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Extracellular tau is toxic to neuronal cells

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Abstract The degeneration of neurons in disorders such as Alzheimer's disease has an immediate consequence, the release of intracellular proteins into the extracellular space. One of these proteins, tau, has proven to be toxic when added to cultured neuronal cells. This toxicity varies according to the degree of protein aggregation. The addition of tau to cultured neuroblastoma cells provoked an increase in the levels of intracellular calcium, which is followed by cell death. We suggest that this phenomenon may be mediated by the interaction of tau with muscarinic receptors, which promotes the liberation of calcium from intracellular stores.

Keywords: Tau protein; Toxicity; Neuron degeneration

1. Introduction

Alzheimer's disease (AD) is characterized by the appearance in the brain of two aberrant structures: extracellular senile plaques and intracellular neurofibrillary tangles (NFT: [1]). While senile plaques are composed of the beta amyloid peptide [2] (for a review, see: [3,4]), the neurofibrillary tangles contain paired helical filaments (PHF) that are formed of hyperphosphorylated isoforms of the microtubule associated protein tau [5] (for a review, see: [6]). The distribution of senile plaques in the brain varies widely, not only between brain regions but also from one individual to another one [7]. In contrast, the formation of neurofibrillary tangles follows a reproducible pattern, whereby they first appear in the entorhinal cortex (EC) and from there, the pathology spreads to surrounding areas like the hippocampus [7]. Indeed, the progress of the formation of tangles correlates with the progress of the disease, that can also be followed by examining the increase of tau phosphorylation [8]. In damaged regions, like the hippocampus, there is an inverse relationship between the number of extracellular tangles and the number of surviving cells [9–11]. This suggests that the neurons, which contain fibrillar lesions and that degenerate, might release the NFT into the extracellular environment [12]. Similarly, upon neuron death, other intracellular components, like tau, could be found in the extracellular space and subsequently in the cerebrospinal fluid [13]. It is not absolutely clear whether intracellular fibrillar tau is toxic to a neuron, since the degeneration of a neuron containing neurofibrillary lesions can take a long time [14], even though tau aggregates are sticky polymers that may sequester different proteins needed for correct cell function [15]. Thus, intracellular NFT toxicity remains under discussion [16–18]. Additionally, the way in which the tau pathology spreads remains unclear [7]. One possible explanation of how the tau pathology could extend in the brain of AD patients following such a reproducible pattern, is that extracellular NFT, or other compounds released by degenerating neuron, may accumulate in the extracellular space and they could be toxic to the surrounding undamaged cells [19]. These toxic compounds could act in a similar way as the extracellular beta amyloid peptide. In this study, we have tested whether extracellular tau or PHF tau are toxic to neuronal cells.

2. Materials and methods

2.1. Tau samples nomenclature

Different tau protein samples will be described in the text as follows: the recombinant tau protein isoform containing 2 N-terminal inserts and 4 microtubule binding repeats (tau 42 [20]) is named as tau; the same isoform of tau bearing the mutations G272V, P301L and R406W [21] as tau_{vlw}; the tau fragment containing the first and third microtubule binding repeats as tau 2R; the tau 42 isoform lacking residues 306–311 as tau $\Delta_{306-311}$; and the tau peptide containing residues 306–311, as tau 306–311.

2.2. Protein purification

The recombinant tau protein isoform (tau 42) containing 2 N-terminal inserts and 4 microtubule binding repeats [20]; the same isoform of tau bearing the mutations G272V, P301L and R406W (tauvlw: [21]); the tau fragment 2R containing the first and third microtubule binding repeats; and the tau 42 isoform lacking residues 306–311 (tau Δ306–311) were isolated as described previously [22]. Phosphorylated isoforms of tau 42 and tauvlw were obtained from an insect cell culture infected with baculovirus expressing these tau proteins [23]. For some experiments, these proteins were dephosphorylated by phosphatase λ [24] according to the manufacturer's recommendations (New England Biolabs, Boston, MA). Tau peptide containing residues 306–311 (tau 306–311) and the Aβ peptide (25–35) were obtained from Neo-MPS (Strasbourg, France). The isolated proteins were fractionated by gel electrophoresis and stained with Coomassie blue. The tau protein content was determined by the BCA assay, at 37 °C. Once the protein content was determined, increasing amounts of this protein were western blotted and their reaction with Ab 7.51 was tested. This reaction of Ab 7.51 was also used as a reference to calculate the protein concentration of other tau preparations.

Filtration chromatography analyses of different tau samples were performed on a Sepharose CL6B column [25] equilibrated in a buffer containing 0.1 M morpholine ethane sulfonate (MES) pH 6.4, 2 mM

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Abbreviations: Aβ, beta amyloid peptide; AD, Alzheimer's disease; EC, entorhinal cortex; MT, microtubule; NFT, neurofibrillary tangles; PHF, paired helical filaments

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$MgCl_2$, 1 M EGTA. The preparation of tau oligomers in the presence of hexafluorisopropanol was carried out as indicated previously for other proteins [26]. The aggregation of tau-heparin filaments was also carried out as indicated previously [22], while Aβ peptide aggregation and the determination of toxicity were carried out as described in [27].

2.3. Antibodies

The antibodies used to detect unpolymerized tau were Ab7.51 (a kind gift of Dr. Wischik, UK) [28], and the 12E8 antibody (Athena Laboratories) which recognizes tau phosphorylated at serine 262 (a kind gift from Dr. Seubert [29]). The antibodies used to detect aggregated tau were PHF-1 and Alz-50 (a kind gift from Dr. Davies [30,31]). Antibodies to characterize the β-tubulin subunit were obtained from Sigma (St. Louis, MO, USA).

2.4. Paired helical filament isolation

Paired helical filaments were isolated as described previously [31]. Isolated PHF and other tau filaments were characterized by electron microscopy [32].

2.5. Cell culture

SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. To assay viability, 1×10^5 cells were seeded on glass coverslips in well plates and after 24 h incubation, they were exposed to different amounts of tau (or its variants) in soluble or aggregated form, for 24 or 48 h. Thirty minutes before performing the experiment, the cells were incubated with 1 mM calcein-AM and 2 μM propidium iodide were Molecular Probes (Eugene, OR). Cell viability was measured by directly counting of the cells labeled with propidium iodide (red fluorescence, dead cells) and calcein-AM (green fluorescence, live cells). Four samples were tested under each condition. We also used a lactatodehydrogenase (LDH) kit (Roche diagnostics, Mannheim, Germany) to test cell viability. Control cells were incubated with the same volume of buffer in each case.

2.6. Calcium dependent fluorescence signal analysis – image acquisition and analysis of the Ca^{2+} response in SH-SY5YS cells

SH-SY5YS cells cultured on coverslips were washed with perfusion buffer (122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM glucose and 20 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer, pH 7.4), and they were loaded with FURA-2AM (7.5 μM) for 45 min at 37 °C. This period facilitated the intracellular hydrolysis of the FURA-2AM. Subsequently, the coverslips were washed with fresh medium and mounted in a superfusion chamber in a NIKON Eclipse TE-2000 microscope. In all experiments the cells were superfused first at 1.2 ml/min with perfusion media and then, with 70 μM ACh in order to establish the functional cell status. When unpolymerized or oligo and polymerized tau proteins were assayed, the perfusion system was stopped. When the experiments were performed with cholinergic antagonists, hexamentonium (10 μM) and atropine (100 μM), the cells were superfused for 2 min before applying ACh or Tau. Incubation with the non-selective voltage dependent calcium channel blocker was carried out by superfusing for 10 min with CdCl2 (50 μM) before the addition of Tau. At the end of each experiment, 70 μM ACh pulses were applied to confirm the viability of the cells under study. Cells were visualized using a Nikon ×40 S Fluor 0.5–1.3 oil lens. The wavelength of the incoming light was filtered to 340 nm and 380 nm with the aid of a monochromator (10 nm bandwidth, Optoscan monocromator, Cairin). These wavelengths corresponded to the fluorescence peaks of Ca^{2+}-saturated and Ca^{2+}-free FURA-2 solutions. The 12-bit images were acquired with an ORCA-ER C 47 42–98 CCD camera from Hamamatsu (Hamamatsu City, Japan) controlled by Metafluor 6.3r6 PC software (Universal Imaging Corp., Cambridge, UK). The exposure time was 150 ms for each wavelength and the changing time <5 ms. The images were acquired continuously and buffered in a fast SCSI disk. The time course data represent the average light intensity in a small elliptical region inside each cell. The background and autofluorescence components were subtracted at each wavelength, and the 340 over 380 nm ratio was calibrated into $[Ca^{2+}]_i$ values using Grynkiewicz's equation [33]. The R_{max}, R_{min} and β parameters were calculated from the spectra of small droplets of fura-2 in Ca^{2+}-saturated and Ca^{2+}-free solutions (100 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris, 10 mM MOPS and 100 μM fura-2. Ca^{2+}-free: plus 2 mM EGTA. Ca^{2+}-saturated: plus 2 mM $CaCl_2$) [34,35]. The in vitro R_{max} and R_{min} were corrected for the differences with the cytosolic environment using the procedure described by Poenie [36,37].

2.7. Statistical analysis

Data correspond to mean values ± S.D. The statistical significance of the results was measured by Student's *t*-test. Differences were considered statistically significant at $P \leqslant 0.05$.

3. Results

3.1. Characterization of soluble and aggregated tau protein

In order to test the possible toxicity of tau on cultured neuroblastoma cells, we first isolated and characterized tau in its soluble form or when polymerized into PHF. We found that the recombinant tau isolated protein existed in part in an oligomerized form, as indicated by chromatography filtration on Sepharose CL6B (Fig. 1A), and thus it behaves like the native tau protein [25]. When the tau protein was incubated in the presence of hexafluorisopropanol, the oligomers found were similar but not identical to those identified previously (Fig. 1B). Indeed, the oligomers generated under these conditions where able to bind to Ab Alz-50 (not shown). In contrast, there were fewer oligomers formed when a tau variant lacking residues 306–311 was analyzed by gel filtration (Fig. 1C). Nevertheless, the 2R variant also elutes in aggregated form (Fig. 1D). The elution volume of the tau oligomers and monomers, together with those of two molecular weight markers, catalase and bovine serum albumin is shown in Fig. 1E.

3.2. Tau promotes death in cultured cells

As previously indicated, neurons containing fibrillar lesions may degenerate and liberate NFT (PHF) and other intracellular components into the extracellular space as ghost tangles or as extracellular proteins. Thus, we tested the effects of exposing cultured cells to PHF. We found that the addition of increasing amounts of PHF was indeed toxic to the cells and promoted a slight increase in cell death (Fig. 2A). Since the main component of PHF is the tau protein, we also examined the effect of this protein in its unaggregated form. Exposing cells to isolated disaggregated tau produced a further increase in cell death when compared to that observed in control cells that were not exposed to tau protein (Fig. 2A). The tau variant lacking residues 306–311 (tau Δ306–311) was also tested in these assays (Fig. 2A). Exposure of the cells to this tau variant produced an increase in cell death when compared with the previous tau preparations was analyzed. Finally, the effects of the tau 2R variant and that of tau peptide containing residues 306–311 were tested (Fig. 2A). It has been shown previously that the toxic effects of unpolymerized and polymerized tau on cell death differ. Indeed, when tau protein (tau 2R) was mixed with heparin to promote its aggregation [22,38], a decrease in its toxicity was observed (see inset Fig. 2B(f)). Moreover, tau toxicity clearly increased over time as seen when the cells were exposed to the different tau variants for 24 or 48 h (Fig. 2B). This was similar to the time dependent toxicity of the Aβ oligomer used as a positive control (Fig. 2B(e)). Also, in the figure has been included a negative control, albumin (see inset of Fig. 2B(e)).

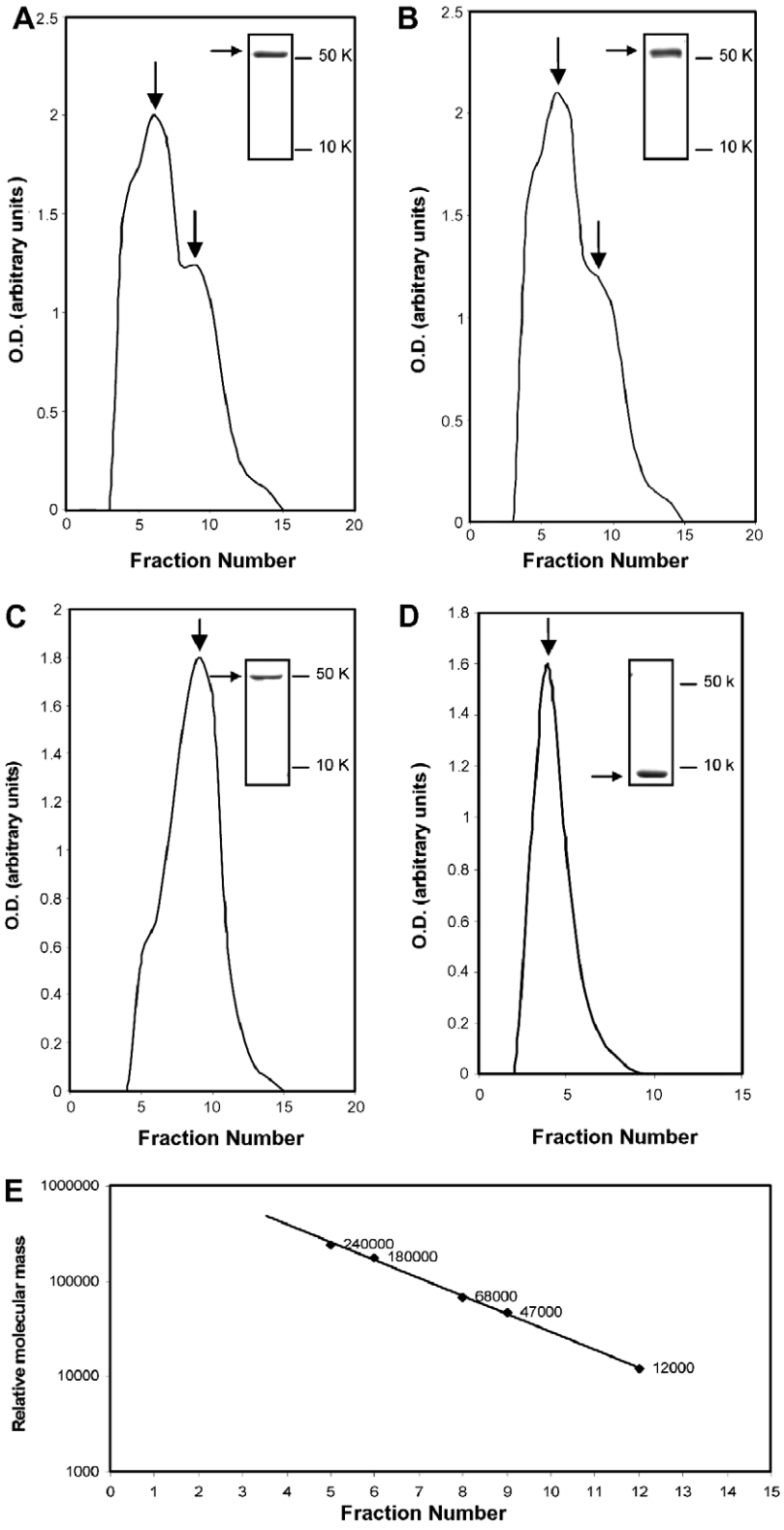

Fig. 1. Size exclusion chromatography of tau samples. Filtration chromatography analyses were performed on a Sepharose CL6B column equilibrated in a buffer containing 0.1 mM (MES) pH 6.4, 2 mM $MgCl_2$, 1 mM EGTA. The elution profiles for: (A) recombinant tau, mainly, with two elution peaks (arrows). (B) Hexafluoroisopropanol induced tau oligomers, (C) tau Δ306–311, and (D) tau 2R, are shown. In (E) the elution fractions for the molecular weight markers catalase (240 kDa), bovine serum albumin (68 kDa) and cytochrome *c* (12 kDa) are shown, together with those of tau oligomers (180 kDa) and tau monomers (47 kDa). Insets in the figures indicate the electrophoretic mobility, in denaturing conditions, of the different used tau fractions (arrows), together with the electrophoretic mobility of molecular weight markers.

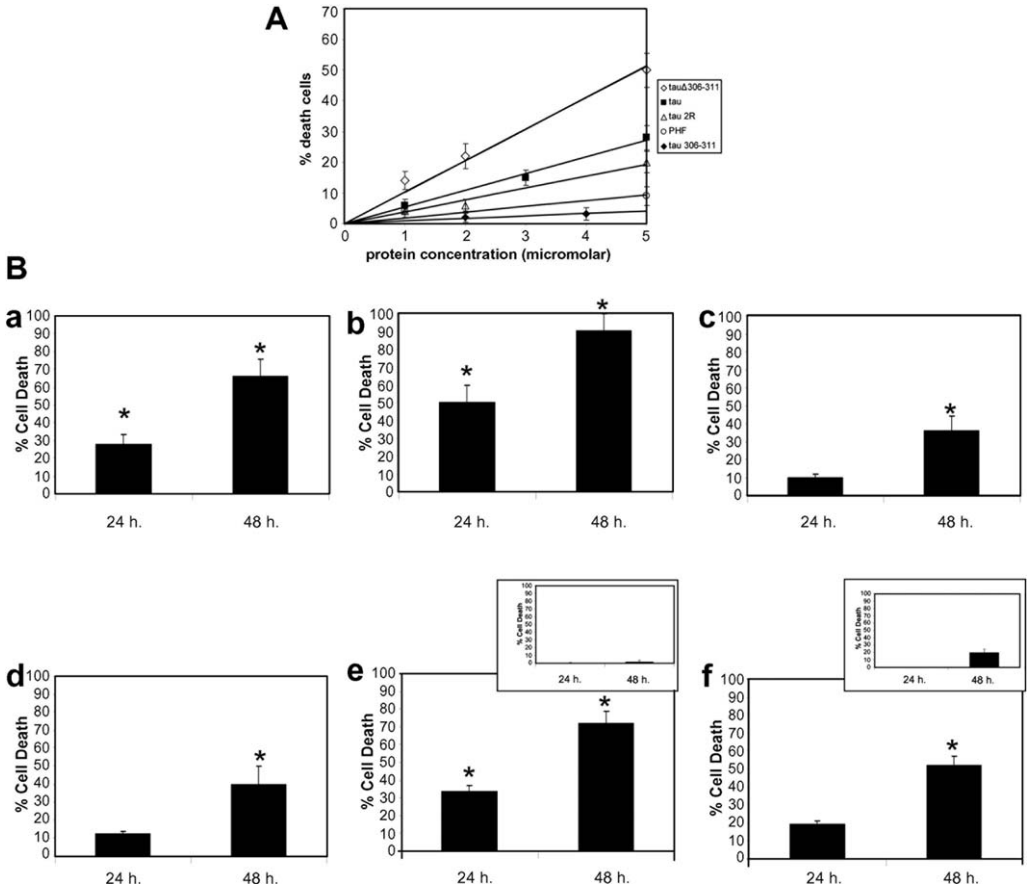

Fig. 2. Toxic effect of tau samples on SH-SY5Y neuroblastoma cells. Cultured SH-SY5Y neuroblastoma cells (see Section 2) were incubated with increasing concentrations of tau preparations. (A) The effect of increasing tau concentrations on cell viability is shown: Recombinant tau (■), tau Δ306–311 (◇), tau 2R (Δ), PHF-tau (○) and tau peptide containing residues 306–311 (◆). (B) The effect of tau at a concentration of 5 μM on cell viability was examined after 24 and 48 h. (a) Recombinant tau, (b) tau Δ306–311, (c) tau peptide containing residues 306–311, (d) PHF-tau, (e) Aβ25–35 polymers (as a positive control, at 20 μM). In the inset the results obtained upon addition of serum albumin at 0.1 mM (negative control), (f) tau 2R. In the inset the results for tau 2R, previously incubated with heparin, are shown. The statistical significance of the results was measured by Student's *t*-test $^{*}P < 0.05$ versus control cells at their respective incubation time in each case.

3.3. Toxicity of tau variants

We tested the effect of phosphorylation on cell toxicity using two tau variants: phosphorylated tau and that of tau bearing mutations present in frontotemporal dementia with Parkinsonism associated to chromosome 17 (see Section 2). These tau variants were first characterized by gel electrophoresis and Western blotting (Fig. 3A). When neuroblastoma cells were exposed to these phosphorylated tau isoforms some differences were observed when compared to that of the unmodified tau isoforms (Fig. 3B). Although, only slight differences were found, a lower toxicity was observed for phosphorylated tau compared to unmodified tau. Nevertheless, both types of tau isoforms are toxic. Again, the toxicity of these tau variants augmented with exposure time (Fig. 3C).

3.4. Changes in the microtubule network of neuroblastoma cells upon addition of tau

We further analyzed the effect of tau proteins on the microtubule network of cultured cells (Fig. 4). The number of assembled microtubules visualized by immunofluorescence appeared to decrease in proportion to the increase in DAPI staining, an effect observed when chromatin condensation occurs prior to cell death (Fig. 4). Since the decrease in microtubule staining may be due to a deregulation of calcium homeostasis in the cell cytoplasm, we studied whether exposure to tau affects the intracellular calcium levels. A rise in intracellular calcium levels could induce an increase in the activity of calcium dependent enzymes like protein kinase C (PKC). Indeed, this kinase phosphorylates serine 262 of the tau protein, a modification that can be identified by the recognition of this phosphoresidue with the 12E8 antibody [39]. Upon addition of recombinant tau protein to neuroblastoma cells, an increase in the phosphorylation of the endogenous tau at serine 262 was clearly observed (Fig. 4B).

3.5. Exposure to Tau deregulates calcium homeostasis in neuroblastoma cells

Since intracellular calcium levels appeared to be affected by exposure of the neurons to tau (Fig. 4), we set out to further test this phenomenon. We examined the effect of adding the previously characterized recombinant tau, tau Δ306–311 variant, recombinant tau treated with hexafluoroisopropanol and PHF-tau, to FURA-2AM loaded SH-SY5Y neuroblastoma cells. This cell line has been studied previously in order to test the toxicity of Aβ oligomers [24]. The mean calcium dependent fluorescence was measured in 20 cells exposed to tau

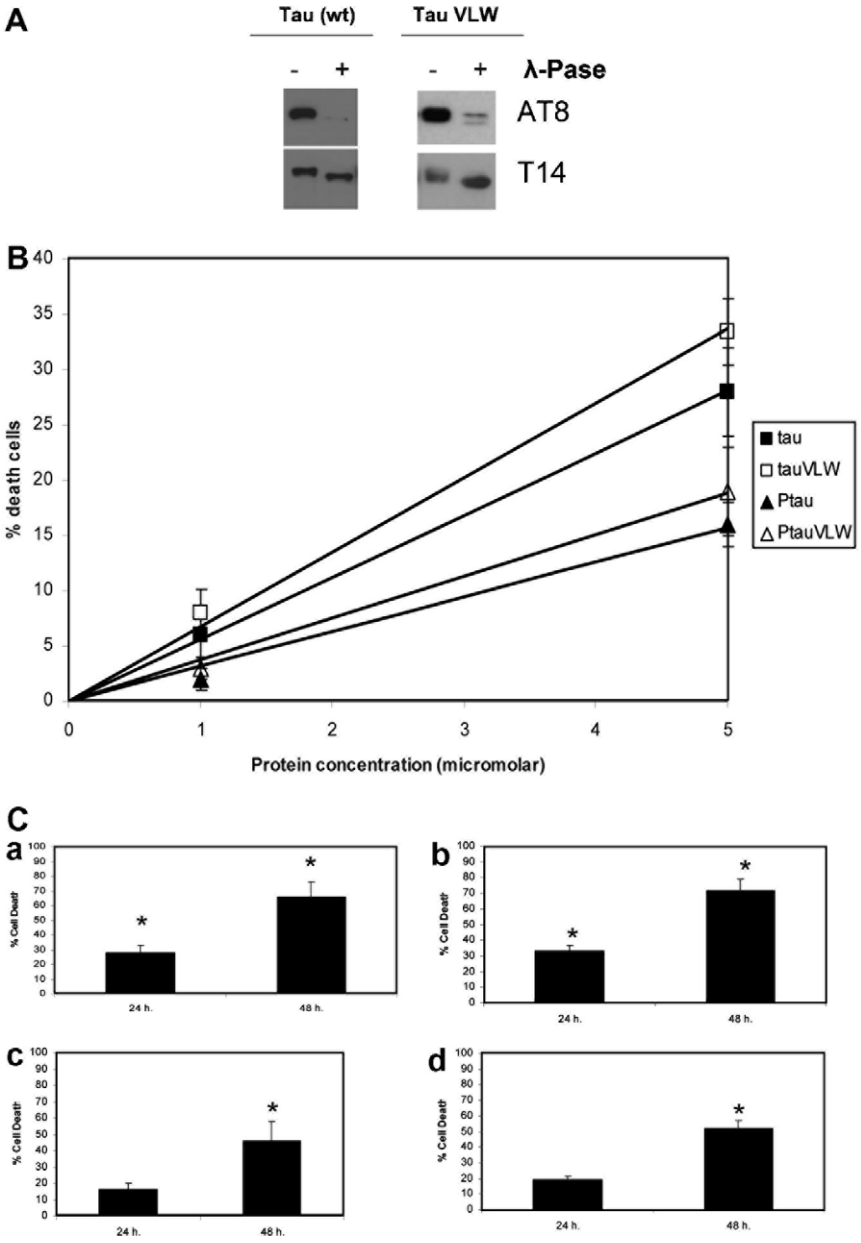

Fig. 3. Toxic effect of tau variants on SH-SY5Y neuroblastoma cells. Cultured SH-SY5Y neuroblastoma cells (see Section 2) were incubated with increasing concentrations of tau preparations, in unmodified or phosphorylated form, isolated from insect cells (overexpressing tau or the tauvlw variant). These expressed tau are present in a hyperphosphorylated form [23]. To isolate them, in their unmodified form, the tau preparations were incubated with phosphatase λ. (A) Untreated or phosphatase treated tau samples were separated by gel electrophoresis, blotted onto nitrocellulose membranes, and probed with the AT8 (which recognizes phosphotau) and T14 (which recognizes tau independent of its phosphorylation state) antibodies. (B) The effect of unmodified tau (■), phosphotau (▲), unmodified tauvlw (□), phosphotauvlw (Δ) on cell viability is shown. (C) As in (B), but the effect of 5 μM tau on cell viability was tested at two different times, 24 × h and 48 h. (a) tau, (b) tauvlw, (c) phosphotau, (d) phosphotauvlw. The statistical significance of the results was measured by Student's *t*-test $^{*}P < 0.05$ versus control cells at their respective incubation time in each case.

proteins and the tau variant, tau Δ306–311, and the positive control ACh all augmented the FURA-2 signal with a peak increase of $\Delta F/F = 3.2 \pm 0.4$ (S.E., Fig. 5A). While exposure to tau treated with hexafluoroisopropanol had a smaller effect, PHF-tau had no effect at all on the intracellular calcium levels.

3.6. A possible mechanism for tau-induced calcium deregulation

Questions still remain regarding the possible mechanism underlying tau toxicity and the increase in cytoplasmic calcium. Several mechanisms might be responsible for the calcium mobilization provoked by tau, including the influx of calcium through membrane channels. To test this possibility, we assessed the effect of tau proteins in the presence of 50 μM cadmium, a non-specific blocker of many calcium-permeable channels [40]. The presence of cadmium had no influence on the alterations in the intracellular calcium signals induced by tau (Fig. 5B(a)). The increase in cytoplasmic calcium provoked by tau could also be due to the activation of cell surface receptors coupled to calcium-influx, such as the nicotinic receptor.

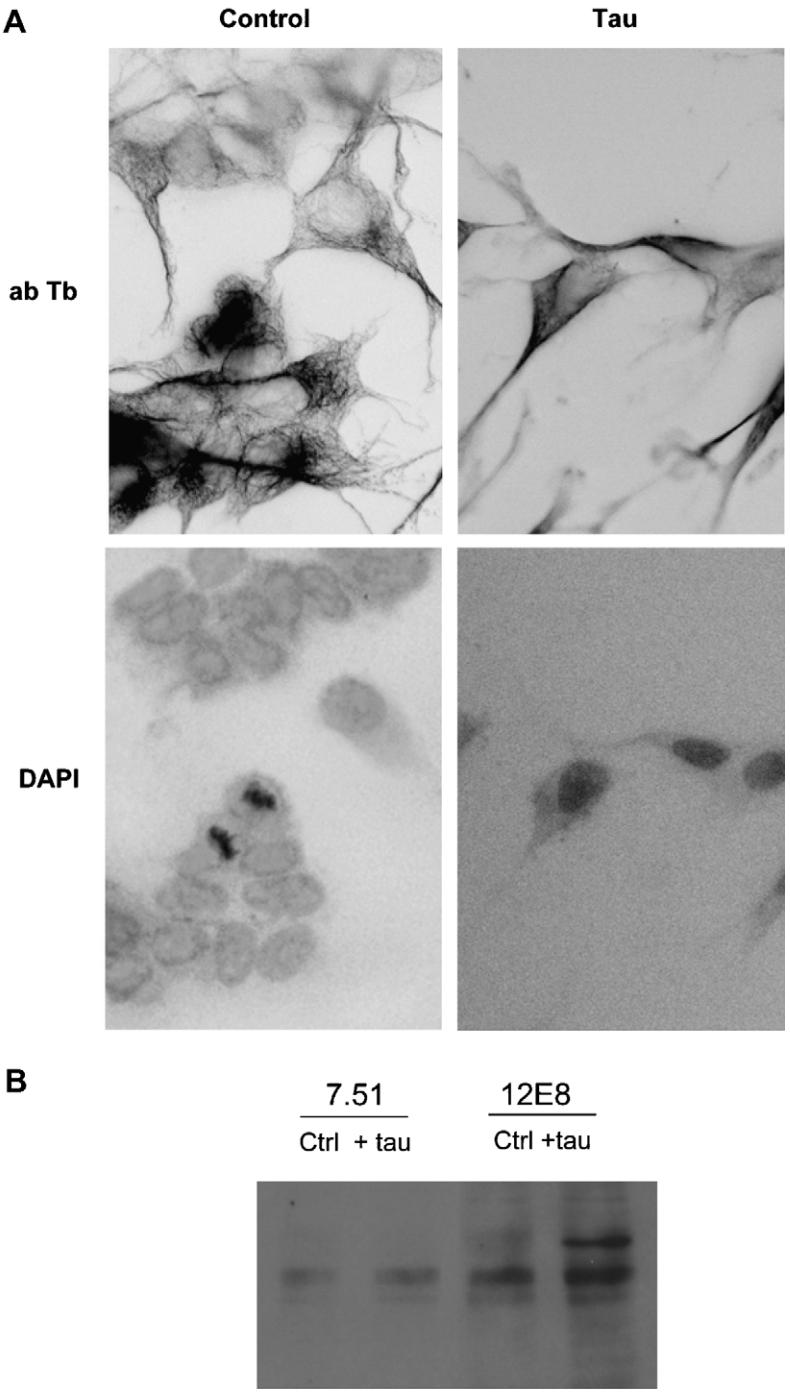

Fig. 4. Effect of tau on microtubule organization. Cultured SH-SY5Y neuroblastoma cells were incubated with tau protein and the effect on microtubule network was tested by immunofluorescence analysis. (A) Tau protein (1 μM) was added to SH-SY5Y cells in culture and the effect on the microtubule network is shown. An impoverished microtubule network was found in cells exposed to tau. In addition, an increase of DAPI staining was observed in the cell nuclei. DAPI mainly binds to condensate chromatin (see mitotic cell in the control) and this increase in DAPI binding to interphase nuclei may be indicative of cell damage in tau treated cells. (B) Western blot probed with an antibody recognising phosphotau (Ab 12E8) on control and tau treated cell extracts. In the same experiment, the total amount of tau determined by its reaction with Ab 7.51 is shown.

Additionally, the increase in calcium could be the result of the calcium liberation from intracellular stores that may be induced by receptors such as those activated by muscarinic agonists. Both nicotinic and muscarinic receptors are thought to be expressed by SH-SY5Y cells [41–43]. However, the presence of the nicotinic antagonist hexamethonium did not appear to affect the changes in calcium homeostasis induced by the different tau variants (Fig. 5B(b)). However, in the presence of the muscarinic antagonist atropine, we found a dramatic decrease in the changes in intracellular calcium promoted by tau protein (Fig. 5B(c)), even that observed when the cells were exposed to the tau Δ306–311 variant. These experiments suggest that the effect of tau is mediated through muscarinic receptors present in the neuroblastoma cells.

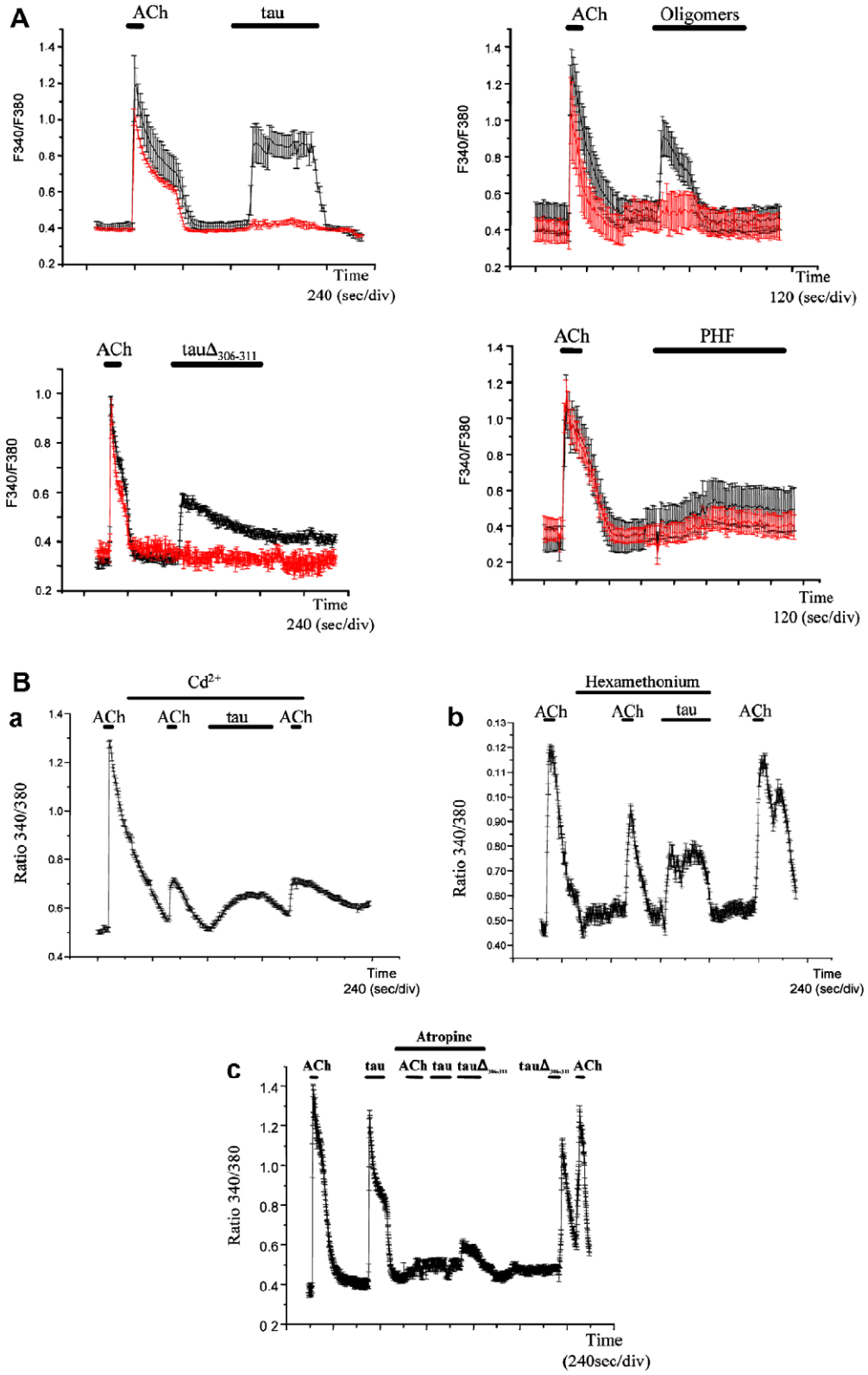

Fig. 5. Tau proteins elevate intracellular free calcium. Cultured SH-SY5Y were incubated with different tau samples, and the changes in intracellular calcium levels were analysed. (A) A time course of calcium dependent fluorescence recorded from cells. FURA-2 loaded SH-SY5Y in response to application of ACh (positive control) followed by that of either recombinant tau (1 μM), hexafluoroisopropanol-induced tau oligomers (1 μM), tau Δ306–311 (1 μM), or PHF-tau. The traces show the average responses from 20 cells. (B) (a) The changes in calcium-dependent fluorescence were recorded after the application of ACh in the absence or presence of 50 μM Cd^{2+} to inhibit Calcium channels. Calcium changes were recorded after the sequential application of ACh and recombinant tau in the presence of the same cation. The trace shows the average responses of 20 cells. (b) A similar experiment to that shown in (a), but in this case hexamethonium (a nicotinic antagonist) was added to the cultures. (c) As in (b), but in this case atropine (a muscarinic receptor antagonist) was addition along with ACh, recombinant tau, and tau Δ306–311.

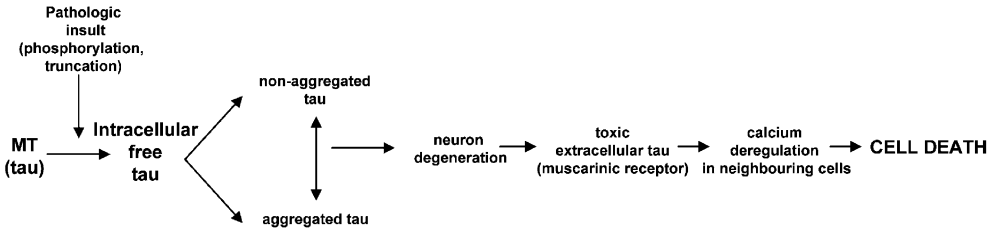

Fig. 6. Model for tau toxicity in neuroblastoma cells. Tau is mainly associated to microtubules inside neurons. Upon modification(s), tau is no longer bound to microtubules and depending on the protein concentration, it can aggregate or not. Upon neuron degeneration, tau accumulates in the extracellular space and it becomes toxic to neighboring cells.

4. Discussion

The results we have obtained indicate initially that the tau protein is toxic when added to neuroblastoma cells in culture. This effect could be relevant to the tau pathology related to Alzheimer's disease since in this disease, tau may accumulate in the extracellular space as a consequence of neuronal death [9]. In turn, this extracellular tau would serve to propagate the cell death that is characteristic of this condition. The cause of the neuronal death related with the tau pathology remains unknown, although it commences in the EC and then spreads to the neighboring neurons.

Under physiological conditions, the tau concentration in a neuron has been calculated to be around 2 μM [44,45] and essentially all tau protein (>95%) is tightly bound to the microtubule (MT) network [45,46]. However, in pathological conditions, tau could be modified by aberrant post-translational events like hyperphosphorylation, truncation, deamidation, etc. [47]. These modifications could lead to tau detaching from MTs and accumulating in a free form. This free tau could pass into the extracellular space when neurons degenerate, where it would be free to diffuse. We could assume that the soma of a neuron containing modified tau is a sphere with a radius of 10 μm, in which tau may be present at a concentration of 2 μM. Upon lysis of the neuron, tau will diffuse in every direction and as such, we can simulate its diffusion by considering virtual spheres. In such a model, at a distance of about 15 μm from the soma of the damaged neurons, the concentration for extracellular tau will be around 130 nM. This concentration was found to be at the limit of tau toxicity. In addition, the distance between the soma of two neurons within a given region, like the dentate gyrus or hippocampus, is about 3–6 μm. Thus, at this distance tau will exert a toxic effect on the cells located close to the degenerating neurons. As indicated, all tau protein is bound to MT in physiological conditions. However, if in the pathological conditions the amount of free tau exceeds that inside the cell, it may reach a critical concentration that has been calculated by some authors to be around 0.5 μM or higher (depending on the different conditions required for tau assembly [45]). At this concentration, the tau protein will start to self aggregate and a change in extracellular toxicity will take place. In fact we found a decrease in the toxicity of PHF-tau compared to that of free tau. Tau toxicity appears to be dependent on its interaction with muscarinic receptors and it appears to involve an increase in intracellular calcium. In turn, among other possible effects the alterations in intracellular calcium homeostasis may result in tau phosphorylation, a modification that could be related to the progress of tau pathology in AD [8]. Tau phosphorylation will facilitate the decrease of tau binding to microtubules, and it could occur through the activation of calcium-dependent protein kinases like PKC or CDK5 (through p25), known to phosphorylate the tau protein at different sites [47].

Thus, our current working hypothesis is summarized in the model shown in Fig. 6. In this model the intracellular levels of tau, that is not bound to MT in degenerating neurons, may appear in the extracellular space (upon neurodegeneration) where it will be toxic to those neighboring cells having muscarinic receptors. These cells will degenerate and their extracellular tau will be released and similarly exert a toxic effect in their surroundings.

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