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Article type : Original Article

# Extracellular tau induces microglial phagocytosis of living neurons in cell cultures

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*Running title:* Tau and neuronal loss

*Keywords:* tau protein, phagocytosis, cell death, neuroinflammation, Alzheimer's disease

**Abbreviations:** Aβ - amyloid-β, AD – Alzheimer's disease, AFM – atomic force microscopy, CGC – cerebellar granule cells, CSF – cerebrospinal fluid; Ara-C - cytosine β-D-arabinofuranoside hydrochloride DIV – days *in vitro*, GSK-3β - glycogen synthase kinase-3β, HEPES - (4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid), LME - L-leucine-methyl-ester, LPS – lipopolysaccharide, MerTK – MER proto-oncogene tyrosine kinase, NFT - neurofibrillary tangles, nSMase - neutral sphingomyelinase-2, PI – propidium iodide, PKC – protein kinase C, PtSer – phosphatidylserine.

**ABSTRACT**

Tau is a microtubule-associated protein, found at high levels in neurons, and the aggregation of which is associated with neurodegeneration. Recently it was found that tau can be actively secreted from neurons, but the effects of extracellular tau on neuronal viability are not clear. In this study, we investigated whether extracellular tau$^{2N4R}$ can cause neurotoxicity in primary cultures of rat brain neurons and glial cells. Cell cultures were examined for neuronal loss, death and phosphatidylserine exposure, as well as for microglial phagocytosis by fluorescence microscopy. Aggregation of tau$^{2N4R}$ was assessed by atomic force microscopy. We found that extracellular addition of tau induced a gradual loss of neurons over 1-2 days, without neuronal necrosis or apoptosis, but accompanied by proliferation of microglia in the neuronal-glial co-cultures. Tau addition caused exposure of the "eat-me" signal phosphatidylserine on the surface of living neurons, and this was prevented by elimination of the microglia or by inhibition of neutral sphingomyelinase. Tau also increased the phagocytic activity of pure microglia, and this was blocked by inhibitors of neutral sphingomyelinase or protein kinase C. The neuronal loss induced by tau was prevented by inhibitors of neutral sphingomyelinase, protein kinase C or the phagocytic receptor MerTK, or by eliminating microglia from the cultures. The data suggest that extracellular tau induces primary phagocytosis of stressed neurons by activated microglia, and identifies multiple ways in which the neuronal loss induced by tau can be prevented.

**INTRODUCTION**

Alzheimer's disease (AD) is a neurodegenerative disorder associated with formation of misfolded protein aggregates in the brain – mainly extracellular amyloid-β (Aβ) and intraneuronal tau – in parallel with microglial activation and neuronal loss (Hardy and Selkoe 2002). For the last few decades formation of fibrillar or soluble oligomeric Aβ aggregates has been considered as a key event in AD pathology, however, the majority of therapeutic trials using treatmemts to decrease brain Aβ levels have failed to benefit patients (Nicoll *et al.* 2003; Orgogozo *et al.* 2003; Matsumoto and

Kohyama 2015). Tau is a microtubule-associated protein which under pathological conditions can be hyper-phosphorylated, cleaved and aggregated into neurofibrillary tangles (NFT) (Iqbal *et al.* 2010). Formation and spreading of intraneuronal NFT correlates with AD stage and cognitive decline (Arriagada *et al.* 1992; Duyckaerts *et al.* 1997; Braak *et al.* 2006), however, neuronal loss in AD brains exceeds the NFT number (Gómez-Isla *et al.* 1997), suggesting that other critical factors for AD pathogenesis and cell death exist. There is evidence that tau may accumulate not only inside neurons but also in extracellular space, cerebrospinal fluids (CSF) and blood serum during AD and other neurodegenerative diseases (Schoonenboom *et al.* 2012; Sengupta *et al.* 2017). It has previously been assumed that the CSF levels of tau and tau/$A\beta_{1\text{-}42}$ reflect the intensity of neuronal damage and AD progression (Saman *et al.* 2012; Steenland *et al.* 2014). Moreover, analysis of AD CSF provided evidence of tau, not only in monomeric form, but also in dimer/trimer and higher aggregates (Sengupta *et al.* 2017). Recent data from several studies have suggested that tau can accumulate in presynaptic terminals of cortical neurons and that neurons can actively secrete full length or fragmented tau (Chai *et al.* 2012; Sokolow *et al.* 2015). Extracellular tau can spread tau pathology through the brain, and be internalized by healthy neurons and affect neuronal functions *in vitro* (Gómez-Ramos *et al.* 2006; Gómez-Ramos *et al.* 2008; Lasagna-Reeves *et al.* 2011) and *in vivo* (Wu *et al.* 2013). Extracellular tau can be toxic to neurons, but the mechanisms by which tau causes cell death are unclear (Sebastián-Serrano *et al.* 2018). It has been shown that extracellular addition of monomeric, full-length tau (and tau-derived construct K18 comprising the repeat domain) cause aggregation of endogenous tau in neuron-like cell cultures and *in vivo* (Michel *et al.* 2014). On the other hand, Manassero *et al*. have reported that microinjection of monomeric full-length tau causes neuronal apoptosis, whereas oligomers promote aggregation of endogenous tau without inducing cell death in human tau-expressing mice (Manassero *et al.* 2017). Extracellular soluble tau oligomers, but not monomers, produced an impairment of long-term potentiation and memory in an AD mouse model (Fá *et al.* 2016; Puzzo *et al.* 2017) and more effectively, than monomers or fibrils, evoked disruption of axonal trafficking (Swanson *et al.* 2017).

AD and other tauopathies are known to be associated with neuroinflammation and microglial activation (Ardura-Fabregat *et al.* 2017; Dani *et al.* 2018; Gerhard *et al.* 2004; Gerhard *et al.* 2006). Microglia are also thought to be implicated in transmission of tau-pathology (de Calignon *et al.*

2012). Microglia isolated from transgenic mice brains and from human brains affected by AD or other tauopathies were found to contain bioactive tau forms that are not synthesized in these cells (Hopp *et al.* 2018). This suggests that extracellular tau can be internalized by microglia which was confirmed by studies *in vivo* and *in vitro* (Bolós *et al.* 2015). Extracellular dephosphorylated forms of tau can activate microglia (Perea *et al.* 2018). In a transgenic tau mouse model, microglia were activated and accumulated around neurons (Sydow *et al.* 2016), and depletion of microglia has been shown to suppress propagation of tau and to reduce excitability in the dentate gyrus in AD mouse models (Asai *et al.* 2015). Activated microglia can be highly phagocytic and phagocytose live neurons if the neurons expose "eat-me" signals on their surface, resulting in neuronal death by primary phagocytosis (Brown and Neher 2014). Recently it has been reported that live neurons containing aggregated P301S-tau activate microglia and are phagocytosed by these microglia (Brelstaff *et al.* 2018). Thus, it is important to understand what factors determine microglial activation in tau-induced pathologies.

It has been previously reported that protein kinase C (PKC) may regulate microglial proliferation and phagocytosis of neurons exposed to Aβ (Castrillo *et al.* 2001; Neniskyte *et al.* 2016), and that activation of protein kinase C in primary rat microglia exposed to LPS was mediated by sphingomyelinases (Akundi *et al.* 2005). Abnormal sphingolipid metabolism and activation of neutral sphingomyelinase-2 (nSMase-2) has been observed in brain ischemia, inflammation, AD and other neurodegenerative disorders (Shamseddine *et al.* 2015). Recently it has been shown that reduction of exosome secretion by nSMase2 inhibition improved pathology and cognition in an amyloid mouse model of AD (Dinkins *et al.* 2016). However, it is unclear whether and how nSMase2 or PKC are involved in tau pathology.

In this study, we investigated the effects of various aggregation forms of extracellular full-length tau 2N4R isoform ($tau^{2N4R}$) on primary mixed neuronal/glial and pure microglial cell cultures and sought to determine whether nSMase and PKC are involved. We found that extracellular $tau^{2N4R}$ independently of its aggregation state induced loss of most neurons without an increase in apparent necrosis or apoptosis, and was accompanied by increased microglial proliferation and phagocytosis. This suggests that extracellular $tau^{2N4R}$ induces primary phagocytosis of stressed-but-viable neurons mediated by activated microglia.

## METHODS

### *Materials*

Synthetic $A\beta_{1-42}$ was obtained from Bachem (Cat #401447), isolectin GS-IB4 from *Griffonia simplicifolia* conjugated with Alexa Fluor488 (Cat#I21411) and Griess reagent kit (Cat #G7921) from Invitrogen (ThermoFisher Scientific), Pierce Chromogenic Endotoxin Quant Kit (Cat #39552) from Thermofisher, TNF-α (Rat) ELISA kit (Cat #abx574582) from Abbexa, poly-(L)-lysine (Cat #3438-100-01) from R&D systems, Ro32-0432 (Cat #557525), TDZD8 (Cat #361540) and UNC569 (Cat #445835) from Calbiochem, Ro31-8220 (Cat #R136), GW4869 (Cat #D1692), Go6983 (Cat #G1918), Annexin V-Cy3.18 conjugate (Cat #A4963), latex beads (carboxylate modified, polysterene, 2 μm) (Cat #L3030), polymyxin B (Cat #P4932), LPS from *E.Coli* 0111:B4 (Cat #L2630) and O26:B6 (Cat #L2654), propidium iodide (Cat #P4170), Hoechst 33342 (Cat #14533), L-leucine-methyl ester hydrochloride (Cat #L1002), cytosine β-D-arabinofuranoside hydrochloride (Cat #C6645) from Sigma-Aldrich. Cell culture reagents DMEM Glutamax (Cat #32430), fetal bovine serum (Cat #A3160502), horse serum (Cat #16050), penicillin-streptomycin (Cat #15140122), Versene solution (Cat #15040066) and chemically competent cells E. coli BL21 Star™ (DE3) strain (Cat #C601003) were purchased from Invitrogen or Gibco (ThermoFisher Scientific), DEAE Sepharose Fast Flow (Cat #17070901), CM Sepharose Fast Flow (Cat #17071901), XK 26/20 column (Cat # 28988948), Superdex 200 resin (Cat #11570804) were obtained from GE Healthcare and Model Tap300Al-G from Budget Sensors.

### *Expression and purification of recombinant tau*

The pRK172 DNA construct expressing full-length tau 2N4R isoform ($tau^{2N4R}$) (Goedert and Jakes 1990) was generously provided by Dr. Michel Goedert (MRC Laboratory of Molecular Biology, Cambridge, UK). Recombinant $tau^{2N4R}$ protein was expressed in E. coli BL21 Star™ (DE3) strain and purified similar as described previously (Goedert and Jakes 1990), with some modifications. Briefly, cell pellet was resuspended in the 100 ml of ice-cold buffer solution A (25 mM Tris, 10 mM EDTA, 0.1 mM PMSF, 0.1 mM DTT, pH 7.4), homogenized and disrupted by sonication. Cell pellet was centrifuged at 25,000 g (4°C) for 30 min. The supernatant was loaded onto DEAE Sepharose Fast Flow column. Collected flow through was mixed with 20 ml of CM Sepharose

Fast Flow, and incubated for 30 min at 4°C. The resin was loaded onto XK 26/20 column. The column was washed with 5 column volumes of buffer solution A and the protein was eluted at 3 ml/min flow rate using a 5-column-volume linear gradient of 0-400 M NaCl. Collected factions were tested by SDS-PAGE. Fractions containing tau protein were combined. The protein was salted out using ammonium sulfate (50 % saturation) and collected by centrifugation at 20000 g (4°C) for 30 min. The precipitate was dissolved in buffer solution B (20 mM sodium phosphate, 0.1 mM PMSF, 0.1 mM DTT, pH 7.4). The solution was loaded (0.5 ml injection loop) onto Tricorn 10/600 column packed with Superdex 200 resin, and the protein was eluted at 1 ml/min using buffer solution B. Collected fractions (2 ml) were checked by SDS-PAGE. Fractions containing monomeric tau protein were combined. The buffer was exchanged to water, the samples were lyophilized and stored at -20°C until further use.

Content of bacterial endotoxin in purified tau preparations was determined using endotoxin level quantification kit Pierce Chromogenic Endotoxin Quant Kit and LPS from E.Coli 0111:B4 and O26:B6.

***Tau$^{2N4R}$ preparations and their characterization***

For fresh, monomeric preparations, lyophilized recombinant tau$^{2N4R}$ was dissolved in 10 mM HEPES buffer and immediately added to cell growth media at 0.138 mg/ml concentration (3 μM, $M_r$=45 900 Da theorethical molecular weight).

For preparation of tau aggregates two protocols were used. First, tau$^{2N4R}$ oligomers were prepared according to (Lasagna-Reeves *et al.* 2010) with some modifications. Recombinant tau$^{2N4R}$ was suspended in 10 mM HEPES buffer (pH 7.4) at 1 mg/ml concentration and incubated at room temperature for 24 h. In the second protocol, tau$^{2N4R}$ solution prepared by the first protocol was supplemented with A$\beta_{1-42}$ oligomers (dilution 1:140, vol./vol.) 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 low speed.

Soluble A$\beta_{1-42}$ oligomers (4-10 nm) were prepared as described Cizas et al., 2010. Briefly, 1 mg of peptide was dissolved in of 1,1,1,3,3,3-hexafluorisopropanol (HFIP) for 30–60 min at room temperature and then was 10-fold diluted with double-distilled water in siliconized Eppendorf tubes. After 10–20 min of

incubation at room temperature, the solution was centrifuged for 15 min at 12,000 rpm. HFIP was evaporated in open vials for 16 h at 18 $^{o}$C in a water heater thermostat and then the solution was incubated for 24 h at room temperature with shaking.

The morphology of tau$^{2N4R}$ aggregates in preparations was characterized using the atomic force microscopy (Dimension Icon, Bruker AXS, Germany), scanning probe work station operating in the tapping mode. Model Tap300Al-G (f = 300 kHz, k = 40 N/m) microcantilevers were used in this work. 20 µl of 0.5 mg/ml tau$^{2N4R}$ sample solution was spotted on mica, incubated at room temperature for 10 min, rinsed with distilled water (Millipore, Inc.) and blown dry with filtered (<0.2 µm) nitrogen stream. When necessary, aggregates were adsorbed to the mica surface and hardened by cross-linking with glutaraldehyde. For this, 30 µl phosphate buffer containing 0.5% (w/v) glutaraldehyde was added to adsorbed tau aggregates and incubated for 30–60 s. Then a glutaraldehyde containing buffer was replaced at least 1-5 times by 30 µl phosphate buffer. Cross-linked tau aggregates were imaged in the same way as non-fixed ones in tapping mode AFM imaging. Images were acquired at scan rates between 0.5 and 1 Hz with resolution of 1024x1024 pixels. The morphology of surface was estimated by measuring the profile of the sample along the x-y plane of the AFM image. The mean height was estimated using Gwyddion software (Czech metrology institute, RRID:SCR_015583).

***Primary neuronal-glial and pure microglial cultures***

Cell cultures were prepared from 5-7-day-old Wistar rats (RGD Cat# 13508588, RRID:RGD_13508588) of both sexes as described in (Bal-Price and Brown 2001). 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 the concentration of $CO_2$ in

the air followed by cervical dislocation. Brains from three-four animals were pooled together and used for one cell culture preparation. In total, about 90 rats were used.

After removal of meninges and blood vessels, cerebellum was dissociated in Versene (1:5000) and centrifuged at 270×g for 5 min. Cells were suspended in growth medium (DMEM Glutamax supplemented with 5 % fetal bovine serum, 5 % horse serum, 13 mM glucose, 20 mM KCl and 1% penicillin/streptomycin) and plated at 500000 cells/ml in 96-well plates coated with 0.001% poly-(L)-lysine. Cells were grown *in vitro* for 5–6 days before exposure to tau. The cultures contained 5.5 ± 0.7 % microglial cells, as assessed by staining with isolectin GS-IB4, 82.3 ± 1.0 % neurons and 11.7 ± 0.6 % astrocytes, as assessed by cellular morphology.

To inhibit proliferation of glial cells CGC cultures were treated with 10 μM of cytosine β- D–arabinofuranoside (Ara-C) at 2 DIV. These cultures contained 97.2 ± 0.4 % neurons, 0.3 ± 0.1 % microglia and 2.4 ± 0.4 % astrocytes. Selective depletion of microglia was achieved by incubating CGC cultures at 6 DIV for 4 hours with 15 mM lysosomotropic agent L-leucine-methyl-ester (LME) (Neniskyte *et al.* 2011). Then the medium was changed with conditioned growth medium from sister cultures. LME treated cultures consisted of 81.5± 2.3 % neuorons, 0.7± 0.2 % microglia and 17.8± 2.2 % astrocytes.

Primary astroglial cultures were obtained from cerebral cortex, as described in (Morkuniene *et al.* 2015). Briefly, brain tissue dissociated in Versene (1:5000) was centrifuged at 290×g for 5 min and suspended in DMEM-Glutamax supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. 15 ml of cell suspension were seeded into 75T flasks coated with 0.001% poly-(L)-lysine. At confluence (7-10 days after initial seeding), pure microglial cell cultures were obtained by mechanically shaking flasks. Then medium with detached cells was centrifuged, cells suspended in fresh growth medium and plated at 100000 cells/ml density into 96-well plates coated with poly-(L)-lysine. Microglial cells were allowed to attach overnight before treatment. The flasks and plates were maintained in humidified atmosphere containing 5% $CO_2$ in the incubator. The purity of the cultures was 88.2 ± 4.2 %.

When inhibitors were used in the experiments, cell cultures were pre-incubated for 30 min with 1 μM Ro31-8220, 1 μM Ro32-0432, 1 μM Gö6983, 4 μM TDZD8, 11 μM GW4869, 50 nM UNC569 before exposure to $tau^{2N4R}$.

***Cell viability and cellular density in cultures assessment***

Cell viability was assessed using fluorescence microscopy (Olympus IX71S1F-3, USA) as described in (Cizas *et al.* 2010). Cellular nuclei were stained with propidium iodide (PI, 7 μM) and Hoechst 33342 (4 μg/ml). Cells with condensed chromatin but PI-negative were considered as apoptotic and PI-positive cells as necrotic. Neurons were recognized according to characteristic shape and morphology using phase contrast microscopy. Microglial cells were identified by Isolectin GS-$IB_4$ conjugated with AlexaFluor488 (7 ng/ml) staining. Neuronal and microglial cell numbers in CGC cultures were assessed by counting specific cells in at least 5 microscopic fields/well and expressed as percentage of specific cells of the total number of cells per field. All quantifications were carried out using ImageJ (RRID:SCR_003070) program.

***Measurements of TNF-α and NO***

After treatment with $tau^{2N4R}$ medium from CGC cultures was collected and used for pro-inflammatory factor quantitative analysis. Cell cultures treated with 100 ng/ml LPS were used as a positive control. TNF-α concentration was determined using commercial ELISA kit according to manufacturer's protocol. Production of NO was evaluated by measuring nitrite levels using commercial Griess Reagent Kit.

***Annexin V staining***

CGC cultures were washed with PBS and incubated with Annexin V-Cy3.18 conjugate (4.5 μg/ml) in binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$ (pH 7.5)) for 10 min at room temperature (Neniskyte *et al.* 2011). After staining cells were washed with binding buffer three times. Cell nuclei were stained with Hoechst 33342 (4 μg/ml) and microglial cells were labelled with isolectin GS-IB4 conjugated with AlexaFluor488 (7 ng/ml). Cells were visualized with fluorescence microscope (Olympus IX71S1F-3, USA) and data were analyzed with ImageJ (RRID:SCR_003070) program. Annexin V-Cy3.18 conjugate fluorescence intensity was analized at least 4-5 microscopic fields. For each microscopic field, the total number of neurons was counted and Annexin V-Cy3.18 fluorescence intensity was normalized to 100 neurons.

***Phagocytosis assay***

Microglial phagocytic capacity was evaluated using latex beads. Pure microglial cultures were incubated with 2 μm carboxylate-modified microspheres at 0.005% (w/v) concentration for 2 hours (37$^{o}$C, 5% $CO_2$) and washed with PBS for several times before visualization as described (Neniskyte *et al.* 2011). Cells were visualized using fluorescent microscope (Olympus IX71S1F-3, USA). All quantifications were carried out using ImageJ (RRID:SCR_003070) program.

***Statistical Analysis***

All data are presented as mean ± standard error (SE) with overlay of individual data points on independent cell culture preparations. Data were tested for normality using Shapiro-Wilk test, and all data passed normality test. 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 (RRID:SCR_003210) 11.0 version software. P values <0.05 were considered significant. This study was not pre-registered. No blinding, randomization, or sample size calculation was performed in this study. There were no exclusions.

***Data accessibility***: The data that support the findings of this study are available from the corresponding author upon the request.

## RESULTS

***Extracellular tau$^{2N4R}$ causes neuronal loss and microglial proliferation.***

Tau protein is normally soluble, however in pathological conditions it may form protein aggregates. To test whether monomeric or aggregated tau$^{2N4R}$ have any influence on neurotoxicity we used three different protein preparations, the structural heterogeneity of which were directly characterized by AFM (Fig. 1). The z-height of the adsorbed tau$^{2N4R}$ (fresh monomeric preparations) was on average less than 3 nm (Fig. 1A, D) while tau$^{2N4R}$ aggregates prepared by incubating tau$^{2N4R}$ solution at room temperature for 24 h or by incubating tau$^{2N4R}$ in the presence of low amount of $A\beta_{1-42}$, had the mean z-height ~2-7 nm and ~3-11 nm, respectively (Fig. 1B, C and D) indicating that levels of aggregation differ among tau$^{2N4R}$ preparations. As previously reported, AFM measurements may underestimate the diameter of protein structures because of sample compression by the AFM tip

(Cizas *et al.* 2010). The purity of recombinant tau protein was confirmed by SDS-PAGE electrophoresis, showing that freshly prepared tau protein gives a single monomeric band (Figure 1S). However, when this tau was incubated for 24 h in growth medium (without serum and cells) we found oligomeric particles of various sizes in all three tau preparations (freshly prepared monomeric, pre-incubated for 24 h in the absence or presence of $A\beta_{42}$) measured by AFM and size exclusion chromatography (Figure 2S, Table 1S). There were no fibrils of tau even after 48 h incubation (Table 1S).

Next we tested whether extracellular $tau^{2N4R}$ can cause neuronal death in CGC cultures. In these experiments, cultures were incubated with various preparations of $tau^{2N4R}$ at 3 μM concentration and neuronal necrosis and apoptosis was evaluated after 24-48 h incubation. As can be seen in Fig. 2A, B, apoptosis was negligible in all conditions tested and there were no statistically significant changes in neuronal necrosis after 24 h and 48 h treatment with various preparations of extracellular $tau^{2N4R}$ compared with control CGC cultures. In all conditions investigated more than 80% of neurons were viable.

Interestingly, although the viability of neurons was not affected by tau, there was a substantial decrease in the number of viable neurons in CGC cultures exposed to various preparations of $tau^{2N4R}$. As shown in Fig. 2A and B the number of viable neurons decreased approximately by 30% after 24 h and by 60% after 48 h treatment independent of tau particle sizes in stock preparations. A lower concentration of $tau^{2N4R}$ (1 μM) caused a smaller reduction in neurons numbers to 84±3% of control cultures after 2 days of treatment, and to 72±11% of control after 3 days, and remained at this level after 5 days of treatment (data not shown). As the effects of 3 μM $tau^{2N4R}$ were more reproducible than 1 μM, 3 μM tau was used in all further experiments.

Numbers of microglial cells in CGC cultures treated with 3 μM $tau^{2N4R}$ for 24 h were similar to those in untreated control cultures but increased approximately by 2-fold compared to control after 48 h incubation, again independently of $tau^{2N4R}$ particle sizes (Fig. 2C).

We also investigated whether tau can be internalized by cells of CGC cultures. Using fluorescently labeled $tau^{2N4R}$ we found that cells in CGC cultures rapidly internalize extracellularly applied protein: after 6 hours of incubation $tau^{2N4R}$ appears in neuronal soma and microglial cells (Figure 3S).

Altogether, these results suggest that extracellularly added tau$^{2N4R}$ induces proliferation of microglia and disappearance of neurons independently of the aggregation state of the added protein. Therefore, in further experiments we used freshly prepared (initially monomeric) tau$^{2N4R}$ solutions, which is the dominant form of tau in CSF (Sengupta et al. 2017).

***Extracellular tau$^{2N4R}$ causes microglia-dependent neurotoxcity in mixed cultures***

To test whether microglial cells contribute to the neuronal loss occurring after exposure to tau$^{2N4R}$, we performed experiments on CGC cultures from which microglial cells were selectively eliminated by incubating them with 15 mM LME for 4 hours, and then freshly prepared tau$^{2N4R}$ (<3 nm, 3 μM) was added for 48 hours. Treatment with LME resulted in elimination of microglial cells whereas neurons and astrocytes remained intact (see Figure 4S). In microglia-depleted cultures, extracellular tau$^{2N4R}$ had no effect on the number of viable and dead (apoptotic and necrotic) cells (Fig. 3), implying that tau$^{2N4R}$ was not directly neurotoxic and that microglial cells were responsible for tau$^{2N4R}$-induced neuronal loss.

In mixed neuronal-glial cultures treated with tau$^{2N4R}$ for 24 hours, we found NeuN-positive neurons with homogenous nuclear staining (Hoechst 33342) inside microglia (Figure 5SB). Notably, cells with nuclear fragmentation or condensation did not react with the anti-NeuN antibody (Figure 5SA). These results suggest that extracellular tau$^{2N4R}$ causes phagocytosis of viable neurons by microglia in primary neuronal-glial cultures.

***Extracellular tau$^{2N4R}$-induced neurotoxic effects are mediated by PKC and neutral sphingomyelinase.***

It has been previously reported that PKC regulates microglial proliferation and phagocytosis of neurons under exposure to bacterial toxins or Aβ (Castrillo *et al.* 2001; Neniskyte *et al.* 2016). To test whether PKC is involved in tau$^{2N4R}$-induced microglial activation and disappearance of neurons from CGC cultures we used selective, cell-permeable inhibitors of PKC - Ro31-8220, Gö6983 and Ro32-0432. Neuronal viability after 48 h incubation was not affected by any of the inhibitors alone (data not shown) or in the presence of tau$^{2N4R}$ (see Figure 6S). As shown in Fig. 4A and B, pre-incubation of cultures with Ro31-8220 or Ro32-0432 prevented tau-induced neuronal loss and

microglial proliferation, while, Gö6983 inhibited the tau-induced microglial proliferation. Ro31-8220 is also known to inhibit GSK-3β activity (Hers *et al.* 1999), therefore, we tested whether pre-incubation of cultures with TDZD8, another selective inhibitor of GSK-3β, prevented tau-induced neuronal loss. As can be seen in Fig. 4A and B, TDZD8 inhibited tau-induced microglial proliferation, but did not inhibit tau-induced neuronal loss, suggesting that GSK-3β is not involved in tau-induced neuronal loss, though microglial proliferation may be dependent on GSK-3β.

It has been previously reported that in some pathological conditions PKC may affect nSMase activity (Kaszkin *et al.* 1998; Clarke and Hannun 2006). To test whether nSMase is involved in $tau^{2N4R}$-induced neurotoxicity, we pre-incubated CGC cultures with GW4869, a selective inhibitor of nSMase, before exposure to $tau^{2N4R}$ for 48 hours. Results presented in Fig. 4A and B show that GW4869 fully prevented $tau^{2N4R}$-induced microglial proliferation and neuronal loss in CGC cultures after 48 h incubation. As with other inhibitors tested, neuronal viability remained unchanged in the presence of $tau^{2N4R}$ + GW4869 or GW4869 compared to control (data not shown).

To test whether MerTK, a microglial receptor that mediates phagocytosis of PtSer-exposed neurons (Neher *et al.* 2013), mediates $tau^{2N4R}$-induced neurotoxicity we incubated CGC neuronal-glial cultures for 48 h with $tau^{2N4R}$ in the presence of UNC569, a selective inhibitor of MerTK. UNC569 substantially prevented $tau^{2N4R}$-induced neuronal loss (Fig. 4A) whereas neuronal viability remained unchanged in all experimental conditions (see Figure 6S). This supports the idea that tau induces neuronal loss by MerTK-mediated phagocytosis of PtSer-exposed neurons. Our data also suggest that microglial proliferation was not necessary for tau-induced neuronal loss.

**Extracellular $tau^{2N4R}$ stimulates the phagocytic activity of microglia, and this is prevented by PKC and nSMase inhibitors.**

One means by which microglia can cause neuronal loss is by phagocytosis, therefore we investigated whether extracellular $tau^{2N4R}$ affects the phagocytic activity of microglia. For this we used pure primary rat microglial cultures (no neurons or astrocytes present), and the phagocytosis assay was performed using 2 μm carboxylate-modified microspheres (that mimic PtSer-exposed cellular structures). $Tau^{2N4R}$ increased microglial phagocytosis of these beads approximately by 4-

fold: from ~2 beads per cell in control to ~8 beads per cell in tau$^{2N4R}$-exposed cell cultures (Fig. 5A, B). Thus, extracellular tau directly and strongly increased phagocytosis by microglia.

To test more directly whether tau-treated microglia have increased phagocytosis, rather than just uptake or binding to the cell surface, we performed experiments using pHrodo conjugated E.coli bioparticles, which become fluorescent only when ingested by cells and acidified within lysosomes. We found that control non-treated microglia exhibited low uptake of pHrodo E.Coli bioparticles (weak fluorescence detected), whereas microglia preincubated with tau$^{2N4R}$ became highly fluorescent (see Figure 7S), indicating that tau$^{2N4R}$ stimulates the phagocytic activity of microglia.

Next, we investigated whether tau-induced phagocytosis by pure microglia was attenuated by selective inhibitors of PKC (Ro32-0432, Gö6983) and nSMase (GW4869). Pre-incubation of microglia with Ro32-0432, Gö6983 and GW4869 completely prevented tau$^{2N4R}$-induced phagocytic activity (Fig. 5B).

**Extracellular tau$^{2N4R}$ has no effect on release of TNF-α and NO.**

Pathogen-activated microglia are known to produce nitric oxide (NO) and pro-inflammatory cytokines such as TNF-α (Kinsner *et al.* 2006), which in turn stimulates microglial proliferation and neuronal loss (Neniskyte *et al.* 2014). In order to test whether tau$^{2N4R}$-induced neurotoxicity is mediated through the release of pro-inflammatory cytokines and/or NO, we measured the level of TNF-α and nitrite (as an indicator of NO production) in cell growth media after 48 h exposure of CGC cultures to tau$^{2N4R}$. We found that the level of TNF-α in CGC cultures after 48 hours incubation with tau$^{2N4R}$ was similar to that in control cultures: 45.6 ± 23.7 pg/ml in tau$^{2N4R}$-treated cultures, respectively, compared to 64.6 ± 19.9 pg/ml in control. Nitrite levels in CGC media after 48 h treatment with tau$^{2N4R}$ were also comparable to control levels: 2.1 ± 0.9 μM compared to 1.16 ± 0.3 μM in control (data of 3 experiments on independent cell culture preparations).

**Extracellular tau$^{2N4R}$ causes microglia-dependent phosphatidylserine exposure via nSMase.**

Microglial phagocytosis of neurons normally requires neuronal exposure of the "eat-me" signal phosphatidylserine (PtSer) on the outer surface of their plasma membranes (Neniskyte *et al.* 2011; Neher *et al.* 2011; Brown and Neher 2014). To determine whether tau$^{2N4R}$ causes neuronal

exposure of PtSer, neuronal-glial cultures were treatment with tau and then stained with fluorescent annexin V that binds to the exposed PtSer. Treatment of the cultures with tau$^{2N4R}$ for 6 h increased PtSer exposure on neurons by 27 ± 5% and treatment for 24h by 62 ± 5% compared to the control group (Fig. 6), indicating that tau$^{2N4R}$ stimulates PtSer externalization on neurons. However, in pure neuronal cultures, treated with Ara-C to eliminate glial cells, tau$^{2N4R}$ did not increase annexin V binding (Fig. 6), indicating that tau did not directly induce PtSer externalization on neurons, but rather induces microglia to cause PtSer exposure on neurons.

Pre-incubation with PKC inhibitors, Ro32-0432 and Gö6983, had no effect on tau$^{2N4R}$-induced PtSer exposure, while GW4869 prevented tau-induced annexin V binding to the neurons, implying that inhibition of nSMase can block tau$^{2N4R}$-induced PtSer signaling and subsequent phagocytosis of neurons.

**The effects of recombinant tau$^{2N4R}$ are not due to presence of bacterial endotoxin.**

In our experiments, we used recombinant tau$^{2N4R}$ that was expressed in bacterial cells. This resulted in some contamination of preparations of tau protein with bacterial endotoxin (LPS) in the concentration range 0.001-0.007% LPS (w/w). Bacterial LPS is also known to cause loss of neurons in CGC cultures (Neher *et al.* 2011). In order to test whether tau-induced neuronal loss was mediated by contaminating LPS we performed experiments in the presence of the antibiotic polymyxin B which binds and inactivates LPS. In the presence of polymyxin B, tau$^{2N4R}$ induced a 60% reduction of neuronal numbers (Fig. 7A) after 48 h treatment, comparable to the level in the absence of polymyxin B (see Fig. 3B), suggesting that the effect of tau$^{2N4R}$ was not caused by contamination of preparations by LPS.

We also tested at what concentration LPS induces neuronal loss in CGC cultures. We did not observe any neuronal loss during 48-72 h incubation of CGCs with 1-10 ng/ml LPS, which correspond to the concentrations of LPS present in 3 μM tau$^{2N4R}$ preparations (Fig. 7B). Statistically significant loss of neurons from CGC cultures was observed only at a 10-fold higher concentration of LPS and longer incubations: there was ~30% loss of neurons after 48 h incubation with 100 ng/ml LPS or about 40% loss of neurons after 72 h incubation with 50 ng/ml LPS (Fig. 7B). Importantly,

LPS-induced loss of neurons was not prevented by GW4869 (Fig. 7B), suggesting that LPS and tau$^{2N4R}$ cause neuronal phagocytosis by different mechanisms.

## DISCUSSION

In the present study, we have demonstrated that various extracellular tau$^{2N4R}$ protein aggregate species (with estimated particle sizes <3 nm, 2-7 nm and 3-11 nm) exhibited time-dependent and size-independent neurotoxicity in CGC cultures. Tau protein-induced neuronal loss occurred without any increase in apoptosis or necrosis, and was accompanied by microglial proliferation. Data presented show that microglial cells are responsible for tau$^{2N4R}$-induced neurotoxicity since (1) neuronal loss was not observed in microglia-depleted CGC cultures, (2) tau did not promote neuronal PtSer exposure in cultures lacking glia and (3) NeuN-positive neuronal nuclei were found inside microglia. In addition, exposure to tau$^{2N4R}$ strongly stimulated microglial phagocytosis measured as uptake of carboxylated beads or *E.coli* bioparticle conjugates in pure microglial cultures. We also observed that tau-induced neuronal loss and microglial proliferation were substantially prevented in the presence of Ro31-8220, Ro32-0432, GW4869 and UNC569, suggesting that neuronal loss was mediated by PKC, nSMase and MerTK. In addition, we demonstrated that extracellular tau$^{2N4R}$ induced nSMase-dependent PtSer exposure on neurons without signs of apparent cell death. Therefore, the data suggest a novel mechanism of extracellular tau-induced nSMase-mediated phagocytosis of neurons by activated microglia, which also involves activation of PKC and MerTK.

Our results indicate that exogenous tau may induce aggressive microglia–mediated disappearance of neurons without any visible direct cell death. This is in line with recent findings showing that extracellular low-n oligomers of tau repeat domain with sizes around 1.6 to 5.4 nm, as well as the recombinant human full length tau protein (ht40 wt, 2N4R), when present in the extracellular medium caused selective synaptotoxicity, but did not affect the viability of SH-SY5Y cells and primary cortical neurons even at high concentrations (1, 5, and 10 μM) (Kaniyappan *et al.* 2017). On the other hand, some authors previously have reported that tau oligomers prepared from full-length tau protein were highly toxic to the SH-SY5Y cells and impaired membrane integrity and viability (Lasagna-Reeves *et al.* 2010; Flach *et al.* 2012). In addition, injection of *in vitro*–prepared tau oligomers into mouse brains caused caspase-mediated cell death leading to memory deficits

(Lasagna-Reeves *et al.* 2011). The preparation conditions of tau species as well as the criteria for the toxicity measurements are heterogeneous. We prepared tau oligomers in HEPES buffer (pH 7.4), ± $A\beta_{1-42}$ oligomers to aid tau aggregation, conditions that are thought to be similar to AD, and did not use any polyanionic additives such as heparin. Recently, Brelstaff et al (2018) found that neurons with intracellular tau filaments were preferentially phagocytosed alive by microglia, and this was mediated by phosphatidylserine exposure on the stressed-but-viable neurons. It is possible that these findings and ours are linked by for example: i) tau leaking from Brelstaff et al's neurons to activate microglial phagocytosis, or ii) extracellular tau in our own study entering neurons to form filaments that stress the neurons. However, it is also possible that these are independent processes induced by extracellular and intracellular tau, both resulting in microglial phagocytosis of otherwise viable neurons (Brelstaff *et al.* 2018).

We observed, that $tau^{2N4R}$ caused externalization of PtSer, a key "eat-me" signal inducing phagocytosis of cells (Neniskyte *et al.* 2011; Neher *et al.* 2011; Brown and Neher 2014), and this effect was sensitive to GW4869, a specific inhibitor of nSMase. In brain, nSMase-2 is the predominant enzyme responsible for the hydrolysis of sphingomyelin to produce ceramide and phosphorylcholine, and is expressed in neurons. Increased activity and expression of nSMase-2 have been associated with neuroinflammation in AD and other pathologies (van Echten-Deckert and Walter 2012). The activity of nSMase-2 and production of ceramides have been linked to the release of exosomes (Trajkovic *et al.* 2008). A recent *in vitro* study has demonstrated that cambinol may inhibit nSMase-2 and suppress extracellular vesicle production while reducing tau seed propagation (Bilousova *et al.* 2018). Depletion of microglial cells and inhibition of exosome production by GW4869 was shown to prevent tau-pathology transmission in PS19 mice expressing P301S human tau (Asai *et al.* 2015). Furthermore, Dinkins and colleges (2016) demonstrated that genetically nSMase2-deficient 5XFAD mice have better cognitive performance and reduced ratios of phosphorylated tau (Dinkins *et al.* 2016). GW4869 is a noncompetitive pharmacological inhibitor of nSMase-2 that is competitive with PtSer binding to nSMase-2 (Luberto *et al.* 2002). The link between activation of nSMase, production of ceramide, activation of caspase 8 and 3, and phosphatidylserine exposure was demonstrated in amyloid peptide-induced apoptosis of dendritic cells (Xuan *et al.*

2010). However, whether extracellular tau-induced nSMase activation, PtSer externalization and microglia stimulation might be associated with exosomes secretion needs further investigations.

TNF-α has been reported to induce phagocytosis of viable neurons (Neniskyte *et al.* 2014), however, we did not find any increase in TNFα release in tau$^{2N4R}$ treated CGC cultures. We found that UNC569, an inhibitor of phagocytic receptor MerTK, recognizing PtSer via opsonin Gas6, prevented loss of neuronal cells in CGC cultures treated with tau$^{2N4R}$. The role of microglial MerTK in phagocytosis of neurons has been shown in several studies describing loss of neurons caused by ischemia (Neher *et al.* 2013) or LPS (Nomura *et al.* 2017). So, MerTK could be a potential target for treating brain disorders characterized by microglia-mediated loss of neurons.

We also found that PKC inhibitors Ro31-8820, Ro32-0432 and Gö6983 possess different neuroprotective effects. This may be related to the fact that these inhibitors may act on different PKC isoforms and different signalling pathways. It has been shown that Ro-31-8220, independently of its effect on PKC, exerts other effects such as inhibition of expression of mitogen-activated protein kinase phosphatase-1, induction of c-Jun expression, activation of Jun N-terminal kinase (Beltman *et al.* 1996), inhibition of GSK-3β activity (Hers *et al.* 1999) and it also acts as immunosuppressive agent (Geiselhart *et al.* 1996). Ro32-0432 was found to inhibit G protein-coupled receptor kinases, again independently of its effect on PKC (Aiyar *et al.* 2000). Currently it is unknown if Gö6983 exerts effects on other kinases, but this inhibitor was shown to inhibit neuronal loss in CGCs induced by Aβ (Neniskyte *et al.* 2016).

In this study, we used tau$^{2N4R}$ at a 3 μM (138 μg/ml) concentration, which is significantly higher than that reported in CSF of AD patients (~300-400 pg/ml) or in wild type/transgenic mice (~50/250 ng/ml) (Yamada *et al.* 2011). However, the intraneuronal tau concentration is estimated to be in the micromolar range (~2 μM) (Gamblin *et al.* 2003; Reynolds *et al.* 2005), and the elimination rate of CSF-tau is very slow (half-life is ~11 days) (Yamada *et al.* 2014). In addition, the level of tau increases several fold in AD brains (Iqbal and Grundke-Iqbal 1997; Khatoon *et al.* 1994), Thus, local tau concentration after enhanced secretion or neuronal death could be significantly higher than mentioned above. Our experiments were carried out with the full-length tau$^{2N4R}$ isoform, which has been reported to be present in AD patients' CSF (Russell *et al.* 2016) and the expression of which is selectively increased in AD brains and correlates with cognitive decline (Chen *et al.* 2017).

We used tau$^{2N4R}$ protein expressed in bacterial cells, which raises the possibility that the effects observed could be related to contaminant LPS. We present several lines of experimental evidence that this is unlikely because: (1) LPS-binding polymyxin B did not remove the effect of tau$^{2N4R}$ preparations on neuronal loss, (2) an order of magnitude higher concentrations of LPS was needed to induce a similar neurotoxicity to tau$^{2N4R}$, (3) nSMase-2 inhibition prevented tau$^{2N4R}$-induced neuronal loss but not LPS-induced neuronal loss, and (4) tau did not induce TNFα or NO release from microglia, whereas LPS does normally increase these signs of microglial activation (Neher *et al.* 2011). However, we can not exclude the possibility that there could be synergistic action of tau$^{2N4R}$ and LPS causing neurotoxic effects. This needs to be elucidated in future experiments.

Taken together, our data show that extracellular tau$^{2N4R}$ can induce phagocytosis of neurons by activated microglia in primary neuronal/glial cultures via activation of nSMase, PKC, MerTK and neuronal PtSer exposure. Neutral SMase may be a useful therapeutic target to prevent neuronal loss in tauopathies such as AD.

--Human subjects --

Involves human subjects:

If yes: Informed consent & ethics approval achieved:

=> if yes, please ensure that the info "Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee." is included in the Methods.

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***Acknowledgements and disclosure of conflict of interests***

This work was supported by the Research Council of Lithuania, Bilateral Exchange Project Joint Research grant S-LJB-18-2 INFLAMTAU. We thank dr Božena Pavliukevičienė for her help with SEC-HPLC experiments. Authors declare no conflict of interests.

***Supporting information***

Additional support information may be found online in the *Supporting Information* section at the end of the article.

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**FIGURE LEGENDS**

**Fig. 1.** Representative tapping mode AFM images of tau$^{2N4R}$ aggregates on mica surface prepared by different protocols. A - topography of fresh preparations of tau$^{2N4R}$; B – topography of tau$^{2N4R}$ incubated at room temperature for 24 h, C – topography of tau$^{2N4R}$ incubated with A$\beta_{1-42}$ oligomers at room temperature for 24 h (for details see Materials and Methods section). D – analysis of z-height profiles of the tau$^{2N4R}$ preparations shown in A, B, and C. For the analysis z-heights were measured from cross section morphologies of the tau$^{2N4R}$ aggregates. Concentration of tau$^{2N4R}$ in solution was 0.5 mg/ml (calculated with respect to a monomer) in all experiments. The colour-code represents the height (Z coordinate) in nm. The lateral size of all images is 3 x 3 μm.

**Fig. 2.** Effects of extracellular tau$^{2N4R}$ on CGC cultures.

A- number of viable, necrotic and apoptotic neurons in CGC cultures after 24 h incubation, and B – after 48 h incubation, C – number of microglial cells in CGC cultures after 24 or 48 h incubation in the presence/absence of various aggregate forms (average z-heights of particles indicated) of tau$^{2N4R}$ at 3 μM concentration (calculated according to monomers). Neuronal death was quantified by nuclei morphology analysis after staining with PI and Hoechst 33342 as described in Materials and Methods section. Neurons with PI-negative nuclei and not exhibiting signs of chromatin condensation were considered as viable. PI-positive neurons were considered as necrotic. Microglial cells were identified by using Isolectin GS-IB4 conjugated with AlexaFluor488. The total number of neurons was quantified in 5 randomly-chosen microscopic fields (at 20× magnification) and averaged for each experiment. There were 226±14 viable, 17±4 necrotic and 1±1 apoptotic neurons in a field of view in the control group. The total number of cells counted per treatment varied between 1000 and 1200 (A and B). Microglial cells (in C) were counted at 10x magnification. The number of microglia in tau-treated cultures was expressed as the percent of the total number of microglia in the control group. One-way ANOVA with Tukey's post hoc indicated changes in number of neurons and microglia. *** - $p<0.001$, ** $p<0.01$, versus control group. All bars represent mean ± SEM (N=5-12 number of experiments on independent cell culture preparation) with overlay of individual data points.

**Fig. 3**. Effects of extracellular tau$^{2N4R}$ on number viable, necrotic and apoptotic neurons in microglia-depleted neuronal-glial cell cultures. To deplete microglia CGC cultures were treated with LME and then exposed to freshly prepared 3 μM tau$^{2N4R}$ for 48 hours. Statistical analysis was performed using One-way ANOVA with Tukey's post hoc; *** - $p<0.001$ versus control group. There were no statistically significant differences between groups in LME treated cultures. All bars represent mean ± SEM (N=3-12 number of experiments on independent cell culture preparation) with overlay of individual data points.

**Fig. 4**. Effects of inhibitors of PKC, GSK-3β, nSMase and MerTK on extracellular tau$^{2N4R}$-induced toxicity in CGC cultures.

A - numbers of neurons and B – numbers of microglia in CGC cultures. Cell cultures were pre-treated with 1 μM Ro31-8220 (protein kinase C), 1 μM Gö6983 (protein kinase C), 1 μM TDZD8 (glycogen synthase kinase 3β), 1 μM Ro32-0432 (protein kinase C), 11 μM GW4869 (neutral sphingomyelinase) and 50 nM UNC569 (Mer tyrosine kinase) inhibitors for 30 min and then treated with freshly prepared 3 μM tau$^{2N4R}$ for 48 hours. Number of neurons and microglia in tau-treated (with/without inhibitors) groups expressed as the percent of the total number of appropriate cells in the control group, which were considered as 100 %. One-way ANOVA with Tukey's post hoc statistical analysis was used to comapre the effects of inhibitors in tau$^{2N4R}$ treated CGC cultures. *** - $p<0.001$, ** - $p<0.01$, * - $p<0.05$ compared to control group, ### - $p<0.001$ compared to tau-treated group. All bars represent mean ± SEM (N=5-12 number of experiments on independent cell culture preparation) with overlay of individual data points.

**Fig. 5.** Effect of extracellular tau$^{2N4R}$ on phagocytic activity of microglia.

A - representative images of control microglial cultures (Control) and microglial cultures exposed to 3 μM tau$^{2N4R}$ (+Tau$^{2N4R}$) and then phagocytosing fluorescent beads. B – statistical data on phagocytosis of fluorescent beads by microglia. Where indicated, pure microglial cell cultures were pre-incubated with 11 μM GW4869 and 1 μM Ro32-0432 for 30 min and then treated with freshly prepared 3 μM tau$^{2N4R}$ for 24 hours. Then carboxylate-modified fluorescent microbeads were added and phagocytosed beads were counted in at least 5 microscopic fields after 2 hours incubation. One-way

ANOVA with Tukey's post hoc statistical analysis; *** - $p<0.001$, compared with control, ### - $p<0.001$ compared to tau-treated group. All bars represent mean ± SEM (N=3-4 number of experiments on independent cell culture preparation) with overlay of individual data points.

**Fig. 6**. Extracellular tau increases phosphatidylserine exposure on neurons. Phosphatidylserine exposure was assed by Annexin Cy3.18 staining as described in Methods. CGC cultures were treated with freshly prepared 3 μM $tau^{2N4R}$ with/without pre-incubation with 1 μM Ro31-8220, 1 μM Go 6983 and 11 μM GW4869 for 30 min. To inhibit proliferation of glial cells, CGC cultures on 2DIV were treated with 10 μM Cytosine β- D –arabinofuranoside (Ara-C). Typical images of CGC cultures labeled with Annexin V-Cy3.18 are presented in Fig.8S. Measured Annexin V-Cy3.18 fluorescence intensity was normalized to 100 neurons. Changes in fluorescence intensity of AnnexinV-Cy3.18 in tau-treated groups (with/wthout inhibitors) were expressed as percent of fluorescence intensity in the control group. One-way ANOVA with Tukey's post hoc statistical analysis was used to compare the effects. *** - $p<0.001$, * - $p<0.05$, compared with control, ### - $p<0.001$ compared to tau-treated group. There were no statistically significant differences between groups in Ara-C treated cultures. All bars represent mean ± SEM (N=3-4 number of experiments on independent cell culture preparation) with overlay of individual data points.

Fig. 7. $Tau^{2N4R}$-induced neuronal loss is not mediated by endotoxin contamination.
A - effects of polymyxin B on $tau^{2N4R}$-induced toxicity in CGC cultures. CGC cultures were treated with freshly prepared 3 μM $tau^{2N4R}$ in the presence of 10 U/ml polymyxin B for 48 h.
B - effects of LPS on neuronal loss in CGC cultures. Where indicated, 11 μM GW4869 was added to CGC cultures prior to LPS. One-way ANOVA with Tukey's post hoc statistical analysis was used to compare the effects. All bars represent mean ± SEM (N=3-6 number of experiments on independent cell culture preparation) with overlay of individual data points. *** - $p<0.001$, ** - $p<0.01$, compared with control; N/S – no statistically significant difference between indicated groups.

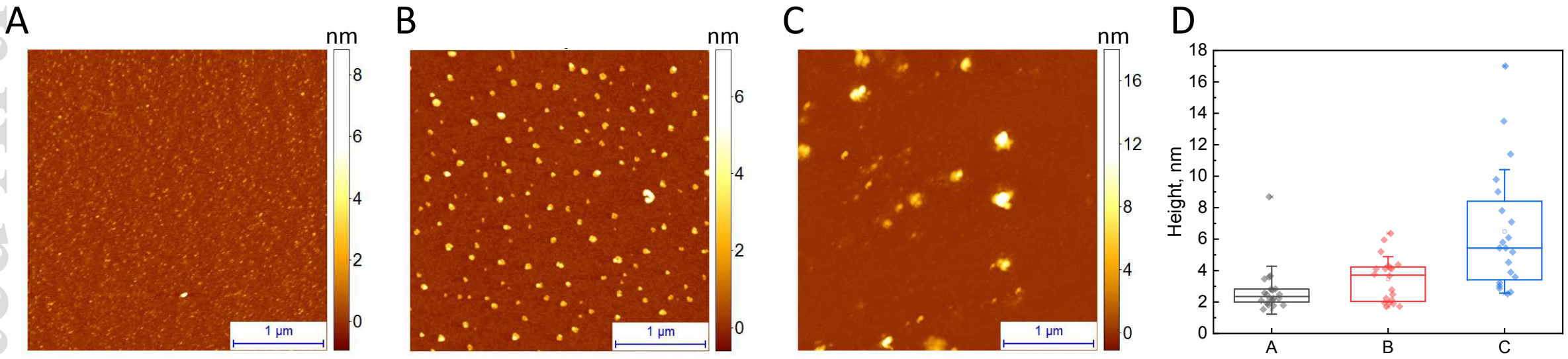





Fig. 1

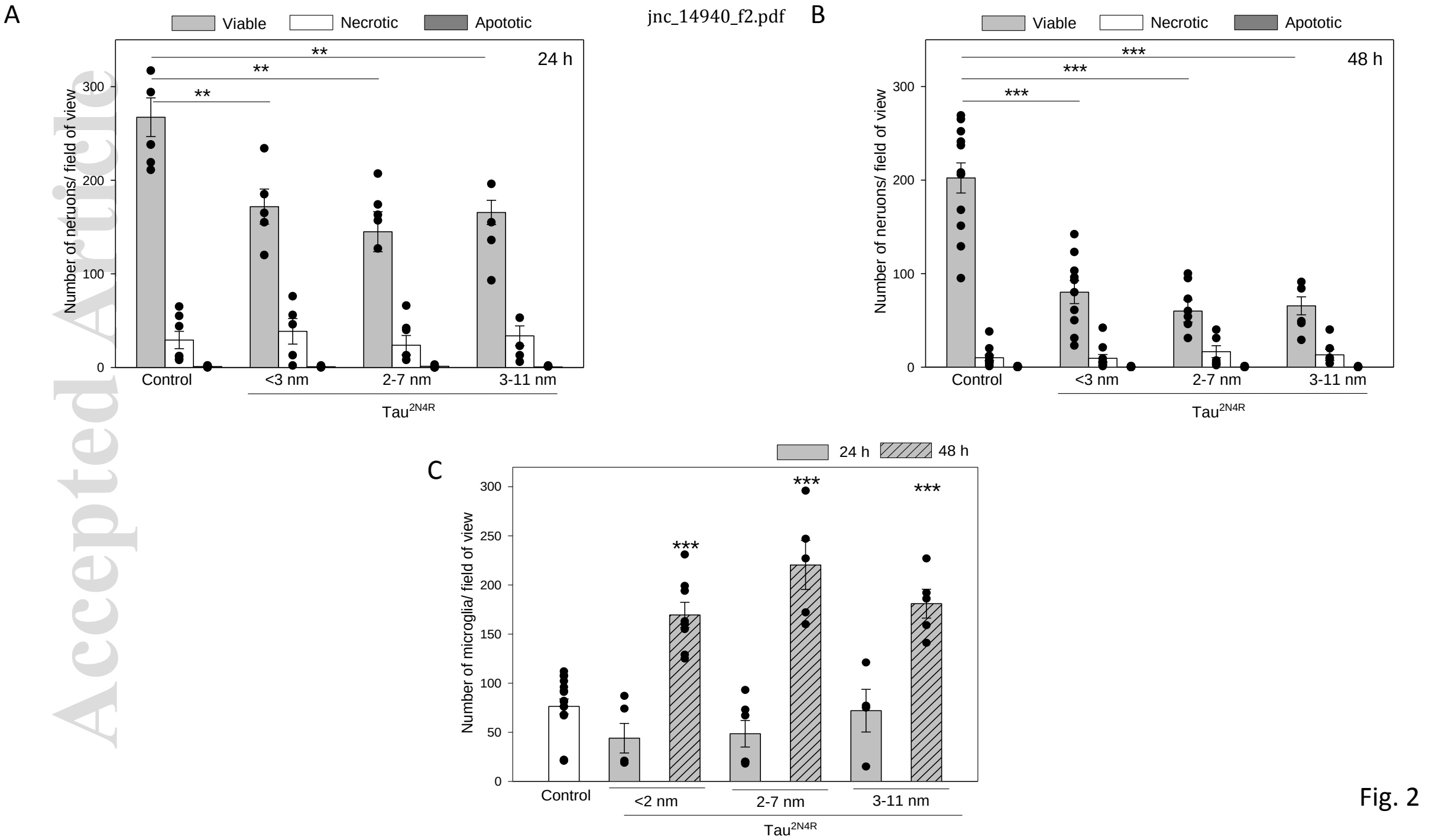



Fig. 2

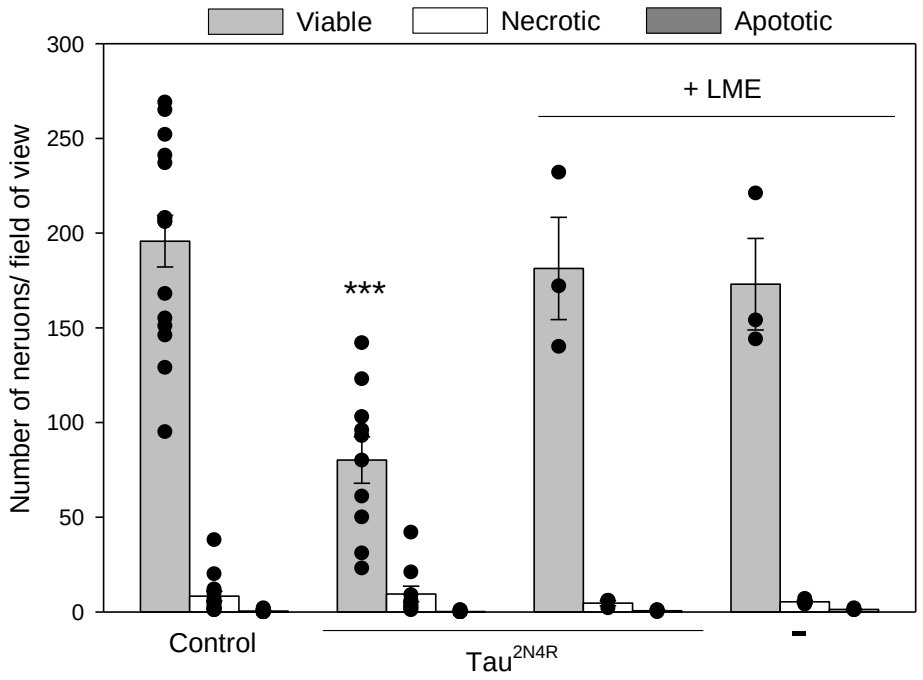
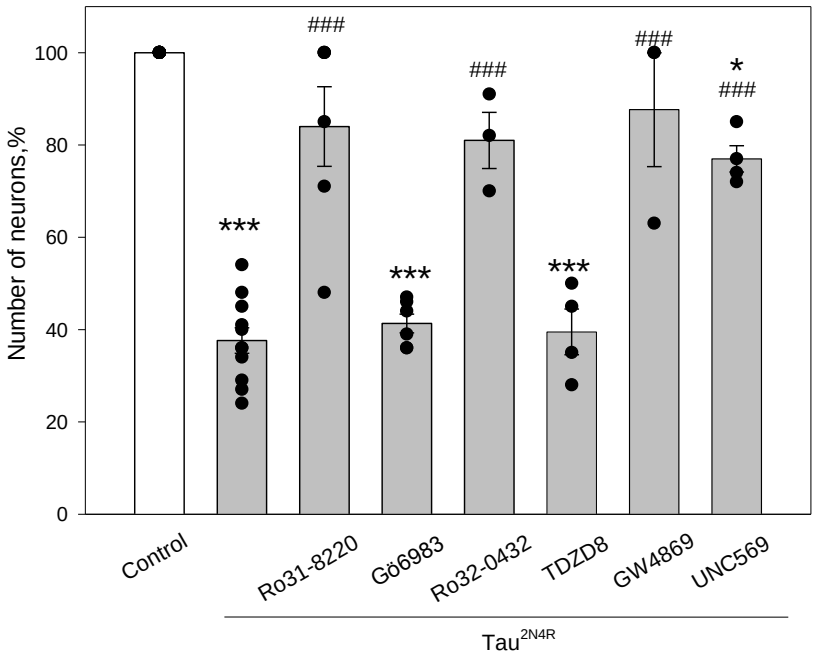







Fig. 3

A

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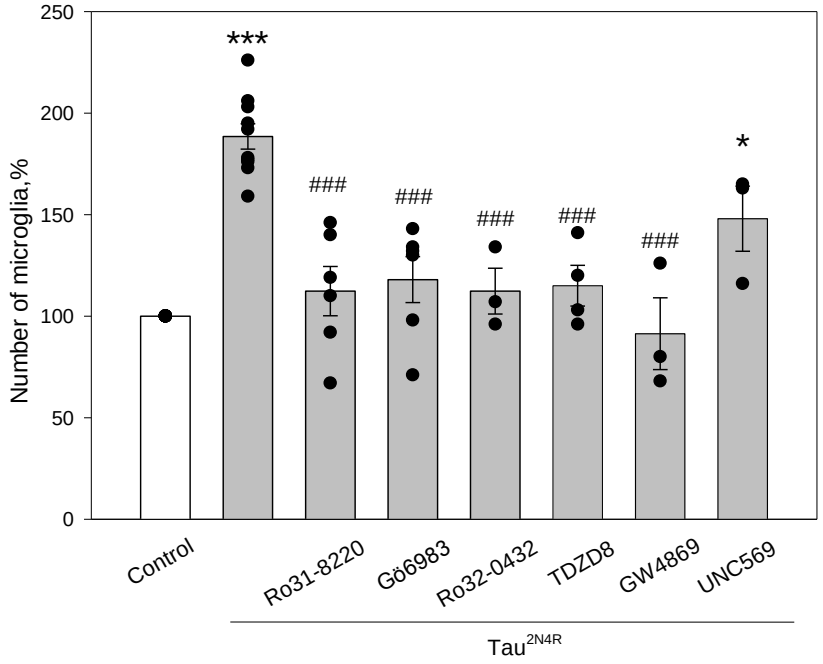
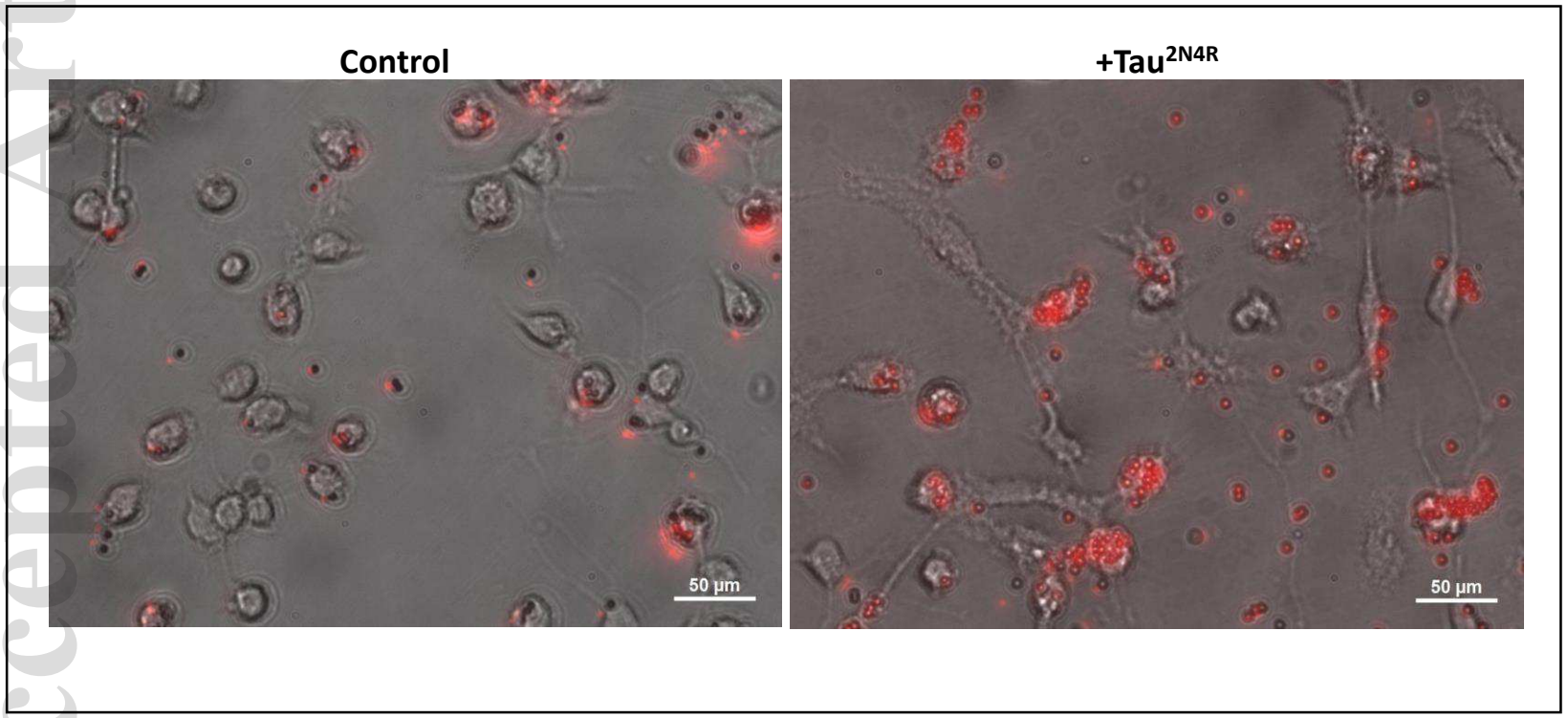





Fig. 4

A

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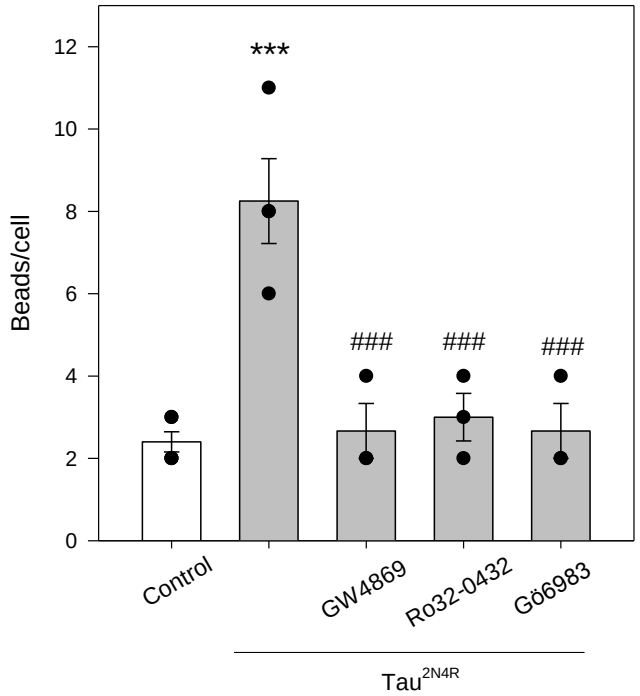




Fig. 5

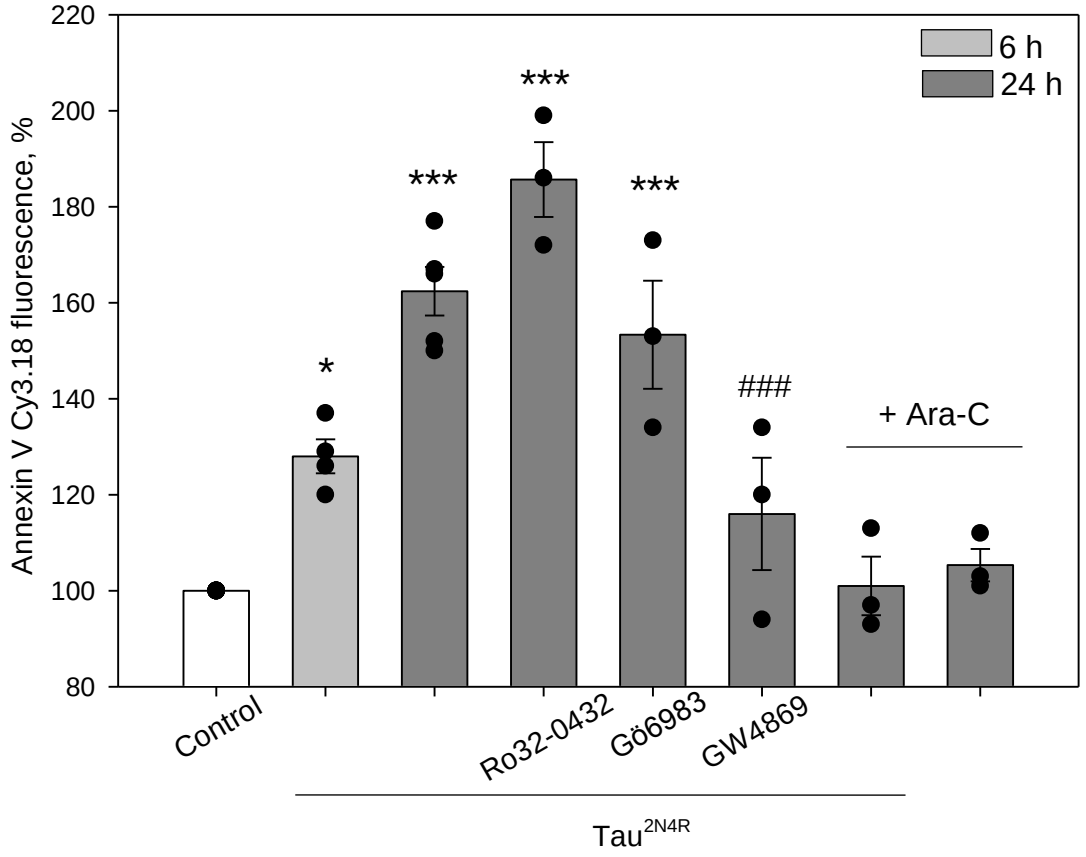




Fig. 6

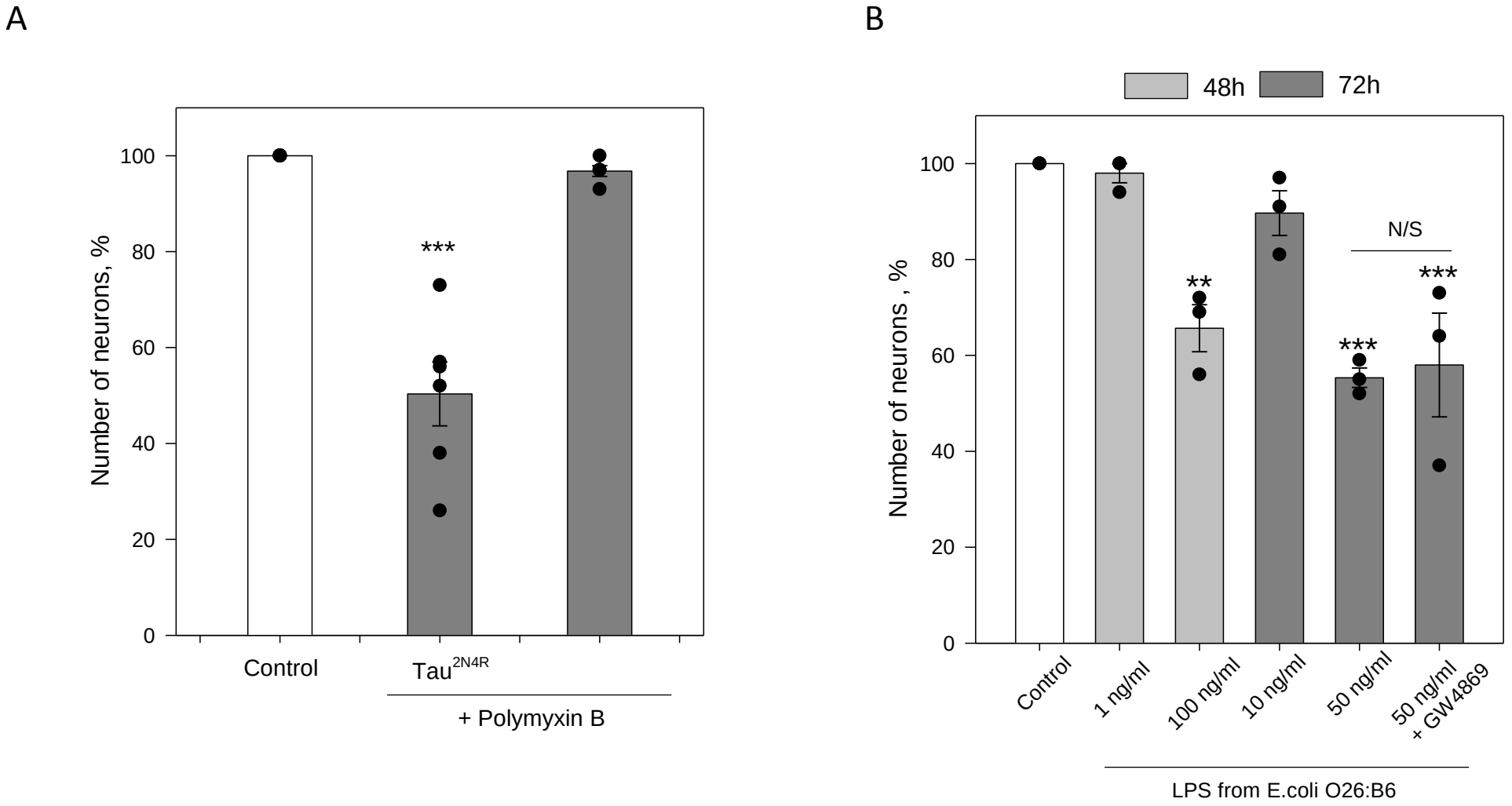




Fig. 7