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Distinct Neurotoxic Effects of Extracellular Tau Species in Primary Neuronal-Glial Cultures

Katryna Pampuscenko[1] · Ramune Morkuniene[1] · Lukas Krasauskas[2] · Vytautas Smirnovas[2] · Taisuke Tomita[3] · Vilmante Borutaite[1]

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Abstract

Recent data from various experimental models support the link between extracellular tau and neurodegeneration; however, the exact mechanisms by which extracellular tau or its modified forms or aggregates cause neuronal death remain unclear. We have previously shown that exogenously applied monomers and oligomers of the longest tau isoform (2N4R) at micromolar concentrations induced microglial phagocytosis of stressed-but-viable neurons in vitro. In this study, we investigated whether extracellular phosphorylated tau^{2N4R} (p-tau^{2N4R}), isoform 1N4R (tau^{1N4R}) and K18 peptide can induce neuronal death or loss in primary neuronal-glial cell cultures. We found that p-tau^{2N4R} at 30 nM concentration induced loss of viable neurons; however, 700 nM p-tau^{2N4R} caused necrosis of both neurons and microglia, and this neuronal death was partially glial cell-dependent. We also found that extracellular tau^{1N4R} oligomers, but not monomers, at 3 μM concentration caused neuronal death in mixed cell cultures: self-assembly tau^{1N4R} dimers-tetramers induced neuronal necrosis and apoptosis, whereas Aβ-promoted tau^{1N4R} oligomers caused glial cell-dependent loss of neurons without signs of increased cell death. Monomeric and pre-aggregated tau peptide containing 4R repeats (K18) had no effect in mixed cultures, suggesting that tau neurotoxicity might be dependent on N-terminal part of the protein. Taken together, our results show that extracellular p-tau^{2N4R} is the most toxic form among investigated tau species inducing loss of neurons at low nanomolar concentrations and that neurotoxicity of tau^{1N4R} is dependent on its aggregation state.

Keywords Tau protein · Cell death · Microglia · Neurons · Alzheimer's disease · Tauopathy

Abbreviations

Aβ	Amyloid-β
$Aβ_{1–42}$	Amyloid-β 1 to 42 amino acid peptide
AD	Alzheimer's disease
Ara-C	Cytosine β- D–arabinofuranoside
BSA	Bovine serum albumin
CGC	Cerebellar granule cells
CSF	Cerebrospinal fluid
DIV	Days in vitro
DMEM	Dulbecco's Modified Eagle Medium
GSK-3β	Glycogen synthase kinase-3β
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid)
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
ISF	Interstitial fluid
MAPT	Microtubule-associated protein tau
MAPK	Mitogen-activated protein kinases
NMDA	N-methyl-D-aspartate
NFT	Neurofibrillary tangles
PHF	Paired helical filaments
PI	Propidium iodide
PVDF	Polyvinylidene fluoride
tau	Tubulin associated unit
tau^{1N4R}	Recombinant full-length tau 1N4R
tau^{2N4R}	Recombinant full-length tau 2N4R
p-tau	Phosphorylated tau
p-tau^{2N4R}	GSK-3β-phosphorylated recombinant full-length tau 2N4R
SDS	Sodium dodecyl sulfate

✉ Katryna Pampuscenko
katryna.pampuscenko@lsmuni.lt

[1] Neuroscience Institute, Lithuanian University of Health Sciences, Kaunas, Lithuania

[2] Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius, Lithuania

[3] Laboratory of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan

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SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
LDS	Lithium dodecyl sulfate

Introduction

Structural changes and aggregation of tau (tubulin associated unit) protein are thought to be involved in pathogenesis of various neurodegenerative diseases including Alzheimer's disease (AD), corticobasal degeneration, Pick's disease, progressive supranuclear palsy and others. Tau is a natively unfolded protein, specifically expressed in neuronal cells. Alternative splicing produces six protein isoforms varying in number of N-terminal inserts (N) and C-terminal repeats (R): 2N4R, 1N4R, 0N4R, 2N3R, 1N3R, and 0N3R [1]. Normally, tau protein stabilizes neuronal microtubules and may act as a signalling protein. However, under pathological conditions, tau may undergo hyperphosphorylation, truncation and aggregation and may detach from microtubules causing disruption of physiological integrity of neurons [2].

Intracellular tau inclusions, known as neurofibrillary tangles (NFT), as well as accumulation of extracellular tau have been detected in brains affected by neurodegenerative processes (for review see [3]). In AD, elevated amounts of tau were also found to be present in cerebrospinal fluid (CSF) and blood of patients [4–6]. Initially the accumulation of tau in CSF of AD patients has been thought to be related to neuronal death [7]. However, since the first report in 2010 [8], more and more studies clearly indicate that tau can be actively secreted by neurons and even glial cells and that changes in extracellular tau levels and composition are linked to pathological conditions [9]. It has been shown that levels of total tau as well as phosphorylated tau (p-tau) in CSF increase substantially with the progression of AD [10–12], and changes in CSF-total-tau levels may occur long before onset of clinical symptoms [13]. While total tau is considered as a non-specific marker for brain damage, p-tau is suggested to reflect NFT pathology allowing to distinguish AD from other dementia [5, 14]. Moreover, in CSF of AD patients, in contrast to healthy individuals, dimeric, trimeric and high molecular weight (> 669 kDa) tau forms have been detected [15]. It has been also reported that CSFs from AD patients contain N-terminal and mid-domain tau fragments [16, 17], R repeats [18–20] and full-length tau [21]. There is evidence that full-length tau and various tau fragments can be secreted and taken up by cells [22] and that pathological tau modifications (such as oligomerization and truncation) may stimulate secretion-internalization cycle potentially promoting transmission of pathology through the brain [23, 24]. Despite the fact that various forms of extracellular tau are detected in brains and CSFs of patients, little is known which of these tau species are most toxic and cause neurodegeneration.

Aggregation/oligomerization of extracellular tau has been suggested as one of the factors contributing to its toxicity. Oligomers of various extracellular tau isoforms have been shown to exert deleterious effects on synaptic function and memory loss [25]. However, the extent of damage in tau oligomer-treated cells may depend on the tau isoform composition [26]. Monomeric extracellular full-length tau, that is generally considered as non-toxic form, may also be involved in the fibrilization of intracellular tau [27, 28]. Thus, the link between aggregation state of tau, isoform, post-translational modification and neurotoxicity remains controversial.

Neuroinflammation and activation of microglia are common features of several tauopathies [29]. It has been shown that microglia take up and secrete tau in vitro and in vivo [30, 31] and thus may accelerate tau propagation between neurons [32]. Microglia from AD brain have been shown to contain tau species that normally are not expressed by these cells [33]. Moreover, tau protein has been shown to activate microglial cells and to enhance their phagocytic activity in various models of neurodegeneration [34–36].

Previously, we have shown that extracellular full-length tau^{2N4R}, independently of its aggregation state, causes microglia-dependent loss of stressed-but-viable neurons [37], and Brelstaff and colleges have reported that neurons in transgenic P301S-tau mice are phagocytosed by microglia [38]. In this study using rat mixed neuronal-glial cell cultures, we compared neuronal-death-inducing effects of 3 extracellular tau species—phosphorylated tau^{2N4R} ($p\text{-}tau^{2N4R}$), isoform 1N4R (tau^{1N4R}) and K18 peptide, a microtubule binding domain composed of four repeats (4R) located in the C-terminal part of the molecule. We found that among tau species investigated, $p\text{-}tau^{2N4R}$, added extracellularly, was the most toxic and induced proliferation of microglia and loss of neurons from cultures already at low, 30 nM concentration, whereas high, 700 nM $p\text{-}tau^{2N4R}$ concentration induced substantial neuronal and microglial necrosis, while a number of microglial cells remained unchanged. The neurotoxic effects of extracellular tau^{1N4R} were detected only at micromolar concentrations and were found to be dependent on oligomerization state, whereas K18 tau fragment of 4R repeats was not toxic to neuronal-glial cultures even at micromolar concentrations.

Methods

Materials and Reagents

NuPAGE LDS sample buffer, NuPAGE 3–8% Tris-Acetate Protein Gel, anti-tau (TAU5) antibody, alkaline phosphatase-conjugated anti-mouse secondary antibody, chemiluminescent CDP-Star substrate, *E. coli* BL21 Star™ (DE3) strain and isolectin GS-IB_4 from *Griffonia simplicifolia* conjugated

with Alexa Fluor488 and TNF-α (Rat) ELISA kit were purchased from *Invitrogen, ThermoFisher Scientific* (USA). Cell culture reagents DMEM Glutamax, foetal bovine serum, horse serum, penicillin-streptomycin and Versene solution were from *Gibco, ThermoFisher Scientific* (USA). Poly-(L)-lysine was from R&D systems (USA). Synthetic $A\beta_{1-42}$ was obtained from *Bachem* (Switzerland), recombinant human GSK-3β-phosphorylated Tau441 from *SignalChem* (Canada) and human Tau K18/Tau PHF Core Protein from *R&D systems* (USA). All other materials were purchased from *Sigma-Aldrich* (USA).

Expression and Purification of Recombinant Tau

The pRK172 DNA construct expressing full-length tau 1N4R isoform (tau^{1N4R}) [39] was generously provided by Dr. Michel Goedert (MRC Laboratory of Molecular Biology). Recombinant tau^{1N4R} protein was expressed in *E. coli* BL21 Star™ (DE3) strain and purified as described previously for tau 2N4R isoform [37].

Cell Cultures and Treatments

Cell cultures were prepared from 5 to 7-day-old Wistar rats of both sexes. Experimental procedures involving animals were undertaken in accordance with the EU Directive 2010/63/EU for animal experiments and the Republic of Lithuania law on the care, keeping and use of experimental animals (approved by Lithuanian State Food and Veterinary Service, ethical approval No. B6 (1.9)-855). Animals were bred and kept under controlled environmental conditions with a 12-h light/12-h dark cycle, at a constant temperature of 22 ± 1 °C. They were housed in plastic cages (one female rat with pups per cage) with water and food ad libitum in the animal breeding and housing facilities of Lithuanian University of Health Sciences. Rats were killed by increasing concentration of CO_2 in the air followed by cervical dislocation.

Primary neuronal-glial cell cultures (also called cerebellar granule cells; CGC) were prepared from rat cerebellum as described [40]. In brief, rat cerebellum was dissociated in Versene (1:5000) solution, centrifuged (270 g × 5 min) and suspended in DMEM Glutamax growth medium supplemented with 5% foetal bovine serum, 5% horse serum, 13 mM glucose, 20 mM KCl and 1% penicillin/streptomycin. Cell cultures were plated in 0.001% poly-(L)-lysine coated 96-well plates at 0.5 mln/ml density and grown for 6–7 days before treatments. These mixed brain cell cultures consisted of 87.2 ± 1.4% neurons and 8.9 ± 1.2% astrocytes (according to cellular and nuclear morphology, and also immunostaining test with NeuN and GFAP, respectively, was performed) and 3.9 ± 0.4% microglia (isolectin-IB_4 positive). To block proliferation of glial cells, cultures were treated with 10 μM cytosine β-D-arabinofuranoside (Ara-C) at 2 DIV. The purity of CGC cultures treated with Ara-C was 96%, with 0.5% microglia and 3.5% astrocytes.

Stock solutions of recombinant human GSK-3β-phosphorylated tau441 ($p\text{-}tau^{2N4R}$) and human tau K18/Tau PHF core protein (K18) were prepared according to manufacturer's recommendations; molar concentrations were calculated using provided information. Different conformations of tau412 (tau^{1N4R}) and tau K18 were prepared as described previously [35, 39, 41]. Briefly, for preparation of fresh, monomeric tau (*I protocol*) recombinant tau^{1N4R} or tau K18 was suspended in 10 mM HEPES buffer (pH 7.4) at 1 mg/ml concentration. In the second protocol (*II*), tau^{1N4R} or tau K18 was suspended in 10 mM HEPES buffer (pH 7.4) at 1 mg/ml and incubated at room temperature for 24 h. In the third protocol (*III*), tau solution prepared by the second protocol was supplemented with $A\beta_{1-42}$ oligomers (ratio of tau/Aβ weight 140:1) followed by pipetting for 1 min. Then, the sample was incubated for 24 h at room temperature with continuous agitation on orbital shaker at a 25 rpm speed. Soluble $A\beta_{1-42}$ oligomers were prepared as described in [42]. All protein solutions were aliquoted and stored at − 80 °C.

Cell Viability Assessment

Cell viability was assessed by propidium iodide (PI; 7 μM) and Hoechst 33342 (4 μg/ml) staining using fluorescence microscope (OLYMPUS IX71S1F-3, USA) as described in [42]. Neuronal cells were distinguished from glial cells by characteristic morphology in phase-contrast images. PI-positive cells were classified as necrotic and cells with condensed chromatin as apoptotic. Microglial cells were stained with isolectin GS-IB_4 conjugated with AlexaFluor488 (7 ng/ml). Neuronal and microglial cell numbers in neuronal-glial cultures were assessed by counting specific cells in at least 5 microscopic fields/well. Neuronal viability was expressed as percentage of specific cells (viable, necrotic, apoptotic) of the total number of neurons per microscopic field. The number of neurons/microglia in tau protein-treated cultures was expressed as the per cent of the total number of neurons/microglia in the control group, which was considered as 100%. All quantifications were carried out using ImageJ program.

Non-reducing SDS-PAGE and Western Blot Analysis

The aggregation state of recombinant human tau^{1N4R} protein was determined by non-reducing SDS-PAGE and Western blot analysis. Tau^{1N4R} protein samples (150 ng) were supplemented with NuPAGE LDS (Invitrogen) sample buffer (without heating and reductive agents) and immediately loaded onto the precast NuPAGE 3–8% Tris-Acetate Protein Gel (Invitrogen). Electrophoretic separation was performed using 50 mM Tricine, 50 mM Tris Base, 0.1% SDS running buffer. Proteins were transferred onto polyvinylidene fluoride

(PVDF; 0.45 μm) membrane using 25 mM Bicine, 25 mM Bis-Tris (free base), 1 mM EDTA transfer buffer. Membrane was blocked with 5% bovine serum albumin BSA (in Tris-buffered saline with 0.05% Tween 20) for 1 h and then incubated with primary antibody TAU5 (0,5 μg/ml) overnight (4 °C). Detection was done using ready-to-use alkaline phosphatase-conjugated anti-mouse secondary antibody (Invitrogen) and chemiluminescent CDP-Star substrate (Invitrogen). Analysis was performed using UVP imaging system and Vision WorksLS Software.

Statistical Analysis

All data are presented as mean ± standard error (SE) of independent cell culture preparations. Statistical comparison between independent experimental groups was performed using a one-way ANOVA followed by a Tukey's test. Statistical analysis was carried out using SigmaPlot (11.0 version software). p values < 0.05 were considered significant.

Results

Extracellular Phosphorylated Tau^{2N4R} Exerts Concentration-Dependent Neurotoxic Effects on Neuronal-Glial Cultures

To test whether extracellular phosphorylated full-length tau^{2N4R} is neurotoxic, we incubated CGC cultures with GSK-3β-phosphorylated tau^{2N4R} (p-tau^{2N4R}) for 48 h. Typical phase-contrast and fluorescence microscopy images of CGC cultures non-treated (control) and treated with 30 nM and 700 nM p-tau^{2N4R} are shown in Fig. 1a. We found that 30 nM p-tau^{2N4R} had no effect on neuronal viability (center panel of Fig. 1a and Fig. 1b) but significantly decreased the total number of neurons by 29% (center panel of Fig.1a and Fig. 1c) compared with control cultures (left panel of Fig. 1a and Fig. 1c). The total number of viable, necrotic and apoptotic neurons was 250 ± 43 cells/field in p-tau-treated cultures compared with 371 ± 31 cells/field in control cultures. Treatment with 30 nM p-tau^{2N4R} also caused microglial proliferation: there was about 2-fold increase in microglial cell numbers in p-tau^{2N4R} group compared with control (green fluorescent isolectin-IB$_4$-labelled cells in the central panel of Fig.1a and Fig. 1d). In contrast, 700 nM p-tau^{2N4R} causes substantial necrosis identified as intensive PI-red fluorescence of nuclei of both neurons and microglia (right panel of Fig. 1a): the number of PI-positive (necrotic) neuronal cells increases up to 76%, whereas the percentage of neurons with condensed chromatin (apoptotic) remains unchanged (Fig. 1b). Importantly, 700 nM p-tau^{2N4R} did not change the total number of neurons in cultures (Fig. 1c) as well as the total number of microglial cells (isolectin-IB$_4$ positive) compared with the control (right panel of Fig. 1a and Fig. 1d). The total number of viable, necrotic and apoptotic neurons was 437 ± 23 cells/field in cultures treated with 700 nM p-tau^{2N4R} which is comparable with numbers in control cultures (see above). Note that most of microglial cells (~95%) were PI-positive necrotic or with unstained nuclei (Fig. 1e). We also tested lower concentrations of p-tau; however, addition of 3 nM p-tau^{2N4R} had no effect on neuronal and microglial viability and cell numbers in CGC cultures during 48-h incubations (data not shown).

To test whether glial cells mediate neurotoxic effects of p-tau^{2N4R} in neuronal-glial cultures, the experiments were performed using CGC cultures treated with 10 μM Ara-C to prevent proliferation of glial cells. After such treatment, cultures contained about 96% neuronal cells. In Ara-C-treated cultures, 30 nM p-tau^{2N4R} had no effect on neuronal viability (Fig. 1b), nor on the total number of neurons in cultures during 48-h incubation (Fig. 1c). Total numbers of viable, necrotic and apoptotic neurons were 432 ± 43 in control and 522 ± 71 in p-tau^{2N4R} group. Addition of 700 nM p-tau^{2N4R} to Ara-C-treated cultures causes 39% neuronal necrosis after 48-h incubation (Fig. 1b), and this effect of p-tau^{2N4R} is about 50% lower than in Ara-C untreated CGC cultures. The percentage of apoptotic cells remained unchanged (Fig. 1b). Viability of neurons in Ara-C-treated cultures is about 55%, and this parameter is also substantially higher than in neuronal-glial cultures without Ara-C treatment (Fig. 1b). The total number of neurons in Ara-C-treated cultures remain unchanged after incubation with 700 nM p-tau^{2N4R} (Fig. 1c) – 483 ± 39 cells/field. These data suggest that glial cells—microglia or/and astrocytes—mediate at least partially the neurotoxic effect of extracellular p-tau^{2N4R}.

Extracellular Tau^{1N4R} Exerts Aggregation State-Dependent Neurotoxic Effects in Neuronal-Glial Cultures

Recently we have shown that tau^{2N4R} exerts aggregation-independent but microglia-mediated neurotoxic effects in CGC cultures [37]. In this study, we investigated whether another isoform tau^{1N4R} can be toxic to neurons and whether neurotoxicity depends on the oligomerization level of tau protein. We used 3 preparations of tau^{1N4R}: fresh, monomeric tau^{1N4R} (I) and tau^{1N4R} incubated for 24 h at room temperature (II) and tau^{1N4R} incubated for 24 h at room temperature in the presence of Aβ$_{1–42}$ oligomers (at 140:1 ratio of tau/Aβ; III). Distribution of monomeric/oligomeric tau species in preparations was confirmed by SDS gel electrophoresis followed by Western blot analysis. As can be seen in Fig. 2, tau^{1N4R} prepared by the *I protocol* gave only ~65 kDa band which represents monomeric form. Incubation of tau^{1N4R} at room temperature for 24 h (*II protocol*) resulted in the formation of ~130 kDa and ~250 kDa species representing dimeric and

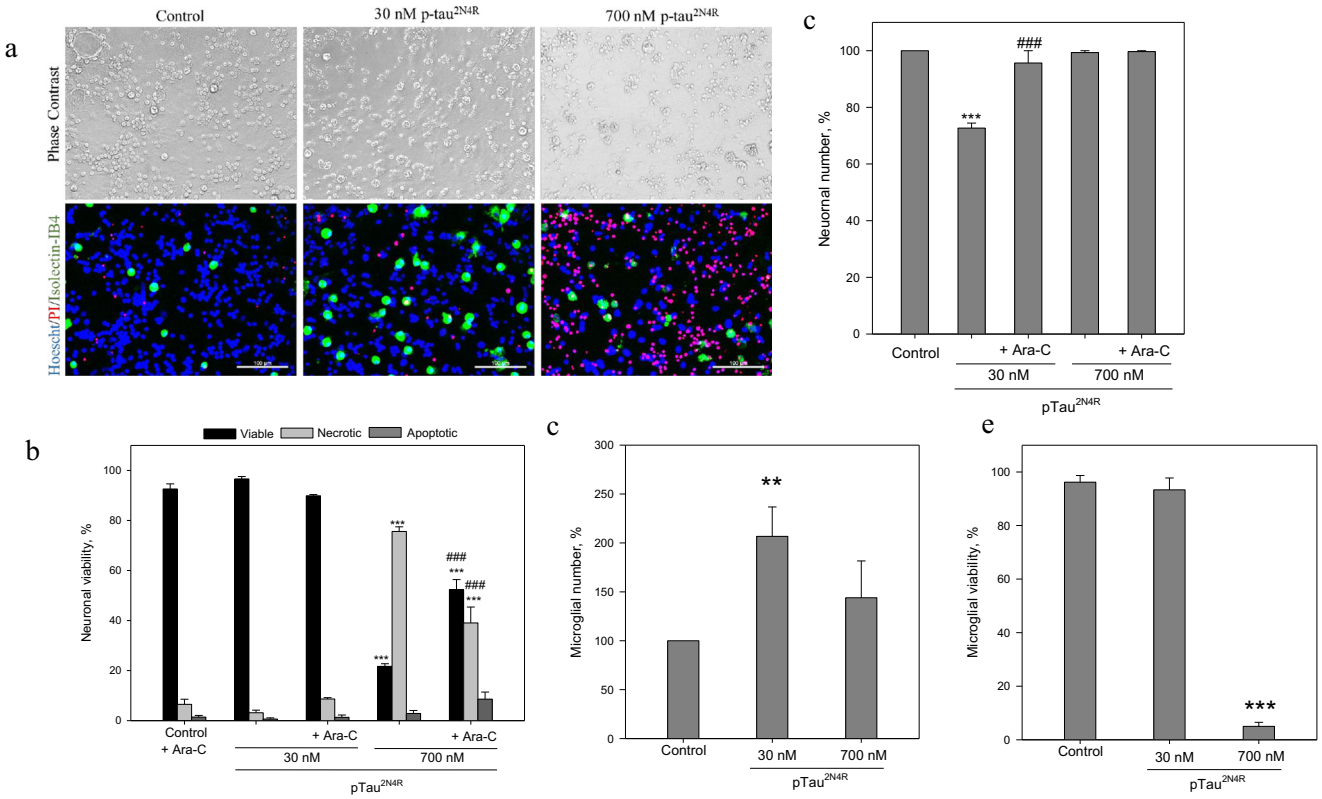

Fig. 1 Effects of phosphorylated tau^{2N4R} protein (p-tau^{2N4R}) on neuronal-glial (CGC) and Ara-C-treated CGC cultures. To inhibit glial cell proliferation neuronal-glial CGC cultures were treated with 10 μM of Ara-C at 2 DIV. Cell cultures were treated with p-tau^{2N4R} for 48 h. **a** Representative images of control and p-tau^{2N4R}-treated cell cultures. Neurons were visualized by phase-contrast microscopy and identified according to characteristic shape and morphology. Cell nuclei were stained with propidium iodide (PI) and Hoechst 33342. PI-positive cells (red) were classified as necrotic, cells showing nuclear shrinkage or fragmentation (Hoechst, bright blue) as apoptotic, and PI-negative cells with homogeneous Hoechst-staining as viable. Microglial cells were labelled with isolectin GS-IB$_4$ -AlexaFluor488 conjugate (green). Scale bars, 100 μm. **b** The effect of p-tau^{2N4R} on neuronal viability. Neuronal viability was expressed as percentage of specific cells (viable, necrotic, apoptotic) of the total number of neurons per field. The total number of neurons was quantified in five randomly chosen microscopic fields (at × 20 magnification) and averaged for each experiment. There were 415 ± 24 viable, 20 ± 6 necrotic and 2 ± 1 apoptotic neurons in a field of view in the control group. **c** The effect of p-tau^{2N4R} on number of neurons in cultures. The total number of neurons (viable, necrotic and apoptotic) was quantified in five randomly chosen microscopic fields. Neuronal number in control group was taken as 100%. **d** The effect of p-tau^{2N4R} on number and **e** viability of microglia in cultures. Microglial cell number presented as percentage of control group (100%). Microglia were counted at × 10 magnification. ***Statistically significant effect ($p < 0.001$) compared with control group, **statistically significant effect ($p < 0.01$) versus control, ###statistically significant effect ($p < 0.001$) compared with p-tau^{2N4R} group. Data are presented as means ± SE for 3 independent experiments

tetrameric forms. And incubation of tau^{1N4R} with Aβ oligomers at room temperature (*III protocol*) generated ~ 130 kDa and 250 kDa and higher molecular weight tau^{1N4R} aggregates.

As shown in Fig. 3, monomeric tau^{1N4R} prepared by the *I protocol* and added to cultures at 3 μM concentration had no effect on neuronal viability (Fig. 3a) and total neuronal cell numbers in cultures (Fig. 3b). However, tau^{1N4R} prepared by the *II protocol* was highly neurotoxic: as shown in Fig. 3a, only 13 ± 5% of neurons remain viable, while percentage of necrotic and apoptotic cells increases to 60 ± 6% and 28 ± 8%, respectively. The total number of neurons in tau^{1N4R} (II)-treated cultures significantly decreases by 34% compared with neuronal numbers in control cultures (Fig. 3b). Tau^{1N4R} prepared by *III protocol* did not change viability of neuronal cells (Fig. 3a); however, it significantly, by 40%, reduced the total number of neurons compared with the control cultures (Fig. 3b). Meanwhile, total numbers of microglial cells during 48-h incubation are similar in all treatment groups—control and treated with tau^{1N4R} prepared by all 3 protocols (Fig. 3c). Microglial viability was also not affected by any of tau^{1N4R} preparations (data not shown). Lower extracellular tau^{1N4R} concentrations, 1–2 μM, prepared by 3 different protocols and added to neuronal-glial cultures for 24 and 48 h, did not induce neuronal death or loss of neurons (data not shown). In these experiments, vehicle controls were not different from control cells (data not shown).

To test the role of glia in tau^{1N4R}-induced neurotoxicity, we performed experiments on Ara-C-treated, 96% neuronal cell cultures. Addition of 3 μM tau^{1N4R} (II protocol) to Ara-C-treated cultures causes 35% necrosis and only 5% apoptosis

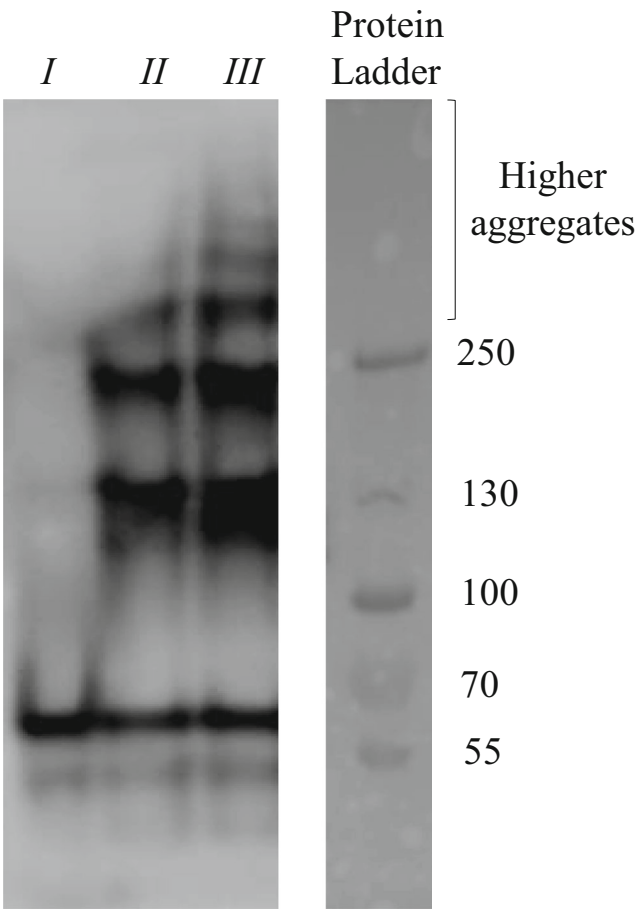

Fig. 2 Representative image of PVDF membrane showing the aggregation of recombinant tau^{1N4R} protein. Recombinant tau^{1N4R} protein samples were prepared by 3 different protocols: tau^{1N4R} protein was suspended in 10 mM HEPES buffer (pH 7.4) at 1 mg/ml concentration (*I protocol*) and was incubated at room temperature for 24 h (*II protocol*) or incubated with Aβ_{1-42} oligomers (ratio tau/Aβ 140:1) (*III protocol*). PVDF membrane was probed with anti-tau (TAU5) antibody. For details, see Methods, Section "Non-reducing SDS-PAGE and Western Blot Analysis". Non-reducing SDS-PAGE and Western blot analysis

of neurons after 48-h incubation (Fig. 3a). These parameters of neuronal death were significantly lower than in mixed neuronal-glial cultures. In accord, neuronal viability after incubation with tau^{1N4R} (II protocol) is about 3 times higher in Ara-C-treated than in Ara-C untreated CGC cultures (Fig. 3a). Addition of tau^{1N4R} (III protocol, 3 μM) had no effect on the viability of neuronal cells in Ara-C-treated cultures (Fig. 3a). Importantly, significant reduction of number of glial cells (from 13 to 4%) in Ara-C-treated cultures prevented tau^{1N4R}-induced decrease in total neuronal number which was observed in mixed neuronal-glial cultures after treatment with tau^{1N4R} prepared by both II and III protocols: total numbers of neurons in Ara-C-treated cultures exposed to tau^{1N4R} are similar as in control group (Fig. 3b), suggesting involvement of glial cells in tau^{1N4R}-induced removal of neurons from cultures.

Taken together, the data suggest that effects of extracellular tau^{1N4R} are highly dependent on its aggregation state: monomeric tau^{1N4R} is not toxic to neuronal-glial cultures, while tau^{1N4R} aggregates generated by II and III protocols were toxic but in different ways—dimers-tetramers of tau^{1N4R} lead to extensive neuronal cell death, whereas tau^{1N4R} dimers-tetramers and higher aggregates induced neuronal loss without apparent signs of death. In the presence of low number of glial cells in Ara-C-treated cultures, neurotoxic effects of tau^{1N4R} aggregates were less pronounced.

Tau K18 Fragment Is Non-toxic to Neuronal-Glial Cultures

Finally, we investigated whether tau 4-repeat domain fragment K18 is neurotoxic when applied extracellularly in mixed neuronal-glial cultures. Similarly as in the case of tau^{1N4R}, 3 different preparations of K18 peptides were added to CGC cultures at 3 μM concentrations and incubated for 48 h. As demonstrated in Fig. 4, regardless of K18 preparation used, the peptide had no effect on neuronal viability and numbers of neurons and microglia in CGC cultures (Fig. 4), suggesting that extracellular tau K18 peptide is not toxic to neuronal and glial cells.

Discussion

In the current study, we demonstrated that p-tau^{2N4R} is the most toxic form of tau compared with tau^{1N4R}, K18 peptide and previously described effects of tau^{2N4R} [37]: p-tau^{2N4R} induced loss of neurons (without apparent signs of cell death) from mixed neuronal-glial cultures at low nanomolar (30 nM) concentration, whereas toxicity of other forms of tau was observed in the range of much higher micromolar concentrations. 30 nM p-tau-induced neuronal loss was accompanied by proliferation of microglia and was prevented in the glia-depleted cultures suggesting that loss of neurons in this case was mediated by glial cells. Similar microglia-mediated neuronal loss was previously demonstrated to occur in CGC cultures treated with tau^{2N4R} but at 3 μM concentration [37]. At 700 nM concentration, p-tau^{2N4R} caused extensive necrosis of neurons. However, in glia-depleted cultures, deleterious effect of 700 nM p-tau^{2N4R} was less pronounced than in mixed cultures, and resulted in higher numbers of viable neurons and less necrosis, suggesting that glial cells might be also involved in p-tau-induced neuronal death. Interestingly, the numerical densities of neurons in cultures treated with 700 nM p-tau^{2N4R} were not affected indicating that dead (necrotic and apoptotic) neurons were not removed from these cultures. This may be at least partially related to death of microglial cells which was also observed in 700 nM p-tau^{2N4R}-treated cultures resulting in lower numbers of microglia than in cultures treated with

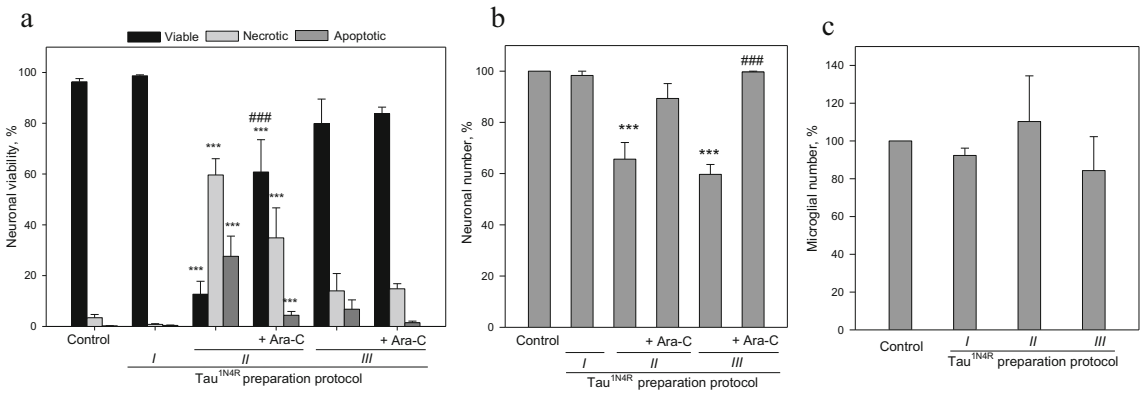

Fig. 3 Neurotoxic effects of tau^{1N4R} in neuronal-glial (CGC) and Ara-C-treated CGC cultures. To induce aggregation, monomeric tau^{1N4R} protein (*I protocol*) was incubated at room temperature for 24 h with (*III protocol*) or without (*II protocol*) $A\beta_{1-42}$ oligomers (ratio tau/Aβ 140:1). Cell cultures were treated with 3 μM tau^{1N4R}. To inhibit glial cell proliferation, neuronal-glial co-cultures were treated with 10 μM of Ara-C at 2 DIV. **a** The effect of tau^{1N4R} on neuronal viability. Neuronal viability was measured by Hoechst33342/propidium iodide staining as described in methods. **b** The effect of tau^{1N4R} on neuronal and **c** microglial cell numbers. Number of neurons and microglia in tau^{1N4R} -treated groups expressed as the percent of the total number of appropriate cells in the control group, which were considered as 100%. ***Statistically significant effect ($p < 0.001$) compared with control group. Data are presented as means ± SE for 3–13 independent experiments

30 nM p-tau^{2N4R}. Toxic effects of p-tau have been previously described in other studies showing that soluble fractions derived from AD hippocampi or transgenic mice brains and rich in p-tau produced reduction in the numbers of viable cells in primary microglia or BV2 cell cultures [43]. It has been also shown that phagocytosis of apoptotic SH-SY5Y cells containing intracellular p-tau species caused microglial death in vitro [43]. Recently, we have shown that CSF from AD patients exhibited distinct neurotoxicity in neuronal-glial co-cultures leading to loss of viable neurons (early stage AD-CSF) or neuronal necrosis and reduction in microglial numbers (middle stage AD-CSF, rich in p-tau) [12].

One may argue that concentrations of p-tau^{2N4R} (30 and 700 nM representing 2 and 50 μg/ml, respectively) used in our study were higher than found in interstitial fluid (ISF) of transgenic mice (~250 ng/ml, total-tau) [44, 45] or in CSFs of patients with neurodegenerative disorders (~300–400 pg/ml, total-tau; ~50–100 pg/ml, p-tau) [10–12]. However, it is not clear what local concentrations of various tau species may be present in the brain under pathological conditions. It has been suggested that since intraneuronal tau concentration was estimated to be in micromolar range (~2 μM) and in AD frontal and temporal cortex tau concentrations were found to be increased by 5–7-folds, the local tau concentrations after cell death might be higher than in CSF or ISF [46–49].

Changes in tau isoform content appear to contribute to cognitive decline in mouse models of neurodegeneration [50], and growing evidence suggests that there is a link between tau predominant isoform and tauopathy [51–53]. In our study, we showed that extracellular tau^{1N4R} exerts aggregation state-dependent neurotoxicity in neuronal-glial cultures distinct from previously described aggregation state-

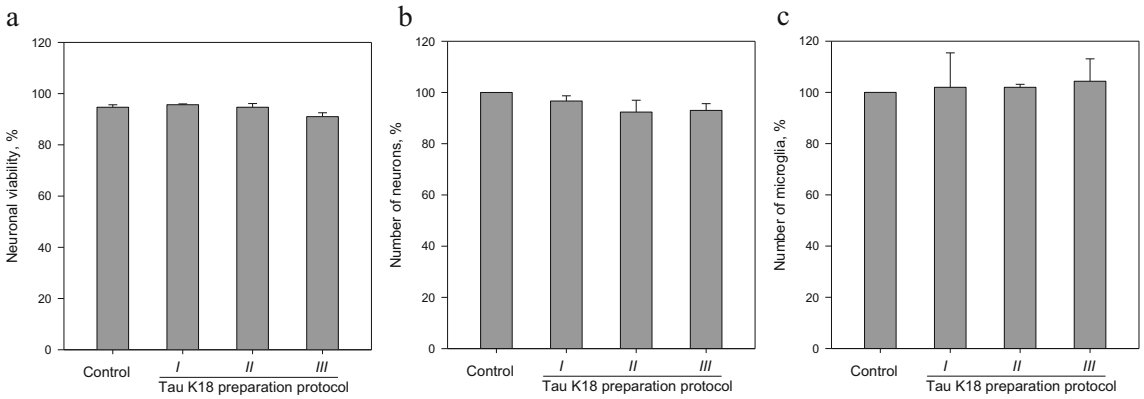

Fig. 4 Tau K18 peptide has no effect in neuronal-glial cultures. Cell cultures were treated with 3 μM tau K18 of different preparations for 48 h as described in Methods. The effect of tau K18 on (**a**) neuronal viability, (**b**) numbers of neuronal and (**c**) microglial cells in cultures. Cell viability was measured by PI and Hoechst 33342 staining and expressed as ratio of viable to total (viable, necrotic, apoptotic) number of neurons. Neuronal and microglial cell number are presented as percentage of control group (100%). There was no statistically significant differences between groups. Data are presented as means ± SE for 3 independent experiments

independent neurotoxicity of the longest tau isoform tau^{2N4R} [37]. In the current study, we demonstrated that tau^{1N4R} oligomers, but not tau^{1N4R} monomers, caused neuronal loss in neuronal-glial cultures: tau^{1N4R} oligomers prepared by II protocol (self-aggregation, dimers-tetramers) caused massive neuronal necrosis and apoptosis that was only partially microglia- and/or astrocyte-dependent. Meanwhile tau^{1N4R} oligomers obtained using III protocol (pre-incubated with trace amounts of Aβ, larger aggregates) caused disappearance of neurons without morphological features of cell death, and this loss was abolished by reduction of glial cells in culture. Different neurotoxicity of tau^{1N4R} oligomers could be explained by distinct size and/or shape of tau species that might affect their internalization [54–57].

Here we show that neurotoxicity of p-tau^{2N4R} and tau^{1N4R} oligomers was significantly prevented by elimination of glial cells, indicating relationship between extracellular tau-caused neuronal loss and neuroinflammatory processes. Several studies have shown that tau can directly activate glial cells [34, 58, 59], and even overexpression of tau protein in neurons caused changes in microglial cells [36, 60]. However, the specific isoforms or oligomeric species of tau involved in neuronal damage and the interaction between neurons and microglia under pathological conditions need to be investigated in more details.

Tau^{2N4R} and tau^{1N4R} differ by the number of N-terminal inserts (*N*) derived from alternative splicing of MAPT transcripts [1]. Since monomeric and pre-aggregated K18 containing 4R fragment had no effect in neuronal-glial cultures, it is possible to speculate that C-terminus of molecule has no influence on extracellular tau neurotoxicity. The role of N-terminal residues in tau-caused neurotoxicity have been described in several studies showing that overexpression and accumulation of the particular N-terminal tau fragments caused mitochondrial dysregulation, NMDA receptor-mediated calpain and ERK/MAPK activation leading to cell death [61, 62]. Tau protein in human CSF appears in isoforms containing 0 N, 1 N and 2 N [63], but whether tau isoform profile changes along with neurodegeneration is unknown.

Conclusions

In summary, our results indicate that neurotoxicity of extracellular tau depends on the isoform as well as on phosphorylation and aggregation state of tau species and involve participation of glial cells. The identification of the link between extracellular tau protein type/state, neuronal dysfunction and glial cell activation status may have potential implications for control of inflammation in neurodegeneration.

Authors' Contributions KP carried out experiments on cell cultures, analysed and wrote the manuscript. LK and VS performed expression and purification of recombinant tau protein. RM planned the experiments, analysed data and wrote the manuscript. TT analysed data and wrote the manuscript. VB initiated, planned and supervised the study, wrote the manuscript. All authors reviewed the final manuscript.

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Availability of Data and Materials The data that support the findings of this study are available from the corresponding author upon request.

Compliance with Ethical Standards

Conflict of Interest Authors declare that they have no conflict of interest.

Ethics Approval Approved by Lithuanian State Food and Veterinary Service, ethical approval No. B6(1.9)-855.

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Code Availability (Software Application or Custom Code) Not applicable.

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