosomes transport by labeling with LC3-GFP (Shemesh and Spira, 2010a).

We compared the transport of the above organelles in control neurons (group a) to that in mt-human-tau expressing neurons (group b), after incubation of the mt-human-tau expressing neurons in 10 nM paclitaxel (group c) and in neurons expressing mt-human-tau (group d) in culture medium containing 100 nM paclitaxel. We previously characterized the axonal transport of SR101- and Lysotracker- and LC3-GFP-labeled organelles in control neurons, and its impedance by mt-human-tau expression (Shemesh and Spira, 2010b) and autophagosomes transport by labeling with LC3-GFP (Shemesh and Spira, 2010a).

Table 1

<table>
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<tr>
<th>Parameter</th>
<th>Experimental group</th>
<th>n</th>
<th>Number of EB3 comets analyzed (24 h, 120 h)</th>
<th>24 h in culture (μm)</th>
<th>120 h in culture (μm)</th>
<th>Change (percentage)</th>
<th>Significance</th>
<th>p value</th>
<th>Alpha</th>
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<tr>
<td>EB3 number per standard AOI</td>
<td>(a) control</td>
<td>5</td>
<td>847, 820</td>
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<td>(b) tau</td>
<td>5</td>
<td>623, 281</td>
<td>124.6</td>
<td>56.22</td>
<td>−54.88%</td>
<td>**</td>
<td>0.0022</td>
<td>0.01</td>
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<td>5</td>
<td>696, 697</td>
<td>139.20</td>
<td>139.40</td>
<td>0.14%</td>
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<td>(d) tau + 100 nM paclitaxel</td>
<td>5</td>
<td>698, 871</td>
<td>169.60</td>
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<td>EB3 length (μm)</td>
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<td>5</td>
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<td>1.153</td>
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<td>1.381</td>
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<td>EB3 velocity (μm/s)</td>
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<td>0.134</td>
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</tbody>
</table>

Four parameters are provided: average EB3 number per standard area of interest, average EB3-GFP comet tail length (in μm), average EB3-GFP comet tail propagation velocity (in μm/s), and the equivalent vector size of the EB3-GFP comets (in μm), in experimental groups a-d. The number of neurons analyzed (n) in each experimental group is 5. The numerical value of each of the 4 parameters is given for measurements conducted at two points in time 48 and 120 h after plating. The first imaging session was conducted 24–44 h after the microinjection of EB3-GFP mRNA to the control neurons (group a), and EB3-GFP + mt-human-tau mRNA microinjection to the experimental groups (b–d). Paclitaxel was added to groups (c) and (d) 1 h after the mRNA microinjection. The second imaging session took place 4 days after plating. Significance levels are indicated as follows: not significant (−); significance level of 95% (**); significance level of 99% (***); significance level of 99.9% (****).
paclitaxel containing culture medium was replaced every 24 h. The neurons were examined twice at 48 and 96 h after the initial addition of paclitaxel to the medium. We found that once the mt-human-tau-induced pathology was established 10 or 100 nM paclitaxel addition did not reverse it (Fig. 8D and E).

Discussion

The present study shows that the mt-human-tau-induced hallmark cellular pathologies of AD in cultured Aplysia neurons are prevented by incubation of the neurons in 10 nM paclitaxel. Higher concentrations of 100 nM paclitaxel do not prevent the unfolding of the pathological processes. Bathing neurons in paclitaxel solution after the establishment of the pathological alterations does not reverse the process.

The principal findings of the present study are consistent with the concept coined approximately 16 years ago by Lee et al. (1994), that MT-stabilizing agents are expected to counteract the MTs destabilizing effects of tau overexpression (Ballatore et al., 2007; Brunden et al., 2010a; Brunden et al., 2009; Michaelis et al., 2002). It is interesting to note however that since 1994 a surprisingly small number of publications successfully followed-up Lee et al. (1994). A Pubmed database search revealed a small number of papers that report on this issue (Brunden et al., 2010b; Butler et al., 2007; Conejero-Goldberg et al., 2008; Michaelis et al., 2005; Zhang et al., 2005). In light of our results which clearly demonstrated that the range of paclitaxel concentration in which beneficial effects are observed is very narrow, it is conceivable that follow-up studies of Lee et al. (1994) yielded mainly negative results, and thus were not published. The subcellular resolution of the observations reported here emphasizes that whereas 10 nM paclitaxel rescues the neurons from undergoing cell biological tau-induced pathologies, 100 nM paclitaxel does not.

On the narrow window of paclitaxel concentrations that protects neurons from undergoing tau-induced degenerative processes

The mechanisms that underlie the differential effects of 10 and 100 nM paclitaxel over mt-human-tau-induced pathology are not known. Below we briefly discuss putative explanations.

Based on live confocal imaging of EB3-GFP comet tails we demonstrated in an earlier study that 10 and 100 nM paclitaxel lead to different degrees of MT polar reorientation and to corresponding degrees of impaired organelle transport (Shemesh and Spira, 2010b). The unexpected observation that paclitaxel, a microtubule stabilizing reagent altered the polar orientations of the MTs rather than stabilizing them, was explained by assuming that the drug generates new MT-nucleation sites in the cell and that these sequester free tubulin dimers that polymerize de-novo to point their plus ends in various directions (De Brabander et al., 1981; Foss et al., 2008; Masurovsky et al., 1981). This in turn leads to impaired organelle transport. Indeed, a positive correlation was found by quantitative analysis of the relations between paclitaxel concentration and the degrees of MT polar mismatching or the levels of impaired organelle transport (Shemesh and Spira, 2010b).

Besides for the differences in the impact of 10 and 100 nM paclitaxel on MT polar orientations and axonal transport (Shemesh and Spira, 2010b), the electron microscope study presented here also revealed differences between the effects of the two paclitaxel concentrations. While 10 nM paclitaxel alone led to a sustained increase in the overall MT density, it did not induce alterations in their spatial distribution (Fig. 5C and D). On the other hand, 100 nM paclitaxel severely compromised the density of MTs (Fig. S1). Thus, 100 nM paclitaxel alone generated damages to the neurons in the form of overall reduced MT density, translocation of the MT to the submembrane domains, formation of axonal bulging and accumulation of subcellular organelles.

Paclitaxel does not reverse the cellular pathology generated by mutant-human-tau

In the above sections we showed that application of 10 nM paclitaxel prior to the onset of mt-human-tau-induced pathologies prevents the unfolding of the cellular processes. We next examined whether application of paclitaxel can reverse established mt-human-tau-induced MT pathologies. To that end we expressed EB3-GFP and mt-human-tau in neurons as described above. Once the neurons revealed clear characteristic mt-human-tau pathologies in the form of chaotic MT polar orientations (Fig. 8A–C) and/or MT translocation to the submembrane domain, we applied 10 nM (n = 12) or 100 nM (n = 4) paclitaxel to the culture medium. In these experiments...
It is well documented that paclitaxel competes with tau on one of its two binding sites to the MTs (Amos and Lowe, 1999; Kar et al., 2003) and acts as a MT-stabilizing reagent. Nevertheless, the process is dose-dependent and it is conceivable that at high paclitaxel concentrations of 100 nM, the MT-stabilizing properties are overwhelmed by other paclitaxel-induced processes. In this respect, it is important to recall that in addition to its MT-stabilizing effects, paclitaxel exhibits non-microtubules associated biological functions (Blagosklonny and Fojo, 1999). For example, in dorsal root ganglion cells, 800 ng/ml paclitaxel (which is the equivalent of ~1 μM), activates calpain, resulting in a decreased concentration of the neuronal calcium sensor-1 (NCS-1), a member of the calcium binding protein family. This, in turn, modulates the inositol-1,4,5-trisphosphate receptor (InsP3R), resulting in reduced InsP3R dependent calcium signaling (Boehmerle et al., 2007). By contrast, in neonatal rat ventricular myocytes, paclitaxel increases the frequency of spontaneous calcium oscillations as a consequence of elevated binding between NCS-1 expression and InsP3R, leading to a release of intracellular calcium (Boehmerle et al., 2006; Zhang et al., 2010). Paclitaxel (100 nM) is also known to activate the transcription factor nuclear factor-kappa B (NF-κB) in various tumor cell lines (Bergstrahl and Ting, 2006; Huang et al., 2000). Furthermore, at concentrations of 10 μM, paclitaxel induces opening of the mitochondrial transition pores (Kidd et al., 2002; Mironov et al., 2005). These observations clearly demonstrate that paclitaxel binds to a variety of target molecules and thus it is conceivable that different doses and different duration of paclitaxel exposure may generate different cell biological cascades.

Translation of the results towards clinical applications of antimitotic drugs in preventing the progress of tauopathies

In view of the present study and since tauopathies develop over months and years (Gasparini et al., 2007) it would be reasonable to further explore the potential use of less toxic, blood–brain barrier (BBB)-penetrating MT-stabilizing reagents to slow down or prevent...
the progress of tauopathies. Recent developments in microtubules stabilizing drugs including epothilones, discodermolide and laulimalide (Florence et al., 2008; Feyen et al., 2008; Kingston, 2009), provide the opportunity to optimize the prevention of neurodegenerative pathologies.

Our study emphasizes the need to understand and establish the quantitative relationships between MT-stabilizing drugs and the endogenous MT-stabilizing molecules such as phosphorylated and unphosphorylated tau isoforms and to further explore the non-microtubule related effects of antimitotic drugs. It is unclear to what extent different types of neurons that undergo tau-induced pathologies, express different isoforms and concentrations of endogenous molecules that directly or indirectly effect MT stabilization and compete with exogenously administered MT-stabilizing reagents. While the selection for potential MT-stabilizing reagents for clinical applications may be complicated by the variability of neuronal phenotypes and their pathological state, MT-stabilizing reagents have the potential to serve as drugs to prevent or slow down the unfolding of tauopathies.

Conclusions

In this article we have shown that 10 nM paclitaxel prevents the unfolding of mt-human-tau-induced pathologies at the cell biological level. Paclitaxel is extensively used at low concentration in the clinics as an anti-neoplastic agent (Jordan and Kamath, 2007).

Since paclitaxel does not cross the BBB and as its beneficial effects are generated within a very narrow concentration range, its potential use for clinical purposes is limited. However, other BBB-penetrating
MT-stabilizing drugs such as epothilones, discodermolide or laulimalide, should be developed.

Supplementary materials related to this article can be found online at doi:10.1016/j.nbd.2011.03.008.

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References


Fig. 8. Once established, mt-human-tau-induced cell pathology cannot be reversed by paclitaxel. A neuron was microinjected with Cerulean mt-human-tau mRNA and EB3-GFP and imaged 48 h after the injection. In this experiment pathological disorientation of the MTs was detected already 48 h after the injection. (A) Cerulean-mt-human-tau and (B) EB3-GFP showing typical loss of the microtubule polar orientation. The angle histogram of the MTs at this region is given in (C). Thereafter, the neuron was incubated for additional 96 h in 10 nM paclitaxel. This did not restore the MT polar orientation. Shown are: images taken 48 h after the application of 10 nM paclitaxel to the culture medium. (D) Cerulean-tau distribution, (E) EB3-GFP, and the MT angle histogram. Note that during the 48 h in 10 nM paclitaxel the MT pathology worsened. Scale bar: 10 μm.

Fig. 7. Paclitaxel (10 nM) rescue the spatial distribution of EB3 comet tails, retrograde axonal transport (A–J) and mitochondrial transport (K–T). (A) A control neuron was microinjected with EB3-GFP mRNA and imaged 96 h later, demonstrating normal MT polar orientations. (B) Retrograde transport of the fluid phase pinocytotic marker SR101 was imaged every 5.7 s. Ten frames were color coded (from blue to red) and displayed as a single image. Note the linear retrograde transport of SR101 labeled vesicles. White spots denote stationary organelles. (C and D) A neuron expressing mt-human-tau-cerulean (not shown) and EB3-GFP (C) for 96 h was incubated with SR101 and color coded (10-frames taken 6.2 s apart, D). The neuron developed typical swellings. Note that the retrograde transport of the SR101 labeled vesicles was impaired. (E and F) A neuron injected with cerulean mt-human-tau and EB3-GFP mRNAs (E) and incubated with 10 nM paclitaxel for 96 h was labeled by SR101. (F) Color coding of retrograde transport of SR101 labeled organelles (10 frames taken 5.7 apart) demonstrated normal retrograde transport. In contrast, neurons injected with Cerulean mt-human-tau and EB3-GFP (G, H and J) mRNAs and were incubated in 100 nM paclitaxel for 96 h revealed impaired retrograde organelle transport. Impairment of organelle transport was observed along the swollen axonal segments (G and H) and narrow segments of neurons incubated in 100 nM paclitaxel (I and J). Color coding of the retrograde transport of SR101 (in H and J are 10 frames taken 6.2 s apart). (K and L) A control neuron was microinjected with the mitochondrial marker hoACTL-GFP and imaged 96 h later. Color coding (as described in the Materials and methods section) of mitochondrial propagation demonstrates bidirectional linear movement of mitochondria along the axon (L). A neuron microinjected with mCherry mt-human-tau mRNA (M) and the mitochondrial marker hoACTL-GFP (N) was imaged 96 h later. Color coding of mitochondrial movement demonstrates that mt-human-tau expression leads to a complete halt of mitochondrial transport (N). To study the effects of paclitaxel on the mitochondrial transport neurons were microinjected with mCherry mt-human-tau mRNAs and the mitochondrial marker. Immediately following injection 10 nM (O and P) or 100 nM paclitaxel (Q–T) was added to the culture medium. (O, Q, and S) Tau distribution in the axons. (P, R, and T) Color coding of mitochondrial movement. Note that whereas mitochondrial transport was normal in the neuron expressing mCherry mt-human tau and exposed to 10 nM paclitaxel, the neurons exposed to 100 nM paclitaxel did not. Scale bars: 10 μm in (j) applies to A–J, 10 μm in (T) applies to K–T.


