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Article

A Combined Cell-Worm Approach to Search for Compounds Counteracting the Toxicity of Tau Oligomers *In Vivo*

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Abstract: A clear relationship between the tau assemblies and toxicity has still to be established. To correlate the tau conformation with its proteotoxic effect *in vivo* we developed an innovative cell-worm-based approach. HEK293 cells expressing tau P301L under a tetracycline-inducible system (HEK T-Rex) were employed to produce different tau assemblies whose proteotoxic potential was evaluated using *C. elegans*. Lysates from cells induced for five days, significantly reduced the worm's locomotor activity. This toxic effect was not related to the total amount of tau produced by cells or to its phosphorylation state but was related to the formation of multimeric tau assemblies, particularly tetrameric ones. We investigated the applicability of this approach for testing compounds acting against oligomeric tau toxicity, using doxycycline (Doxy) as prototype drug. Doxy affected the tau solubility and promoted the disassembly of already formed toxic aggregates in lysates of cells induced for five days. These effects translated into a dose-dependent protective action in *C. elegans*. These findings confirm the validity of the combined HEK T-Rex cells and *C. elegans*-based approach as a platform for pharmacological screening.

Keywords: tauopathy; tau protein; oligomers; proteotoxicity; *C. elegans*; tetracyclines.

1. Introduction

After mutation or post-translational modification, particularly phosphorylation, the microtubule binding protein tau dissociates from microtubules and undergoes aggregation, a process that begins with the formation of soluble aggregates and ends with the deposition in the cytoplasm of cells of large fibrillar hyperphosphorylated aggregates called neurofibrillary tangles (NFTs) [1]. This aggregation process is one of the well-established mechanisms underlying a class of neurodegenerative diseases called tauopathies [1]. Although NFTs are a key pathological hallmark of these diseases, in animal models of tauopathy their presence did not correlate with the neuronal loss and memory decline associated with the disease [2]. Extracellular tau, in the form of oligomers more than fibrillar assemblies, have instead been proposed as the main cause of the onset and progression of tauopathies [3].

On the basis of evidence from different cell-based and animal studies, tau oligomers were proposed as the main cause of the toxicity. Oligomers obtained from sonicated fibrils, but not fibrils themselves, reduced the viability of human neuroblastoma SH-SY5Y cells and their toxicity was counteracted by incubation with tau oligomer-specific T22 antibody [4,5]. In neurons derived from induced pluripotent stem cells tau oligomeric

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assemblies caused neurite degeneration, neuronal loss, and alterations of synaptic transmission and neuronal activity [6]. Tau oligomers can also be internalized by hippocampal neurons, inducing functional alterations and reducing cell viability [7].

Tau oligomers with apparent molecular weights of 140 kDa and 170 kDa were found in the brain of two mouse models of tauopathy, both expressing human tau carrying P301L substitution [8]. This mutation, associated with frontotemporal dementia (FTD), results in a protein more prone to aggregate and became hyperphosphorylated than the wild type [9–12]. In these animals, the cerebral level of oligomers correlated well with the memory impairment associated with the disease progression [8].

Similar tau oligomeric species were found in the cerebral tissues of patients with Alzheimer's disease (AD) and FTD with parkinsonism linked to chromosome 17, underlying their clinical significance [8]. In addition, the injection of tau oligomers, but not monomers or fibrils, into the brain of C57BL/6 mice caused a memory deficit comparable to that observed in mouse models of AD, and was accompanied by neuronal death, synaptic dysfunctions and alterations in mitochondrial function [13].

Among the extracellular multimeric forms of tau, trimers were suggested as the minimum assembly required for cellular uptake, seeding and toxicity in different cell lines and primary neurons [14,15]. However, a clear relationship between the precise tau assembly/assemblies and toxicity remains to be established. Their identification is no trivial matter since they are a priority for the development of new therapeutic strategies, still needed today.

To correlate the proteotoxic effect with the molecular tau assemblies *in vivo* we developed an innovative approach involving HEK293 cells expressing tau P301L under a tetracycline-inducible system (HEK T-Rex) and the nematode *Caenorhabditis elegans*. HEK T-Rex cells have already been used *in vitro* to correlate intracellular tau aggregation and cell viability [16,17]. More recently, HEK T-Rex cells expressing tau P301L tagged with green fluorescent protein (GFP) have been used as a cell-based tau overexpression assay to screen for pharmacological compounds against tau aggregation [18].

Here we assessed the proteotoxic potential of the various tau assemblies produced by HEK T-Rex cells at different times after the induction, using *C. elegans* as biosensor, which we have previously shown can specifically recognize the toxicity of amyloidogenic proteins, including tau [19]. We recently demonstrated that the administration of 1) recombinant tau oligomers, 2) soluble tau assemblies produced in the brain of P301L transgenic mice, and 3) tau species formed in the brain of mice after traumatic brain injury (TBI) [20,21] impaired the worm's motility and synaptic transmission.

We also examined whether this cell-worm-based approach can be employed as experimental platform to search for pharmacological agents to counteract the toxicity of tau oligomers. To this end, we tested the effect of doxycycline (Doxy), a tetracycline with known antibiotic activity which also has pleiotropic effects against various amyloidogenic proteins [22–25]. Doxy was recently reported to reduce amyloid aggregation of recombinant tau, prevent tau seeding and lower the toxicity of tau aggregates *in vitro* [26]. Here we demonstrate for the first time that Doxy protected against the proteotoxic effect of tau oligomers *in vivo*.

This reinforces the rationale for repurposing this drug as an anti-tauopathy agent and confirms the validity of the combined HEK T-Rex cells and *C. elegans*-based approach as a platform for pharmacological screening.

2. Results

2.1- Multimeric tau assemblies from HEK T-rex are proteotoxic for C. elegans

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We recently demonstrated that *C. elegans* recognized as toxic the tau present in brain homogenates from vertebrate models of tauopathy [21]. We therefore applied this approach to investigate the characterization of the tau assemblies responsible for the proteotoxic effect *in vivo*. As the source of tau we employed HEK T-Rex cells expressing, under the control of a tetracycline-inducible system, the human P301L mutated protein fused to a HA tag. Thus, cells were treated with 1 μg/ml Doxy to induce the expression of tau P301L (Induced) (**Fig. 1a**) and harvested immediately before (time 0) and 1, 3 and 5 days later. Control cells were treated with the same volume of 10 mM phosphate buffered saline (PBS), pH 7.4, and harvested at the same time points (Not-induced) (**Fig. 1a**). The induction of tau expression did not affect the viability of Induced cells at any of the time points (**Fig. S1**).

Cell lysates from Not-induced and Induced cells were then administered to *C. elegans* and the locomotor activity of nematodes was evaluated seven days later by counting the number of body bends/min (**Fig. 1b**). Control worms were treated with the same volume of 10 mM PBS, pH 7.4 (Vehicle). Motility was not affected in nematodes treated with lysates of Not-induced cells and cells induced for 0, 1 and 3 days compared to controls (**Fig. 1b**). Only the treatment of nematodes with cell lysates from HEK T-Rex cells induced for 5 days significantly reduced the locomotor activity compared to Not-induced cells at the same time points and Vehicle-fed worms (**Fig. 1b**).

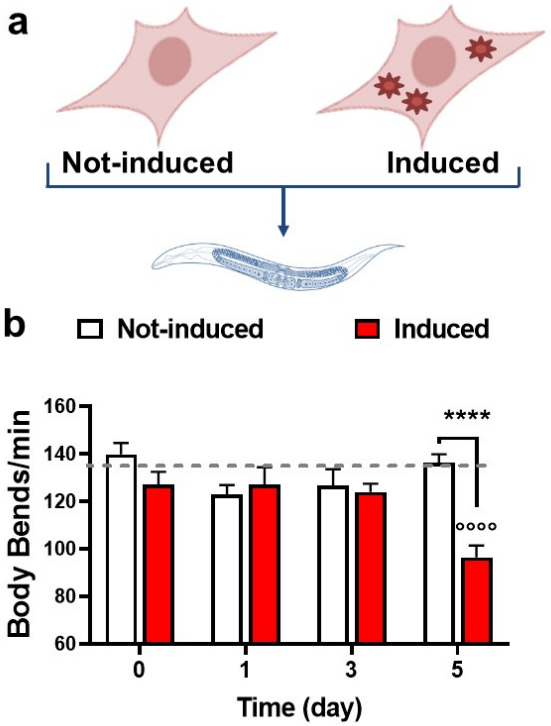

Figure 1. Time-dependent effect of the induction of tau P301L expression in HEK T-Rex cells on *C. elegans* motility. (a) HEK T-REx cells were treated with 1.0 μg/ml Doxy to induce the expression of human tau P301L (Induced) or the same volume of 10 mM PBS, pH 7.4 (Not-induced). (b) Cell lysates were prepared from cells collected immediately after (0) and 1, 3 or 5 days after the treatment and administered to *C. elegans* at the final concentration of 30 μg proteins/100 worms/100 μl. As controls, worms were treated in the same experimental conditions with 10 mM PBS, pH 7.4 (Vehicle) (100 worms/100 μl) (dashed line). Locomotor activity was recorded 7 days after treatment. Data are mean ± SEM (N= 50 worms/group). °°°°p<0.0001 *vs* Vehicle and ****p<0.0001 *vs* Not-induced, one-way ANOVA and Bonferroni's *post hoc* test.

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Experiments were then done to correlate the onset of the locomotor impairment in worms with the amount and conformational state of tau P301L expressed by cells. Western blot analysis was done first to record the level of total tau and phosphorylated tau (P-tau) in lysates of Not-Induced and Induced cells harvested 1, 3 and 5 days after induction. As shown in **Figure 2**, similar amounts of tau and P-tau were produced in Induced cells from 1 to 5 days of induction, indicating that neither the level of the protein nor its degree of phosphorylation correlated with the toxicity in the worms.

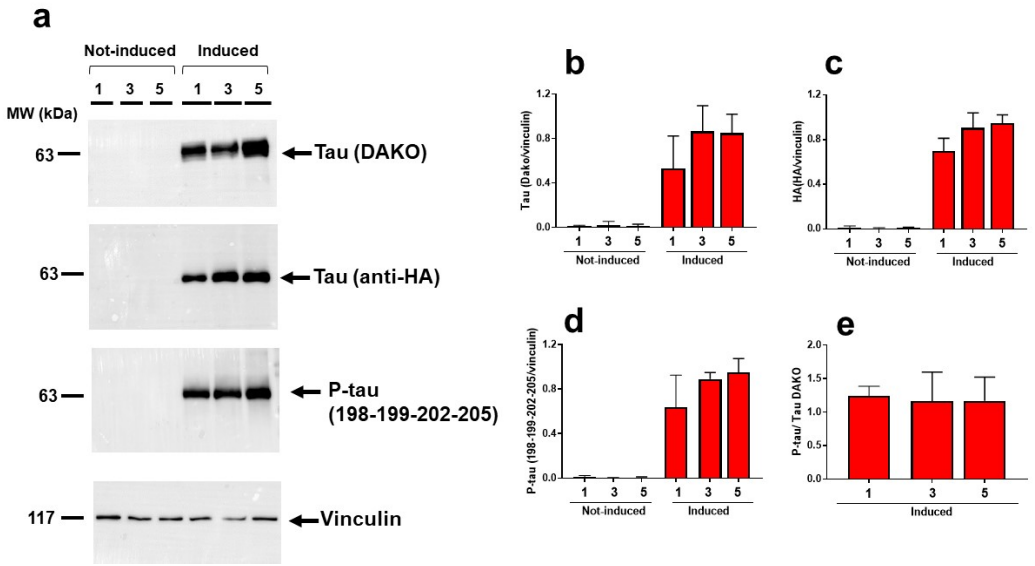

Figure 2. Time-dependent effect of the induction of HEK T-REx cells on the expression and phosphorylation of tau. (a) Representative Western blot of total tau and phosphorylated tau (P-Tau) in lysates of HEK T-Rex cells collected 1, 3 or 5 days after treatment with doxycycline (Induced) or 10 mM PBS, pH 7.4 (Not-induced). Equal amounts of proteins were loaded in each gel lane (30 µg) and immunoblotted with anti-tau (DAKO), anti-HA tag, anti-P-tau (198-199-202-205) or anti-vinculin antibody. (b, c) Total tau quantification expressed as the mean volume of (b) DAKO and (c) HA tag band immunoreactivity/vinculin. (d) P-tau band immunoreactivity/vinculin band immunoreactivity. Data are mean ± SD (N=3). (e) Ratio of the immunoreactivity signal of P-tau/vinculin to Tau DAKO/vinculin of Induced cells. Data are the mean volume of the immunoreactive band/Vinculin ± SD from three independent experiments.

To confirm the specific role of tau in the toxic effect in *C. elegans*, lysates of Not-Induced cells and cells Induced for 5 days were immunodepleted of tau before administration to worms (**Fig. 3**). The tau P301L expressed by HEK T-Rex cells were tagged with HA, so tau in the lysates was immunoprecipitated by incubation with an anti-HA tag antibody *[27]*. Cell lysates were incubated in the same experimental conditions with an anti-GFP antibody as negative control [28]. Lysates were analyzed by Western blot before (**Fig. S2**) and after the immunoprecipitation to confirm tau depletion (**Fig. 3a-b**) and then were given to *C. elegans*. Tau immunodepletion abolished the motility defect caused by the lysates from cells induced for 5 days (**Fig. 3c**), indicating that tau had a key role in the toxic effect in *C. elegans*.

We then examined whether this toxic effect could be related to changes in the solubility of the tau P301L expressed. Detergent solubility assays were done on lysates of cells induced for 3 and 5 days to determine the levels of soluble and insoluble tau assemblies. After 5 days of induction, the cells contained a significantly higher percentage

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of insoluble tau than cells induced for 3 days (**Fig. 4**), indicating that the toxicity in worms may be related to a change in protein solubility.

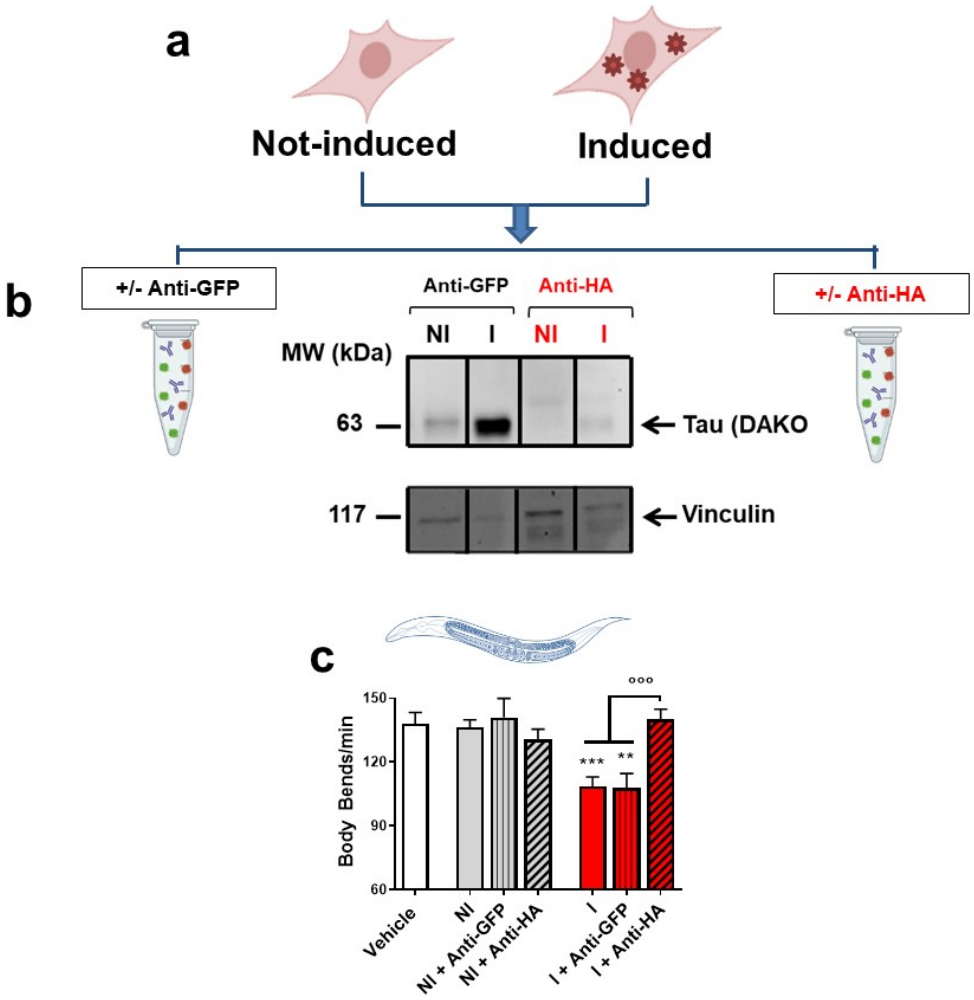

Figure 3. Tau immunoprecipitation abolishes the toxicity. (a) Lysates from HEK T-REx cells (10 µg proteins) induced (I) or not induced (NI) for 5 days were immunoprecipitated with (b) 1 µg of an anti-GFP antibody (+GFP) or anti-HA tag antibody (+ anti-HA). Tau in post-immunoprecipitation lysates was measured by Western blot analysis. Equal amounts of cell lysates (20 µl) were loaded in each gel lane and immunoblotted with anti-tau antibody (DAKO) or anti-vinculin antibody. (c) The immunoprecipitated (+ anti-GFP or + anti-HA) or untreated cell lysates were administered to worms (50 µl cell lysate/50 worms). Control worms were treated in the same experimental conditions (50 µl/50 worms) with 10 mM PBS, pH 7.4 (Vehicle), Locomotor activity was recorded 7 days after treatment. Data are mean ± SEM (N= 50 worms/group). ** $p<0.001$ and ***$p<0.0005$ *vs* Vehicle, °°°$p<0.001$ *vs* Induced and Induced + anti-GFP, one-way ANOVA and Bonferroni's *post hoc* test.

To gain information on the characterization of tau assemblies responsible for toxicity, lysates of cells induced for 3 and 5 days were subjected to Western blot analysis using T22 antibody, to specifically recognize oligomeric tau [4]. Oligomers were detected in lysates of cells induced for 3 days and their levels were significantly higher in lysates of cells induced for 5 days (**Fig. 5a-b**). This observation was further supported by semi-denaturing

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8% SDS-PAGE gel analysis followed by Western blot (**Fig. 5c-d**). Lysate of cells induced for 3 days had a more intense tau monomer band at 63 kDa than the cells induced for 5 days, which showed an increase of the immunoreactive signal corresponding to the oligomeric molecular weight tau species (**Fig. 5c**). Separation on a native PAGE 3-8% gradient gel indicated the tau oligomeric bands at ~ 180 kDa in the lysates of cells induced for 3 days and at ~ 240 kDa in the lysates of cells induced for 5 days (**Fig. 5d**).

These findings indicate that the expression of tau P301L by HEK T-Rex cells resulted in the formation of multimeric tau assemblies with time and suggested that only those with molecular weight resembling the tetrameric protein mediate the proteotoxic effect in *C. elegans*.

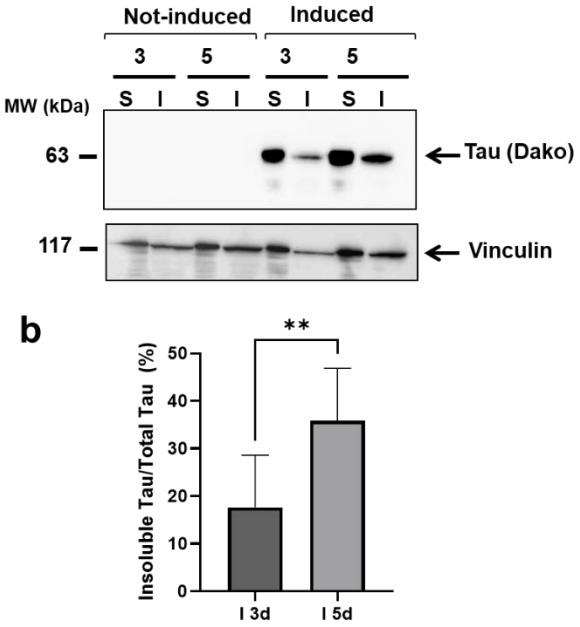

Figure 4. Detergent insolubility assay of tau P301L produced by HEK T-Rex cells 3 and 5 days after induction. Lysates were prepared from HEK T-Rex cells collected 3 or 5 days after induction (Induced) or treated with 10 mM PBS, pH 7.4 (Not-induced). (a) Representative Western blotting showing the detergent insolubility assay of soluble (S) and insoluble (I) fractions probed with anti-tau DAKO antibody or anti-vinculin antibody. (b) Tau quantification in the S and I fractions expressed as the mean percentage immunoreactivity of the DAKO signal in the insoluble fraction/total Tau (soluble + insoluble fraction). Data are mean ± SD (N=8) ** $p< 0.01$ and *** $p<0.001$, one-way ANOVA and Bonferroni's *post hoc* test.

2.2- Doxy protected from the toxicity induced by multimeric tau

We investigated whether *C. elegans* can be employed to test compounds protecting against oligomeric tau toxicity. We tested the effect of Doxy which, at 100 μM, prevented the seeding of tau and counteracted the toxicity of aggregates in SH-SY5Y cells [26]. Although we used Doxy in our experimental condition to induce tau expression in HEK T-Rex cells, the concentration of 1.95 μM we used was certainly lower than that used for pharmacological studies. Furthermore, for the *C. elegans* experiments we used lysates from cells collected five days after the induction in which the residual Doxy concentration

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is likely to be close to zero. Lysates of HEK T-Rex cells in which the tau expression was induced for five days were incubated for 2 h with different concentrations of Doxy before being administered to the worms.

Doxy protected worms in a dose-dependent manner from the toxic effect of cell lysate, with a half-maximal inhibitory concentration (IC50) of 8.06 μM **(Figure 6a)**. At the optimal concentration of 50 μM, Doxy completely abolished the neuromuscular impairment induced by cell lysates in the worms (**Fig. 6b**).

The protective effect of Doxy cannot be ascribed to an effect on the total levels of tau and P-tau in cell lysates (**Fig. S3**) but to its ability to reduce the level of insoluble tau, as indicated by the detergent insolubility assay **(Fig. 6 c-d**). Analysis of samples under semi-denaturing conditions indicated that Doxy reduced the intensity of the immunoreactive signals corresponding to the tau monomeric band at 63 kDa as well as tau oligomeric bands at ~ 240 kDa, responsible for the toxicity (**Fig. 6e**). From these data we concluded that Doxy exerts a protective effect against the toxicity induced in *C. elegans* by tau oligomers, affecting their conformational state.

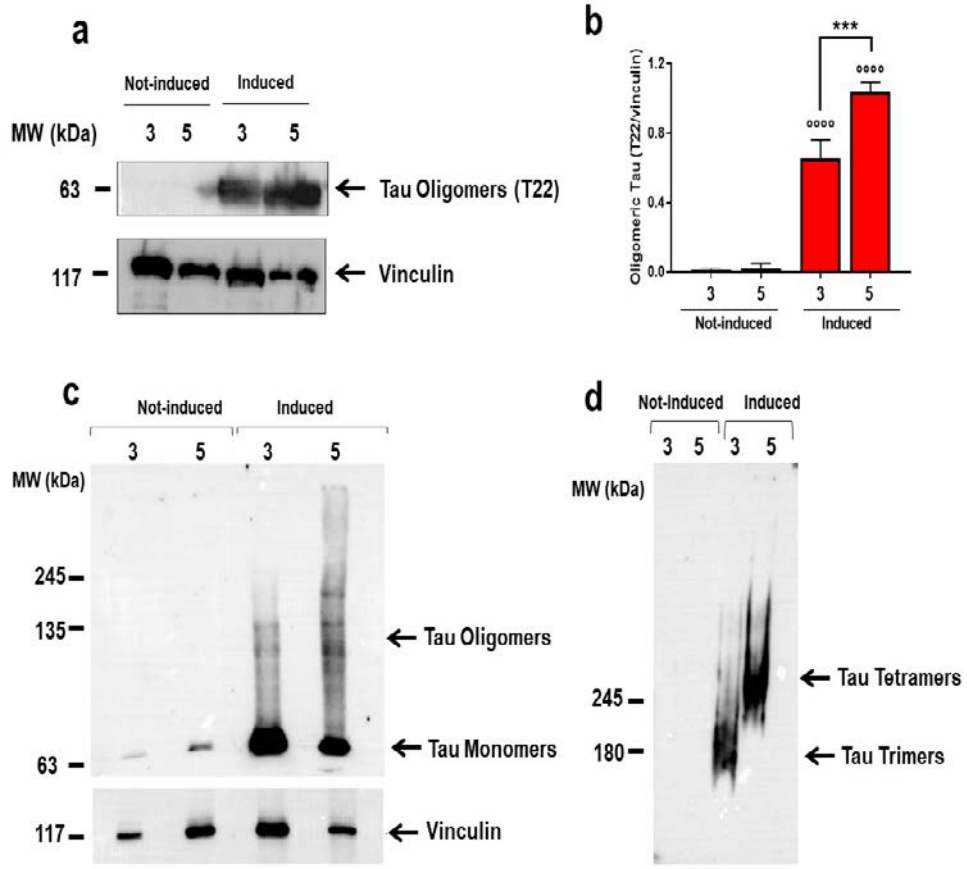

Figure 5. Tau P301L oligomers in HEK T-Rex induced cells. (a) Representative Western blot of oligomeric tau in lysates of HEK T-Rex cells collected 3 or 5 days after treatment with doxycycline (Induced) or 10 mM PBS, pH 7.4 (Not-induced). Equal amount of proteins were loaded in each gel lane (30 μg) and immunoblotted with anti-oligomeric tau (T22) or anti-vinculin antibody. (b) Oligomeric tau quantification expressed as the mean volume of the T22 band immunoreactivity/vinculin. Data are mean ± SD (N=3). °°°°p<0.0001 *vs* Not-induced at the corresponding time point and *** p<0.0005, one-way ANOVA and Bonferroni's *post hoc* test. (c, d) Equal amounts of proteins (30 μg) of cell lysates were loaded in each lane of (c) semi-denaturing gel or (d) native PAGE 3-8% gradient gel and immunoblotted with anti-tau DAKO antibody or anti-vinculin antibody.

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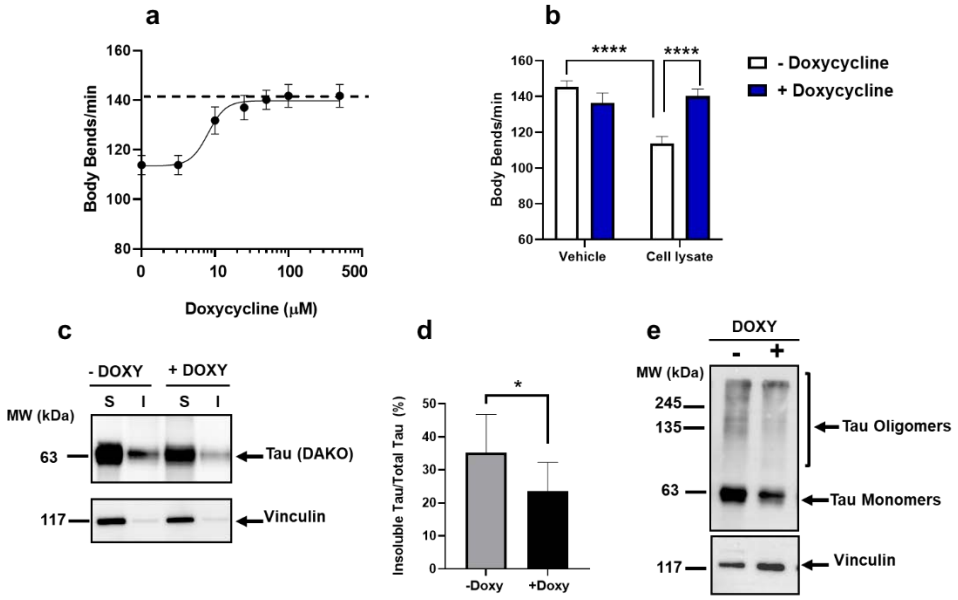

Figure 6. **Doxy protected from the toxicity induced by oligomeric tau.** (a) Dose–response effect of Doxy on the locomotor dysfunction induced in worms by lysates of HEK T-Rex cells induced for five days. Worms were fed for 2 h with 30 μg of cell lysates previously incubated for 2h with 0–200 μM doxycycline. Control worms received the same volume of 10 mM PBS, pH 7.4 alone (*dashed line*). Locomotor activity was determined 7 days after the treatment. Each value is the mean ± SEM, $N = 40$. (b) Body bends of worms treated with lysates of HEK T-Rex cell induced for 5 days (Cell lysate) or 10 mM PBS, pH 7.4 (Vehicle) in the presence (blue bars) or absence (white bars) of 50 μM Doxy. Locomotor activity was determined 7 days after the treatment. Data are the mean ± SEM, N= 45. ****p<0.0001 and ***p<0.001, one-way ANOVA and Bonferroni's *post hoc* test. Interaction cell lysate/Doxy= p<0.0001, two-way ANOVA and Bonferroni's *post hoc* test. (c) Representative Western blotting of the detergent insolubility assay of soluble (S) and insoluble (I) fractions of lysates of cells induced for 5 days and incubated for 2 h in the absence (- DOXY) or presence of 50 μM doxycycline (+ DOXY) and probed with anti-tau DAKO antibody or anti-vinculin antibody. (d) Tau quantification in the S and I fractions expressed as the mean immunoreactivity of the DAKO signal in the insoluble fraction/total Tau (soluble + insoluble fractions) immunoreactivity. Data are mean ± SD (N=8 **** p<0.0001, one-way ANOVA and Bonferroni's post hoc test. (e) Equal amounts of proteins (30 μg) of lysate of cells induced for 5 days and incubated for 2 h without (- DOXY) or with 50 μM doxycycline (+ DOXY) were loaded in each lane of semi-denaturing gel and immunoblotted with anti-tau DAKO antibody or anti-vinculin antibody.

3. Discussion

We developed a new experimental approach based on the combined use of HEK T-Rex cells and *C. elegans* to produce multimeric tau assemblies and demonstrate their toxicity *in vivo*. HEK293 cells expressing full-length tau isoforms under the control of tetracycline have already been employed to investigate the mechanisms of protein aggregation and the involvement of phosphorylation in the conformational changes [17,18]. We show that the overexpression of tau in these cells for 1 up to 5 days did not result in any significant change of the phosphorylation status of the protein but caused a time-dependent formation of multimeric insoluble assemblies. These findings indicate that HEK T-Rex, already employed for studies on tau fibrillization, can also be used to study the first phases of aggregation. The use of cells instead of recombinant protein as the source of tau offers the advantage of avoiding extraction and purification methods for

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the isolation of tau oligomers at different molecular weights, whose standardization and reproducibility is not so simple.

Only HEK T-Rex cell lysates containing tau with a molecular weight of ~ 240 kDa, resembling the tetrameric protein, has a toxic effect when administered to *C. elegans*, inducing a motility defect, whose specificity was validated by tau immunodepletion studies. Previous *in vitro* studies indicated trimeric tau assemblies as the minimal unit responsible for the toxicity [14,15]. In our conditions, lysates from HEK T-Rex cells induced for 3 days, mainly containing tau trimers, did not have any toxic effect in worms, it cannot be excluded that *in vivo* higher concentration of trimers or larger tau conformers are required for a proteotoxic effect. The neuromuscular defects with lysates of cells induced for five days were comparable to those obtained when worms were treated with brain homogenates from mice modeling genetic or sporadic forms of tauopathy [21]. These data suggest that multimeric insoluble assemblies similar to those formed in HEK T-Rex 5 days after induction are also present in the brain of P301L mice and TBI mice.

The mechanisms of the neuromuscular dysfunction caused by tau assemblies remain to be elucidated. We hypothesized that, similarly to oligomers of other misfolded proteins, tau oligomers too, once ingested by *C. elegans*, can be absorbed by the gut and diffuse in various tissues, affecting neuromuscular function [29,30].

We then investigated whether this cell-worm-based approach can be applied to studies aimed at discovering drugs to interfere with tau oligomeric toxicity. *C. elegans* has already been proposed as a valuable *in vivo* model for screening of compounds against different protein misfolding diseases [19,30] and the observations from these studies have already been translated into clinical applications [19,31].

As a prototype anti-amyloidogenic compound we used Doxy, which inhibits aggregation and oligomerization of different misfolded proteins *in vitro* and *in vivo* [32–37]. Recent findings with *in vitro* recombinant tau indicate that this compound can interfere with the aggregation of protein, the exposure of hydrophobic residues, and cell toxicity [26].

Our findings indicate for the first time that Doxy can also affect tau solubility and promotes the disassembly of already formed toxic aggregates *ex vivo*. These effects, which translated into a dose-dependent protective action in *C. elegans*, suggest that tetracyclines could be potential compounds for the treatment of tauopathies. In a strategy of "drug repurposing", this old class of compounds offer promising safe and inexpensive therapy, backed by data demonstrating their ability to pass the blood-brain barrier [38]. The ability of tetracyclines to interact with oligomers of different amyloidogenic proteins, including amyloid β and tau, is an added value and not a limit, particularly if the idea of the conformation-dependent rather than sequence-dependent role of the protein is considered relevant for the onset of a central amyloidogenic disease.

4. Materials and Methods

4.1 HEK T-Rex cells

Human embryonic kidney (HEK) 293 cells were engineered to obtain HEK T-Rex cells expressing human tau P301L (Tau P301L) tagged with human influenza hemagglutinin (HA), under the control of a Tet-promoter (Avila et al., Polymerization of tau peptides into fibrillar structures. The effect of FTDP-17 mutations. FEBS Lett. 1999 Mar 5;446(1):199-202). Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) with high glucose and pyruvate) (Gibco, Merck, Italy) containing 10% tetracycline-free Fetal Bovine Serum (FBS,), 1% Glutamax (Gibco, Merck, Italy), 5 μg/mL blasticidin (Gibco, Merck, Italy) and 100 μg/mL hygromycin B (Invitrogen, Italy company).

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To induce the stable expression of Tau P301L, HEK T-Rex cells were plated in 12-well plates (Corning® Costar® TC-Treated Multiple Well Plates, Merck, Italy) at 0.1 x 10^6 cells/well and after 24 h were treated with 1.0 μg/mL doxycycline hyclate (Sigma Aldrich, Italy) diluted in DMEM (Induced cells). Control cells were treated with the same volume of DMEM (Not-induced cells). Cells were then incubated at 37°C in 5% CO2 in air, and 3 and 5 days later cells were harvested in 10 mM phosphate buffered saline (PBS), centrifuged at 150 x g for 5 min at 4°C; the pellet was collected and stored at -80°C until use.
Experiments were also run to assess the effect of tau expression on cell viability. HEK T-Rex cells, 24 h after plating in 12-well plates at 0.1 x 106 cells/well, were treated with 1.0 μg/mL Doxy diluted in DMEM (Induced cells) and cell viability was determined 1, 3 and 5 days later in MTT and lactate dehydrogenase (LDH) assays. The MTT assay was done with thiazolyl blue tetrazolium bromide (Sigma Aldrich, Italy). The release of LDH was determined on 25 μL of cell media (Sigma Aldrich, Italy).
Cell viability was assessed by incubating cells with 1:10 Alamar Blue reagent (Invitrogen, Italy) in culture medium (without FBS) for 2 h at 37°C. Fluorescence was then measured with a spectrofluorimeter (TECAN plate reader, Infinite M200, Switzerland) using λ excitation 560 nm and λ emission 590 nm. Not-induced cells treated with DMEM and collected at the same time points were used as controls.

4.2 Immunochemical analysis

Cell pellets were homogenized in 50 μL of 50 mM Tris-HCl, pH 7.5, containing 50 mM KCl and 10 mM $MgCl_2$). Protein concentration was quantified with Pierce BCA Protein Assay kit (Life Technologies, Italy). Samples were analyzed by immunoblotting using 10% SDS-PAGE gel and Western blotting. After heating the samples at 95°C for 10 min in sample buffer containing 5% β-mercaptoethanol (1:1 v/v, Bio-Rad, Milan, Italy), 30 μg of the proteins were loaded in each lane of the gel. The membranes were blocked with 10 mM Tris-HCl solution, pH 7.5, containing 100 mM NaCl, 0.1% (v/v) Tween 20, 5% (w/v) low-fat dry milk powder and 2% (w/v) bovine serum albumin, and incubated overnight with the anti-human Tau rabbit polyclonal antibody (1:1000, DAKO, Glostrup, Denmark), anti-HA tag rabbit polyclonal antibody (1: 2000, Abcam, Cambridge, UK), anti-oligomeric tau rabbit monoclonal antibody T22 (1: 20000, Merck, Milan, Italy), anti-phospho tau antibody anti-tau paired 198, 199, 202, 205 (1:2000, Abcam) or anti-vinculin mouse monoclonal antibody (1:5000, Merck). Anti-mouse IgG peroxidase conjugate (1:10000, Sigma Aldrich, Milan, Italy) and anti-rabbit IgG peroxidase conjugate (1:10000, Sigma Aldrich) were used as secondary antibodies. Recombinant human tau-441 isoform (h-tau 41, Abcam) was used as the standard.
To characterize protein aggregates samples were diluted in loading buffer composed of 25 mM Tris solution containing 200 mM glycine, 0.2% SDS, 5% glycerol and 0.025% bromophenol blue, and 30 μg of proteins were loaded in each lane of 2.5% stacking and 6% resolving polyacrylamide gels (semi-denaturing gel). In addition, samples were diluted in 62.5 M Tris solution containing 40% glycerol and 0.001% bromophenol blue, and 30 μg of proteins were loaded into NuPAGE™ 3 to 8% Tris-Acetate, 1.0-1.5 mm, Mini Protein Gels (Thermo Fisher Scientific) (Native gel). At the end of electrophoresis gels were blotted onto Polyvinylidene fluoride (PVDF) membrane, blocked as described before and incubated overnight with the anti-human Tau rabbit polyclonal antibody (1:1000), anti-oligomeric tau rabbit monoclonal antibody T22 (1: 20000) or anti-vinculin mouse monoclonal antibody (1:5000). Anti-mouse IgG peroxidase conjugate (1:10000) and anti-rabbit IgG peroxidase conjugate (1:10000) were used as secondary antibodies.

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The mean volumes of immunoreactive bands were recorded using Image Lab™ software (Bio-Rad). The data were expressed as the mean of the immunoreactive bands/volume of total vinculin-stained proteins in the spot ± SD.

4.3 Detergent insolubility assay

Cells lysates were analyzed with an adapted detergent insolubility assay [39]. Briefly, cell lysates (50 µg) were incubated for 20 min at 4°C in 50 mM Tris-HCl solution, pH 7.5, containing 0.5% Triton X-100, 0.5% NP-40 and 0.5% sodium deoxycholate. Samples were centrifuged at 150 x *g* at 4°C for 5 min, the supernatants were collected and centrifuged at 100000 x g at 4°C for 50 min. The supernatant (Soluble fraction) and the pellet (Insoluble fraction) were collected and analyzed in 10% SDS-Page gel. The soluble fraction was heated at 95°C for 10 min in 0.5 M Tris-HCl solution, pH 6.8, containing 10% SDS, 12% β-2-mercaptoethanol, 50% glycerol and 0.001% bromophenol blue, and 50 µg of proteins were loaded in each gel lane. The insoluble fraction was suspended in 1 mL of 10 mM PBS and centrifuged at 50000 x g at 4°C for 30 min. The supernatant was collected, heated at 95°C for 10 min in 1 M Tris-HCl solution, pH 6.8, containing 20% SDS, 24% β-2-mercaptoethanol, 50% glycerol and 0.001% bromophenol blue, and 50 µg of proteins were loaded in each gel lane. At the end of electrophoresis gels were blotted onto PVDF membrane, blocked with 10 mM Tris-HCl solution, pH 7.5, containing 100 mM NaCl, 0.1% Tween 20, 5% low fat dry milk powder and 2% bovine serum albumin, and incubated overnight with anti-human Tau rabbit polyclonal antibody (1:1000) or anti-vinculin mouse monoclonal antibody (1:5000). Peroxidase-conjugated anti-mouse and anti-rabbit IgG (1:10000) were used as secondary antibodies. The mean volumes of immunoreactive bands were quantified as described before. The data were expressed as the mean of the immunoreactive bands/volume of total vinculin-stained proteins in the spot ± SD.

4.4 Tau immunoprecipitation

Cells lysates were diluted in 10 mM PBS, pH 7.4, containing 0.1% TritonX-100, to obtain a solution of 10 µg of proteins/100 µL. Samples were then incubated overnight at 4°C under orbital shaking, with 1 µg of anti-HA tag rabbit polyclonal antibody (Abcam, Cambridge, UK) or anti-Green Fluorescent Protein (GFP) mouse monoclonal antibody (B-2, Santa Cruz Biotechnology Inc., Heidelberg Germany). At the end of incubation, samples were mixed with 20 µL of Protein A and protein G Resin beads (Genespin Srl, Milan, Italy), then incubated for 2h at 4°C under orbital shaking. Samples were centrifuged at 150 x *g* for 5 min at 4°C, the supernatant was collected and centrifuged again at 100 x *g* for 5 min at 4°C. The supernatants were then analyzed by SDS-PAGE and Western blot to determine the amount of tau in immunoprecipitated samples (output). Thirty µg of cell lysates were analyzed in the same experimental condition to determine the level of tau before immunoprecipitation (input). Twenty µL of input and output samples were analyzed as described before and incubated overnight with anti-human Tau rabbit polyclonal antibody (1:1000) or anti-vinculin mouse monoclonal antibody (1:5000). Peroxidase-conjugated anti-mouse and anti-rabbit IgG (1:10000) were used as secondary antibodies. Input and output samples were also employed for *C. elegans* experiments.

4.5 *C. elegans* studies

Bristol N2 nematodes were obtained from the *Caenorhabditis elegans* Genetic Center (CGC, University of Minnesota, Minneapolis, MN, USA) and propagated at 20°C on solid Nematode Growth Medium (NGM) seeded with *E. coli* OP50 (CGC) for food. Age-

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synchronized animals were obtained by the bleaching technique [40]. *C. elegans* at the first larval stage were transferred to fresh NGM plates and grown at 20°C. At L3-L4 larval stage nematodes were collected with 10 mM PBS, pH 7.4, centrifuged, and washed twice with PBS to eliminate bacteria. Worms were incubated for 2h at room temperature with orbital shaking, in the absence of *E. coli*, with lysates of Not-induced or Induced cells (30 µg protein/100 worms/100 µL of 10 mM PBS, pH 7.4). Control worms were incubated with 10 mM PBS, pH 7.4 (100 worms/100 µL).

Worms were then plated onto NGM plates seeded with OP50 *E. coli*, grown at 20°C and transferred every day for six days to new NGM plates seeded with *E. coli* to avoid overlapping generations [21]. The locomotor activity of nematodes was scored seven days after the treatment by counting the number of left–right movements in one minute in liquid (body bends/min) [41,42]. All behavioral evaluations were done blinded.

In similar experiments worms were incubated with cell lysates previously immunoprecipitated with anti-HA tag rabbit polyclonal antibody or anti-GFP mouse monoclonal antibody as previously described (30 µg protein/100 worms/100 µl).

4.6 Effect of Doxy

To investigate the effect of Doxy on the toxicity, worms at L3-L4 larval stage, were fed for 2 h at room temperature with orbital shaking, in the absence of *E. coli*, with cell lysates of HEK T-Rex cells induced for 5 days (30 µg protein/100 worms/100 µl of 10 mM PBS, pH 7.4) containing or not 0–200 µM doxycycline hyclate (Sigma Aldrich). Control worms were incubated with 10 mM PBS, pH 7.4 (100 worms/100 µl). At the end of incubation, worms were plated onto NGM plates seeded with OP50 *E. coli*, grown at 20°C and transferred every day for 6 days to new NGM plates seeded with *E. coli* to avoid overlapping generations. The locomotor activity of nematodes was scored 7 days after the treatment as described before.

Lysates of HEK T-Rex cells (30 µg protein/100 µl of 10 mM PBS, pH 7.4) incubated or not with 50 µM Doxy for 2 h at 20°C in orbital shacking, were analyzed trough detergent insolubility assay or semi-denaturing gels as described before. Membranes were blotted with anti-human Tau rabbit polyclonal antibody (1:1000) or anti-vinculin mouse monoclonal antibody (1:5000). Peroxidase-conjugated anti-mouse and anti-rabbit IgG (1:10000) were used as secondary antibodies

4.7 Statistical analysis

The data were analyzed using GraphPad Prism 8.0 software (CA, USA) by Student's t-test, one-way or two-way ANOVA and Bonferroni's or Tukey's *post hoc* test. A p value < 0.05 was considered significant.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Tau P301L expression did not affect cell viability; Figure S2: Western blot of cell lysates prior to immunoprecipitation and used for the input; Figure S3: Effect of doxycycline on tau expression.

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Data Availability Statement: Data supporting reported results can be requested from the corresponding authors.

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