Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59988R3
Full Title:	Modulation of Tau Subcellular Localization as a Tool to Investigate the Expression of Disease-Related Genes
Keywords:	Nuclear Tau; differentiated SH-SY5Y; nuclear fractions; localization signals; Subcellular fractionation; Western Blot
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Pisa, Italy

TITLE:

Modulation of Tau Subcellular Localization as a Tool to Investigate the Expression of Disease-Related Genes

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KEYWORDS:

nuclear Tau, differentiated SH-SY5Y, nuclear fractions, localization signals, subcellular fractionation, western blot

SUMMARY:

Tau is a neuronal protein present both in the cytoplasm, where it binds microtubules, and in the nucleus, where it exerts unconventional functions including the modulation of Alzheimer's disease-related genes. Here, we describe a method to investigate the function of nuclear Tau while excluding any interferences coming from cytoplasmic Tau.

ABSTRACT:

Tau is a microtubule binding protein expressed in neurons and its main known function is related to the maintenance of cytoskeletal stability. However, recent evidence indicated that Tau is present also in other subcellular compartments including the nucleus where it is implicated in DNA protection, in rRNA transcription, in the mobility of retrotransposons and in the structural organization of the nucleolus. We have recently demonstrated that nuclear Tau is involved in the expression of the VGluT1 gene, suggesting a molecular mechanism that could explain the pathological increase of glutamate release in the early stages of Alzheimer's disease. Until recently, the involvement of nuclear Tau in modulating the expression of target genes has been relatively uncertain and ambiguous due to technical limitations that prevented the exclusion of the contribution of cytoplasmic Tau or the effect of other downstream factors not related to nuclear Tau. To overcome this uncertainty, we developed a method to study the expression of target genes specifically modulated by the nuclear Tau protein. We employed a protocol that couples the use of localization signals and the subcellular fractionation, allowing the exclusion of the interference from the cytoplasmic Tau molecules. Most notably, the protocol is easy and is composed of classic and reliable methods

that are broadly applicable to study the nuclear function of Tau in other cell types and cellular conditions.

INTRODUCTION:

The functions of Tau protein in the nucleus have garnered significant interest in recent years, as it has been shown to be closely associated with nucleic acids[1–6]. Indeed, a recent genome-wide study demonstrated that Tau binds genic and intergenic DNA sequences in vivo[7]. A role in nucleolar organization has been suggested[8–11]. Moreover, Tau has been proposed to be involved in DNA protection from oxidative and hyperthermic stress[5,10,12,13], whereas mutated Tau has been linked to chromosome instability and aneuploidy[14–16].

Until now, the challenges in studying the functions of Tau in the nuclear compartment remained almost unsolved due to the difficulties in dissecting the specific contribution of nuclear Tau from the contribution of cytoplasmic Tau. Moreover, the functions attributed to Tau molecules in the nuclear compartment, up to now, are only correlative because they lack an unequivocal demonstration of the direct involvement of nuclear Tau proteins. Indeed, the involvement of Tau in the mobility of retrotransposons or in the rRNA transcription or in DNA protection[11,12,17–19] might be also explained by the contribution of cytoplasmic Tau or by the effect of other downstream factors not related to nuclear Tau.

Here, we provide a method that can solve this issue by exploiting a classical procedure to isolate the nuclear compartment combined with the use of Tau constructs 0N4R tagged with nuclear localization (NLS) or nuclear export signals (NES). This approach eliminates the complex issues related to possible artefacts due to the spillover of Tau molecules from the cytoplasmic compartment. Moreover, Tau-NLS and Tau-NES constructs induce the enrichment or the exclusion of Tau molecules from the nuclear compartment, respectively, providing positive and negative controls for the involvement of nuclear Tau molecules in a specific function. The protocol is technically easy and it is composed of classic and reliable methods that are broadly applicable to study the nuclear function of Tau in other cell types, differentiated or not, such as cancer cells that reactivate Tau expression[20,21]. Moreover, it might be applied also to other proteins that are present in both the cytoplasm and the nucleus in order to dissect biological functions related to different compartments.

PROTOCOL:

1. Cell culture

1.1. Culture SH-SY5Y cells (human neuroblastoma cell line, CRL-2266) in complete medium (Dulbecco's modified Eagle medium:nutrient mixture F12 [DMEM/F-12] supplemented with 10% fetal bovine serum [FBS], 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin). Maintain the cells in an incubator at 37 °C and 5% CO_2. Grow cells in 10 cm plates and split when confluent.

2. Cell differentiation

2.1. To differentiate SH-SY5Y cells, the day after the plating, add 10 µM retinoic acid (RA) to complete medium for 5 days.

2.2. The sixth day replace medium with differentiation medium: DMEM/F-12 supplemented with 50 ng/mL BDNF, 2 mM L-glutamine. Do not add FBS or antibiotics.

2.3. Grow the cells in differentiation medium for 3 days.

3. Chimeric constructs cloning

3.1. Generate Tau-NLS construct by cloning by restriction enzyme digestion in frame at the 3' end of Tau sequence 0N4R (383aa) the 3xNLS(SV40NLS):5'-CCAAAAAAGAAGAGAAAGGTAGATCCAAAAAAGAAGAGAAAGGTAGATCCAAAAAAGAAGAGAAAGGTA-3'.

NOTE: The 3xNLS at the 3' end of Tau is cloned into the pCMV-Tau plasmid for mammalian expression exploiting the XhoI and BamHI restriction sites into the multicloning site (MCS).

3.2. Generate Tau-NES construct by cloning by restriction enzyme digestion in frame at the 3' end of Tau sequence 0N4R (383aa) the NES sequence: 5'-AGTGAGCTGCAGAACAAGCTGGAAGAGTTGGATCTGGACTCGTACAA-3'.

NOTE: The NES at the 3' end of Tau is cloned into the pCMV-Tau plasmid for mammalian expression exploiting the EcoRI and BamHI restriction sites into the MCS.

3.3. Transform DH5alpha *E. coli* strain with 100 ng of DNA from step 3.1 or 3.2 and plate cells on LB-Agar plates with 100 mg/mL ampicillin. Let grow overnight at 37 °C.

3.4. Pick a single colony and spike the cells into 5 mL of LB with ampicillin. Let the cells grow at 37 °C in agitation overnight.

3.5. Extract plasmid with a DNA miniprep (**Table of Materials**) and sequence to verify the constructs.

3.6. Transform DH5alpha *E. coli* strain with the sequence verified constructs and plate cells on LB-Agar plates with ampicillin. Let grow overnight at 37 °C.

3.7. Pick a single colony and spike the cells into 5 mL of LB with ampicillin. Let the cells grow at 37 °C in agitation for 2 h.

3.8. Put the cells from step 3.7 in 200 mL of LB with ampicillin. Let grow overnight at 37 °C.

3.9. Pellet the cells at 3,500 x *g* for 10 min at 4 °C

3.10. Extract plasmid with a DNA maxiprep (**Table of Materials**).

4. Cell transfection

4.1. Seed 400,000 cells from step 1.1 in 6-well plates or 20,000 cells in 8-well chamber slides. Plate four samples: control cells to be transfected with an empty vector, cells to be transfected with untagged Tau, cells to be transfected with Tau-NLS and cells to be transfected with Tau-NES.

4.2. The day after seeding transfect 400 ng of DNA for each well using the cationic lipids (**Table of Materials**) in 6-well plates or 200 ng of DNA for each well for 8-well chamber slides, according to the manufacturer's instructions.

4.2.1. Incubate the DNA and the cationic lipids separately in 250 µL (for 6-well plates) or 25 µL (for 8-well chamber slides) of reduced serum medium for 5 min at RT. Then combine them to generate the DNA-lipid complex and incubate for 20 min.

4.2.2. Replace the culture medium with 2 mL (for 6-well plates) or 250 µL (for 8-well chamber slides) of fresh complete culture medium. Add the DNA-lipid complex to the cells and incubate at 37 °C overnight.

4.3. Alternatively, transfect DNA with the cationic reagent polyethylenimine (PEI).

4.3.1. Mix 2 µg of DNA and 6 µL of PEI with 200 µL of complete culture medium (for each well in 6-well plates), or 1 µg of DNA and 3 µL of PEI with 100 µL of complete culture medium (for each well in 8-well chamber slides), vortex and incubate for 10 min at RT.

4.3.2. Add the mix to the cells and add 1.8 mL of complete culture medium per well in 6-well plates or 150 µL of complete culture medium per well in 8-well chamber slides to reach plating volume.

4.4. Change the medium the day after transfection and add the differentiation media as described in step 2.2.

5. Immunofluorescence

5.1. Remove the culture medium and rinse cells with 1x PBS. Fix cells with 100% ice cold methanol for 3 min without shaking. Remove the fixing solution and wash briefly with 1x PBS.

5.2. Permeabilize with 0.1% non-ionic surfactant in 1x PBS for 5 min at room temperature (RT). Briefly, wash with 1x PBS, 3 times.

5.3. Incubate cells with blocking buffer (0.1% Tween 20 and 1% BSA in PBS) for 30 min at RT on an orbital shaker.

5.4. Incubate with appropriate primary antibodies (e.g., mouse monoclonal anti-Tau13 antibody) diluted 1:500 in blocking buffer overnight at 4 °C on an orbital shaker. Remove the antibody solution and wash, briefly, with 1x PBS.

5.5. Incubate with secondary antibodies conjugated to fluorophore (e.g., goat anti-mouse antibodies conjugated to Alexa Fluor 633) diluted 1:500 in blocking buffer for 1 h at RT. Remove the antibody solution and wash briefly with 1x PBS 3 times.

5.6. To stain nuclei, incubate with DAPI diluted 1:20,000 in blocking buffer for 10 min at RT. Wash with 1x PBS 3 times. Mount coverslips on a slide using antifade mounting medium.

6. Western blot

6.1. To collect the cell pellet from step 4.4, remove the medium, and wash cells with PBS. Incubate with 500 µL of 0.1% trypsin for 4 min at 37 °C. Add an equal volume of complete medium and resuspend cells.

6.2. Collect cells in a tube and centrifuge at 500 x *g* for 5 min. At the end of centrifugation carefully remove the supernatant. Add 1 mL of PBS, centrifuge at 500 x *g* for 5 min and carefully remove the supernatant. Store cell pellets on ice for immediate use or freeze at -80 °C for long-term storage.

6.3. For total protein extracts, incubate the cell pellet for 30 min on ice in lysis buffer (20 mM Tris-HCl pH 8, 20 mM NaCl, 10% glycerol, 1% octylphenoxy poly(ethyleneoxy)ethanol, branched [**Table of Materials**], 10 mM EDTA) supplemented with protease and phosphatase inhibitors. According to the abundance of the pellet, use 50 µL to 100 µL of lysis buffer.

6.3.1. Centrifuge the extract at 16,000 x *g* for 15 min. Collect the supernatant and quantify the protein concentration by any standard quantification assay. Prepare the protein samples for the SDS-PAGE by mixing 20 µg of proteins with 5 µL of 4x Laemmli buffer in a total volume of 20 µL and boil at 100 °C for 5 min.

NOTE: The sample can be stored at -20 °C.

6.4. For subcellular fractionations, resuspend cells from step 6.2 in complete medium, and collect 1 x 10^6 cells per each sample. Centrifuge at 500 x *g* for 10 min to obtain cell pellets for the following steps.

6.4.1. To isolate subcellular compartments, fractionate according to kit specifications. To isolate each fraction, incubate the cell pellet from step 6.4 with the corresponding buffer, centrifuge, collect the supernatant and add the next buffer to the pellet as described in 6.4.1.1-6.4.1.5. Add in order cytoplasmic extraction buffer, membrane extraction buffer, nuclear extraction buffer, nuclear extraction buffer supplemented with 5 mM $CaCl_2$ and 3 U/µL micrococcal nuclease and cytoskeletal extraction buffer.

NOTE: All buffers must be supplemented with protease inhibitors. Scale buffer volumes according to the volume of the cell pellet.

6.4.1.1. To isolate the cytosolic fraction, incubate cell pellets in 100 µL of ice-cold cytoplasmic extraction buffer supplemented with protease inhibitors at 4 °C with gentle mixing for 10 min. Centrifuge at 500 x *g* at 4 °C for 5 min and transfer the supernatant to pre-chilled tubes.

6.4.1.2. Add 100 µL of ice-cold membrane extraction buffer supplemented with protease inhibitors to the pellet from step 6.4.1.1, and incubate at 4 °C with gentle mixing for 10 min. Centrifuge at 3,000 x *g* at 4 °C for 5 min and collect the supernatant.

6.4.1.3. For the soluble nuclear fraction, add 50 µL of nuclear extraction buffer supplemented with protease inhibitors to the pellet from step 6.4.1.2, and vortex. Incubate at 4 °C for 30 min, centrifuge at 5,000 x *g* at 4 °C for 5 min, and collect the supernatant.

6.4.1.4. For the insoluble nuclear fraction, add 50 µL of nuclear extraction buffer supplemented with protease inhibitors, $CaCl_2$ and micrococcal nuclease to the pellet from step 6.4.1.3, and vortex. Incubate at 37 °C for 5 min, and then vortex again. Centrifuge at 16,000 x *g* at RT for 5 min and collect the supernatant.

6.4.1.5. For the cytoskeletal fraction, add 50 µL of cytoskeletal extraction buffer supplemented with protease inhibitors to the pellet from step 6.4.1.4, and vortex. Incubate 10 min at RT. Centrifuge the tube at 16,000 x *g* for 5 min, collect the supernatant and discard the pellet.

NOTE: Scale buffer volumes according to the cell volume, as indicated in the kit protocol. Refer to the kit protocol for further details on incubation and centrifugation time and temperature[22–29]. Alternatively, use any standard methods[30] that, by using detergents and by increasing ionic strength and centrifugation speed, separates the cytosolic, the membrane-bound, the cytoskeletal and the nuclear fractions. Separate the soluble nuclear fraction and the insoluble nuclear fraction by exploiting standard nuclear extraction buffers. The sample can be stored at -20 °C.

6.4.2. For the SDS-PAGE, add 7 µL of 4x Laemmli buffer to 20 µL of subcellular fractions obtained from steps 6.4.1.1−6.4.1.5, boil at 100 °C for 5 min.

6.5. Load samples on an acrylamide gel and perform electrophoresis at a constant voltage of 120 V. Transfer proteins to nitrocellulose membrane at 250 mA for 90 min.

6.6. Check proper protein gel electrophoresis and successful blotting by incubating the membrane for 5 min in Ponceau staining solution. Rinse the membrane in distilled water until the background is clean. Remove the stain by continued washing with Tris buffered saline with Tween 20 (TBST) for 10 min on a shaker.

6.6. Incubate the membrane with blocking solution (5% milk in TBST) for 1 h at RT on shaker. Wash 3 times with TBST for 5 min.

6.7. Hybridize the membrane with the primary antibody in blocking solution (1% milk in TBST) overnight at 4 °C. Wash 3 times with TBST for 5 min.

6.8. Hybridize the membrane with the HRP-conjugated secondary antibody in blocking solution for 1 h at RT. Wash 3 times with TBST for 5 min.

6.9. Detect the protein band using chemiluminescence. Quantify the intensity of Western Blot bands by ImageJ. Normalize protein expression onto the product of a housekeeping gene: histone H2B for the nuclear soluble and insoluble fraction, GAPDH for the cytoplasmic fraction and for total extracts.

REPRESENTATIVE RESULTS:

The strategy used to dissect the impact of nuclear Tau in gene expression avoiding the contribution of cytoplasmic Tau proteins has been depicted in **Figure 1**. Briefly, Tau proteins tagged with NLS or NES are accumulated in or excluded from the nuclear compartment, respectively. The functional effect of this unbalance is the alteration of the gene expression measured as the product of the VGluT1 gene.

Following the protocol description, SH-SY5Y cells were treated with RA for 5 days and then with BDNF for 3 days in order to obtain post-mitotic neuron-like cells (**Figure 2**). In the absence of RA and BDNF, undifferentiated SH-SY5Y cells assume a rounder morphology and form cell clumps. As expected, starting the differentiation protocol, clumps unwind and cells spread out neurites; at the end of differentiation, cells are uniformly distributed and interconnected via a network of branched neurites.

The day after seeding, cells have been transfected with Tau-NLS or Tau-NES plasmids (section 4.2) with cationic lipids. For cells expressing Tau-NLS or Tau-NES constructs, Tau subcellular localization can be detected by immunofluorescence with anti-Tau antibodies. Depending on the efficiency of transfection, cells display a strong nuclear staining merging with the DAPI signal or a cytoplasmic staining with empty nuclei if they are successfully transfected with Tau-NLS or Tau-NES, respectively (**Figure 3**). The lack of these specific signals indicates an inefficient transfection.

To analyse the proteins enriched in different subcellular compartment, cells were collected and counted in order to process equal amounts of cells per sample. Any standard fractionation method that exploits increasing detergent and ionic strength and increasing centrifugation speed can be used to separate the cellular compartments from one another and thus isolate the cytosolic, the membrane-bound, the cytoskeletal and the nuclear fractions.

Once the nuclei have been isolated, the nuclear soluble fraction and the chromatin bound fraction were separated by adding 3 U/µL of micrococcal nuclease and 5 mM $CaCl_2$.

For Western blot analysis, equal volumes of cytoplasmic and membrane fractions and half volumes of the other fractions have been loaded on a gradient precast acrylamide gel, to correct for the different amount of buffer added at each step.

To verify the efficient separation of different fractions, the Western blot exploiting the following antibodies has been performed: anti-GADPH (present in all fractions except the cytoskeleton and particularly enriched in the cytoplasmic fraction); anti-actin (particularly enriched in the cytoskeletal fraction); anti-tubulin (particularly enriched in Cytoplasmic and cytoskeletal fractions); anti-H2B (enriched in the nuclear fractions) (**Figure 4**).

An enrichment of these markers in different subcellular fractions indicates that the fractionation is not well performed. It must be noted that any protocol for subcellular fractionation might present a 10-15% of contamination between fractions.

Once verified the successful fractionation of the sample, the Western blot has been performed to check the signal of Tau in the nuclear compartment and the VGluT1 signal in the total extract (**Figure 5**). While untagged Tau is detectable in all fractions, Tau-NLS is strongly enriched in the nuclear compartment and it is poorly detectable in the cytoplasmic fraction. On the contrary, Tau-NES is enriched in the cytoplasmic fraction and it is less detectable in the nuclear fraction. The presence of a small amount of Tau-NES into the soluble nuclear fraction has to be expected since, like the endogenous Tau, it is translocated into the nucleus and once into the nuclear compartment the nuclear export signal allows its translocation to the cytoplasm. The detection of a different enrichment for these two fusion proteins might indicate a problem in the efficiency of transfection or in the cloning of constructs or in fractionation.

Quantitative analysis of Western blot can be done using ImageJ. Values are normalized for the housekeeping gene specific for each fraction (GAPDH for cytoplasmic fraction; histone H2B for soluble nuclear and chromatin-bound fractions).

The graph in **Figure 5B** reports the ratio of Tau in the soluble nuclear fraction and cytoplasmic fraction to highlight that Tau-NLS is highly enriched in the soluble nuclear fraction (SNF) while Tau-NES is decreased. Moreover, Tau-NLS is enriched in the chromatin-bound fraction (CBF) with respect to the cytoplasmic fraction (CF) while Tau-NES is decreased. SNF/CF = 1 and CBF/CF = 1 correspond to Tau ratio in control cells. The endogenous Tau is weakly detectable in all fractions as expected. The graph in **Figure 5C** reports the quantification of VGluT1 expression in the total extracts of samples expressing different amount of nuclear Tau. In cells expressing Tau-NES, VGluT1 expression is comparable to the baseline expression in control cells. On the contrary, in cells expressing untagged Tau or Tau-NLS, the expression of VGluT1 is more than doubled.

FIGURE LEGENDS:

Figure 1: Graphical representation of the strategy used to allow a nuclear or a cytoplasmic accumulation of Tau. Tau-NLS is accumulated in the nuclear compartment while Tau-NES is excluded. The experimental readout is the modulation of the VGluT1 expression.

Figure 2: Representative undifferentiated and differentiated cell culture. Image of undifferentiated SH-SY5Y (left), cells differentiated by RA (middle) and differentiated by RA and BDNF (right). Scale bar = 100 µm.

Figure 3: Representative image of Tau subcellular enrichment by immunofluorescence. Image of cells untransfected or expressing untagged Tau, Tau-NES or Tau-NLS constructs. Tau signal has been obtained by immunofluorescence (red), nuclei signal has been obtained by DAPI staining (blue), merged images are reported. Scale bar = 10 µm.

Figure 4: Representative detection of proteins enriched in subcellular fractions by Western blot. Western blot of subcellular fractions from SH-SY5Y cells. CF = cytoplasmic fraction; MF = membrane fraction; SNF = soluble nuclear fraction; CBF = chromatin bound fraction; CKF = cytoskeletal fraction.

Figure 5: Representative detection of nuclear Tau and VGluT1 proteins. (**A**) Western blot of Tau protein detected in the nuclear and cytoplasmic fractions. (**B**) The graph reports the ratio of Tau in the nuclear fractions and cytoplasmic fraction. The values have been normalized on the endogenous Tau. SNF/CF = 1 and CBF/CF = 1 corresponds to endogenous Tau ratio in control cells. (**C**) Western blot of VGluT1 protein. (**D**) The graph reports the quantification of VGluT1 expression in the total extracts of samples expressing different amount of nuclear Tau. Kruskal-Wallis ANOVA and Mann-Whitney test; *** $p < 0.001$, **** $p < 0.0001$, n.s. $p > 0.05$. All results are shown as mean ± SEM from at least three independent experiments. This representative figure has been modified from Siano et al.[31].

DISCUSSION:

We describe a method to measure the impact of nuclear Tau protein on gene expression. With this protocol the contribution of cytoplasmic Tau is strongly limited. Critical steps of this protocol are the following: the differentiation of human neuroblastoma SH-SY5Y cells, the subcellular fractionation and the localization of Tau protein in the nuclear compartment.

First, as shown in the representative results section, the differentiation of SH-SY5Y cells by adding RA and BDNF is crucial to obtain a good preparation of neuron-like cells in culture. The density of cells seeded is particularly important since a lower density might impact cell proliferation. Moreover, for experiments that need a high number of cells, like cellular fractionation and Western blot, it is important to note that the BDNF differentiation step blocks the cellular proliferation to allow the terminal differentiation, thus limiting the number of cells in culture. Alternative differentiation protocols use only RA or NGF instead of BDNF. However, while adding BDNF after RA allows to reach a better morphological differentiation[32,33], NGF induces a weaker neurite outgrowth in SH-SY5Y cells[34]. Moreover, it has been extensively demonstrated that the combination of RA and BDNF allows to obtain a homogeneous neuronal population with expression of neuronal markers and decreased proliferation[35]. For this reason, the differentiation protocol exploited here combines RA and BDNF.

However, the procedure reported to dissect the role of Tau in different subcellular compartments can be used also for undifferentiated cells or for different cell types.

The subcellular fractionation is a very critical step and it is crucial to have enough starting material: a commercial kit requires only 1 x 10^6 cells, whereas other procedures may need a much higher starting quantity. Moreover, the use of a kit with standard buffers and steps guarantees the reproducibility of the experiment that is unavoidable and essential. However, since the composition of buffers is often proprietary, they might contain detergents which may alter the function of the protein of interest and it might be difficult to optimize the isolation of the fractions. Moreover, even in the best condition, there might be a 10-15% of contamination between fractions. A poor yield from each fraction could be overcome by increasing the incubation time in extraction buffers of specific fractions.

Since the functions of nuclear Tau have gained significant interest in recent years, it is particularly important to provide a reliable method to dissect the function of Tau in different cellular compartments. Coupling the subcellular fractionation, with the expression of Tau constructs specifically directed or excluded from the nucleus, allows one to finely tune the amount of Tau in different compartments.

A critical step in this part of the protocol is the cloning of Tau tagged with nuclear localization signal or with the nuclear export signal. The efficiency of the NLS is guaranteed by the presence of a 3XNLS consensus sequence from the SV40 virus. The nuclear translocation of the protein can be easily checked by immunofluorescence, and the lack of signal into the nucleus might be due to an incorrect cloning or to an inefficient transfection. On the contrary, the nuclear export is guaranteed by the NES consensus sequence. In this case, the immunofluorescence allows checking of the export of Tau from the nucleus. However, a weak nuclear signal is not to be excluded since Tau-NES protein enters the nucleus and then, due to the NES sequence, it is exported into the cytosol.

Up to now, the function of nuclear Tau has been studied only by correlative approaches that do not assure its direct involvement. The protocol here described, provide the first approach allowing to clearly discriminate the specific function of Tau into the nuclear compartment. As previously demonstrated, the endogenous Tau does not affect the results obtained by this protocol. Indeed, the same experiment performed in non-neuronal cells that do not express endogenous Tau, leads to VGluT1 altered expression. We applied this protocol to study the expression of disease-related genes[31]. Anyhow, it could be exploited also to investigate other nuclear Tau functions, such as the involvement on DNA damage, the interaction with nuclear cofactors or with the chromatin.

ACKNOWLEDGMENTS:
This work was supported by grants from Scuola Normale Superiore (SNS14_B_DIPRIMIO; SNS16_B_DIPRIMIO).

DISCLOSURES:
The authors have nothing to disclose.

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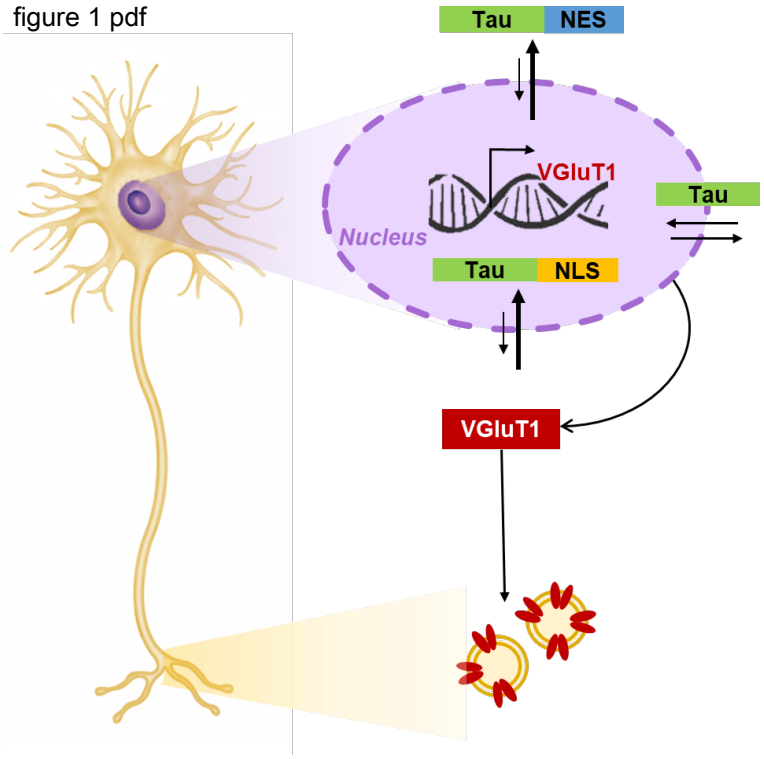

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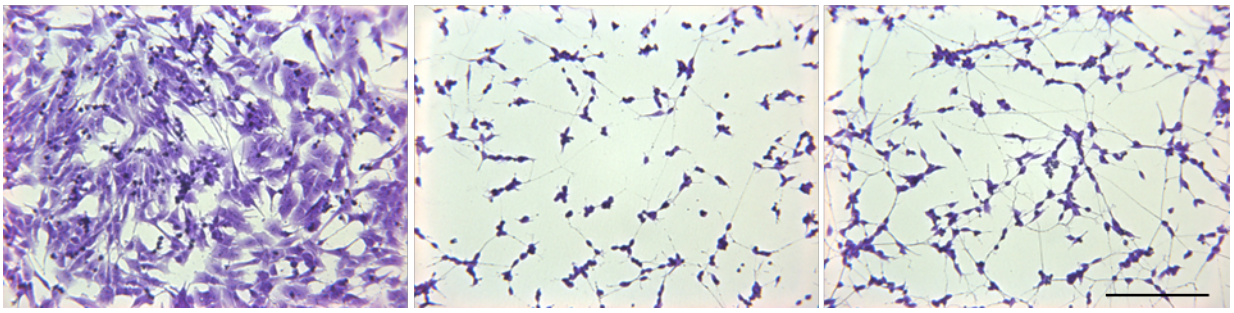

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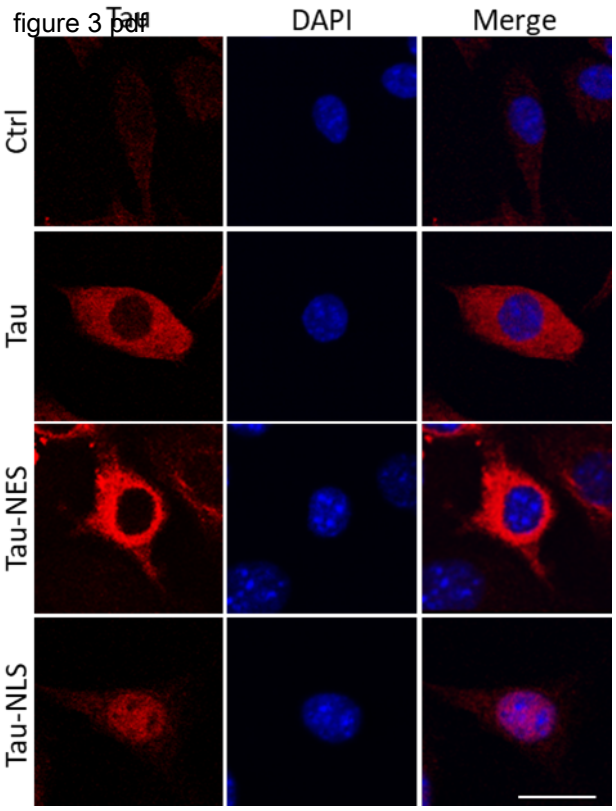

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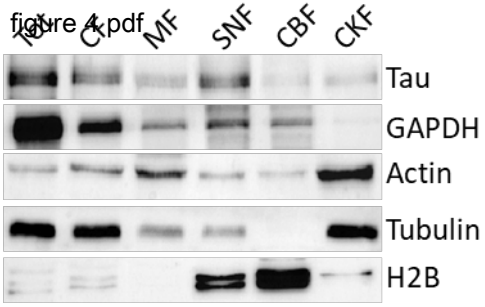

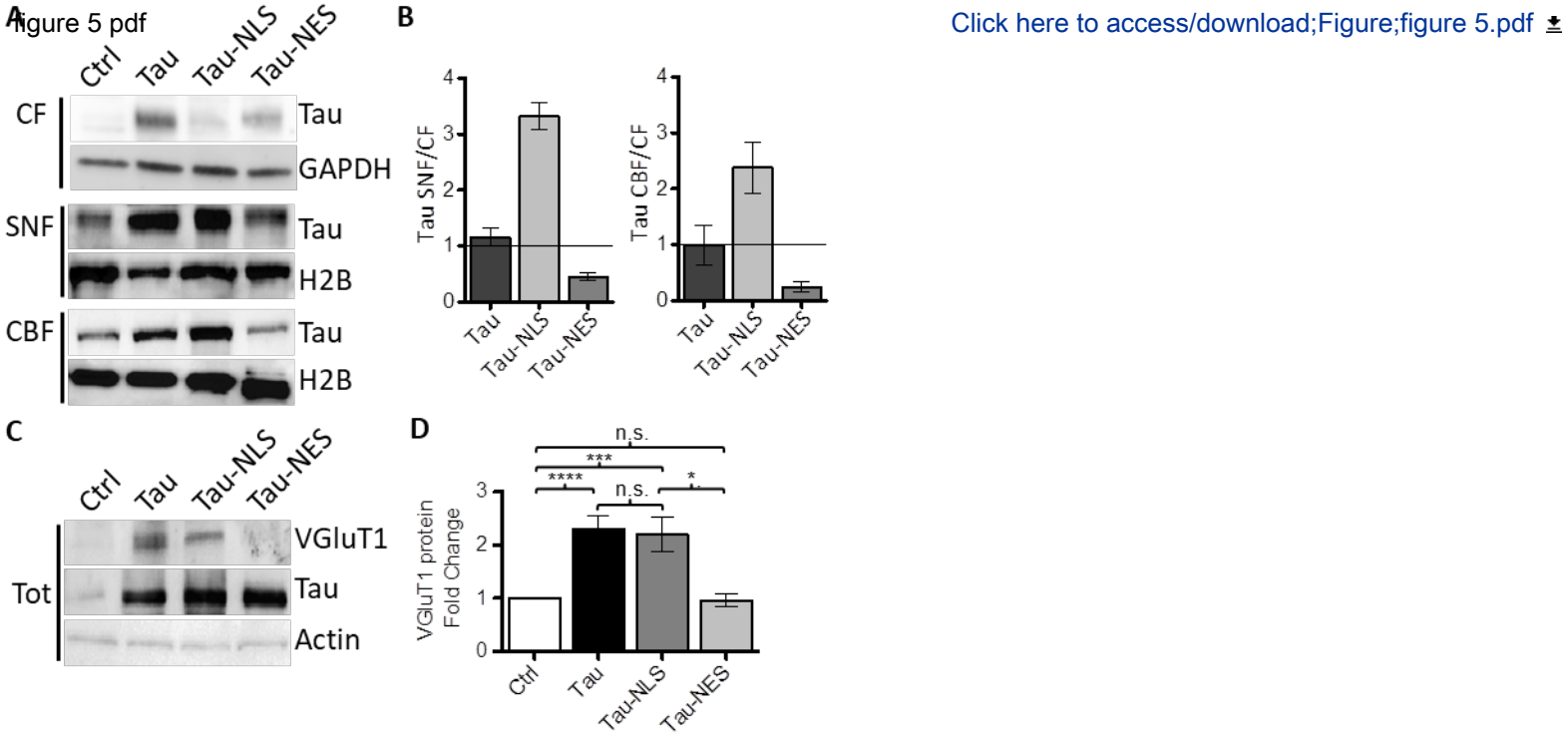

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Alexa Fluor 633 goat anti-mouse IgG	Life Technologies	A21050	IF 1:500
anti Actin Antibody	BETHYL LABORATOI	A300-485A	anti-rabbit WB 1:10000
anti GAPDH Antibody	Fitzgerald Industrie	10R-G109a	anti-mouse WB 1:10000
anti H2B Antibody	Abcam	ab1790	anti-rabbit WB 1:15000
anti Tau-13 Antibody	Santa Cruz Biotechnology	sc-21796	anti-mouse WB 1:1000; IF 1:500
anti Tubulin alpha Antibody	Thermo Fisher Scientific	PA5-16891	anti-mouse WB 1:5000
anti VGluT1 Antibody	Sigma-Aldrich	AMAb91041	anti-mouse WB 1:500
BCA Protein Assay Kit	Euroclone	EMPO14500	
BDNF	Alomone Labs	B-250	
Blotting-Grade Blocker	Biorad	1706404	Non-fat dry milk
BOVIN SERUM ALBUMIN	Sigma-Aldrich	A4503-50g	
cOmplete Mini	Roche	11836170001	protease inhibitor
Criterion TGX 4-20% Stain Free, 10 well	Biorad	5678093	
DAPI	Thermo Fisher Scientific	62247	
DMEM/F-12	GIBCO	21331-020	
Dulbecco's Modified Eagle's Medium Low Glucose	Euroclone	ECM0060L	
EDTA	Sigma-Aldrich	0390-100ml	pH=8 0.5M
Foetal Bovine Serum	Euroclone	EC50182L	
Glycerol	Sigma-Aldrich	G5516-500ml	
Goat anti-mouse IgG-HPR	Santa Cruz Biotechnology	sc-2005	WB 1:1000
Goat anti-rabbit IgG-HPR	Santa Cruz Biotechnology	sc-2004	WB 1:1000
IGEPAL CA-630	Sigma-Aldrich	I8896-50ml	Octylphenoxy poly(ethyleneoxy)ethanol
Immobilon Western	MERCK	WBKLS0500	

Lab-Tech Chamber slide 8 well glass slide	nunc	177402	
L-glutamine	Euroclone	ECB3000D	100X
Lipofectamine 2000 transfection reagent	Thermo Fisher Scientific	12566014	cationic lipid
Methanol	Sigma-Aldrich	322415-6X1L	
$MgCl_2$	Sigma-Aldrich	M8266-100G	
NaCl	Sigma-Aldrich	S3014-1kg	
Opti-MEM reduced serum medium	Gibco	31985070	
PEI	Sigma-Aldrich	40,872-7	
Penicillin/Streptomycin	Thermo Fisher Scientific	15140122	10,000 U/ml, 100ml
Phosphate Buffered Saline (Dulbecco A)	OXOID	BR0014G	
PhosStop	Roche	4906837001	phosphatase inhibitor
QIAGEN Plasmid Maxi Kit	Qiagen	12163	Step 3.10
Retinoic acid	Sigma-Aldrich	R2625-100mg	
Subcellular Protein Fractionation Kit for cultured cells	Thermo Fisher Scientific	78840	
Supported Nitrocellulose membrane	Biorad	1620097	
TC-Plate 6well	SARSTEDT	833,920	
TCS SP2 laser scanning confocal microscope	Leica	N/A	
Triton x-100	Sigma-Aldrich	X100-500ml	Non-ionic surfactant
Trypsin-EDTA	Thermo Fisher Scientific	15400054	0.50%
Tween-20	Sigma-Aldrich	P9416-100ml	
VECTASHIELD antifade mounting medium	Vector Laboratories	H-1000	

Wizard *Plus* SV Minipreps DNA Purification Systems	Promega	A1330	Step 3.5

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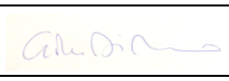
Signature:	
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