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Pseudophosphorylation of Tau at distinct epitopes or the presence of the P301L mutation targets the microtubule-associated protein Tau to dendritic spines

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ABSTRACT

Alzheimer's disease is characterized by the accumulation of amyloid-β (Aβ) and Tau in the brain. In mature neurons, Tau is concentrated in the axon and found at lower levels in the dendrite where it is required for targeting Fyn to the spines. Here Fyn mediates Aβ toxicity, which is vastly abrogated when Tau is either deleted or a truncated form of Tau (Tau^{1-255}) is co-expressed. Interestingly, MAP2, a microtubule-binding protein with mainly dendritic localization that shares Fyn-binding motifs with Tau, does not mediate Aβ's synaptic toxicity in the absence of Tau. Here we show in hippocampal neurons that endogenous Tau enters the entire spine, albeit at low levels, whereas MAP2 only enters its neck or is restricted to the dendritic shaft. Based on an extensive mutagenesis study, we also reveal that the spine localization of Tau is facilitated by deletion of the microtubule-binding repeat domain. When distinct phosphorylation sites (AT180–T231/S235, 12E8–S262/S356, PHF1–S396/S404) were pseudophosphorylated (with glutamic acid, using alanine replacements as controls), Tau targeting to spines was markedly increased, whereas the pseudophosphorylation of the late phospho-epitope S422 had no effect. In determining the role physiological Fyn has in the spine localization of Tau, we found that neither were endogenous Tau levels reduced in Fyn knockout compared with wild-type synaptosomal brain fractions nor was the spine localization of over-expressed pseudophosphorylated or P301L Tau. This demonstrates that although Fyn targeting to the spine is Tau dependent, elevated levels of phosphorylated Tau or P301L Tau can enter the spine in a Fyn-independent manner.

1. Introduction

Alzheimer's disease (AD), as the most common form of dementia [2], is characterized by two hallmark lesions, plaques that contain the amyloid-β (Aβ) peptide as their major constituent, and neurofibrillary tangles that are mainly composed of heavily phosphorylated forms of the microtubule-associated protein (MAP) Tau [15]. Tau is an unusual protein because it contains 80 serine and threonine residues as well as 5 tyrosine residues [7]. More than a dozen of these sites are already phosphorylated under physiological conditions, although to a varying degree. In AD, Tau is hyperphosphorylated, which means that some of these residues display increased phosphorylation, whereas others are *de novo* phosphorylated [28]. To date, most research has focused on serine/threonine- rather than tyrosine-directed phosphorylation, assisted by the early availability of antibodies that recognize distinct phosphorylated serine and threonine epitopes of Tau.

Tau is up-regulated during neuronal development. The fetal form of Tau is initially distributed evenly in the cell body and neurites (Fig. 1A–H). When axons emerge and neurons become polarized, Tau becomes enriched in axons. In mammalian neurons, this axonal sorting takes place within 1–2 weeks [41]. In mature neurons, under physiological conditions, the majority of Tau is localized to the axon, whereas MAP2, another member of the MAP family, is mainly localized to the dendrite. However, as we have reported recently for brains of adult mice, Tau is also localized, albeit at lower levels, to the dendrite where it serves an important function that cannot be fulfilled by MAP2 [20]. Here Tau is required for the targeting of the Src kinase Fyn to dendritic spines where Fyn phosphorylates the NMDA receptor, a prerequisite for recruiting the postsynaptic density (95kD) protein (PSD95) into a protein complex. This complex then mediates the excitotoxic signaling that is triggered by Aβ, the preferential binding site of which is at the synapse [24]. However, Tau also has a physiological role in the spine where it is thought to participate in spine edification [8]. Furthermore, synaptic activation has been shown to induce the translocation of Tau to the synaptic compartment, where it interacts with filamentous actin

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; FTD, frontotemporal dementia; MAP, microtubule-associated protein; MBR, microtubule-binding repeat; PSD, postsynaptic density; SH, Src homology; SIM, structured illumination microscopy

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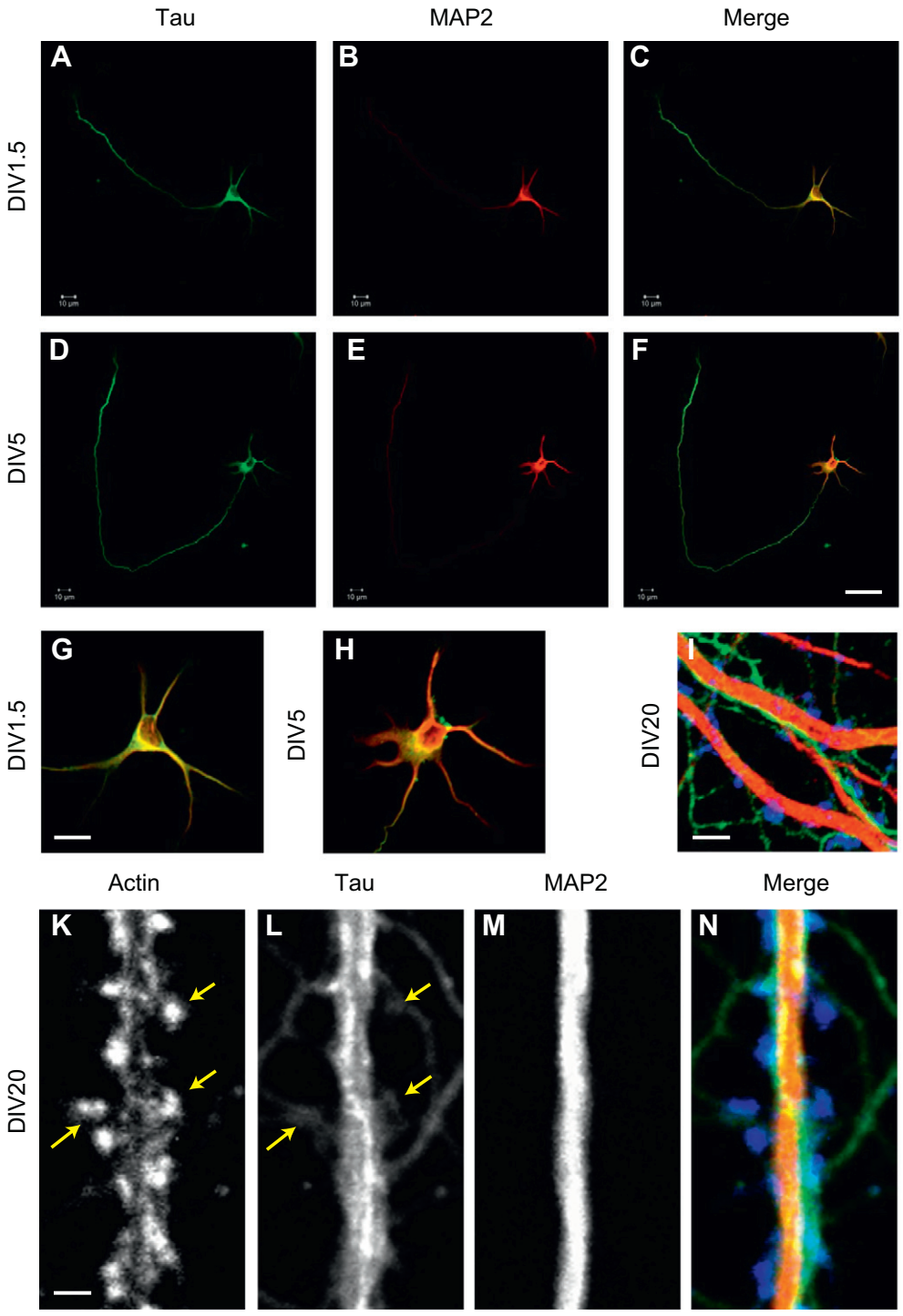

Fig. 1. Tau and MAP2 segregate with advanced primary neuron development; when spines have formed, endogenous MAP2 and Tau are both localized to dendrites, but whereas Tau enters the spines, MAP2 only enters the spine neck. (A–H) With advanced time in culture, primary hippocampal neurons from wild-type mice undergo a process of compartmentalization of Tau and MAP2 (DIV1.5, A–C, G; DIV5, D–F, H; close-ups of cell bodies in G, H). (I) At DIV20, Tau is more concentrated in axons, whereas MAP2 is concentrated in dendrites (Tau in green; MAP2 in red, and actin in blue). (K–N) Using actin to visualize spines (K), localization reveals that unlike Tau, that enters the head of spines (indicated by arrows) (L), MAP2 only enters the neck, although only at low frequency (M). (N) Merged image with Tau (in green), MAP2 (in red), and actin (in blue). DIV, days in vitro. Scale bar: A–F (25 μm), G, H (10 μm), I–N (2 μm).

[13]. The same study also demonstrated that a short exposure of cortical cultures to oligomeric Aβ induces the mislocalization of Tau into the spines under resting conditions and abrogates its subsequent activity-dependent synaptic translocation. Together, these findings suggest differences in synaptic Tau dynamics depending on whether it is physiologically or pathologically phosphorylated.

In the absence of Tau or when a form of Tau is expressed that accumulates in the cell soma and is excluded from the dendrite (such as the 255 amino acid-long ΔTau that contains the projection domain but lacks the microtubule-binding repeats; MBRs), Fyn is prevented from entering the spine, as a result of which Aβ can neither directly nor indirectly excitotoxically signal through the NMDA receptor [20]. This finding has resulted in several models that aim to explain how Aβ, Tau and Fyn interact in the postsynapse to cause damage to neurons [10,19].

Fyn contains an amino-terminal Src-homology (SH) region with acylation sites, a unique domain, an SH3 domain (with which it

interacts with PXXP motifs), an SH2 domain (with which it interacts with phospho-tyrosine residues), an SH1/kinase domain, and a carboxy-terminal regulatory tail [23]. Tau interacts with Fyn at least in two ways. First, the Y18 residue of Tau is phosphorylated by Fyn, with the phosphorylated motif interacting with Fyn via the latter's SH2 domain [36]. Second, Tau contains seven PXXP motifs that are localized amino-terminal of the microtubule-binding domain and are arranged consecutively. Of these motifs, the seventh has been found to be crucial for the interaction with the SH3 domain of Fyn and other Src non-receptor tyrosine kinases [3,20,27]. Interestingly, this motif is also critical for the interaction of Tau with the protein phosphatase PP2A [32]. Why the mainly dendritic protein MAP2 cannot compensate for the absence of Tau in Tau knockout (Tau^{KO}) mice and target Fyn to dendritic spines is not understood, particularly as both MAP2 and Tau efficiently bind Fyn [26,32,39].

In recent years, several groups have investigated the role of Tau at the postsynapse [10]. Aβ was found to change the phosphorylation status of Tau and alter its subcellular localization [40,42], with a specific role reported for the S262 epitope of Tau (a site recognized by the antibody 12E8) [29]. How the Tau/Fyn-interaction is regulated and the relative role of the phospho-Y18/SH2 and PXXP/SH3 interactions are not fully understood. It has been shown that tyrosine phosphorylation of Tau has a role in disease progression [3], possibly by triggering down-stream phosphorylation of serine and threonine residues. In the present study, we expressed truncated, pseudophosphorylated and alanine-mutated (non-phosphorylatable) forms of Tau in primary hippocampal cultures from C57BL/6 wild-type and Fyn knockout (Fyn^{KO}) mice to determine what dictates the localization of Tau to the spine.

2. Materials and methods

2.1. Mouse strains and animal ethics

The mouse strains used in this study included C57BL/6, Tau^{KO} [35], Fyn^{KO} [34], and Tau transgenic pR5 mice that express the P301L mutation found in familial cases of frontotemporal dementia (FTD) [11]. Animal experimentation was approved by the Animal Ethics Committee of the University of Queensland (approval number QBI/027/12/NHMRC).

2.2. Primary culture

Hippocampal neurons from embryonic day (E) 18 wild-type or Fyn^{KO} mouse embryos were plated onto poly-D-lysine-coated coverslips in a 12-well plate at a density of 5,000 cells/well [12]. The plating medium was based on Neurobasal medium (Invitrogen), supplemented with 5% fetal bovine serum (Hyclone), 2% B27, 2 mM Glutamax (Invitrogen), and 50 U/ml penicillin/ streptomycin (Invitrogen). This was changed to serum-free neurobasal medium 24 h post-seeding, and half the medium was changed twice a week.

2.3. Mutagenesis and molecular cloning

Tau mutant constructs were generated by site-directed mutagenesis, using a pENTR donor vector containing full-length human 2N4R Tau (hTau40, 441aa) as a template. To introduce a V5 tag at the carboxy-terminus, Gateway cloning was used, and the Tau cassette was transferred from the pENTR donor vectors into the pcDNA6.2/V5-DEST vector, using LR clonase (Invitrogen). The following expression vectors were generated: ΔTau (Δ256-441), ΔPXXP (Δ176-242); ΔMTB (Δ256-367); P301L, Y18A; mutants of the AT180 epitope (T231A/S235A and T231E/S235E); mutants of the 12E8 epitope (S262A/S356A and S262E/S356E); mutants of the PHF1 epitope (S396A/S404A and S396E/S404E); and mutants of the serine 422 epitope (S422A and S422E). In addition, an additional myc-tagged construct was generated for AT180A (T231A/S235A). For an overview of the Tau phosphorylation sites used in this study, see Chen et al. [7]. The MAP2c-EGFP plasmid was kindly provided by Dr. Estelle Sontag (University of Newcastle, Australia). We thank Dr. Xiaobing Yuan (Department of Neurobiology and Anatomy, Drexel University College of Medicine, USA) for the EGFP-α-tubulin plasmid.

2.4. Immunocytochemistry and imaging analysis

Neurons were transfected at 18 days in vitro (DIV18) using Lipofectamine 2000 (Invitrogen). To visualize endogenous Tau and MAP2, images were also taken at DIV1.5 and DIV5. A red fluorescent protein (tagRFP, in short: RFP; Evogen) expression vector was co-transfected to visualize neurons fully, i.e., including the spines. Forty-eight hours after the transfection, cells were washed once with PBS, fixed with 4% paraformaldehyde/4% sucrose for 10 min, permeabilized with 0.2% Triton X-100 for 10 min, then blocked for 1 h in 10% goat serum, followed by incubation overnight at 4 °C. Primary antibodies were mouse anti-myc (Sigma, 1:500), rabbit anti-V5 (Sigma, 1:500), rabbit anti-human Tau (Dako, 1:500), rabbit anti-MAP2 (Millipore; 1:500), mouse anti-Tau5 (Millipore, 1:500), and mouse anti-PSD95 (Millipore, 1:500). Secondary antibodies were Alexa Fluor (488, 555, 647)-labeled goat anti-mouse and goat anti-rabbit IgG antibodies (Invitrogen). We further used Alexa Fluor647-labeled phalloidin (Invitrogen) to detect actin. RFP was co-transfected as a control. Fluorescence images were taken with a 20 or 60× objective on a Zeiss LSM710 laser scanning confocal microscope. Super-resolution structured illumination microscopy (SIM) images were taken with a 60X objective on a Zeiss ELYRA PS.1 SIM/STORM microscope.

2.5. Synaptosomal preparations and western blot analysis

Synaptosomes were prepared from dissected forebrains ($n = 4$ for the each genotypes) using a modified volume-adjusted method based on a previously published protocol [20]. In brief, brain tissue was homogenized in sucrose buffer (1 ml/60 mg tissue, 0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$) using a dounce homogenizer (800 rpm, 10 strokes). The brain homogenate was centrifuged at 1,400*g* for 10 min. Two hundred microliters of the supernatant was collected as the "whole brain lysate," and the pellet containing cell debris and nuclei was discarded. The remaining supernatant was then centrifuged at 14,000*g* for 20 min to obtain the crude synaptosomal fraction in the pellet. The pellet was then re-suspended in 2 ml sucrose buffer, layered over a discontinuous Ficoll gradient (4 ml of both 5% and 12%), followed by centrifugation at 80,000*g* for 1 h (SW41 Ti Rotor, Swinging Bucket, Beckman). A milky interface containing the synaptosomes was recovered from the layer between the 5% and 12% Ficoll layers; this was topped-up with sucrose buffer to 3 ml and centrifuged at 45,000*g* for 25 min to pellet the synaptosomes (TLA-100 Rotor, Fixed Angle, Beckman). Purified synaptosomes were then sequentially extracted with buffers of increasing stringency: pH 6 (40 mM Tris–HCl, 2% Triton X-100, 0.5 mM $CaCl_2$), pH 8 (20 mM Tris–HCl, 1% Triton X-100), and SDS (5% SDS) as previously described [30]. The SDS buffer extract contains strongly PSD-associated proteins. All solutions contained protease inhibitors (Complete Protease Inhibitor Cocktail tablets, Roche) and phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4, and 2 mM $Na_4P_2O_7$, all from Sigma). All centrifugations were carried out at 4 °C. For western blot analysis, the following primary antibodies were used: total Tau (Dako, polyclonal, 1:5,000), Fyn (BD Biosciences, 1:2,000), PSD95 (Millipore, 1:2,000), MAP2 (Sigma, monoclonal, 1:1,000), and actin (monoclonal, Millipore, clone C4, 1:5,000).

2.6. Image and data analysis

Image analysis was carried out using Zen (Zeiss imaging) and Image-J (NIH imaging) software. For each group, in three independent experiments, at least 500 spines were analyzed from more than 20 neurons. For the analysis, dendritic branches were selected from secondary

dendrites. For each spine, a region of interest (ROI) in the spine head and one in the dendritic shaft were determined in the RFP channel, and also used for the Tau channel. Furthermore, for a subset of the samples, we determined the mean intensity in the spine as a function of the mean intensity in the dendritic branch, followed by a Pearson correlation analysis. This single neuron analysis was done to rule out that the spine localization of Tau is simply a reflection of higher expression levels. The average intensity of Tau was measured in the ROI, subtracting the background of the image. The localization of the different tagged proteins to spines was determined by obtaining the ratio of the mean intensity in the spine to the mean intensity in the dendritic shaft for Tau, followed by normalization of the value obtained for RFP that distributes evenly throughout the dendrite and the spines. An unpaired *t*-test was used for statistical analysis.

3. Results

3.1. Endogenous Tau enters the dendritic spine, whereas MAP2 is confined to its neck

Both Tau and MAP2 can be easily detected in primary neuronal cultures irrespective of their phosphorylation state. This differs from the immunohistochemical staining of wild-type mouse tissue, in which endogenous Tau, different from MAP2, is difficult to detect possibly because it is so tightly bound to microtubules that it cannot be labeled by Tau-specific antibodies [14]. As a primary hippocampal neuron differentiates, Tau and MAP2 begin to compartmentalize as shown for the processes and cell bodies with images taken from DIV1.5 (1.5 days in vitro) and DIV5 neurons, and Tau becomes enriched in axons whereas MAP2 becomes enriched in dendrites (Fig. 1A–H). A close-up is shown at DIV20, with Tau being concentrated in the axon and MAP2 in the dendrite (Fig. 1I). Given the distribution of Tau and MAP2, it is not understood why the predominantly dendritic MAP2 cannot compensate for the absence of the primarily axonal Tau in Tau^{KO} mice, thereby targeting Fyn to dendritic spines, particularly because both MAP2 and Tau efficiently bind Fyn. By analyzing DIV20 wild-type hippocampal cultures and using actin staining via phalloidin to visualize the spines, we found that both endogenous MAP2 and Tau are localized to dendrites; however, whereas Tau entered the spine head, MAP2 mostly remained localized in the dendritic shaft, entering the neck of only 6.5 % of spines (analyzing 200 neurons) and never the spine head (Fig. 1K–N). This might explain why MAP2 cannot compensate for Tau in localizing Fyn to the spine head.

3.2. Over-expressed MAP2 is mainly excluded from spines, whereas P301L Tau is enriched

We next aimed to determine whether over-expression of MAP2 would target it to the spine. We also investigated how the spine localization of Tau is affected by the presence of the P301L mutation, by deleting functional domains of Tau, and by either introducing pseudophosphorylation sites or over-expressing constructs in which critical phosphorylation residues had been mutated to alanine to abrogate phosphorylation (Fig. 2). All constructs were generated using the longest human Tau isoform, hTau40, as a template, and by adding a V5-tag at the carboxy-terminus (except for MAP2c and α-tubulin that were tagged with EGFP).

For all transfections, using triplicates, more than 500 spines were counted from at least 20 secondary branches. We found that even full-length MAP2c, although targeted to the dendrite, was mainly excluded from spines, meaning that it was either not efficiently targeted to the spine head or not efficiently retained. This finding was obtained by comparing the distribution in the spine with that in the dendritic branch, using RFP that distributes throughout the entire cytoplasm and into the spines for normalization (Figs. 3A and 6A). As a control, we included EGFP-tagged α-tubulin (Fig. 3B). When full-length Tau was over-expressed, it was targeted to dendritic spine heads at relatively low levels compared with RFP, whereas MAP2c showed no localization at all (Figs. 3C and 6A). Confirming previous findings [17,20], P301L Tau was found to be targeted to the spine at a five-fold higher rate than non-mutated, wild-type Tau ($P < 0.001$) (Figs. 3D and 6B). A Pearson correlation analysis revealed that the increased targeting of P301L Tau to spines is not because of an increased expression of P301L Tau compared with wild-type Tau (wild-type Tau: $r = 0.79$, $P = 0.011$; P301L Tau: $r = 0.72$, $P = 0.015$).

To obtain a higher resolution of Tau localization, structured illumination microscopy (SIM) was used. This revealed that Tau in P301L Tau over-expressing neurons does not co-localize with PSD95 but may instead form a scaffold that supports the PSD (Fig. 3E–G).

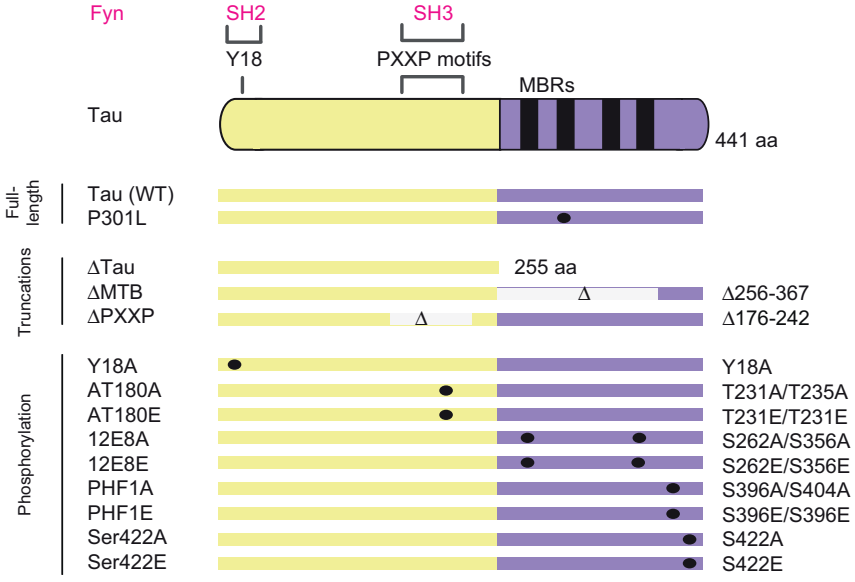

Fig. 2. Tau mutation/truncation constructs. Tau constructs generated to determine the role of critical epitopes in the localization of Tau to spines. The longest human Tau isoform, hTau40, was used as a template. This contains four microtubule-binding domains (MBRs) that are indicated in black. Fyn has been shown to bind Tau via its SH3 domain and, in a phosphorylation-dependent manner, via its SH2 domain. All Tau constructs contain a short V5-tag.

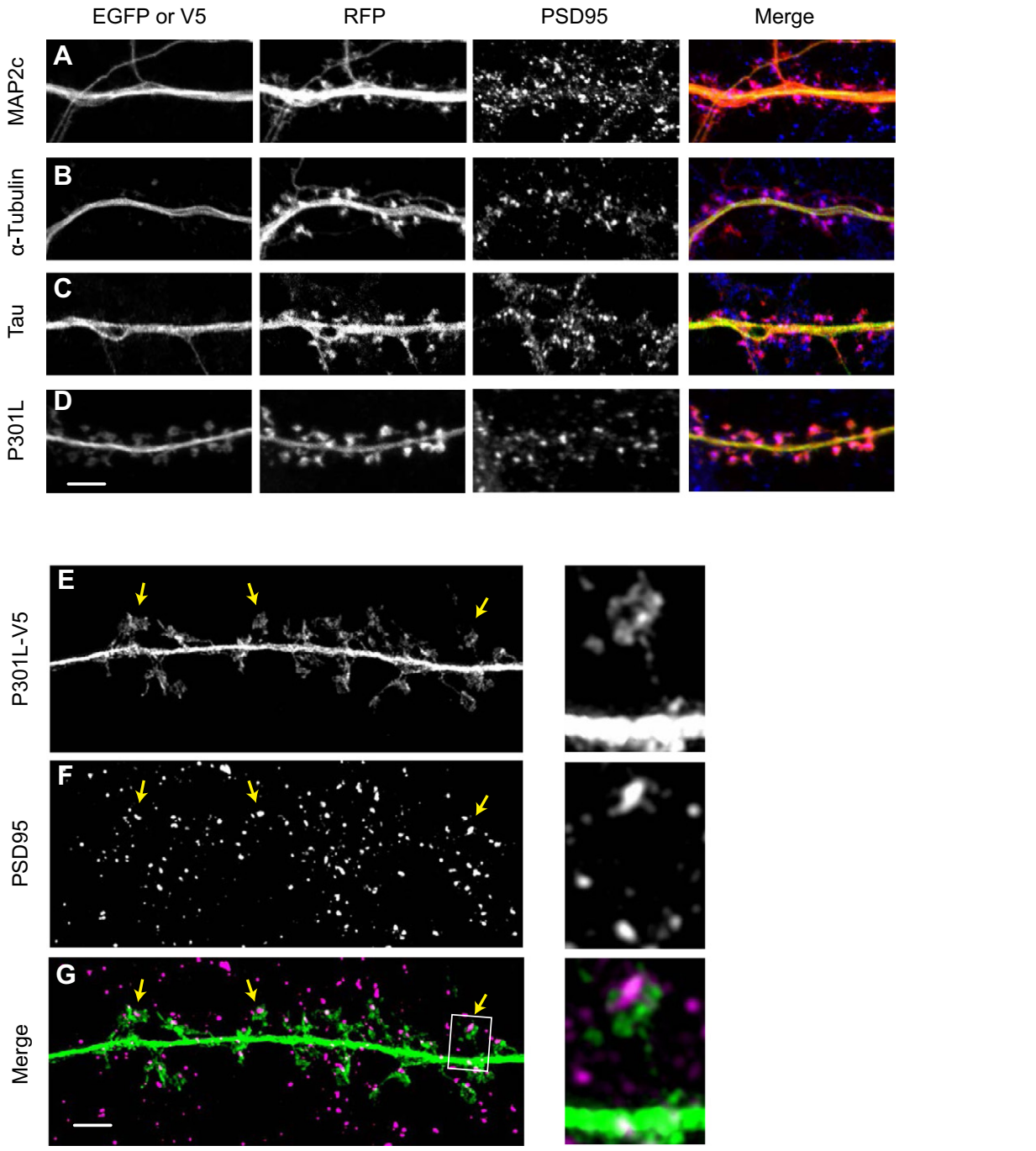

Fig. 3. Over-expressed MAP2 is mainly excluded from spines, whereas P301L Tau is enriched in spines. Wild-type neurons co-transfected with different V5-tagged expression constructs together with RFP to reveal dendrites and spines. (A) When full-length MAP2 tagged with EGFP is over-expressed, it is targeted to the dendrite but excluded from spines, which are visualized with RFP. (B) EGFP-tagged α-tubulin is included as control. (C) When full-length Tau is over-expressed, it is targeted to the dendrite but mostly excluded from spines. (D) In contrast, P301L Tau is targeted at high levels to the spine. (E–G) Structured illumination microscopy (SIM) was used to determine that when P301L Tau is over-expressed (E) it does not co-localize with PSD95 (F) in the spine (G, merge). A close-up is shown on the right for the area marked by a white box in G. Scale bar: 2 μm.

3.3. Spine localization of mutants lacking PXXP motifs or the microtubule-binding domain

We next compared the localization of different Tau truncation mutants to that of full-length Tau (Fig. 4A): ΔTau that comprises the projection domain and contains the first 255 amino acids of the Tau protein (based on the longest human Tau isoform) (Fig. 4B), ΔMTB that lacks the MBRs but contains the projection domain as well as the carboxy-terminus of the protein (Fig. 4C), and ΔPXXP that lacks the seven PXXP binding motifs, including those that interact with the SH3 domain of Fyn (Fig. 4D). By comparing the localization with that of full-length Tau, we observed a reduced localization of ΔTau to the dendrite, with a very low signal detectable beyond the primary dendrite (Fig. 4B). This extends previous findings obtained *in vivo* [20]. ΔMTB was found to distribute as freely as RFP (P = n.s.), including into spines, suggesting that the binding of Tau to microtubules hinders its free distribution (Figs. 4C and 6A). The PXXP motifs of Tau are critical for the interaction of Fyn with Tau (Fig. 2). However, we found under conditions with physiological Fyn levels and activity that deletion of the PXXP motifs did not cause a difference in the targeting of Tau to the spine from that of the full-length construct (P = n.s.) (Figs. 4D and 6).

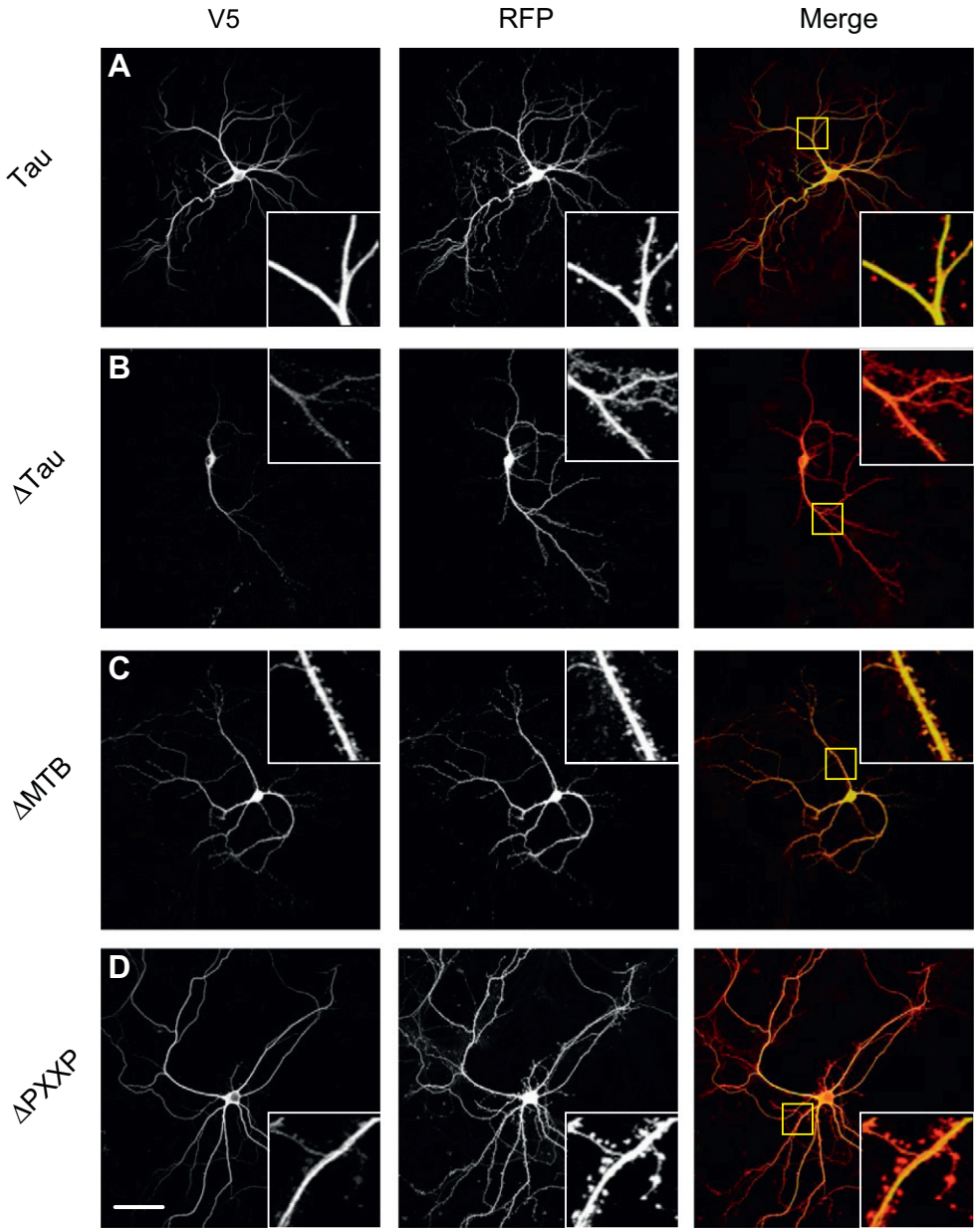

Fig. 4. Spine localization is facilitated by the deletion of the microtubule-binding domain. (A) When wild-type Tau is over-expressed, it distributes throughout the neuron but is mostly excluded from the spines (see close-up, and RFP localization that was used as a positive control for spine localization). (B) ΔTau displays reduced localization to the dendrite, with a very low signal detectable beyond the primary dendrite. (C) ΔMTB lacks the region containing the MBRs and distributes freely, including into spines. (D) Deletion of the PXXP binding sites that interact with the SH3 domain of Fyn does not result in spine localization that is different from that of wild-type Tau. Scale bar: 20 μm.

3.4. Pseudophosphorylation of distinct sites (AT180, 12E8, PHF1) alone is sufficient to target Tau to spines, different from the pseudophosphorylation of the late phospho-epitope S422

Several of the "classical" phosphorylation epitopes of Tau (e.g. AT180) are defined by two amino acid residues (T231/S235), and hence, for our study, both sites were mutated together. To assess the role of distinct phosphorylation sites in spine localization, we generated a series of constructs by changing serine, threonine, and tyrosine residues either to alanine (A) to prevent phosphorylation, or to negatively charged glutamate residues (E) in order to mimic phosphorylation. We thus over-expressed a series of Tau constructs that were tagged at the carboxy-terminus with a V5-tag (Fig. 2): Y18A (mutating the tyrosine residue that is phosphorylated by Fyn) (Fig. 5A), AT180A (Fig. 5B), AT180E (Fig. 5C), 12E8A (Fig. 5D), 12E8E (Fig. 5E), PHF1A (Fig. 5F), PHF1E (Fig. 5G), S422A (Fig. 5H), and S422E (Fig. 5I). RFP was used to visualize the spines, with the relative quantification shown in Fig. 6A. A Pearson correlation analysis revealed that, e.g., the increased targeting of AT180E Tau to spines is not because of an increased expression of this mutant compared with that of AT180A (AT180E Tau: $r = 0.81$, $P = 0.008$; AT180A Tau: $r = 0.84$, $P = 0.004$) (Fig. 6C). The analysis revealed that all A mutants were precluded from entering the spines beyond the levels found for non-mutant, full-length Tau. Of the E mutants, all but the S422E mutant showed an increase in spine localization that was in the order of 4- to 5.5-fold. Together these findings indicate that (i) phosphorylation levels are low under normal conditions, (ii) introducing a pseudophosphorylation at one site is sufficient to target Tau to the spine, and (iii) S422 behaves differently from the other investigated phospho-epitopes, possibly reflecting the fact, as shown in animal studies, that this is an epitope that is phosphorylated relatively late in disease (i.e., when Tau filaments have already formed) [1]. The S422 site may thus have no role in

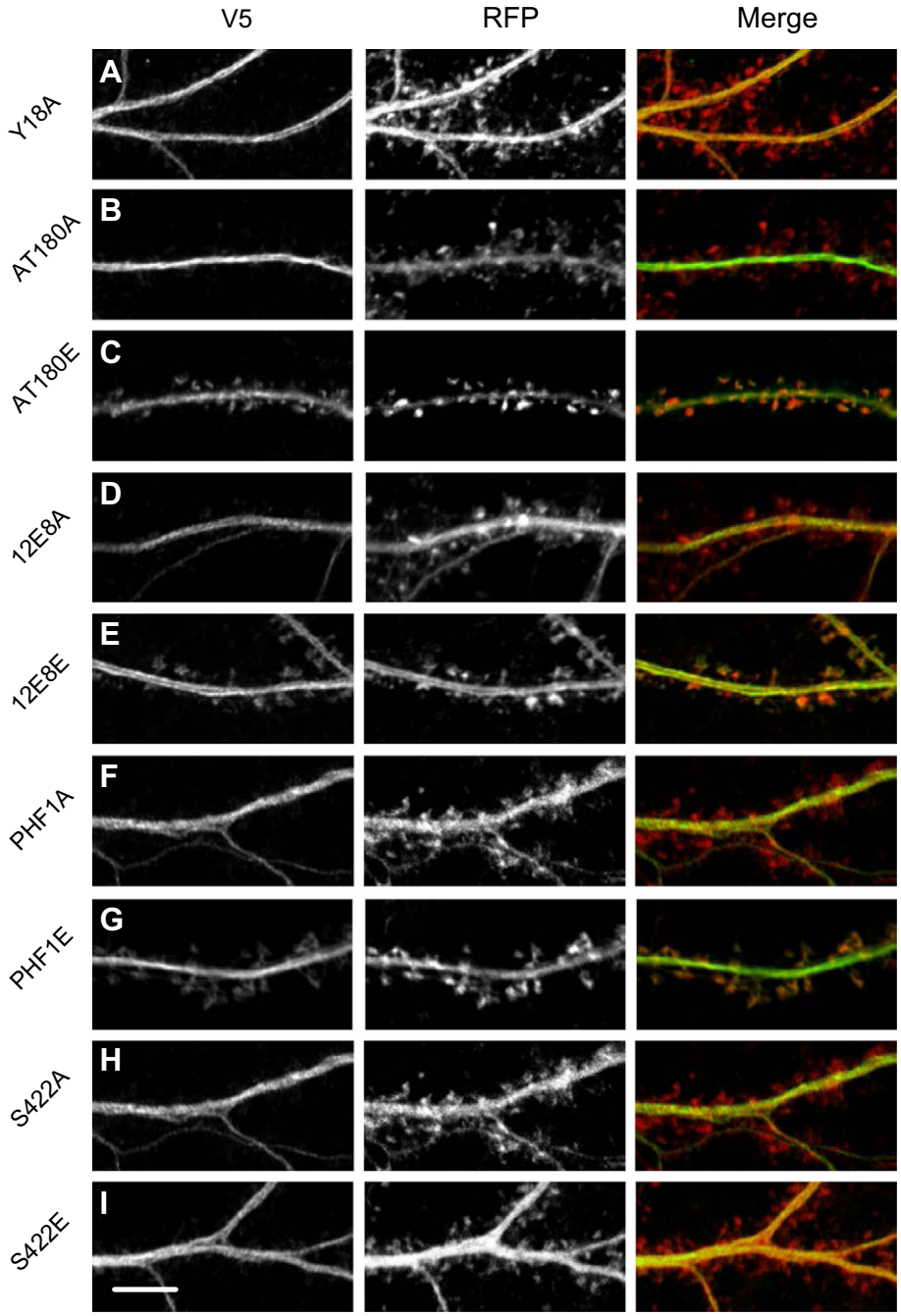

Fig. 5. Pseudophosphorylation of distinct sites (AT180, 12E8, PHF1) alone is sufficient to target Tau to spines, different from the pseudophosphorylation of the late phospho-epitope S422. Tau was over-expressed in primary neuronal cultures, with spine targeting determined for different V5-tagged Tau constructs: Y18A (A), AT180A (B), AT180E (C), 12E8A (D), 12E8E (E), PHF1A (F), PHF1E (G), S422A (H), and S422E (I). RFP was used to visualize the spines. Merged images are shown for Tau (in green) and RFP (in red). Scale bar: 4 μm.

regulating the subcellular distribution of Tau, different from Y18, T231/S235 (AT180), S262/S356 (12E8), and S396/S404 (PHF1). The strongest localization effect (when considering the A/E ratio) was seen for the AT180 epitope, which was therefore analyzed further.

3.5. AT180E Tau is targeted to the spine whereas AT180A is excluded from it

Interestingly, the AT180 epitope (a site that is hyperphosphorylated in AD) overlaps with the seventh PXXP motif ($pT^{231}PPKS^{235}P$) that has a role in the interaction of Tau with both Fyn and the protein phosphatase PP2A [32]. We co-transfected wild-type neurons with a V5-tagged AT180E construct and a myc-tagged AT180A construct and performed immunocytochemistry and confocal microscopy. The A and E channels were exposed to the laser power so as to obtain an unsaturated and comparable signal intensity in the branch, allowing for a comparison of the signal intensity in the spine (Fig. 7). Using RFP as a marker, we confirmed that even under co-expression conditions, AT180A is mainly excluded from spines, whereas a significant proportion of AT180E in the same neurons is localized to the spines.

3.6. Absence of Fyn does not abolish Tau targeting to spine

Tau has previously been shown to be required for the targeting of Fyn to the dendritic compartment and, more specifically, the spine [20]. To determine whether the opposite relationship also exists, namely, that Tau localization to spines is Fyn dependent, we determined for a

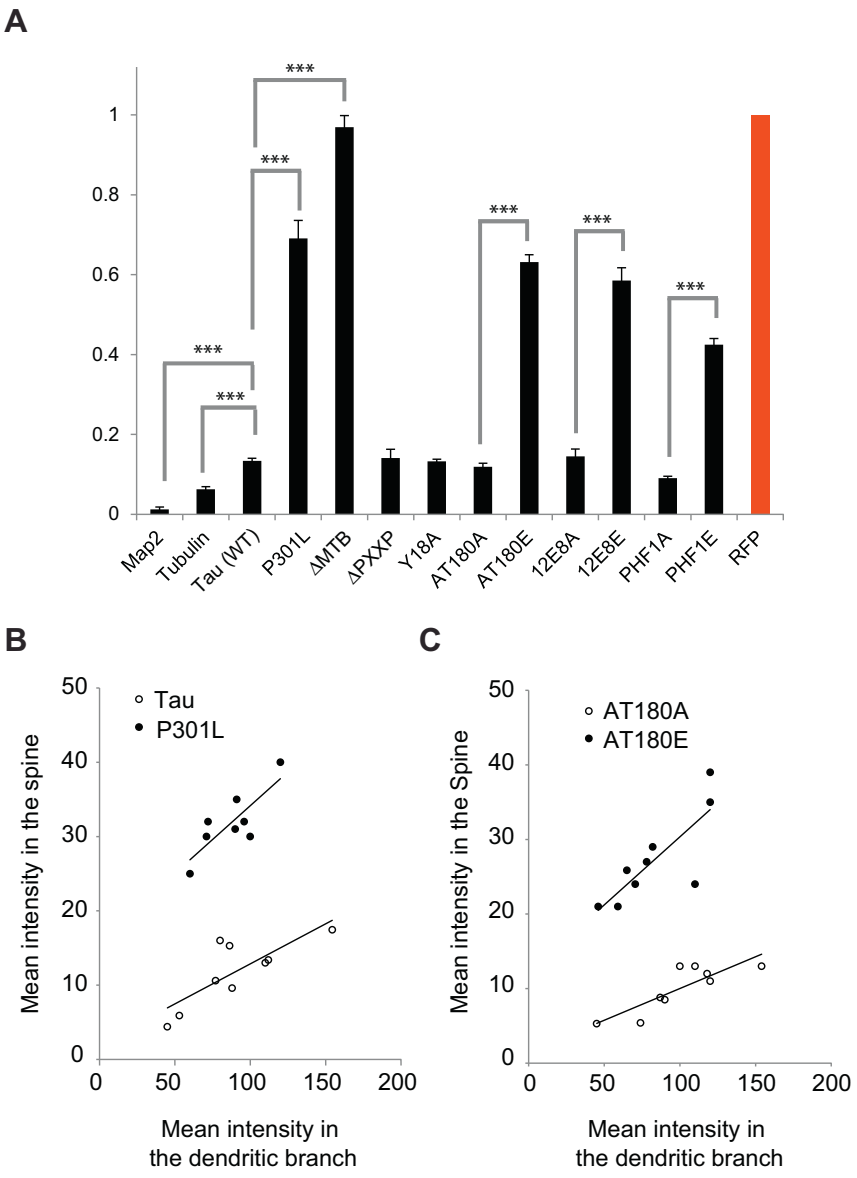

Fig. 6. Quantification of the spine targeting ratio of MAP2, wild-type Tau and Tau mutants. (A) Quantification of relative spine levels by determining the ratio of the mean intensity in the spine compared to the dendrite for each transfectant, followed by normalization of the value obtained for RFP, which distributes evenly throughout the dendrite and the spines. For each experimental group, in three independent experiments, at least 500 spines were analyzed from more than 20 neurons each (***$P < 0.001$). (B, C) Pearson correlation coefficient analysis of two representative pairs of transfected neurons, with the imaging done at the single neuron level (wild-type Tau: $r = 0.79$, $P = 0.011$; P301L Tau: $r = 0.72$, $P = 0.015$; AT180E Tau: $r = 0.81$, $P = 0.008$; AT180A Tau: $r = 0.84$, $P = 0.004$).

series of mutants with prevalent spine localization (P301L, AT180E, 12E8E, and PHF1E) whether this localization was altered compared to wild-type neurons, when the Tau mutants were over-expressed in Fyn^{KO} primary neuronal cultures (Fig. 8A). We also included the AT180A mutant that in wild-type neurons is mainly excluded from the spine (Fig. 5 and 7). We found that spine localization was not affected by deletion of Fyn. In the case of the P301L Tau mutant, there was a slight reduction in spine localization when Fyn was absent, although the biological significance of this finding remains to be determined (Fig. 8B).

Using biochemical methods, we have previously reported that levels of synaptic Fyn are reduced by 73% and 62% in synaptosomal PSD95 fractions obtained from pooled hippocampi of ΔTau transgenic and Tau^{KO} mice, respectively, compared to wild-type controls [19,20]. We thus obtained brain extracts and synaptosomal PSD fractions by sucrose fractionation from total forebrains of wild-type, Fyn^{KO}, and P301L mutant Tau transgenic pR5 mice and analyzed these for levels of Fyn, Tau, MAP2, actin, and PSD95 (Fig. 9). Loading equal amounts of protein (10 μg), the analysis revealed that neither total (normalized for actin, $P = 0.75$) nor synaptosomal levels (normalized for PSD95, $P = 0.12$) of Tau differed when Fyn^{KO} was compared with wild-type mice (Fig. 9). While this analysis demonstrates that the absence of Fyn does not abolish the targeting of Tau to the spines, it does not rule out that Fyn has a role when this kinase is over-activated as is the case in AD. We also found confirming the above spine localization analyses that whereas MAP2 was present in total extracts, it was absent from the synaptosomal preparation.

4. Discussion

In the present study, we expressed different truncated, pseudo-phosphorylated, and alanine-mutated forms of the microtubule-associated protein Tau in primary hippocampal neurons to determine what factors dictate the localization of Tau to dendritic spines, as well as the role of Fyn in this process. We also compared the spine localization

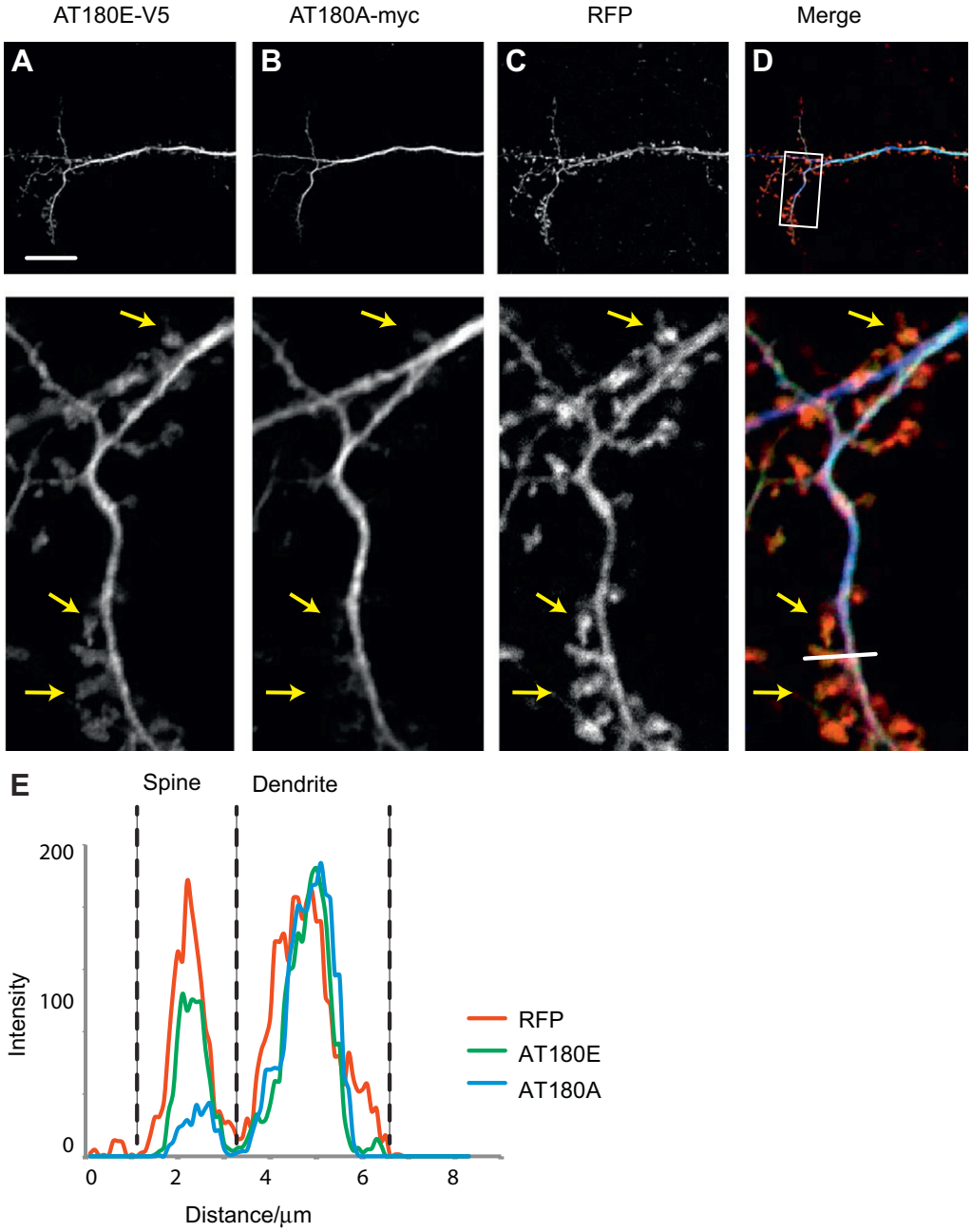

Fig. 7. AT180E Tau is targeted to the spine whereas AT180A is excluded from it. Wild-type neurons were co-transfected with AT180E-V5 and AT180A-myc followed by immunocytochemistry and confocal microscopy. The A and E channels were exposed to the laser power so as to obtain a comparable signal intensity in the branch, thereby allowing for a comparison of the signal intensity in the spine. (A) AT180E-V5, (B) AT180A-myc, (C) RFP, and (D) merge. A close-up is shown in the second row of images for the area marked by a box in D, with arrows pointing at spines. (E) Line profile of a selected spine (see section in close-up below D) revealing that AT180A is mainly excluded from the spine whereas a significant fraction of AT180E is localized to the spine (indicated by arrows). Scale bar: 10 μm (A–D).

of both endogenous and over-expressed Tau with that of MAP2, a microtubule-binding protein that is mainly localized to the dendritic compartment of mature neurons.

We first found in mature primary hippocampal cultures obtained from wild-type mice that endogenous Tau is targeted to the spine, albeit at low levels. This extends our previous findings, obtained in brain sections and by fractionation, that a small proportion of Tau is localized to the dendritic compartment under physiological conditions [20]. Interestingly, there is evidence for brain region-specific differences in this localization, with Hoffmann and colleagues reporting the presence of hyperphosphorylated Tau in dendritic spiny protrusions of CA3 hippocampal neurons, but not in the dendritic spines of pyramidal neurons of cortical layer V [16]. The same study also demonstrated the absence of Tau from the spines of neurons in which the somatodendritic compartment was almost completely filled with filamentous hyperphosphorylated Tau. Although the reason for this brain region specificity is not understood, differences in the principal cytoskeletal elements in hippocampal versus cortical spines may have a role. Unlike dendritic spines of cortical pyramidal neurons, which have an actin-based cytoskeleton, the CA3 thorny excrescences occasionally contain microtubules [9,33]. Indeed, in our confocal analysis of hippocampal cultures, we found low levels of tubulin staining in the dendritic spines (data not shown).

We next analyzed MAP2 as the "typical" dendritic representative of the MAP family. Interestingly, MAP2 was mostly restricted to the dendritic shaft and only occasionally entered the neck of the spine,

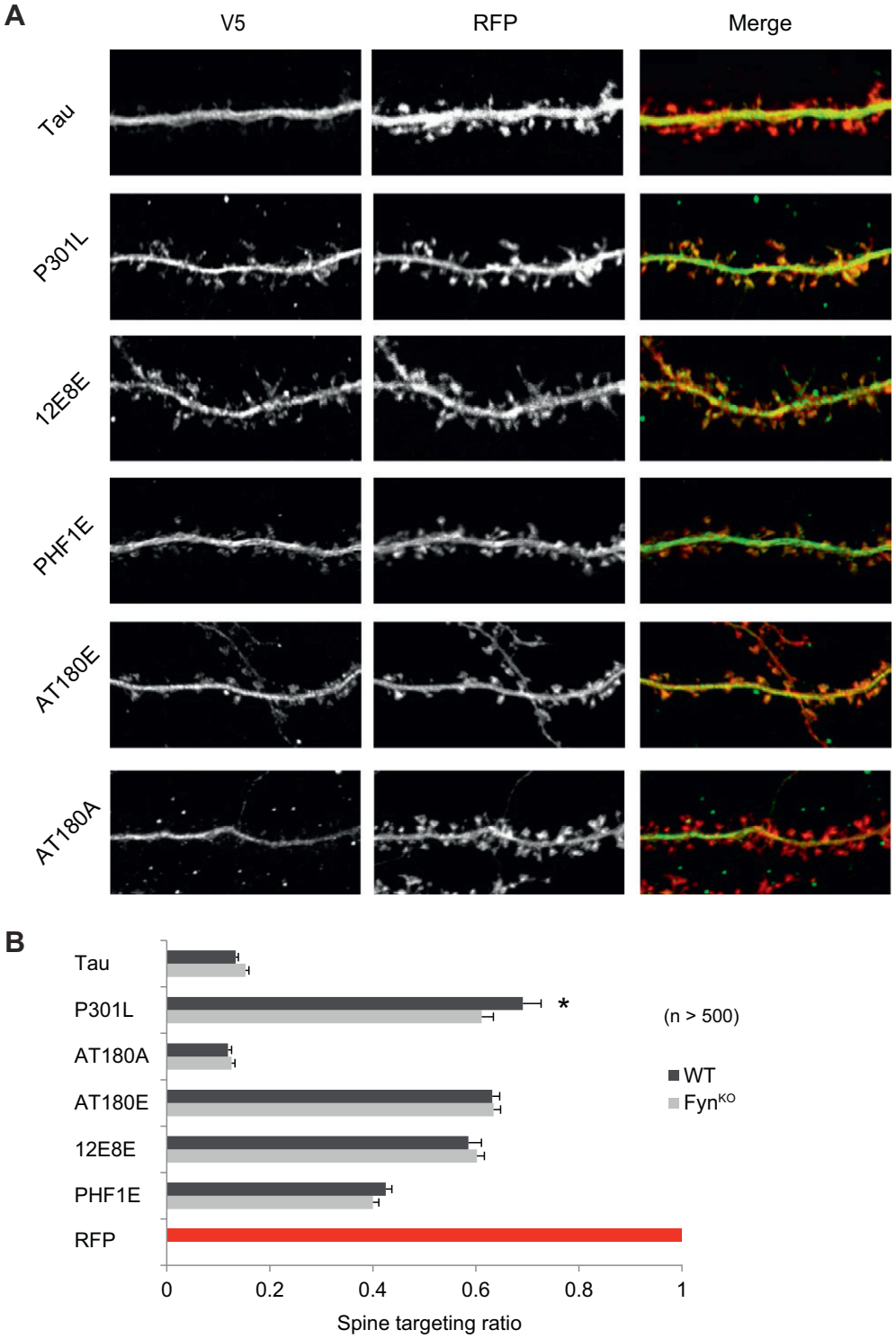

Fig. 8. Absence of Fyn does not abolish Tau targeting to spine. (A) When Tau is over-expressed in Fyn^{KO} primary neuronal cultures, the localization of phospho-Tau mutants does not differ from that in wild-type neurons. (B) For each experimental group, in three independent experiments, at least 500 spines were analyzed from more than 20 neurons each. A slight reduction in spine localization is seen for P301L Tau when Fyn is absent (*$P < 0.05$).

whereas Tau entered the spine head. This finding is supported by the western blot analysis, which revealed an absence of MAP2 in the synaptosomal fraction. Together this would explain why MAP2 cannot compensate for the absence of Tau in Tau^{KO} mice in targeting the tyrosine kinase Fyn to dendritic spines, despite the fact that both Tau and MAP2 share crucial binding sites for Fyn and the serine/threonine-directed phosphatase PP2A, which both directly and indirectly regulates Tau phosphorylation [21,26,32,39]. What remains unexplained is why MAP2 does not enter the spine head. Even by increasing the expression levels by over-expressing MAP2, no strong staining intensity was revealed in the spine head, which indicates that MAP2 is either not efficiently targeted or not efficiently retained. Because MAP2 is a relatively large scaffolding protein, it might be bound to the microtubules in the dendritic branches more tightly than Tau, such that it that it is less dynamic and cannot be released efficiently into the spine.

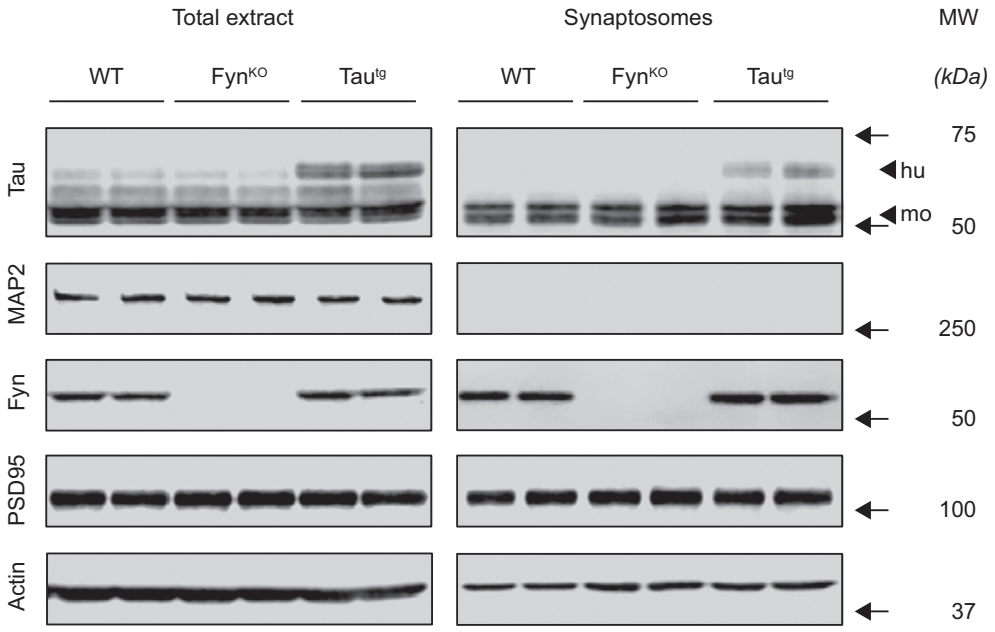

Fig. 9. Absence of Fyn does not affect levels of Tau in synaptosomal fraction. (A) Total brain extracts and synaptosomal PSD95 fractions obtained by sucrose fractionation from wild-type (WT), Fyn knockout (Fyn^{KO}), and P301L mutant Tau transgenic pR5 mice (Tau^{tg}) analyzed for the presence of Tau, MAP2, Fyn, PSD95, and actin. Human (hu) and endogenous mouse (mo) Tau are indicated by arrows. Neither total (normalized for actin, $P = 0.75$) nor synaptosomal Tau levels (normalized for PSD95, $P = 0.12$) are reduced in wild-type compared to Fyn^{KO} extracts.

We also found that, unlike over-expressed wild-type human Tau, FTD-linked mutant P301L Tau is efficiently targeted to the spine head. This might explain why expression of this P301L mutant form of Tau in transgenic mice, combined with Aβ, causes a particularly strong pathology and early lethality [19]. Strong effects of mutant Tau on spines have been demonstrated in several studies. For example, expression of mutant Tau *in vivo* has been shown to affect spine morphology and eventually cause spine loss [22], a phenotype that is augmented by Aβ [4,6]. In P301S Tau mice, the fraction of thin spines was found to be strongly reduced, whereas that of mushroom spines was found to be increased compared to controls [16]. Another study reported that, even at an early age, P301S Tau transgenic mice are characterized by a lower average spine density, length, area and volume compared with wild-type mice, concomitant with an impaired learning at this age in the Morris water maze [38]. Together with our findings this reveals that FTD mutant forms of Tau accumulate in the spine, which results in morphological changes and, subsequently, spine loss.

Our hippocampal primary culture study also revealed that a mutant form of Tau that only comprises the projection domain (ΔTau) does not travel efficiently beyond the primary dendrite, supporting previous findings of dendritic exclusion of ΔTau [20,37]. A second deletion construct, ΔMTB, lacked the MBRs but contained the projection domain as well as the carboxy-terminus. This form of Tau was found to distribute freely, including into the spines, suggesting that trapping of Tau by binding to microtubules, the major interaction partner of Tau [31], hinders its free distribution throughout the neuron. When we deleted the PXXP motifs of Tau (a domain retained in the ΔTau mutant and required for interaction with the SH3 domain of Fyn), the localization of this construct to spines did not differ from that of wild-type Tau. We had expected that the spine targeting ratio would be further reduced but because this ratio is already low for wild-type Tau our method may not have been sensitive enough to reveal a difference.

Although truncation of Tau is increasingly being shown to have a role in pathogenesis [43], phosphorylation remains an important post-translational modification of Tau, and hyperphosphorylated Tau has a critical role in AD pathogenesis. In our mutagenesis study, we showed that Tau is massively targeted to the spine when the AT180, 12E8, and PHF1 phospho-epitopes are pseudophosphorylated, using alanine mutant forms as a control. Targeting occurred to a similar degree as for the P301L mutant. Interestingly, the S422 epitope behaved differently, as both the A and the E mutants revealed the same weak localization to spines. We deliberately did not use GFP-tagged versions of Tau, as we have found that tagging Tau with GFP instead of V5 abrogates the exclusion of spine localization seen for V5-tagged wild-type Tau (data not shown). Hoover and colleagues, in contrast, used GFP-tagged Tau for expression and in addition expressed the shorter human 0N4R isoform of Tau [17], whereas we expressed the longest isoform, 2N4R, tagged with V5. In the former study, 14 serine and threonine residues were simultaneously changed to either alanine (termed AP), or glutamate (termed E14), resulting in an approximately four-fold increase in the spine localization of E14 Tau and a four-fold decrease in the localization of AP compared to wild-type Tau. In our study, we refrained from mutating 14 sites simultaneously and rather mutated individual sites, although we had previously generated transgenic mice expressing E10-mutated Tau [18]. Our approach of mutating single phospho-epitopes allowed us to demonstrate that not all sites mediate spine localization equally (AT180, 12E8, and PHF1 do, whereas S422 does not), and to show that introducing a single pseudophosphorylation epitope is sufficient to target Tau efficiently to the spine. One would conclude that in primary hippocampal neurons Tau is normally not highly phosphorylated because, if it were, phosphorylation at any epitope that had not been mutated to alanine should overcome the exclusion conferred to these mutant forms as far as spine localization is concerned. Interestingly, the S422E mutant is not efficiently targeted to the spine nor does its localization differ from that of the A mutant, suggesting that phosphorylation of this epitope has no role in subcellular Tau targeting. This is supported by the notion that this epitope is only phosphorylated under pathological conditions, with evidence from animal studies suggesting that this site is phosphorylated only when Tau filaments have formed, i.e., relatively late in the AD disease process [1].

We have previously shown that Tau is critically required to target Fyn to dendritic spines where this kinase phosphorylates the NMDA receptor, a prerequisite for recruiting PSD95 into a protein complex that then mediates the excitotoxic signaling triggered by Aβ [20]. In our study, we therefore aimed to determine whether Fyn has a role in

targeting endogenous Tau to the synaptosomal compartment. We found that the absence of Fyn did not abolish spine targeting of Tau. With Fyn activation having a prominent role in AD [5], our study does, however, not imply that Fyn has no role in phosphorylating and targeting of Tau in a pathological context. How Tau is localized in the presence of over-activated Fyn has not been addressed in our study. Also, as far as the interaction between Fyn and Tau are concerned, it remains to be determined whether Tau and Fyn form a complex when traveling into the spine or whether their effect is mediated independent of a direct interaction. Possible scenarios as to how these two molecules might interact have been discussed previously [25].

5. Conclusion

In conclusion, we have shown that endogenous Tau and MAP2 display differences in spine localization, with Tau entering the spine head, whereas MAP2 only enters the neck and does so infrequently. We have also revealed a crucial role for the MBR domain in the targeting of Tau to the spine and have further demonstrated that spine targeting depends on distinct phosphorylation sites and can occur independent of Fyn. This does not rule out, that in the presence of activated Fyn, Tau's targeting to the dendritic spine is modulated.

Acknowledgments

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