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Visualizing the microtubule-associated protein tau in the nucleus

LU Jing[1], LI Ting[2], HE RongQiao[2], BARTLETT Perry F[1] & GÖTZ Jürgen[1*]

[1]*Clem Jones Centre for Ageing Dementia Research, Queensland Brain Institute, The University of Queensland, Brisbane, Queensland 4072, Australia;*
[2]*Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China*

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Although tau is mainly known as an axonal microtubule-associated protein, many studies indicate that it is not restricted to this subcellular compartment. Assessing tau's subcellular distribution, however, is not trivial as is evident from transgenic mouse studies. When human tau is over-expressed, it can be immunohistochemically localized to axons and the somatodendritic domain, modeling what is found in neurodegenerative diseases such as Alzheimer's disease. Yet, in wild-type mice, despite its abundance, tau is difficult to visualize even in the axon. It is even more challenging to detect this protein in the nucleus, where tau has been proposed to protect DNA from damage. To establish a framework for future studies into tau's nuclear functions, we compared several methods to visualize endogenous nuclear tau in cell lines and mouse brain. While depending on the fixation and permeabilization protocol, we were able to detect nuclear tau in SH-SY5Y human neuroblastoma cells, we failed to do so in N2a murine neuroblastoma cells. As a second method we used subcellular fractionation of mouse tissue and found that in the nucleus tau is mainly present in a hypophosphorylated form. When either full-length or truncated human tau was expressed, both accumulated in the cytoplasm, but were also found in the nuclear fraction. Because subcellular fractionation methods have their limitations, we finally isolated nuclei to probe for nuclear tau and found that the nuclei were free of cytoplasmic contamination. Together our analysis identifies several protocols for detecting tau in the nucleus where it is found in a less phosphorylated form.

Alzheimer's disease, fractionation, microtubule-associated protein, neuroblastoma, nucleus, phosphorylation, tau

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A major interest in the protein tau stems from the fact that it forms insoluble aggregates in many neurodegenerative diseases including Alzheimer's disease (AD) [1]. Tau belongs to the family of microtubule-associated proteins (MAPs), with tau being the prototypical axonal and MAP2 the prototypical dendritic family member [2]. MAPs have a central role in neuronal integrity [3]. While tau has a major function in the axon, early on it was recognized that this protein has also important physiological functions in other compartments, such as the dendrite, where low concentrations of tau facilitate the targeting of the tyrosine-directed kinase Fyn to the dendritic spines [4]. The dendrite is not the only 'non-canonical' subcellular compartment to which tau has been localized; in fact, studies to localize tau to cell-types other than the neuron and subcellular compartments other than the axon date back to the early 1990s when the dephosphorylation-dependent anti-tau antibody Tau-1 was used to visualize nuclear tau in some but not all neuroblastoma cell lines investigated [5].

Whether there are also changes to nuclear tau in AD is unknown, but interestingly, RNA splicing alterations have been identified, suggesting that the nucleus needs to be factored in when pathogenesis of AD is studied [6]. When analyzing nuclear tau, human cell lines (JC, CG, LA-N-5,

*Corresponding author (email: j.goetz@uq.edu.au)

HeLa, and SH-SY5Y) consistently showed nucleolar Tau as detected with the Tau-1 antibody, while a rodent neuroblastoma cell line (N2a) consistently failed to do so [5,7,8]. Two human cell lines (JC and CG) were further analyzed by subcellular fractionation followed by formic acid (FA) extraction to enrich for insoluble proteins. One cell line (CG) failed to reveal cytoplasmic tau, and tau was only observed in the nuclear FA fraction, while a second cell line (JC) revealed tau in both the soluble and FA fraction [5]. Cytoplasmic tau was shown not to localize to microtubules [8]. Together this was interpreted such as that tau exists in the nucleus in a less soluble form. Subsequently, using human brain tissue, isolated nuclei were used to demonstrate the insolubility of nuclear tau [9]. It was argued that nuclear tau is highly insoluble as much as tau is in the paired helical filaments (PHFs) that are found in the tau tangles in AD brain. Furthermore, it was suggested at the time that in AD, tau would even form PHFs in nuclei. It was speculated that the nucleus might contain factors that are capable of inducing PHF formation, and when these nuclear PHFs are released into the cytoplasm that they would act as a catalyzer of tau aggregation and subsequent neurodegeneration. A nuclear role for tau was extended to human non-neuronal cells, demonstrating shared structural and functional features of nuclear and cytoplasmic tau [10]. Tau is a phosphoprotein, with hyperphosphorylation causing its aggregation [11]. It is therefore not unreasonable to assume that subcellular targeting in general depends on phosphorylation. Similar to plasma membrane-localized tau being less phosphorylated [4,12], there is evidence that oxidative and heat stress causes tau to enter the nucleus in a dephosphorylated form [13]. Whether under physiological conditions the phosphorylation status of nuclear tau differs from that of non-nuclear tau is not known.

In analyzing tau distribution, in particular its localization to the nucleus, a review of the literature and our own experience show that tau is not easily detected in this compartment. In general, tau is difficult to visualize by immunohistochemistry in mouse brain unless the protein is over-expressed by transgenic approaches. While this reveals axonal and somatodendritic localization, it is difficult on non-transgenic sections to even detect axonally localized tau, and it is even more challenging to visualize nuclear tau. To visualize the latter, the dephosphorylation-dependent anti-tau antibody Tau-1 has been widely used, while most other antibodies failed to detect nuclear tau, possibly reflecting the phosphorylation status of tau in the nucleus. Different experimental systems, protocols and antibodies have been used in the past, providing conflicting results.

The purpose of our study was therefore to establish a set of robust methods to detect and visualize nuclear tau, including tau knockout samples as controls. Robust methods of visualizing nuclear tau are a prerequisite in order to obtain a better understanding of the role nuclear tau has in neurons, and to get a handle on what regulates the allocation of tau to different subcellular compartments. Therefore, we validated a range of detection methods in cell culture and *in vivo*. Together our study shows that tau can be detected in the nucleus in human SH-SY5Y but not murine N2a neuroblastoma cells, and that detection depends on a specific protocol. Fractionation revealed that tau in the nucleus is less phosphorylated than in the cytoplasm. Finally, we found that the isolation of intact nuclei is an elegant method to visualize and analyze nuclear tau.

1 Materials and methods

1.1 Cell culture and immunohistochemistry

SH-SY5Y human and N2a murine neuroblastoma cells were cultured in DMEM medium (Life Technologies, Melbourne, VIC, Australia) supplemented with 10% FBS (Hyclone). Cells were plated at 1×10^5 mL^{-1} into 12 well plates that were plated with poly-D-lysine-coated coverslips. After 24 h, the medium was replaced by DMEM/1% FBS. After 24 h of serum deprivation, the vast majority of cells were in G1 stage. The cells were fixed and permeabilized according to previously established procedures (Table 1), followed by incubation with the dephosphorylation-dependent anti-tau antibody Tau-1 (Millipore, Billerica, MA, USA; 1:200) and the phosphorylation-independent anti-Tau antibody Dako Tau (Dako, Glostrup, Denmark; 1:200). Secondary antibodies were goat anti-rabbit and goat anti-mouse conjugated to Alexa Fluor 488 and 568, respectively (Life Technologies, USA). DNA was counterstained with DAPI (Sigma, St Louis, MO, USA). Images were taken with a Zeiss LSM 510 inverted confocal microscope.

Specifically, the following six methods were validated (Table 1):

Method I [5]. After washing with PBS, the cells were fixed with 3% formaldehyde for 30 min at 24°C, then permeabilized with 0.5% Triton X-100 in PBS (PBST) for 5 min, rinsed twice in PBS, reduced in 0.5 μg mL^{-1} $NaBH_4$ in PBS for 4 min, and blocked with 1% BSA in PBS for 10 min. Incubation with primary antibodies was done at 4°C in 1% BSA overnight. Incubation with secondary antibodies was done at 37°C for 30 min in PBS. Preparations were counterstained with DAPI before mounting with mounting medium (Dako).

Method II [10]. After washing with PBS, the cells were fixed with pre-cooled methanol at –20°C for 15 min. After rinsing with PBS three times, the cells were incubated with primary antibodies in 2% BSA. After washing with PBS, secondary antibodies were applied in 2% BSA.

Method III [14]. The cells were first fixed with 1% PFA for 20 min, and then with methanol for 10 min. The cells were permeabilized with 0.2% PBST, before blocking with 5% BSA. Antibodies were used in the same way as for Method I except that 5% instead of 1% BSA was used for the reaction buffer.

Table 1 Methods used previously to visualize nuclear tau protein in various experimental systems[a)]

Method	Fixation	Permeabilization	Blocking buffer	Experimental system	Antibody	Ref.
I	3% formaldehyde	0.5% PBST	1% BSA	Human neuroblastoma cells (JC, CG)	Tau-1	[5]
II	Methanol	N.D.	2% BSA	Human Huh-7 hepatoma cells, human fibroblasts	Tau-1	[10]
III	1% PFA and Methanol	0.1% PBST	5% BSA	Human fibroblasts, HeLa cells (human)	Tau-1	[14]
IV	Methanol (Step 2)	0.2% PBST (Step 1)	2% BSA	Primary cortical mouse neurons	Tau-1, Tau3R	[13]
V	4% PFA	0.1% PBST	5% NGS	Murine neuroblastoma cells (N2a), formaldehyde-treated	phospho-T181, phospho-S396	[15]
VI	4% PFA	N.D.	2% NGS+ 3% BSA+ 0.1% PBST	Mouse brain section	Tau isoform-specific antibodies	[16]

a) The methods are numbered consecutively (I–VI), based on the publication year of the respective study. Abbreviations used: PFA, paraformaldehyde; PBST, PBS, Triton-X-100; BSA, bovine serum albumin; NGS, normal goat serum; N.D., not done; Tau3R, antibody specific for 3R tau; phospho-T181, phospho-S396; see opposite order in IV. Note that for method IV, cells were heat-shocked, while for method V, they were formaldehyde-treated.

Method IV [13]. After washing with PBS, the cells were first permeabilized with 0.2% PBST for 5 min (Sultan and colleagues had permeabilized for 10 min, but that seemed too harsh to us as under these conditions most cells came off), then the cells were fixed in cold methanol for 10 min at −20°C. The cells were washed with PBS before blocking with 2% BSA. Antibodies were used as for Method I except that 2% BSA was used for the reaction buffer.

Method V [15]. After washing with PBS, the cells were fixed with 4% PFA, followed by permeabilization with 0.1% PBST. The cells were blocked with 5% NGS (normal goat serum) before the antibodies were applied as described in Method I, with 5% NGS being used for the reaction buffer.

Method VI [16]. After washing with PBS, the cells were fixed with 4% PFA, then blocked directly with 3% BSA and 0.1% NGS containing 0.1% Triton X-100 in PBS. Then the antibodies were used in the same way as for Method I except that 3% BSA and 0.1% NGS containing 0.1% Triton X-100 in PBS was used for the reaction buffer.

1.2 Animal models and ethics

C57BL/6 mice, tau knockout mice [20], pR5 mice that express human P301L mutant full-length tau [21], and ΔTau74 mice that express a truncated form of wild-type human tau that lacks the microtubule-binding domain [4], were all supplied by UQ Biological Resources. The animal experimentation was approved by the Animal Ethics Committees of the University of Queensland (approval number QBI/027/12/NHMRC).

1.3 Subcellular fractionation, sequential extraction and Western blot analysis

Brain tissue from 3-month-old male mice was fractionated into cytoplasmic and nuclear fractions [17], followed by dephosphorylation of the extracts with λ protein phosphatase (New England Biolabs, Hitchin, UK) according to the manufacturer's instructions. To obtain fractions of increasing insolubility, a sequential extraction was performed with the nuclear fraction. High salt reassembly buffer (RAB) (0.1 mol L^{-1} Mes, 1 mmol L^{-1} EGTA, 0.5 mmol L^{-1} MgSO4, 0.75 mol L^{-1} NaCl, 0.02 mol L^{-1} NaF, 1 mmol L^{-1} PMSF, 0.1% complete protease inhibitor (Roche), pH 7.0) was used to generate the RAB-soluble fractions. The resulting pellets were extracted with radioimmunoprecipitation assay (RIPA) buffer (50 mmol L^{-1} Tris, 150 mmol L^{-1} NaCl, 1% Nonidet P-40, 5 mmol L^{-1} EDTA, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0) and centrifuged to generate RIPA-soluble samples. Finally, the RIPA-insoluble pellets were re-extracted with 70% formic acid (FA) to recover the most insoluble cytoskeletal aggregates. This was followed by dephosphorylation with λ protein phosphatase. Western blotting was performed as described previously [18]. To determine the phosphorylation of tau [19], the dephosphorylation step was omitted and brain extracts were immunoblotted with antibodies AT8 (specific for pS202/pT205 of tau, 1:2000; Thermo Scientific, USA), AT180 (specific for pT231/pS235, 1:2000; Thermo Scientific), AT270 (specific for pT181, 1:2000; Thermo Scientific), and 12E8 (specific for pS262/pS356, kind gift of Dr. Peter Seubert, Neotope Inc., Palo Alto, CA, USA), followed by incubation with IRDye fluorescent secondary antibodies (LI-COR, Lincoln, NE, USA; 1:10000) and detection using the Infrared Imaging system (LI-COR). Quantification was done with the Odyssey Application Software Version 3.0, and for statistical analysis, Prism 6 was used for plotting and statistics. The data were expressed as mean±standard deviation (SD). A two-tailed *t*-test was carried out to determine the differ-

ence of tau phosphorylation between nuclear and cytoplasmic fractions. **, $P<0.01$; ***, $P<0.001$.

1.4 Isolation of nuclei and confocal analysis

Nuclei were isolated from brains of 3-month-old C57BL/6 mice and tau knockout mice [20], using a nuclei isolation kit (Sigma, St Louis, MO, USA; NUC101-1KT). The nuclei were first identified with DAPI staining using a fluorescent microscope (Olympus CKX41, Japan). A suspension of 1–2 million/mL nuclei was generated and cyto-spun with a Cellspin I centrifuge (THARMAC, Germany) onto glass slides. Cytospot used 100 μL per slide, centrifuging at 2000 r min^{-1} for 2 min. Once the slides were dry, they were kept in the fridge until further use. Immunostaining was done as previously reported [9,15], except that the nuclei were permeabilized with 0.2% PBST after fixation. Nuclei were stained for GAPDH (Millipore; Cat. ABS16, 1:200), tubulin (Sigma; Cat. T6074, 1:200), Dako Tau and Lamin B (Abcam; Cat. ab16048, 1:200). The same secondary antibodies were used as for immunocytochemistry. Alternatively, nuclei can also be incubated directly with the antibodies in Eppendorf tubes, centrifuged at 250×*g* for 5 min, washed, and then evenly spread onto glass slides for mounting. This method better preserves the nuclear morphology than cytospinning on slides but consumes many more nuclei. Images were taken with a Zeiss LSM 510 inverted confocal microscope. Z-stacks were obtained to demonstrate the intactness of the nuclei using either Lamin B staining or staining for tau and Lamin B. The latter was further reconstructed with Imaris surpass and spot modules and colocalization of tau and DNA was calculated with the coloc module (Imaris, Bitplane, St. Paul, MN, USA).

2 Results

2.1 Nuclear staining for tau is cell type- and fixation-dependent

In order to obtain a method that would allow the visualization of nuclear tau in tissue culture cells, we validated several protocols that had previously demonstrated endogenous tau in the nucleus (Table 1). Several of these protocols had used the antibody Tau-1 that detects tau when not phosphorylated at the AT8 phospho-epitope Ser202/Thr205. Two of the methods (IV and V) subjected the cells to either heat-shock or formaldehyde treatment which caused tau to localize to the nucleus [13,15]. For our comparative analysis, we used undifferentiated human SH-SY5Y and murine N2a cells. Both cell lines are widely used in the field. SH-SY5Y cells can be neuronally differentiated, using retinoic acid [22]. When kept undifferentiated, they differ from fibroblasts by having a bipolar morphology, as is also found for N2a cells [15,23]. We synchronized the cells by serum-deprivation, followed by processing according to the six protocols I–VI, using Tau-1, Dako Tau (a phosphorylation-independent pan-neuronal anti-tau antibody) and the nuclear marker DAPI (in blue) for visualization (Figures 1 and 2). The six protocols vary with regards to fixation, permeabilization, and blocking buffer used (Table 1). In SH-SY5Y cells (Figure 1), for all six protocols, Tau-1 staining was confined to the nucleus. Reactivity with Tau-1 was confined to the nucleus, and Dako Tau reactivity was absent at all when protocol I was used. Protocol II revealed a strong cytoplasmic and a punctate non-overlapping staining with Tau-1 for the nucleus. Protocol III revealed a very weak punctate, diffuse staining for Dako Tau in the nucleus and cytoplasm, and nuclear staining with Tau-1. Protocol IV revealed a punctate Dako Tau staining. Note that because of the reverse order of fixation and permeabilization, the bright-field images for IV look different compared with the other five methods. Of all protocols, protocols V and VI yielded the strongest overall staining, with pronounced focal staining for Tau-1 in the nucleus, and a strong staining for Dako Tau in the cytoplasm. Different from SH-SY5Y cells, N2a cells failed to reveal nuclear tau staining, both with Dako Tau and Tau-1 (Figure 2). Dako Tau revealed a strong cytoplasmic staining for all protocols but IV that revealed a subtle punctate staining. Interestingly, Tau-1 led to strong, diffuse cytoplasmic staining with protocols II, III, V and VI. There is evidence for very slight nuclear Dako Tau staining when using methods II and V. While we have not applied a systematic approach to test all combinations of fixation, permeabilization, and blocking buffer, for practical purposes protocol V worked best in visualizing nuclear and cytoplasmic tau in human SH-SY5Y cells. The data also show that N2a cells fail to present with nuclear tau within the limit of our detection method.

2.2 In mouse brain both endogenous and transgenic tau are in the nuclear fraction

We have previously analyzed the distribution and localization of the three major murine tau isoforms in the adult brain, 0N4R, 1N4R and 2N4R [16]. This revealed that the brain is the only organ to express tau at significant levels, with 0N4R being the predominant isoform in the 2-month-old adult. Subcellular fractionation of the brain showed that the 1N isoform is over-represented in the soluble nuclear fraction. This is in agreement with the immunohistochemical analysis as the 1N isoform strongly localizes to the neuronal nucleus, although it is also found in cell bodies and dendrites, but not axons. The 0N isoform was mainly found in cell bodies and axons, whereas nuclei and dendrites were only slightly stained with the 0N antibody. The 2N isoform was found to be highly expressed in axons and in cell bodies, with a detectable expression in dendrites and a very slight expression in nuclei. The 2N isoform that was unde-

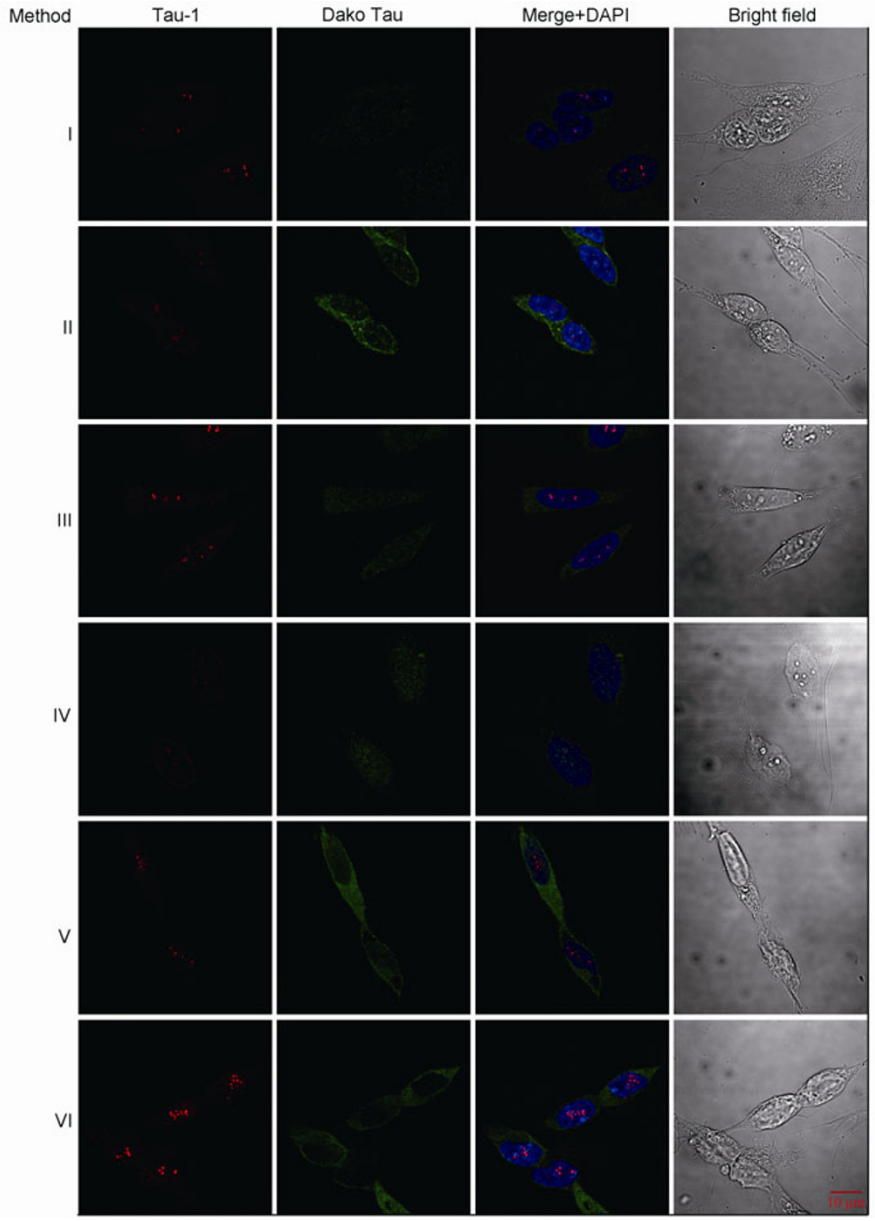

Figure 1 Visualizing endogenous nuclear Tau by immunofluorescence in SH-SY5Y cells. SH-SY5Y human neuroblastoma cells were synchronized by serum deprivation and then processed using the fixation, permeabilization and blocking conditions listed in Table 1, employing antibodies Tau-1 (in red) and Dako Tau (green) as well as the nuclear marker DAPI (blue) for detection. Note that because of the reverse order of fixation and permeabilization, the bright-field images obtained with protocol IV look different from those obtained with the other five methods.

tectable at P0, in adult brain was mainly found localized to cell bodies and dendrites [16].

Here, we determined which isoforms are nuclear by performing a crude fractionation of adult wild-type (C57BL/6) mouse brain, obtaining a nuclear (N), cytoplasmic (C) and total (T) fraction from three mice each. Our analysis confirmed previous findings that all three murine tau isoforms are also found in the nuclear fraction (Figure 3A). Our previous isoform study only analyzed RAB fractions, using dephosphorylated tau [16]. To determine the solubility of nuclear tau, here, we performed a sequential extraction that allowed us to enrich for increasingly insoluble proteins. We found all three isoforms in the nuclear RAB and in the less soluble RIPA fraction, but not the FA fraction (Figure 3B). This indicates that in the nucleus tau exists in different states of solubility. We next determined whether transgenically expressed human tau, which has been shown to be enriched in the somatodendritic domain [24], would also be found in the nuclear fraction. As a negative control for antibody specificity we used tau knockout mice [20]. We analyzed two tau transgenic mouse strains; pR5 mice that express human P301L mutant full-length tau [21], and ΔTau74 mice that express a truncated form of wild-type human tau that lacks the microtubule-binding domain [4]. We found

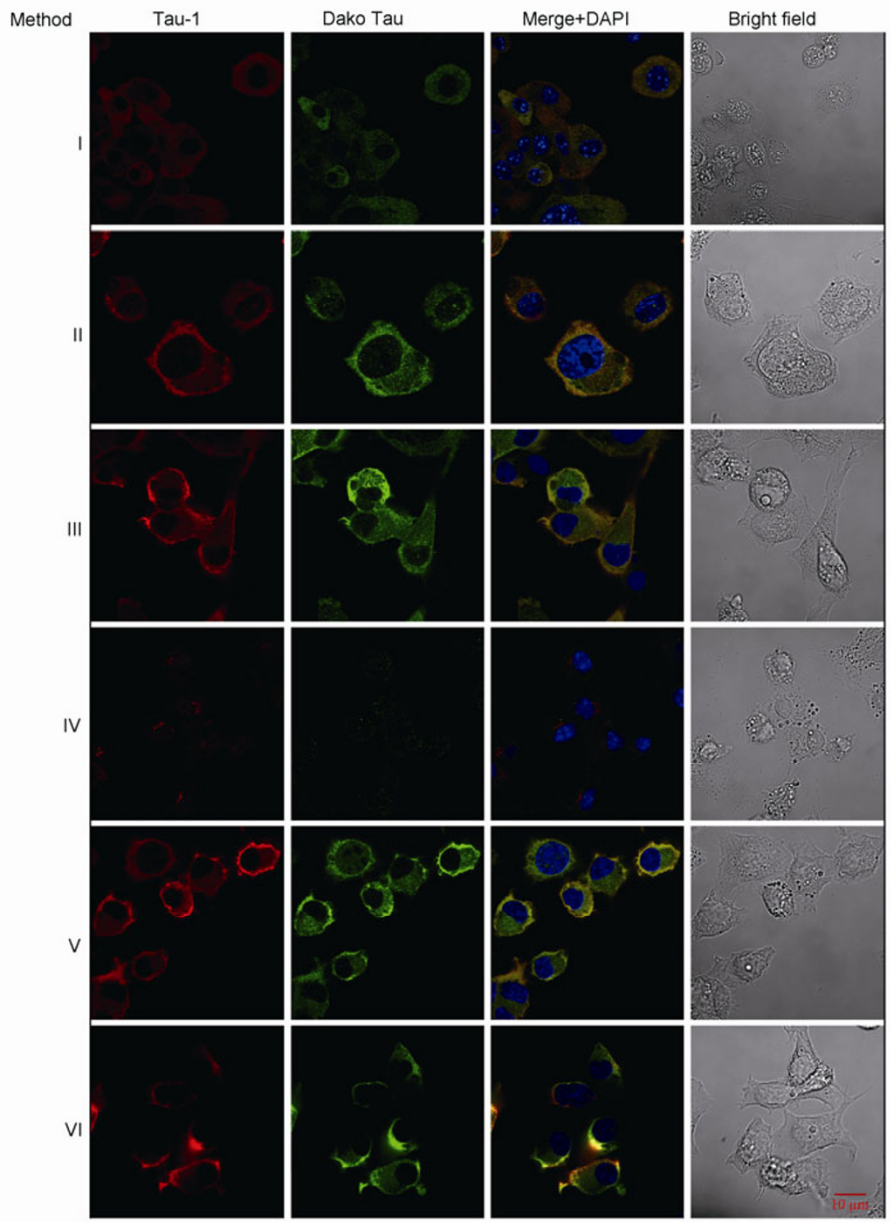

Figure 2 Visualizing endogenous nuclear Tau by immunofluorescence in N2a cells. N2a murine neuroblastoma cells were synchronized by serum deprivation and then processed using the methods listed in Table 1, employing antibodies Tau-1 (in red) and Dako Tau (green) as well as DAPI (blue) for detection. Different from SH-SY5Y cells, N2a cells virtually do not express tau in nuclei. Note: Because of the reverse order of fixation and permeabilization, the bright-field images obtained with protocol IV look different from those obtained with the other five methods.

that both full-length and truncated human tau not only accumulate in the cytoplasmic but also in the nuclear fraction (Figure 3C).

2.3 Subcellular fractionation reveals dephosphorylated tau in the nucleus

Tau is a phospho-protein under physiological conditions. In the adult brain, there are two to three moles of phosphate per mole of tau [11], and this ratio is increased in the developing brain [25]. Because tau is mainly localized to the cytoplasm, we set out to determine whether tau would be phosphorylated to a similar degree in the nucleus as has been found for the cytoplasm. We therefore analyzed the nuclear and cytoplasmic fractions of 3-month-old C57BL/6 mice using the phospho-tau antibodies AT8, AT180, AT270 and 12E8, and found that nuclear tau is largely dephosphorylated (Figure 4A). We quantified the degree of hypophosphorylation of nuclear tau by determining the ratio of phosphorylated tau to total tau levels in the respective fractions (Figure 4B). Together this shows that in adult wild-type mouse brain, tau is strongly dephosphorylated in the nucleus compared to the cytoplasm. Whether this applies to all phospho-epitopes of tau remains to be determined.

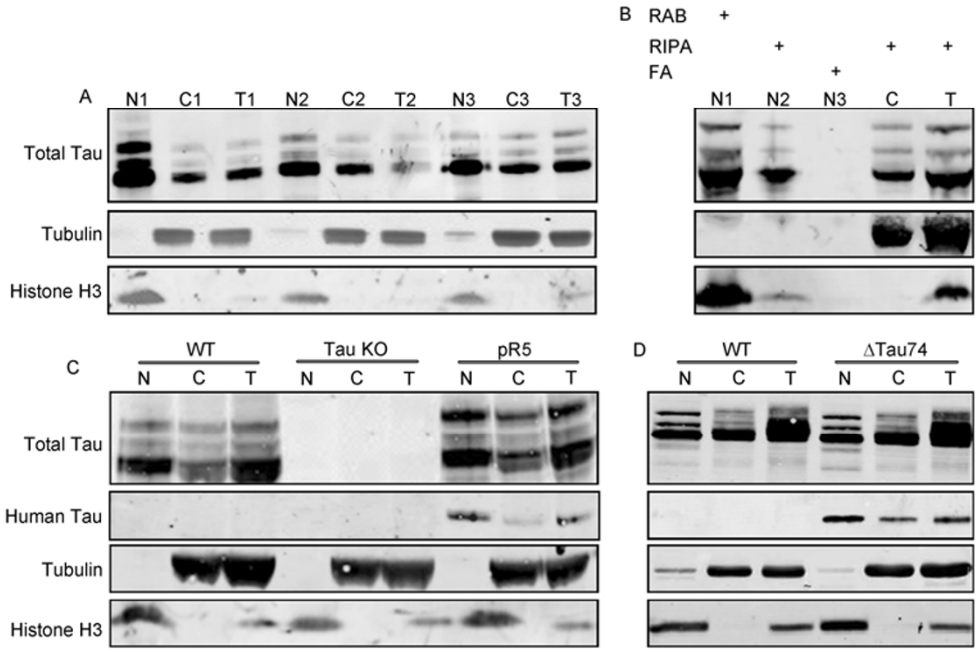

Figure 3 Western blot analysis of subcellular fractionations of brain tissue detects nuclear tau. A, Subcellular fractionation of three wild-type mouse brains into nuclear (N) and cytoplasmic (C) fraction, followed by dephosphorylation. T, total lysate. Tubulin was used as cytoplasmic and histone H3 as nuclear marker throughout. B, Differential extraction of the nuclear fraction into increasingly insoluble fractions reveals all three tau isoforms in the RAB (high salt reassembly buffer) fraction N1, and the RIPA (radioimmunoprecipitation assay buffer) fraction N2, but not the FA (formic acid) fraction N3. C, Fractionation of brains from human P301L mutant tau transgenic pR5 mice, using Tau KO (knockout) mice as controls, reveals transgenic tau in nuclear fraction. D, Analysis of brain fractions from ΔTau74 mice that over-express the projection domain of human tau (and lack the microtubule-binding domain) reveals ΔTau in nuclear fraction.

2.4 Tau can be detected in nuclei isolated from mouse brain

Because there is the possibility that, due to their biochemical properties, cytoplasmic proteins might contaminate the nuclear fraction, we used an alternative method where this potentially confounding factor is ruled out. We therefore isolated intact nuclei from 3-month-old C57BL/6 wild-type and tau knockout brains, followed by staining with tubulin and GAPDH (representing cytoplasmic markers that should be absent), DAPI (nuclear marker), tau, and Lamin B (nuclear envelope marker) (Figure 5). We firstly found for tubulin, GAPDH and DAPI, that the protocol isolated nuclei that were virtually free of any contamination of cytoplasmic markers (Figure 5A). We next performed Z-stacking to demonstrate that the nuclei were intact, as demonstrated by staining with Lamin B and DAPI (Figure 5B). As was evident from the different layers, the Lamin B-labelled nuclear envelope was intact. The staining further revealed that the nuclei were obtained without contamination of cytoplasmic proteins. To reveal nuclear tau staining in wild-type nuclei, we used DAPI and tau staining (Figure 5C). Tau was stained in many but not all nuclei from mouse brain, because not all cell-types in brain are neurons that express tau at substantial levels. As a negative control, we included nuclei from tau knockout brains. Finally, we obtained 3D images reconstructed with Imaris that demonstrated a segregation of DAPI (heterochromatin DNA) and tau staining in nuclei of wild-type mice (Figure 5D). The Imaris workstation was used to reconstruct three-dimensional images to show the relative position of tau and DNA. The Pearson coefficient was calculated, demonstrating that tau and DAPI are most likely not co-localized. However, this does not imply that tau does not interact with DNA. Because tau is localized to the less DAPI-reactive euchromatin, it can still interact with DNA and confer protection from stress. Future studies will investigate whether nuclear tau levels and/or their distribution differ depending on the neuronal cell-type.

3 Discussion

There is an increasing interest in non-canonical functions of tau, a protein traditionally allocated to the axon where it binds to and stabilizes microtubules [26]. The new interest extends to the nucleus where tau is believed to have an important scaffolding function [13,27]. Because, in the past, data on nuclear tau expression have been inconsistent, in part because of different fixation methods and experimental systems, we validated three principal methods: immunocytochemical staining of human and murine neuroblastoma cell lines testing six protocols, subcellular fractionation of wild-type and human tau transgenic mouse brain, and isolation of nuclei from mouse brain.

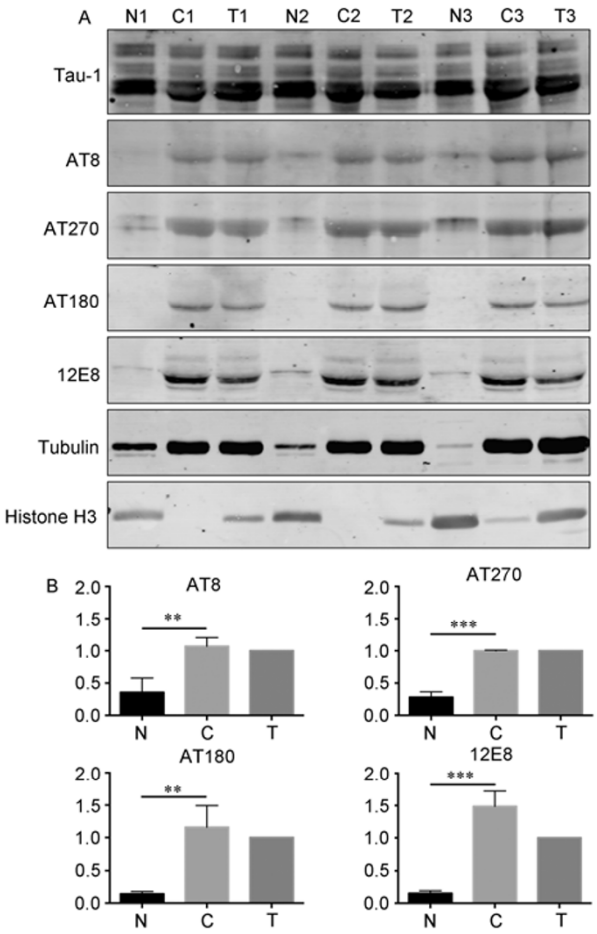

Figure 4 Hypophosphorylation of nuclear tau. A, Analysis of Tau phospho-epitopes AT8, AT180, AT270, and 12E8. B, Quantification of the relative phosphorylation levels in the nucleus, normalizing for total tau. Nuclear (N), cytoplasmic (C) fraction and total lysate (T). Tubulin was used as cytoplasmic and histone H3 as nuclear marker. Data was expressed as mean±SD. **, $P<0.01$; ***, $P<0.001$.

All three methods revealed nuclear tau. Applying the first approach, we found that N2a murine neuroblastoma cells did not contain detectable levels of tau in the nucleus, whereas SH-SY5Y neuroblastoma cells that are human-derived did. For SH-SY5Y cells, not all protocols enabled us to visualize nuclear tau equally well. Rather, we found that fixing the cells with 4% PFA, permeabilizing them with 0.1% PBST and blocking them with 5% NGS yielded the best results, with Dako Tau detecting both cytoplasmic and to some degree nuclear tau, and Tau-1 exclusively detecting nuclear tau, with a pattern that did not overlap with Dako Tau. Our data confirm previous findings that indicated differences in nuclear tau localization between human and murine cell-types [5]. They further suggest that tau exists in different conformations and that the different epitopes are accessible to the respective antibodies in a compartment-specific manner. In other words, the data suggest that while tau is tightly bound to microtubules in axons, in the nucleus it is also tightly bound, although to different proteins, making tau less accessible to antibodies. This notion is supported by the finding that in formaldehyde-treated N2a cells, tau can be detected in the nucleus only with some of the tested tau antibodies [15]. Likewise, in heat shock-treated cortical primary neurons, tau could be visualized in nuclei using either the 3R tau-specific antibody Tau3R or with Tau-1 [13].

Being nuclearly localized, tau was found to be dephosphorylated whereas work by others implies that thermal and other forms of stress cause the opposite, i.e., hyperphosphorylation of tau [15,28]. We found under unstressed conditions that tau is dephosphorylated in the nucleus, reminiscent of what has been reported for both membrane-associated and secreted tau [29].

Crude subcellular fractionation, our second method, showed that all three major adult tau isoforms (0N4R, 1N4R and 2N4R) are present in both the cytoplasmic and nuclear fraction, and that a sequential extraction using RAB, RIPA and FA identifies nuclear tau both in the soluble RAB and in the insoluble (or at least less soluble) RIPA fraction. We did not find tau in the highly insoluble FA fraction. These findings support our previous findings by subcellular fractionation that the 1N4R isoform of tau is under-represented in the chromatin-associated and cytoskeletal fraction and over-represented in the soluble nuclear fraction [16]. Subcellular fractionation is to some degree a crude method and proteins can purify in particular fractions without necessarily being in the corresponding subcellular compartment, simply because their biochemical properties caused them to co-purify with that particular fraction. Here, a novel method might help that performs a proteomic mapping in living cells using spatially restricted enzymatic mapping, with ascorbate peroxidase (APEX) being the tag [30].

As a third method we directly isolated nuclei. Lamin B staining and 3D reconstruction revealed that the nuclei we had isolated were intact. In our view, this is the most convincing evidence for tau being localized in nuclei, especially as we have been using tau knockout nuclei as a control that failed to demonstrate nuclear tau.

What are the functions of nuclear tau? Nuclear tau has been localized to nucleoli and to the nucleolar organizing regions (NORs) where it may have a role in the synthesis and transcription of rRNA [5,31]. A putative role is also in nucleolar organization by binding to pericentromeric DNA [14]. The punctate pattern seen for Tau-1 in our immunocytochemical study in SH-SY5Y cells is reminiscent of that of nucleoli although this has not been further investigated. *In vitro* studies further revealed that purified tau binds to AT-rich sequences in the minor groove of DNA [27]. Using an electrophoretic mobility shift assay (EMSA) it was shown that both the proline-rich domain and the microtubule-binding domain contribute to the interaction of tau with DNA. Tau has further been shown to increase the melting temperature of DNA [32]. Finally, DNA was found

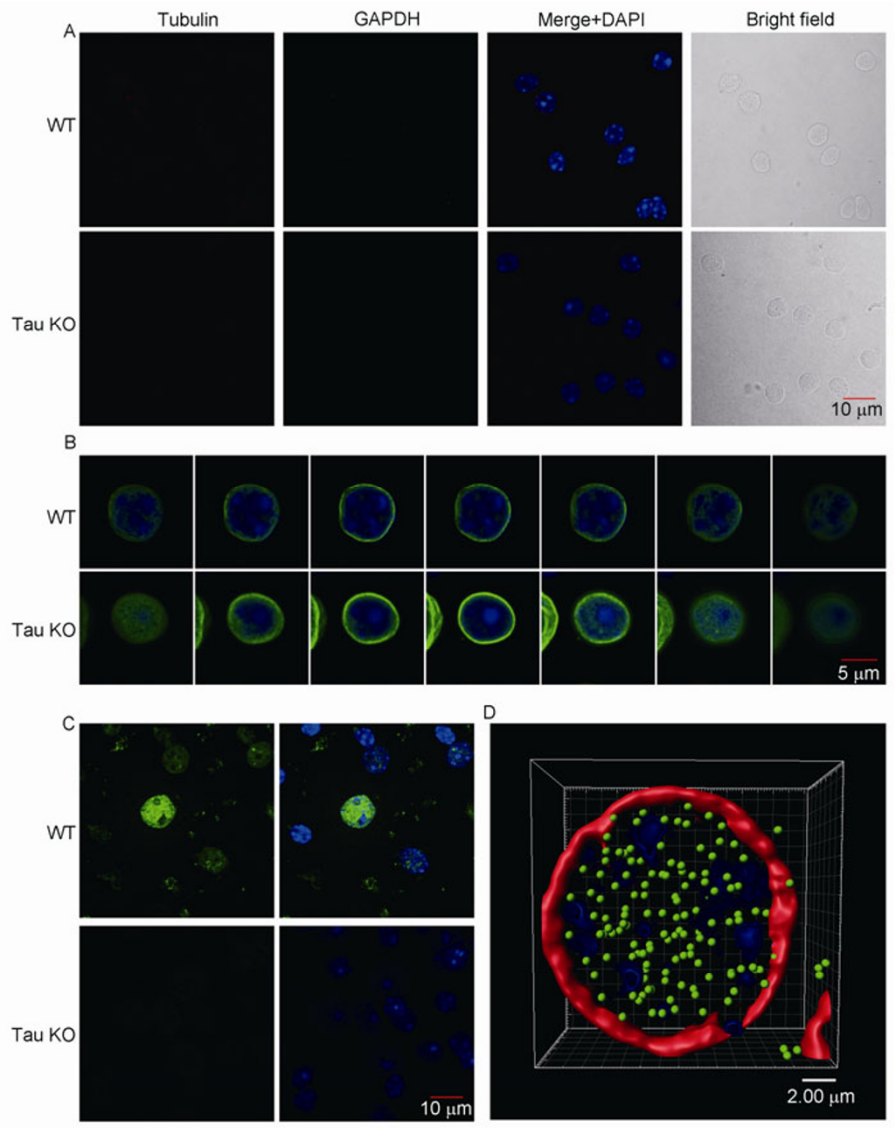

Figure 5 Tau can be detected in nuclei isolated from wild-type mouse brain, using tau knockout (KO) samples as negative control. A, Isolated nuclei (DAPI; blue) were tested with antibodies for tubulin and GAPDH revealing no contamination with cytoplasmic proteins. B, Z-stack imaging of wild-type and tau knockout nuclei showing that the nuclei are intact. Lamin B (green), DAPI (blue). C, Images showing nuclei stained with Dako Tau antibody (green). Top row, wild-type; bottom row, tau KO. D, 3D image reconstruction with Imaris surpass and spot modules, and colocalization calculation with coloc module. Tau (green) distributes independently from DNA (blue). Lamin B (red). Pearson's coefficient, −0.067.

to be protected from free radical damage by tau [27]. In taking this a step further, work in primary neuronal cultures suggested that tau has a role in protecting DNA in the nucleus from damage such as heat shock or oxidative stress [13]. This protective function was correlated with an increased binding of tau to DNA via its AT-rich minor groove. Interestingly, when this interaction was inhibited with the antibiotic netropsin, the unbound tau did not stay in the nuclear fraction. Whether shuttling of tau into the nucleus requires specific cargo proteins remains to be determined. Tau has also a role in the nucleo-cytoplasmic relocalization of nuclear proteins such as the splicing factor SFPQ; however, whether this relocalization is governed by nuclear tau remains to be determined [33].

In conclusion, we have tested six protocols for the visualization of nuclear tau in cultured cells of which several revealed prominent nuclear staining using antibody Tau-1 in SH-SY5Y cells. No nuclear tau was found in N2a cells cultured under normal conditions. Crude fractionation of wild-type mouse brain reveals tau in the soluble RAB and to a lesser extent, the less soluble RIPA, but not the insoluble FA fraction. Tau in the nuclear fraction is hypophosphorylated compared with the cytoplasm. Finally, we present the isolation of nuclei as a viable method to both visualize nuclear tau and investigate its role in the nuclear architecture. Together, this insight will assist in dissecting the role of nuclear tau and in developing a tau-based therapy in AD [34,35].

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