CHAPTER

Methods related to studying tau fragmentation 13

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Abstract

Several tau posttranslational modifications have been implicated in neuronal degeneration. Among them, tau fragmentation has been identified not only in brain samples obtained from Alzheimer's disease (AD) and related disorder subjects but also in AD culture and animal models. Some of these tau fragments have not been extensively studied. In contrast, data obtained recently showed that tau fragmentation mediated by enhanced or abnormal calpain, caspase 2, caspase 3, and asparagine endopeptidase activity results in the formation of toxic fragments. These cleaved tau forms induce neuronal death, synapse loss, and/or behavioral deficits. Here, we described protease activity assays and methods to study the effects of tau fragments on neuronal viability.

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1 INTRODUCTION

Cellular protein levels are the result of the balance between synthesis and degradation. Proteolysis is responsible for the degradation of dysfunctional, misfolded, aberrant, or aggregated proteins in order to keep this homeostasis. Protease activation is tightly regulated to prevent massive proteolytic activity and to ensure cell viability from development to aging. On the other hand, abnormal proteolysis, either primary or secondary to a disease process could have deleterious effects in eukaryotic cells (Nijholt, De Kimpe, Elfrink, Hoozemans, & Scheper, 2011; Tanaka & Matsuda, 2014). This seems to be the case in Alzheimer's disease (AD) and other degenerative diseases characterized by the aggregation and accumulation of abnormal and/or truncated proteins. Thus, the pathological cleavage of the amyloid precursor protein by β and γ secretases leads to the generation of beta-amyloid (Aβ), the main component of senile plaques (Glenner & Wong, 1984; Gotz, Eckert, Matamales, Ittner, & Liu, 2011; Hardy & Selkoe, 2002; Hyman, Van Hoesen, Damasio, & Barnes, 1984; Selkoe, 1994; Yankner, Lu, & Loerch, 2008). Oligomeric Aβ triggers neuronal degeneration followed by cell death in affected brain areas (Gotz et al., 2011; Hardy & Selkoe, 2002; Selkoe, 1994; Yankner et al., 2008). The microtubule-associated protein (MAP) tau seems to play an essential role as mediator of Aβ-induced neurodegeneration (Grundke-Iqbal et al., 1986; Huang, Wy, & Zhou, 2016; Rapoport, Dawson, Binder, Vitek, & Ferreira, 2002; Reifert, Hartung-Cranston, & Feinstein, 2011; Roberson et al., 2007; Vossel et al., 2010). The mechanisms by which tau mediates Aβ toxicity have not been completely elucidated. However, a growing body of evidence suggests that Aβ induces posttranslational modifications of tau that result in the loss of its normal functions and/or the gain of pathological ones. Recently, several studies have addressed the role of tau fragmentation in neurodegenerative diseases (Canu et al., 1998; Gamblin et al., 2003; Lang, Riherd-Methner, & Ferreira, 2014; Park & Ferreira, 2005; Reifert et al., 2011; Reinecke et al., 2011; Zhang et al., 2014; Zhao et al., 2016). The presence of multiple forms of cleaved tau has been described in brain samples obtained from AD subjects as well as in AD culture and animal models (Ferreira & Bigio, 2011; Gamblin et al., 2003; Lang et al., 2014; Park & Ferreira, 2005; Reifert et al., 2011; Reinecke et al., 2011; Zhang et al., 2014; Zhao et al., 2016). While some of these tau fragments might be only markers of degeneration, others do induce neuronal death. The latter is the case for tau fragments or truncated forms of this MAP generated as the result of calpain, caspase 3, caspase 2, and/or asparagine endopetidase (AEP) activity (Fig. 1). Tau cleavage mediated by calpain is an early and conserved event in tauopathies including AD, frontotemporal dementia with parkinsonism linked to chromosome 17, corticobasal degeneration, progressive supranuclear palsy, tangle-predominant, and Pick disease among others (Ferreira & Bigio, 2011). It precedes tau phosphorylation and leads to the generation of a 17-kDa fragment ($tau_{45–230}$) (Fig. 1A). The expression of this fragment in otherwise healthy hippocampal neurons induced neuronal cell death (Park & Ferreira, 2005; Park, Tournell, Sinjoanu, & Ferreira, 2007;

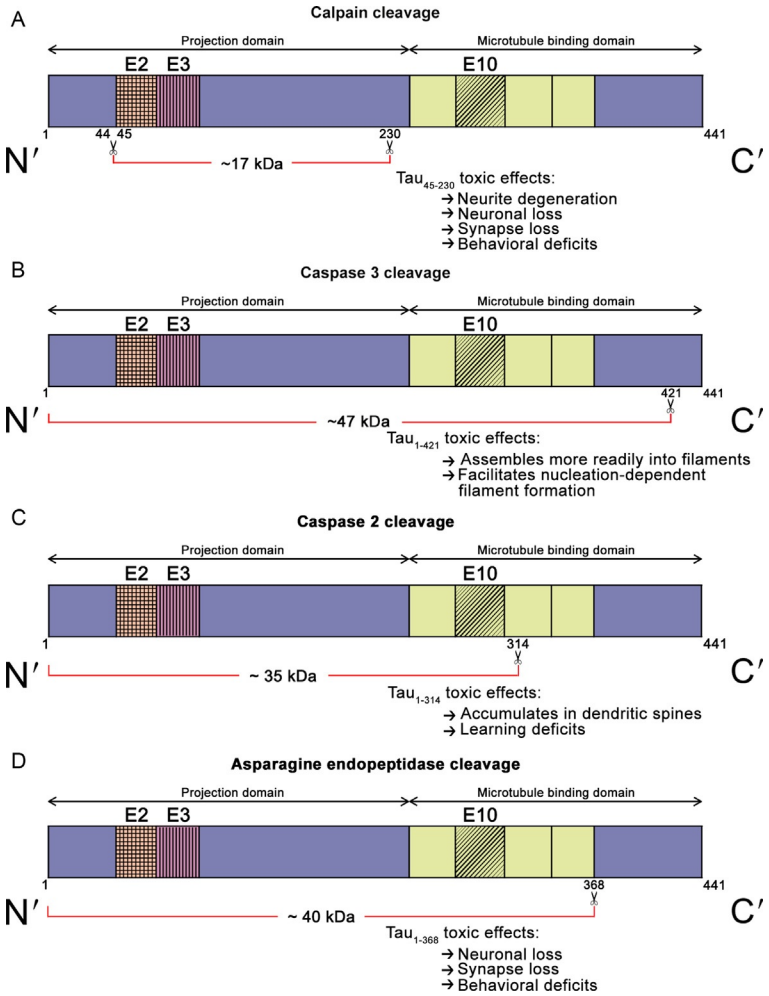

FIG. 1

Tau fragmentation mediated by calpain, caspase 3, caspase 2, and asparagine endopeptidase. Schematic representation of human adult tau isoform protein showing cleavage sites for calpain (A), caspase 3 (B), caspase 2 (C), and asparagine endopeptidase (D) involved in the generation of tau toxic fragments or truncated forms. The molecular weight of such tau fragments and their toxic effects are also indicated. Exons 2 (E2), 3 (E3), and 10 (E10) expressed only in adult tau isoforms are highlighted.

Sinjoanu et al., 2008). Similar results have been described when $tau_{45–230}$ was expressed in nonneuronal cells as well as in *Drosophila* retina (Park & Ferreira, 2005; Reinecke et al., 2011). Furthermore, the analysis of the phenotype of $tau_{45–230}$ transgenic mice showed enhanced cell death in the pyramidal cell layer

of the hippocampus and significant synapse loss in this brain area (Lang et al., 2014). These morphological changes correlated with functional defects including memory deficiency- and anxiety-like behavior (Lang et al., 2014).

Caspases, cysteine proteases largely involved in apoptosis, are also involved in tau fragmentation. In contrast to calpain-mediated tau cleavage, the enzymatic activity of caspases results in large truncated forms of tau (Chung et al., 2001; Fasulo et al., 2000; Gamblin et al., 2003; Rissman et al., 2004). Caspase 3 cleaves tau at amino acid 421 generating a truncated form of ~47 kDa molecular weight (Fig. 1B). $Tau_{1–421}$ assembled more readily into filaments than full-length tau and facilitated nucleation-dependent filament formation (Chung et al., 2001; Fasulo et al., 2000; Gamblin et al., 2003; Rissman et al., 2004). This truncated tau form was highly localized in neurofibrillary tangles (Gamblin et al., 2003). Caspase 2 cleaves tau at amino acid 314 generating a truncated form of ~35 kDa molecular weight (Fig. 1C). $Tau_{1–314}$ was detected in brain samples obtained from AD subjects. in vivo and in vitro studies showed that $tau_{1–314}$ aggregated at lower levels than full-length tau. However, it's missorting into dendritic spines in central neurons at early stages of the disease process contributed to learning deficits (Zhao et al., 2016).

As in the case of caspases, AEP cleavage also generates truncated tau forms (Fig. 1D). This lysosomal cysteine protease cleaves tau at both N255 and N368. The longest (~40 kDa) truncated tau form ($tau_{1–368}$) has been detected in AD brain samples. In addition, in vitro studies showed that $tau_{1–368}$ readily aggregated and decreased the microtubule stabilizing activity of this MAP as compared to full-length tau (Zhang et al., 2014). The expression of this truncated tau form induced apoptosis, synapse loss, and cognitive deficits in a mouse model; a phenotype that closely resembled the one observed in $tau_{45–230}$ transgenic mice (Lang et al., 2014; Zhang et al., 2014). Together, the data briefly reviewed earlier strongly suggest that tau fragmentation plays an important role in the mechanisms leading to neuronal degeneration. Therefore, it would be of interest to identify the complement of proteases capable of cleaving tau into toxic fragments. Such studies will require the use of experimental approaches similar to those applied for the characterization of the abnormal or enhanced calpain, caspases, or AEP activity leading to tau fragmentation described earlier.

Here, we described step-by-step methods to study protease activity in neurons that develop in culture or in situ as well as in vitro conditions. We also included detailed protocols for nucleofection of tau fragments into neurons in order to study their toxic effects.

2 METHODS

2.1 PROTEASE ACTIVITY ASSAYS

The activity of a protease could be analyzed using tissue lysates or cell extracts prepared from primary cultures or cell lines. For this purpose, colorimetric or fluorogenic assays use synthetic protease substrates conjugated with *p*-nitroanilide (*p*-NA)

and 7-amino-4-methylcoumarin (AMC), respectively. *p*-NA and AMC are released upon cleavage and measured using a spectrophotometer or a microtiter plate reader. It is worth mentioning that although these assays provide a sensitive and accurate measurement of protease activity, different factors including the specificity of the substrate and the amounts of total protein and/or number of cells used could affect the results. It is recommended that all these assays are performed in duplicates or triplicates as well as using samples with different protein concentrations. Protease activity could be quantified using *p*-NA or AMC calibration curves. Alternatively, results could be displayed in relative units.

The specific proteolytic activity of a protease on tau proteins could also be assessed by performing in vitro assays using recombinant proteins followed by Western blot analysis for the identification of proteolytic fragments (Laemmli, 1070; Towbin, Staehelin, & Gordon, 1979).

Here, we describe the methods commonly used to determine calpain, caspase 3, caspase 2, and AEP activity.

2.1.1 Protease activity assays using cultured cells or tissue lysates

2.1.1.1 Calpain activity assay

1. Harvest cells (2×10^6 cells) in 150 µL of homogenization buffer (170 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA) in water)
2. Pellet cells by centrifugation at $500 \times g$ for 5 min at 4°C
3. Resuspend cells in 100 µL of extraction buffer (5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 20 mM Tris–HCl, pH 7.4) and incubate on ice for 20 min. Gently mix the samples by pipetting up and down
4. Centrifuge at $10{,}000 \times g$ for 5 min in a microcentrifuge at 4°C
5. Transfer the supernatant to a new eppendorf tube and place on ice
6. Determine protein concentration by the modified Lowry method (Bensadoun & Weinstein, 1976; Lowry, Rosebrough, Farr, & Randall, 1951)
7. Dilute the cell lysate (50–200 µg) in 85 µL of extraction buffer. For positive controls, use 1–2 µL of active calpain (Calbiochem, 1 µg/µL) and bring the volume to 85 µL with extraction buffer. For negative controls, add 1 µL of 1 mM calpain inhibitor Z-LLY-FMK to the treated cell lysate
8. Add 10 µL of 10× reaction buffer (1×: 50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 30 mM NaCl, and 5 mM dithiothreitol (DTT)) and 5 µL of 2 mM calpain substrate Ac-LLY-AMC (Calpain activity assay kit, Fluorogenic, Calbiochem, San Diego, CA) to each well in a 96-well plate (fluorescence friendly)
9. Incubate at 37°C for 1 h in the dark
10. Read the fluorescence at an excitation wavelength of 360–380 nm and an emission wavelength of 440–460 nm using a fluorescence microplate reader

Notes:

- Always use freshly prepared or thawed DTT in buffers
- Enzymes should be stored at −70°C to maintain activity
- For quantification purposes, the fluorescence values determined in the presence of the calpain inhibitor should be subtracted from the sample readings

2.1.1.2 Caspase 3 activity assay

1. Harvest cells (2×10^6 cells) in phosphate buffer saline (PBS)
2. Centrifuge at $500 \times g \times 5$ min
3. Resuspend the pellet in extraction buffer (50 mM HEPES, 5 mM DTT, 0.1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.4)
4. Incubate on ice for 10 min
5. Centrifuge at $10{,}000 \times g \times 5$ min
6. Transfer the supernatant to a new eppendorf tube and place on ice
7. Determine protein concentration using the modified Lowry method (Bensadoun & Weinstein, 1976; Lowry et al., 1951)
8. Dilute 100–200 μg of the cytosolic extract into 50 μL of assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4). Transfer to a 96-well plate
9. Add 50 μL of assay buffer/well and 10 μL/well of caspase-3 fluorometric substrate (0.3 mM Ac-DEVD-AMC, Calbiochem. Final concentration: 30 μM)
10. Cover the plate and incubate at 37°C for 2 h
11. Read the plate using a fluorescence plate reader measuring excitation at 360 nm and emission at 460 nm

Notes:

- Always use freshly prepared or thawed DTT in the cell lysis buffer
- Enzymes should be stored at −70°C to maintain activity
- Determine background readings from well containing only cell lysates and buffers
- Subtract background readings from sample readings before calculating caspase 3 activity

2.1.1.3 Caspase 2 activity assay

1. Harvest cells (2×10^6 cells) in PBS
2. Centrifuge at $500 \times g \times 5$ min
3. Resuspend the pellet in chilled extraction buffer (50 mM HEPES, 10 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, pH 7.4)
4. Incubate on ice for 10 min
5. Centrifuge at $10{,}000 \times g \times 5$ min
6. Transfer supernatant (cytosolic extract) to a fresh tube and keep on ice
7. Determine protein concentration using a modified Lowry method (Bensadoun & Weinstein, 1976; Lowry et al., 1951)
8. Dilute 100–200 μg of the cytosolic extract into 50 μL assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4). Transfer to a 96-well plate
9. Add 50 μL assay buffer

10. Add 5 μL/well of caspase 2 fluorometric substrate (4 mM VDVAD-AMC, Abcam, Cambridge, MA) (Final concentration: 200 μM)
11. Cover the plate and incubate at 37°C for 1–2 h
12. Read samples using a fluorescence plate reader measuring excitation at 360 nm and emission at 460 nm

Notes:
- Always use freshly prepared or thawed DTT in the cell lysis buffer
- Enzymes should be stored at −70°C to maintain activity
- Determine background readings from well containing only cell lysates and buffers
- Subtract background readings from sample readings before calculating caspase 2 activity

2.1.1.4 AEP activity assay

1. Harvest cells (2×10^6 cells) in cold PBS
2. Centrifuge at $500 \times g \times 5$ min
3. Resuspend the pellet in assay buffer (20 mM citric acid, 60 mM Na_2HPO_4, 1 mM EDTA, 0.1% CHAPS, and 1 mM DTT, pH 6.0)
4. Incubate on ice for 10–30 min
5. Centrifuge at $10{,}000 \times g \times 5$ min
6. Transfer supernatant (cytosolic extract) to a fresh tube and keep on ice
7. Determine protein concentration using the modified Lowry method (Bensadoun & Weinstein, 1976; Lowry et al., 1951)
8. Dilute 100–200 μg of the cytosolic extract in 50 μL of assay buffer. Transfer to a 96-well plate
9. Add 50 μL assay buffer (final volume 100 μL)
10. Add AEP substrate Z-Ala-Ala-Asn-AMC (Bachem, Torrance, CA) at a final concentration of 20 μM
11. Cover the plate and incubate at 37°C for 1–2 h
12. Quantify AMC released by substrate cleavage by measuring at 535/587 nm in a fluorescence plate reader

Notes:
- As positive controls, use aspartate standards (100 mM; Abcam, Cambridge, MA)
- Assay buffer and substrate must be equilibrated at room temperature
- It is recommended to measure activity after the initial 10 min of incubation

2.1.2 In vitro protease activity assays

2.1.2.1 In vitro calpain activity assay

1. Dissolve tau recombinant proteins (6 μg) in 50 μL of buffer containing 20 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, DTT 10 mM, 2 mM phenylmethylsulfonyl fluoride (PMSF)

2. Add 10 μL of reaction buffer (50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 30 mM NaCl, and 5 mM DTT) and 1 μg/U calpain (EMD Bioscences, Inc., San Diego, CA) diluted in calpain solvent (20 mM imidazole, 5 mM beta mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 30% glycerol, pH to 6.8)
3. Incubate for 1 h at 30°C in water bath
4. Add equivalent volume of Laemmli 2 × buffer to stop the reaction and boil for 10 min (Laemmli, 1970)
5. Separate tau fragments by SDS-PAGE followed by Western blot analysis using specific tau antibodies (i.e., clone tau5, diluted 1:1000, Abcam) (Laemmli, 1970; Towbin et al., 1979)

2.1.2.2 In vitro caspase 2, caspase3, and AEP assays

1. Incubate recombinant tau proteins (6 μg) diluted as described earlier with recombinant caspase 2 (5 μg/mL), caspase 3 (5 μg/mL) (G-Biosciences) in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) or with recombinant AEP protein (Novoprotein, 5 μg/mL) in AEP buffer (50 mM sodium citrate, 5 mM DTT, 0.1% CHAPS, and 0.5% Triton X-100, pH 6.0)
2. Incubate for 1 h at 37°C
3. Add equivalent volume of Laemmli 2 × buffer to stop the reaction and boil for 10 min (Laemmli, 1970)
4. Separate tau fragments by SDS-PAGE followed by Western blot analysis using specific tau antibodies (i.e., clone tau5, diluted 1:1000, Abcam) (Laemmli, 1970; Towbin et al., 1979)

2.2 DETERMINATION OF TAU FRAGMENT TOXICITY

Tau fragments should be tested to differentiate cleaved tau forms that are markers of differentiation from those that do have toxic effects, and therefore, actively participate in the mechanisms leading to neuronal loss. Potential toxic effects of tau fragments could be easily tested by expressing them in cultured neurons (Banker & Goslin, 1998; Ferreira & Loomis, 1998; Park & Ferreira, 2005). Several transfection methods (i.e., chemical-based and electroporation methods) have been described to transfect primary neurons. However, cultured neurons are notoriously difficult to transfect and showed poor transfection efficiency. A decade ago, a new method of gene transfer technology named nucleofection was described enabling experiments with primary neurons. Nucleofection combines optimized electroporation- and chemical-based transfection techniques, and provides higher transfection efficiency than other transfection methods when using central neurons. Later, we described the nucleofection method to express tau fragments in primary neurons. We also described assays to determine cell viability in cultured neurons nucleofected with cDNA encoding for different tau fragments.

2.2.1 Nucleofection of tau fragment cDNA into dissociated hippocampal neurons

1. Sacrifice an embryonic day 18 (E18) pregnant Sprague–Dawley rat
2. Working in an horizontal laminar hood, remove the fetuses from the uteri, and remove their brains
3. Place the brains in a sterile culture dish containing 5 mL of calcium- and magnesium-free Hanks balanced salt solution, supplemented with 9.9 mM HEPES, and 1 U/100 μg of penicillin (1 U)/streptomycin (100 μg) in water (BSS)
4. Under a dissecting microscope, remove the meninges of each hemisphere and dissect the hippocampi
5. Place the hippocampi in a sterile 60 mm dish containing 3–4 mL BSS
6. Transfer the hippocampi to a sterile conical tube containing 5 mL 0.25% trypsin in BSS
7. Incubate in a water bath at 37°C for 15 min. Gently remove the trypsin solution and add 5 mL of BSS and let stand for 5 min at room temperature.
8. Repeat twice more and finally add 0.5 mL of BSS per embryo dissected
9. Using a Pasteur pipette followed by a fire-polished pipette (the tip diameter of which should be half of the regular diameter), gently dissociate the cells
10. Determine the density of the cells using a hemocytometer
11. Centrifuge dissociated hippocampal neuronal suspension (2×10^6 cells) at 500 $g \times 6$ min
12. Remove supernatant completely without disturbing the soft neuronal pellet
13. Resuspend the neurons in the premixed nucleofection solution (82 μL of nucleofection solution and 18 μL of supplement solution, Lonza, Allendale, NJ). Avoid the formation of air bubbles
14. Remove the 100 μL of cell suspension and mix with cDNA (1–5 μg cDNA in up to 15 μL of deionized water or Tris/HCl 1 mM EDTA 1 mM, pH 8.0. DNA purity should be measured by the ratio of absorbance (A) at 260–280 nm. The A_{260}:A_{280} ratio should be at or above 1.8)
15. Add the mixture to the cuvette avoiding the formation of air bubbles
16. Place the cuvette in the nucleofector™ and start the machine using the program O-03
17. Remove the suspension using a transfer pipette and dilute the cells in warmed plating medium (MEM10) containing Minimum Essential Medium (Invitrogen), 10% heat-inactivated horse serum, 0.6% glucose, and penicillin (1 U)/streptomycin (100 μg)
18. Plate neurons on Carolina™ Assistant-Brant Cover glasses (12 mm) prepared as described later
19. Four hours after plating, place coverslips cell-side-down into dishes containing cultured astrocytes prepared as described later

20. Keep in maintenance medium (N2 medium, Banker & Goslin, 1998; Ferreira & Loomis, 1998) containing MEM, 0.6% D-glucose, 0.1% ovalbumin, 1 mM sodium pyruvate, 5 μg/mL insulin, 20 nM progesterone, 100 μM putrescine, 30 nM selenium dioxide, 100 μg/mL apo-transferrin) until processed for cell viability assays

Notes:
- The experimental protocol should be approved by the Institution Animal Care and Use Committee in accordance with United States Public Health Service regulations and applicable deferral and local laws before performing any experiments using vertebrate animals
- Embryonic day 16 (E16) pregnant mice could also be used
- Nucleofection solution should be used at room temperature
- Do not exceed 150 μL of final volume in the nucleofection cuvette
- Dilute the nucleofected neurons immediately in warm culture medium
- As positive control, use neurons nucleofected using a GFP-plasmid

2.2.2 Preparation of coverslips

1. Coverslips manufactured specifically from a Deutsche Spiegelglass microsheet are required for the survival of neurons
2. Wash coverslips in concentrated nitric acid for 72 h
3. Rinse coverslips in deionized water 3 × 1 h each
4. Heat sterilize coverslips for 8 h at 225°C
5. Place coverslips in 60 mm sterile culture dishes under the laminar flow, apply two paraplast dots (Sigma) in the periphery of the coverslips (see also Banker & Goslin, 1998). These paraplast dots will separate them from the monolayer of cultured astrocytes
6. Coat them with poly-L-lysine hydrobromide (Sigma, 1 mg/mL in borate buffer, pH 8.5)
7. Wash coverslips 3 × 1 h in sterile milliQ water
8. After removing the final wash, add 5 mL of MEM10
9. Store the dishes in an incubator for 24 h before plating

2.2.3 Preparation of astrocyte cultures

1. Proceed as described earlier for the preparation of hippocampal cultures (Section 2.1, steps 1–3)
2. Under a dissecting microscope, remove the meninges of each hemisphere and dissect the cerebral cortex
3. Cut the cortex in small pieces (2–4 mm) and transfer the tissue to a sterile conical tube containing 15 mL 0.25% trypsin solution in BSS
4. Incubate in a water bath at 37°C for 30–35 min
5. Using a sterile transfer pipette, aspirate the tissue and transfer it to a 50 mL sterile conical tube containing MEM10

6. Pipette up and down 10–12 times with a Pasteur pipette to triturate the tissue
7. Spin this mixture at $500 \times g \times 10$ min
8. Resuspend the pellet in 20 mL of fresh MEM10
9. Using a fire-polished Pasteur pipette, dissociate the cells until the solution becomes homogenous
10. Determine the cell density using a hemocytometer and dilute the cell suspension to approximately 200,000 cells/mL and plate them into 60 mm dishes
11. Incubate cultures at 37°C with 5% CO_2

Notes:
- The glial cultures should be fed once a week with fresh MEM10 until they reach 75% confluence
- Replace MEM10 medium with N2 medium at least 24 h before the transfer of coverslips with neurons onto the glia monolayer

2.2.4 Neuronal viability assays

Here, we described two methods to determine the effects of tau fragments on neuronal viability. The fluorescence cell viability assay identifies neurons with damaged membranes mainly due to necrosis. In contrast, the TUNEL assay specifically identifies neurons undergoing apoptosis.

2.2.4.1 Fluorescence cell viability assay

This assay permits the simultaneous detection of live and dead neurons using probes that recognize intracellular esterase activity and plasma membrane integrity, respectively. These probes are: calcein AM, a nonfluorescent cell permeant that is converted to fluorescent by intracellular esterase activity and is retained within live cells (green fluorescence in live cells); and ethidium homodimer (EtD-1) that enters neurons with damaged membranes and binds to nucleic acids enhancing its fluorescence in dead cells (red fluorescence in dead cells).

To assess cell viability, proceed as follows:

1. Allow the LIVE-DEAD determination reagents (Thermo Fischer Scientific, Waltham, MA) to warm up to room temperature
2. Wash coverslips containing hippocampal neurons with sterile PBS at room temperature to remove serum esterase activity
3. Prepare 2.5 mL of a reagent solution containing 4 μM EtD-1 and 2 μM calcein in PBS per 35 mm dish (Thermo Fisher Scientific). Vortex.
4. Incubate coverslips in the solution for 20 min at 37°C
5. Rinse coverslips with PBS
6. Mount coverslips on clean glass slides without mounting medium
7. View the labeled cells under the fluorescence microscope immediately. Count live and dead cells per field × 10 fields from three different culture preparations and calculate the ratio live/dead cells per experimental condition

2.2.4.2 TUNEL assay

TUNEL fluorescence assay is a well-established, fast, and simple nonradioactive technique to detect and quantify neurons undergoing apoptosis. It detects free 3′-OH termini in single-stranded breaks in high-molecular-weight nuclear DNA fragments. The presence of these groups is considered an established marker of apoptosis. This method permits the differentiation of cell death by apoptosis from necrosis (see earlier). In combination with immunostaining using a neuron-specific tubulin antibody, this method allows one to establish a ratio of TUNEL(+) neurons/total number of neurons under a given experimental condition.

To perform a TUNEL assay, proceed as follows:

1. Fix cells in 4% paraformaldehyde in PBS containing 0.12 mM sucrose for 15 min
2. Wash coverslips in PBS
3. Permeabilize cells for 2 min at room temperature using freshly prepared 0.1% Triton X-100 in 0.1% sodium citrate buffer, pH 6.0
4. Wash coverslips 2 × 5 min in PBS to stop reaction
5. Block nonspecific binding with 10% bovine serum albumin (BSA) in PBS for 1 h at room temperature
6. Incubate coverslips in a humidified lightproof container with a solution containing class III neuron-specific tubulin antibody (clone Tuj1, Sigma, at 1:1000 dilution) overnight at 4°C
7. Rinse coverslips 3 × 10 min in PBS
8. Incubate coverslips in a solution containing 100 μL TUNEL label solution (fluorescein-labeled nucleotide mixture solution, In Situ Cell death Detection Kit, Sigma) plus 50 μL enzyme solution (terminal deoxynucleotidyl transferase from calf thymus, In Situ Cell death Detection Kit, Sigma) and 2 μL of AlexaFluor (1:500, Molecular Probes) anti-mouse IgG or label solution only (negative control) for 1 h at 37°C
9. Rinse coverslips 3 × 10 min in PBS
10. Mount coverslips cell-side down on clean glass slides using a fluorescence-compatible mounting medium (i.e., Vectashield, Vector Laboratories, Burlingame, CA)

3 CONCLUSION

We described here simple methods to detect the activity of proteases involved in the generation of tau fragments implicated in neurodegeneration. We also presented here methods to assess the toxic effects of such fragments in neurons. Similar methods could be used in the future as more tau fragments are identified.

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