CHAPTER

Assessing the degradation of tau in primary neurons: The role of autophagy

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Changyi Ji[1], Maoping Tang[1], Gail V.W. Johnson[2]

University of Rochester, Rochester, NY, Unites States

[2]Corresponding author: e-mail address: gail_johnsonvoll@urmc.rochester.edu

CHAPTER OUTLINE

[1]These two authors contributed equally.

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Abstract

Tau is a neuronal cytosolic, highly regulated protein. Although first identified as a protein that binds and stabilizes microtubules, it is now clear that tau plays numerous other roles in neurons. In addition to its key physiological roles in neuronal structure and function, tau is also involved in the pathogenesis of Alzheimer's disease and numerous other neurodegenerative disorders. In all tauopathies, there are pathogenic accumulations of tau. Given that tau homeostasis requires a balance of synthesis and degradation, understanding the pathways that mediate tau clearance and regulate this process in the disease state is of fundamental importance.

In neurons, macroautophagy (referred to as autophagy in this chapter) plays a pivotal role in clearing damaged or misfolded proteins under normal conditions. However, in the disease state autophagy is impaired and tau may not be efficiently targeted for degradation which contributes to the increases in pathological tau species. Therefore, establishing model systems that allow for the analysis of tau clearance by autophagy and quantitative assessment of interventions that increase autophagy and tau clearance are needed. Of particular importance is the use of primary neurons as a model system, as they are more reflective of the relevant in vivo autophagy pathway than clonal or immortalized cell models.

In this chapter we present detailed methods for the preparation of neurons, immunoblotting and imaging analyses, genetic and pharmacological manipulation of autophagy with analyses, and methods to quantitatively measure changes in tau and phospho-tau levels.

1 INTRODUCTION

Tau is an important cytoskeleton protein enriched in neurons. The health of the neuron depends on its ability to maintain the appropriate levels of functional tau, which is accomplished by balancing synthetic and degradative processes. In neurons, constitutive macroautophagy (which is referred to as autophagy in this chapter) is crucial for maintaining proteostasis (Boland et al., 2008; Maday & Holzbaur, 2016), and tau is cleared through this pathway (Fan et al., 2016; Kruger, Wang, Kumar, & Mandelkow, 2012; Lei, Brizzee, & Johnson, 2014). In addition to the autophagy machinery, autophagy adaptors as well as chaperones and cochaperones are of fundamental importance in facilitating the clearance of soluble proteins such as tau (Jo et al., 2014; Salminen et al., 2012; Salminen, Ojala, Kaarniranta, Hiltunen, & Soininen, 2011). Therefore, when studying the turnover of tau by autophagy it is important to not only measure the presence of autophagy indicators such as the levels of LC3II (Mizushima, Yoshimori, & Levine, 2010) but also to examine the

expression of adaptors (e.g., p62), chaperones, or cochaperones, in addition to changes in tau and phospho-tau levels. Caution also needs to be exercised in terms of selecting autophagy activators for use in neuronal systems. Although nutrient deprivation (starvation) and mTOR inhibition (treatment with rapamycin or torin 1) induce autophagy in other cell types, the data indicate that they do not effectively upregulate autophagy in neurons (Maday & Holzbaur, 2016). However, proteasome inhibition reliably increased autophagy in neurons (Kruger et al., 2012; Lei et al., 2014), and therefore in this chapter we use proteasome inhibition with MG132 to induce autophagy as an example.

In many neurodegenerative diseases, such as Alzheimer's disease being the most prevalent, a defining hallmark is the accumulation of tau aggregates. These aggregates of tau are abnormally posttranslationally modified, with phosphorylation being the most studied, and are highly insoluble. Although tau aggregates are a defining neuropathological feature of these diseases (cumulatively described as "tauopathies"), there is increasing evidence that these insoluble accumulations of tau are not the primary toxic species but rather pathologically modified monomers and/or oligomers are the likely culprits. The reason(s) why these pathological species of tau accumulate have not been clearly defined; however, decrease in the efficiency of the clearance pathway(s) is likely a contributing factor. Indeed, it has been well documented that in Alzheimer's disease there are autophagy deficits that likely occur early in the pathogenic process (Nixon et al., 2005; Nixon & Yang, 2011, 2012). Given that tau is an autophagy substrate, it is reasonable to speculate that these deficits contribute to the increases in pathological tau species in the disease state. Indeed, several studies have shown that increasing autophagy in tauopathy mouse models results in reduced tau pathology and has beneficial outcomes (Congdon et al., 2012; Schaeffer & Goedert, 2012). Therefore, the establishment of relevant models and approaches to increase our understanding of the turnover of tau by autophagy, as well as to analyze interventions that can increase tau turnover is of great importance.

In this chapter we describe methods to analyze the turnover of tau by autophagy in primary cortical neurons. We provide detailed information about the preparation and use of neuronal cultures for both immunoblot analyses as well as imaging, and how the data are analyzed.

2 MATERIALS

2.1 PRIMARY NEURON CULTURE AND MEDIA

1. Cold packs
2. Dissecting microscope
3. Dissecting tools: dissecting scissors (Roboz, 3.5″, Cat# RS-5840), fine forceps (45° angle, Dumont style 5/45 and curved thin, Dumont style 7)
4. MEM medium: GIBCO (Cat# 11095-080), 500 mL

5. Neurobasal medium: GIBCO (Cat# 21103-049), 500 mL
6. B27 supplement: GIBCO (Cat# 17504-044), 10 mL, 50 × concentrate
7. 200 mM L-Glutamax: GIBCO (Cat# 35050-061)
8. DNase I: Sigma (Cat# DN25) 100 mg. Need to make 100 mg/mL stock in solvent containing 20 mM Na-acetate, pH 6.5, 5 mM $CaCl_2$, 0.1 mM PMSF, and 50% glycerol. Filter-sterilize the solution and store it at −20°C
9. AraC: cytosine β-D-arabinofuranoside, hydrochloride, Sigma (Cat# C6645). A 40 mM stock solution is made in sterile, double-distilled water (ddH_2O) and can be stored in aliquots at −20°C
10. Primocin: Invivogen (Cat# ant-pm-1)
11. Fetal bovine serum (FBS): GIBCO (Cat# 10437)
12. Hank's balance salt solution (HBSS): VWR (Cat# 02-0121-0500)
13. 0.25% Trypsin with EDTA: Corning (Cat# 25053CI)
14. PBS: VWR (Cat# 02-0119-0500)
15. Poly-D-lysine hydrobromide, high molecular weight, Corning (Cat# 354210). Need to make 2.5 mg/mL stock in sterile ddH_2O according to the manufacturer's instruction. Aliquot and store at −20°C
16. 190 Proof ethanol
17. Tissue culture dishes or multiwell plates (Greiner Bio-one)
18. Cover slips, 1.5 thickness
19. Sterile plastic pipettes (5 mL, 10 mL)
20. Sterile conical tubes (15 mL, 50 mL)

2.2 WESTERN BLOTS OF PHOSPHO-TAU, TOTAL TAU, AND AUTOPHAGY MARKERS

1. RIPA lysis buffer (radioimmunoprecipitation assay buffer)

   150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris–HCl, pH 8.0 with protease inhibitors (aprotinin, leupeptin, and pepstatin all at final concentrations of 10 μg/mL; 1 mM PMSF) and phosphatase inhibitors (Na_3VO_4, 1 mM; NaF, 5 mM final concentrations). The buffer without the protease/phosphatase inhibitors can be stored at 4°C. Aprotinin, leupeptin, and pepstatin can be made up as 1000 × stocks in DMSO and stored in aliquots at −20°C. PMSF is made up as a 100 × stock in isopropanol and stored at −20°C. Na_3VO_4 and NaF are made up as 500 × and 200 × stocks in ddH_2O, respectively, and stored at −20°C.
2. 5 × Laemmli loading buffer

   10% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, 50 mM Tris–HCl. Check the pH and adjust to 6.8.
3. 30% Acrylamide/0.8% Bis

   For 1 L: Add 300 g acrylamide and 8 g Bis-acrylamide to 1 L of ddH_2O. After everything is dissolved, filter the solution and store at 4°C.

   Note: Wear a mask and gloves when weighing acrylamide. It is a cumulative neurotoxin!

4. 1.5 M Tris–Cl, pH 8.8
   For 1 L: Add 181.5 g of Tris–base to 800 mL of ddH_2O and adjust the pH to 8.8 with HCl. Bring up the volume to 1 L and store at 4°C.
5. 0.5 M Tris–Cl, pH 6.8
   For 1 L: Add 60 g of Tris–base to 800 mL of ddH_2O and adjust the pH to 6.8 with HCl. Bring up the volume to 1 L and store at 4°C.
6. 10% Ammonium persulfate (APS)
   For 10 mL: Add 1 g of APS to 10 mL of ddH_2O. After everything is dissolved, filter the solution and store at −20°C.
7. 10% Acrylamide gel recipe example (for tau and p62; for LC3 the recipe should be adjusted to 16% acrylamide)

	30% Acrylamide, 0.8% Bis	1.5 M Tris (pH 8.8)	0.5 M Tris (pH 6.8)	ddH_2O	10% APS	TEMED
Running gel (15 mL/2 gels 1.5 mm space)	5.0 mL	3.75 mL	—	6.25 mL	75 µL	10 µL
Stacking gel (5%, 6 mL/2 gels)	1.0 mL	—	1.5 mL	3.5 mL	70 µL	10 µL

8. Running buffer (Tris–glycine/SDS)
   25 mM Tris–base, 190 mM glycine, 0.1% SDS. Do not need to adjust pH. However, the pH should be ~8.1–8.5.
9. Transfer buffer (wet)
   25 mM Tris–base, 190 mM glycine, 10% methanol. Do not need to adjust pH. However, the pH should be ~8.1–8.5.
10. 1 × Tris-buffered saline with Tween-20 (TBS-T)
    20 mM Tris, 137 mM NaCl, 0.05% Tween-20, pH 7.6.
11. Blocking buffer
    5% Nonfat milk (or 5% BSA) in 1 × TBS-T buffer.
12. Luminol solution (LS)
    Dissolve 5 mg of Luminol (Enzo, Cat# 610-002) into 1 mL of DMSO (1.25 mM). Then add this to 19 mL of 0.1 M Tris, pH 8.6. Keep in the dark at 4°C.
13. Enhance solution (ES)
    Dissolve 11 mg of *para*-hydroxy coumaric acid (Sigma, Cat# C9008) into 10 mL of DMSO. Keep in the dark and at room temperature.
14. Autoradiography film (Lab Scientific, Inc., Cat# XAR ALF2025), an autoradiography cassette (VWR, Cat# 95039-986), and access to a dark room and developer. Alternatively a chemiluminescence imaging system (e.g., VWR CHEMI only, Cat# VWR1730-1471) can be used.

15. Antibodies

    Primary antibodies: PHF1 (mouse monoclonal, epitope: phosphoSer396/404, 1:5000, generous gift from Dr. P. Davies [Litwin Zucker Center for Alzheimer's Research]), total tau (rabbit polyclonal, 1:10,000, Dako, Cat# A0024), p62 (rabbit polyclonal, 1:1000, CST, Cat# 5114), LC3 (rabbit polyclonal, 1:5000, Novus, Cat# NB100-2220), β-actin (mouse monoclonal, 1:10,000, ThermoFisher, Cat# MA5-15739).

    Secondary antibodies: goat anti-rabbit IgG-HRP conjugate (Jackson ImmunoResearch, Cat# 1111-005-045, 1:5000), goat anti-mouse IgG-HRP conjugate (BioRad, Cat# 17606516, 1:5000).

16. Reagents

    MG132 (Sigma-Aldrich, Cat# C2211) prepared as a 1 mM stock in DMSO stored in aliquots at −80°C. Prior to use a 200 μM stock solution is prepared in Neurobasal medium which can be stored at −20°C.

    NH_4Cl (FisherScientific, Cat# A661), 5 M stock solution in H_2O stored at −20°C.

2.3 ASSESS AUTOPHAGY VACUOLE NUMBER AND COLOCALIZATION BETWEEN PHOSPHO-TAU AND AUTOPHAGY

1. mCherry-hLC3B-pcDNA3.1 (Addgene, Plasmid #40827)
2. pEGFP-C1 (from Clontech, but has been discontinued; alternative plasmids with fluorescent tags can be obtained through Addgene, e.g., see http://www.addgene.org/browse/article/979/)
3. GFP-tau constructs: GFP-T4, GFP-T4-2EM, GFP-T4-2EC. T4 is a human tau containing four microtubule-binding repeats but without exons 2 and 3. Mutant constructs T4-2EM (T231E/S235E) and T4-2EC (S396E/S404E) were generated by mutating the indicated threonine or serine to glutamic acid using the QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). All the mutations were confirmed by DNA sequencing analysis (Ding, Matthews, & Johnson, 2006). These constructs in pcDNA3.1(−) were used as templates and the inserts were amplified by PCR using primers with *Eco*RI and *Kpn*I sites and subcloned in frame into pEGFP-C1. All GFP constructs were confirmed by sequencing. Alternatively, pRK5-EGFP-Tau (Addgene, Plasmid #46904) could be used instead of GFP-T4
4. Lipofectamine® 2000 Transfection Reagent (ThermoScientific, Cat# 11668027)
5. Fixing solution (4% paraformaldehyde (16% paraformaldehyde, VWR, Cat# 100503-914)/4% sucrose in PBS)
6. ProLong® Diamond Antifade Mountant (Life Technologies, Cat# P36961)
7. Confocal microscope (which is usually available on a fee for service basis in a core facility of a university)
8. Fiji ImageJ (https://imagej.net/Fiji/Downloads)

3 METHODS

3.1 COATING PLATES AND COVERSLIPS WITH POLY-D-LYSINE

Note: All manipulations must be carried out using sterile procedures.

1. Pretreat glass coverslips in a glass beaker, soak the glass coverslips in 190 proof ethanol overnight, flame to dry, and put them into a clean glass petri dish with a cover. Sterilize by autoclaving. Transfer sterile coverslips into a clean 10 cm tissue culture dish. Arrange them properly to minimize overlapping.

   Note: We routinely use 18 mm diameter round #1.5 thickness coverslips (Denville Scientific Inc., Cat# M0714 (1002548)). Tissue culture grade dishes do not need to be pretreated.
2. Dilute poly-D-lysine (2.5 mg/mL stock) to 50 μg/mL in sterile PBS. Add appropriate amount of diluted poly-D-lysine onto coverslips or culture dishes. Cover dishes and incubate at room temperature overnight in a sterile hood.

   Note: Make sure there is enough solution to cover every coverslip and the bottom of the dish, and the surface does not dry out overnight. Alternatively, dishes with poly-D-lysine solution can be incubated at 37°C in a cell culture incubator.
3. Next day, rinse the coated coverslips and/or dishes with PBS three times.
4. After the final wash, add half volume of plating media to the coated coverslips and/or dishes and precondition the dishes in an incubator (37°C, 5% CO_2). Unused dishes can be wrapped with parafilm after PBS is completely removed and stored at 4°C for up to 4 weeks.

3.2 CORTICAL NEURON CULTURE

3.2.1 Dissection (note that no animal work can be performed without formal approval from the appropriate Institutional Animal Care and Use Committee (IACUC))

1. Euthanize a pregnant Sprague-Dawley rat, embryonic day 18 by an approved method from Institutional Animal Care and Use Committee (IACUC).

   Saturate the abdomen of the rat with 70% ethanol before incision. Cut through the abdominal skin and muscle to expose the uterus. Remove the uterus and transfer it into a sterile 10 cm dish.

   Note: Clean tools as necessary throughout the dissection process by soaking them in 70% ethanol and then rinse with sterile double-distilled water.
2. Open placenta to separate embryonic sac. Remove individual fetuses and put into a 6 cm dish with ice-cold HBSS. Remove brains from each fetus and place them into 33 mm dishes with ice-cold HBSS. Keep dishes with fetuses and brains on flat ice packs for all procedures.

3. Under the dissection microscope, remove meninges and dissect cortices using the fine forceps. Transfer cortices to a sterile 33 mm dishes containing 2 mL of ice-cold HBSS.

   Note: Make sure the cortical tissues are submerged under HBSS throughout the dissection process.

3.2.2 Digestion and plating

Note: The subsequent steps are performed in a laminar flow hood using aseptic techniques.

1. Transfer cortices into an empty 33 mm dish and mince them with a dissecting scissor.
2. Add 1 mL of prewarmed 0.25% trypsin (37°C) and then use a 5 mL serological pipet to transfer the tissue into a sterile 50 mL conical tube. Use another 1 mL of trypsin to rinse the dish and transfer all tissues. Bring the total volume of trypsin up to 5 mL for every four brains. Scale up and down the final volume of trypsin according to the number of brains.
3. Transfer the tube with trypsin and tissue into a 37°C water bath and incubate for 20 min. Every 10 min gently shake the tube.
4. After trypsin digestion, add an equal volume of plating medium (MEM, 10% FBS, 10 mM glucose, Primocin) to stop the action of trypsin.
5. Add 20 μL DNase I (100 mg/mL) to each 10 mL solution. Swirl the tube to mix thoroughly. Triturate the tissues into a single cell suspension with a 10 mL serological pipet. Avoid making bubbles by pipetting against the tube wall.
6. After pipetting up and down ~15–20 times, let the solution sit for 2 min. Transfer cell suspension into a new 50 mL conical tube.
7. Count the number of neurons using a hemocytometer (FisherScientific, Cat# 0267151B). Dilute cell suspension into a desired amount in MEM plating medium (MEM, 5% FBS, 10 mM glucose, Primocin). Add the diluted cells into the preconditioned coverslips or dishes. Plating densities for each experiment are given below.
8. Keep primary neuron culture at 37°C, 5% CO_2.

3.2.3 Maintenance

1. 4–24 h after plating neurons, change the plating medium into growth medium (Neurobasal, 1 × B27, 0.25 mM Glutamax).

   Note: Gently aspirate the plating medium off and replenish with fresh growth medium. Avoid exposing neurons to room air for too long.
2. On day 4 postplating, change half of growth medium containing 1.5 μM AraC to inhibit glia proliferation.
3. Thereafter, change one-third of medium with fresh growth medium without AraC every 3–4 days.

3.3 WESTERN BLOTTING FOR AUTOPHAGY MARKERS AND TAU EXPRESSION

3.3.1 Activate autophagy with MG132 and block autophagic flux with NH_4Cl

1. For these studies neurons are plated at a density of 5×10^5 cells per 60 mm plate.
2. At DIV 8, treat neurons with 1 μM MG132 in the presence or absence of 2.5 mM NH_4Cl. There are four groups: untreated control, MG132, NH_4Cl, and MG132 + NH_4Cl. The MG132 and NH_4Cl are added to the neurons at the same time. Return dishes to the incubator at 37°C, 5% CO_2.
3. After 16 h treatment, neurons are collected for western blotting.

3.3.2 Make cell lysates

1. Place the culture dishes on ice and wash twice with ice-cold PBS.
2. Aspirate the PBS and then add ice-cold RIPA lysis buffer (100 μL per 5×10^6 cells/60 mm dish).
3. Scrape adherent cells off the dish using a plastic cell scraper and then gently transfer the cell suspension into a precooled 1.5 mL microcentrifuge tube with a p200 pipettor.
4. Sonicate (Misonix Inc S-3000 Misonix Sonicator Ultrasonic Cell Disruptor) each sample at 0.5 W for 10 s.
5. Spin at $16000 \times g$ for 10 min at 4°C.
6. Gently transfer the supernatant into a fresh 1.5 mL microcentrifuge tube and keep on ice.
7. Determine the protein concentration using the BCA assay (Pierce BCA Protein Assay Kit, ThermoFisher, Cat# 3225). Based on the protein concentration, dilute samples appropriately with 5 × SDS (Laemmli) loading buffer. The recommend final protein concentration is 1 μg/μL.

3.3.3 Running the gel and transferring to nitrocellulose

1. Denature the samples at 95°C for 10 min. Cool the tubes and spin briefly.
2. Load equal amounts of protein into the wells of the SDS-PAGE gel; molecular weight markers (Fisher BioReagents™ EZ-Run™ Prestained *Rec* Protein Ladder, Cat# BP3603500) should also be run in a lane.
3. Run the gel at 25–35 mA/gel. Stop the gel when needed, usually after 1–2 h.
4. Cut the filter paper (clean cotton cellulose fiber; or use precut filters, e.g., ThermoFisher, Cat# 88600) and nitrocellulose membrane (BioTrace™ NT Nitrocellulose Transfer Membranes, Pall Laboratory, Cat# 66485) to size.
5. Remove gel sandwiches from the electrophoresis box. Gently pry apart gel plates. Peel gel away from glass plate.
6. Assemble transfer sandwich (cassette, two foam pads (BioRad, Cat# 1703933), two pieces of filter paper, nitrocellulose, and gel). Transfer proteins to nitrocellulose at 100 V for 1 h on ice.

3.3.4 Blotting the membrane

1. Remove the membrane from the transfer cassette and place it in a container with blocking buffer for 1 h at room temperature with rocking.
2. Add the primary antibody diluted in blocking solution. Incubate at 4°C overnight.
3. Wash the membrane three times with 1 × TBS-T, 10 min for each wash.
4. Add secondary antibody diluted in the blocking solution to the membrane and incubate at room temperature for 1 h with rocking.
5. Wash the membrane three times with 1 × TBS-T, 10 min for each wash.
6. Develop the membrane with ECL solution. In a 1.5 mL tube, mix 1 mL of LS, 1 μL of 30% H_2O_2, and 10 μL of ES, and add onto the membrane, incubate for ~30 s with rocking. Pick up the blot with forceps, touch the edges to a Kimwipe to remove excess ECL, and place between two sheets of clear plastic in an autoradiography cassette. In a darkroom expose the blot to the film and develop. Exposure times are usually 15 s to 1 min but can be varied as needed. If available a chemiluminescence imaging system can be used instead of film.
7. Blots always need to be reprobed for loading controls. In this chapter we use β-actin as the loading control; however, other proteins such as GAPDH can also be used. Representative immunoblots are shown in Fig. 1.

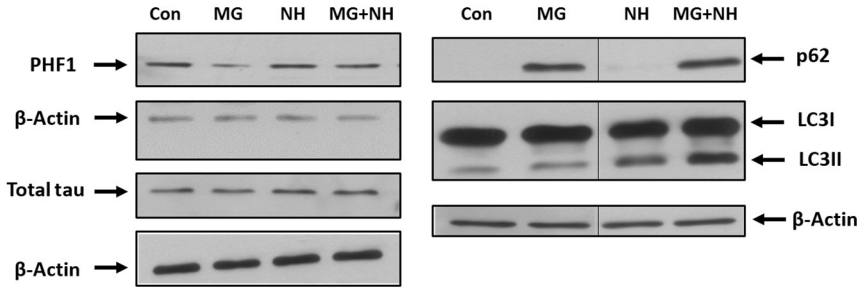

FIG. 1

Western blot analysis of tau clearance and autophagy markers. Western blot of PHF1, total tau, p62, LC3-I/LC3-II in untreated neurons or neurons treated with 1 μM MG132 (MG), 2.5 mM NH_4Cl (NH), or 1 μM MG132 + 2.5 mM NH_4Cl (MG + NH) for 16 h. Five micrograms of protein was loaded for the PHF1 and tau immunoblots, and 20 μg protein for p62 and LC3. The blots were reprobed for β-actin as a loading control. The *vertical line* on the p62, LC3, and β-actin blots indicates that intervening lanes were removed; however, the data shown are from a single membrane/exposure. These data show that increasing autophagy by proteasome inhibition (Korolchuk, Menzies, & Rubinsztein, 2010; Kruger et al., 2012) (MG132 treatment) results in a decrease in the levels of tau phosphorylated at Ser396/404 (PHF1) concomitant with an increase in p62. Treatment with the lysosomotropic reagent NH_4Cl inhibits lysosomal acidification and the degradation of LC3II (Mizushima et al., 2010) as well as the degradation of phospho-tau. Inhibition of autophagic degradative processes is required to confirm that the reagent used to active autophagy is actually causing tau degradation through this pathway.

3.3.5 Quantitation of immunoblot data

1. Exposure times are critical as it is important to maintain the signal within the linear range and not overexpose the films.
2. Films are scanned and saved as .tif files for analysis.
3. Densitometric analyses can be carried out using ImageJ. LiCor also offers free software for western blot analyses (https://www.licor.com/bio/products/software/image_studio_lite/).
4. Data should be normalized to loading controls, and for quantitative analyses a minimum of three biological replicates is needed.

3.4 ASSESS AUTOPHAGIC VACUOLE NUMBER USING AN MCHERRY-LC3 REPORTER AND EVALUATE THE COLOCALIZATION OF TAU AND AUTOPHAGY VESICLES

3.4.1 Cotransfect GFP-C1, GFP-T4, GFP-T4-2EC, or GFP-T4-2EM and mCherry-LC3 reporter into neurons

1. Plate 3×10^5 neurons into each well of 12-well plates containing 18 mm coverslips. At DIV 8, neurons are cotransfected with GFP-C1 and mCherry-LC3, GFP-T4 and LC3-mCherry, GFP-T4-2EC and mCherry-LC3, or GFP-T4-2EM and mCherry-LC3. Before transfection, remove half of the media and save as conditioned media. If just determining autophagic vacuole number, the neurons can be transfected with mCherry-LC3 only.
2. For each well, add 3 μL Lipofectamine 2000 to 37.5 μL MEM medium without serum. Mix gently and incubate for 5 min at room temperature.
3. For each well, add 750 ng of mCherry-LC3 reporter plasmid and 750 ng of either GFP-C1, GFP-T4, GFP-T4-2EC, or GFP-T4-2EM plasmid, separately, into 37.5 μL MEM medium without serum.
4. Combine the diluted DNA with the diluted Lipofectamine 2000. The ratio of the Lipofectamine (in μL) to total DNA (in μg) is 2:1.
5. Mix gently and incubate the mixture at room temperature for 10 min.
6. Apply the total 75 μL DNA/Lipofectamine mixture into each well. Shake the plates gently.
7. After 5 h, remove Lipofectamine by changing the media with half fresh neuron growth medium and half conditioned medium.

3.4.2 MG132 treatment and fixing the coverslips for imaging

1. 48 h after transfection, incubate neurons with or without 0.4 μM MG132.
2. After 3 h of treatment, wash neurons with warm PBS twice.
3. Fix cells in PBS containing 4% paraformaldehyde and 4% sucrose for 5 min at room temperature.
4. Aspirate the fixing solution, wash neurons with PBS three times, 5 min for each wash.
5. Add 1 drop of ProLong Diamond Antifade Mountant onto each glass slide.

6. Remove excess liquid from coverslip, and place the coverslip sample side down onto the mounting medium.
7. Cure overnight at room temperature in the dark.
8. Seal the edges with nail polish. Store the slides at 4°C in the dark until imaging.

3.4.3 Analysis of autophagy vesicle numbers by confocal microscope

1. Take a series of z-stack images with slice size at 0.4 μm using a confocal microscope with appropriate settings for mCherry. We use an Olympus FV1000 Confocal Laser Scanning Microscope with 40× oil-immersion objective. Save the whole stack of original images as an .oib file.
2. Import the raw images into Fiji ImageJ software using the plugin LOCI.
3. Generate maximum projections of the z-stacks of the entire field ("Image" → "Stacks" → "Z project").
4. Choose a region without neuronal soma and processes ("Edit" → "Selection"). Measure the fluorescence intensity in this empty area ("Analyze" → "Measure") and use it as background intensity.
5. Subtract the respective mean background intensity from the entire field ("Process" → "Math" → "Subtract").
6. Change the image format to 8-bit ("Image" → "Type" → "8 bit").
7. Choose a region of interest to be measured, for example, a single soma or a portion of a given process.
8. Adjust threshold and create a binary mask of the region of interest ("Image" → "Adjust" → "Threshold").
9. Segregate the binary mask by watershed method ("Process" → "Binary" → "Watershed").
10. Count the particle number or measure the total LC3 area using the analyze particles function ("Analyze" → "Analyze particles").

3.4.4 Analysis of colocalization between tau and autophagic vacuoles by confocal microscope

1. Take a serial of z-stack images as in Section 3.4.3 with appropriate settings for eGFP and mCherry. Save the raw data as an .oib file.
2. Import the raw images into Fiji ImageJ.
3. Subtract the background as in Section 3.4.3 for each channel.
4. Choose a region of interest.
5. Generate intensity plots of the GFP constructs and LC3-mCherry at a given line of interest (line width of 1 pixel) using the plot profile tool ("Analyze" → "Plot profile"). Normalize the intensity value for a given channel by subtracting the respective mean background and dividing the dataset by this maximum. Representative images and quantitated data are shown in Figs. 2 and 3.

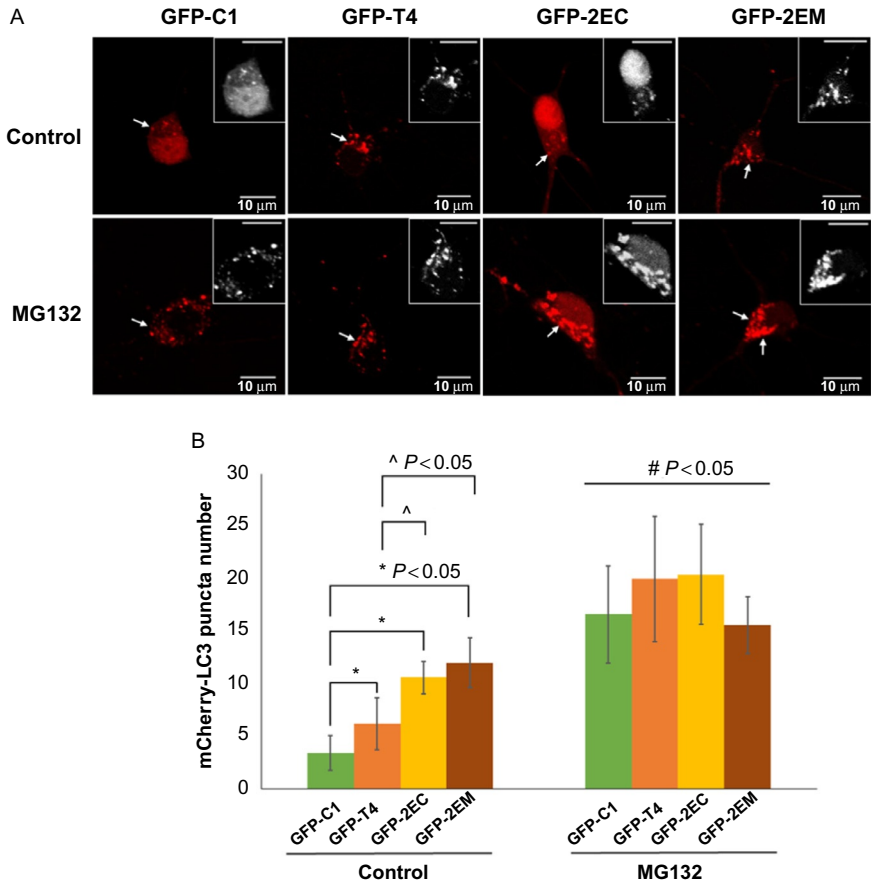

FIG. 2

Autophagy induction in primary cortical neurons. (A) mCherry-LC3 puncta in the soma of neurons cotransfected with GFP-C1 (negative control), GFP-T4 (tau), GFP-T4-2EC (GFP-2EC, phospho-tau mimetic), or GFP-T4-2EM (GFP-2EM, phospho-tau mimetic) in the presence or absence of MG132 (0.4 μm) treatment for 3 h. *Arrows* indicate LC3-labeled puncta that are representative of autophagic vacuoles. Scale bar: 10 μm. (B) Quantitation of mCherry-LC3 area normalized to the soma area (puncta size of those that were counted was 0.2–5 μm) mean ± SEM; $n = 5$ neurons from one experiment. The mCherry-LC3 puncta number between two groups as indicated was evaluated for statistical significance using unpaired Student's *t*-test. $^{*}P < 0.05$ indicated significance when compared GFP-C1 with GFP-T4, GFP-2EC, or GFP-2EM in the control group, respectively. $\hat{}P < 0.05$ indicated significance when comparing GFP-T4 with GFP-2EC or GFP-2EM in the control group, respectively. $\#P < 0.05$ indicated significance between each treatment in the MG132 group and their counterpart in the control group. These data suggest that expression of tau and especially phospho-tau mimetics increases autophagy. These are representative data for illustration purposes. For quantitative studies at least three biological replicates are needed.

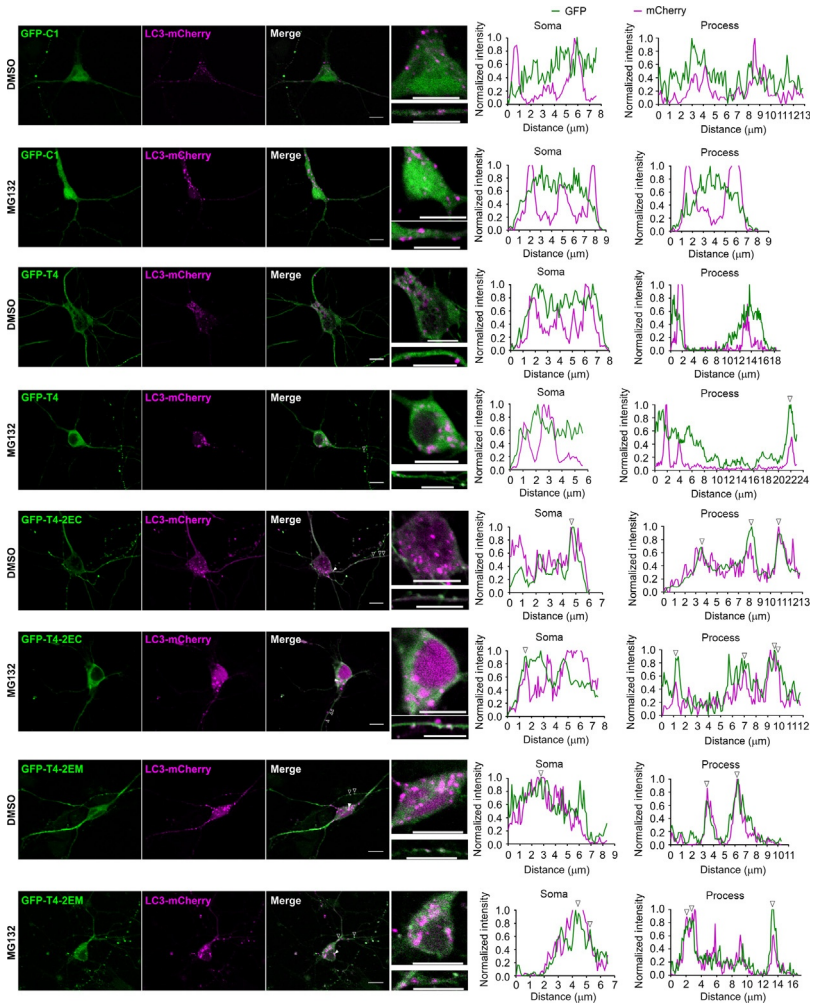

FIG. 3

Localization of phosphorylated tau in autophagic vesicles. mCherry-LC3 was cotransfected with GFP-C1, GFP-T4, GFP-T4-2EC, or GFP-T4-2EM into primary cortical neurons. 48 h after transfection, neurons were treated with either DMSO or 0.4 μm MG132 for 3 h before fixing. Colocalization between LC3 and different tau species was assessed by intensity analysis along defined line of interests (line width equals 1 pixel). Corresponding line scans are shown on the *right*. *Arrowheads* on the images and line scans denote overlapping peaks of fluorescence intensity. Scale bars: 10 μm. As indicated by the overlapped fluorescence peaks, GFP-T4-2EC and GFP-T4-2EM colocalize with mCherry-LC3-labeled autophagic vesicles in both neuronal soma and process in the presence or absence of MG132 treatment. This suggests phosphorylated tau species are targeted to autophagy in both basal and stimulated conditions. As a negative control, GFP alone (GFP-C1) does not form vesicle-like structures nor colocalize with mCherry-LC3. However, GFP-T4 intensity does overlap with mCherry-LC3-labeled vesicles when autophagy is stimulated by MG132 in the transfected neurons. This result suggests a subset of tau is targeted to autophagy, most likely phosphorylated tau species.

4 CONCLUSION

Neurons are dependent on autophagy to maintain cellular homeostasis and tau, and in particular phospho-tau is an autophagic substrate. Here we present the methodology to prepare primary rat cortical neurons and measure autophagy and the clearance of phospho-tau. We show how to carry out western blot analyses and also use imaging techniques to demonstrate the targeting of tau to autophagy. The procedures presented here can be generalized to analyze pharmacological or genetic interventions that modulate autophagy and tau clearance in neurons.

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