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ORIGINAL COMMUNICATION

MAPT mutations in amyotrophic lateral sclerosis: clinical, neuropathological and functional insights

Sibylle De Bertier[1] · Géraldine Lautrette[2] · Maria-del-Mar Amador[1,3] · Tomoko Miki[4] · Séverine Boillée[1] · Christian Stefan Lobsiger[1] · Delphine Bohl[1] · Frederic Darios[1] · Selma Machat[2] · Mathilde Duchesne[5] · Patrick Vourc'h[6,7] · Anne-Laure Fauret-Amsellem[8] · Philippe Corcia[6,9] · Nathalie Guy[10] · Philippe Couratier[2] · Danielle Seilhean[1,4] · Stéphanie Millecamps[1]

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Abstract

Background Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are part of a well-established disease continuum, underpinned by TDP43-pathology. In contrast, the clinical manifestations of Tau-linked disorders are typically limited to cognitive phenotypes or atypical parkinsonism, although few reports describe motor neuron involvement associated with *MAPT* (microtubule-associated protein Tau) mutations. This study aimed to investigate the contribution of *MAPT* to the ALS phenotype.

Methods We analyzed a whole-exome sequencing database comprising 470 ALS patients and explored the pathogenicity of the identified variants through familial, clinical, neuropathological, and cellular studies.

Results We identified two missense variants in the Tau repeat domains: the novel p.I308T variant, in a patient with early-onset ALS, and the p.P364S mutation in three families with spinal- or respiratory-onset ALS. Segregation of this mutation with disease could be confirmed in two affected cousins. The observation of p.P364S patient's tissue showed accumulations of hyperphosphorylated Tau in various brain regions, prominent in the motor cortex with Lewy body-like inclusions, along with a C-terminal cleaved form of Tau in muscle. In NSC-34 motor neuron cells expressing p.I308T or p.P364S mutants, Tau was discontinuous along the neurites, with clusters of mitochondria resulting from impaired mitochondrial motility.

Conclusion These findings expand the molecular understanding of ALS to include *MAPT* mutations. *MAPT* analysis should be incorporated into ALS genetic screening, particularly in patients with a familial history of the disease. Recognizing the full spectrum of *MAPT*-linked neurodegenerative diseases is of considerable interest, given the ongoing efforts to develop *MAPT*-targeted therapies.

Keywords Motor neuron disease · ALS · Tauopathies · Genetics · Mutation · MAPT · TAU

Introduction

MAPT (microtubule-associated protein Tau) encodes for Tau protein, which promotes the assembly and the stability of microtubule network in differentiated neurons, through the presence of four repeated (R1–R4) microtubule-binding domains (MBDs), with partial repeated amino acid sequence. Six isoforms of Tau, generated by alternate splicing, exist in the adult brain [1], containing either 4 (4R-Tau) or 3 (3R-Tau) MBDs (Fig. 1A). Unbalanced amounts of 4R and/or 3R-Tau isoforms are typical and characteristic features detected in the brain tissue of several Tauopathies including Alzheimer's disease (AD), cortico-basal degeneration (CBD), Pick's disease, progressive supranuclear palsy (PSP), and fronto-temporal lobe degeneration (FTLD). In these diseases, the pathogenic aggregated and hyperphosphorylated Tau into paired helical filament is specifically detected in neurons and/or glial cells of various brain regions. Tauopathies linked degenerative disorders are all characterized by cognitive symptoms, sometimes associated with parkinsonism, depending on the topography of the Tau deposits [2]. Mutations in *MAPT* have

Sibylle De Bertier, Géraldine Lautrette and Maria-del-Mar Amador contributed equally to this work.

Philippe Corcia, Nathalie Guy, Philippe Couratier, Danielle Seilhean, Stéphanie Millecamps jointly directed this study.

Extended author information available on the last page of the article

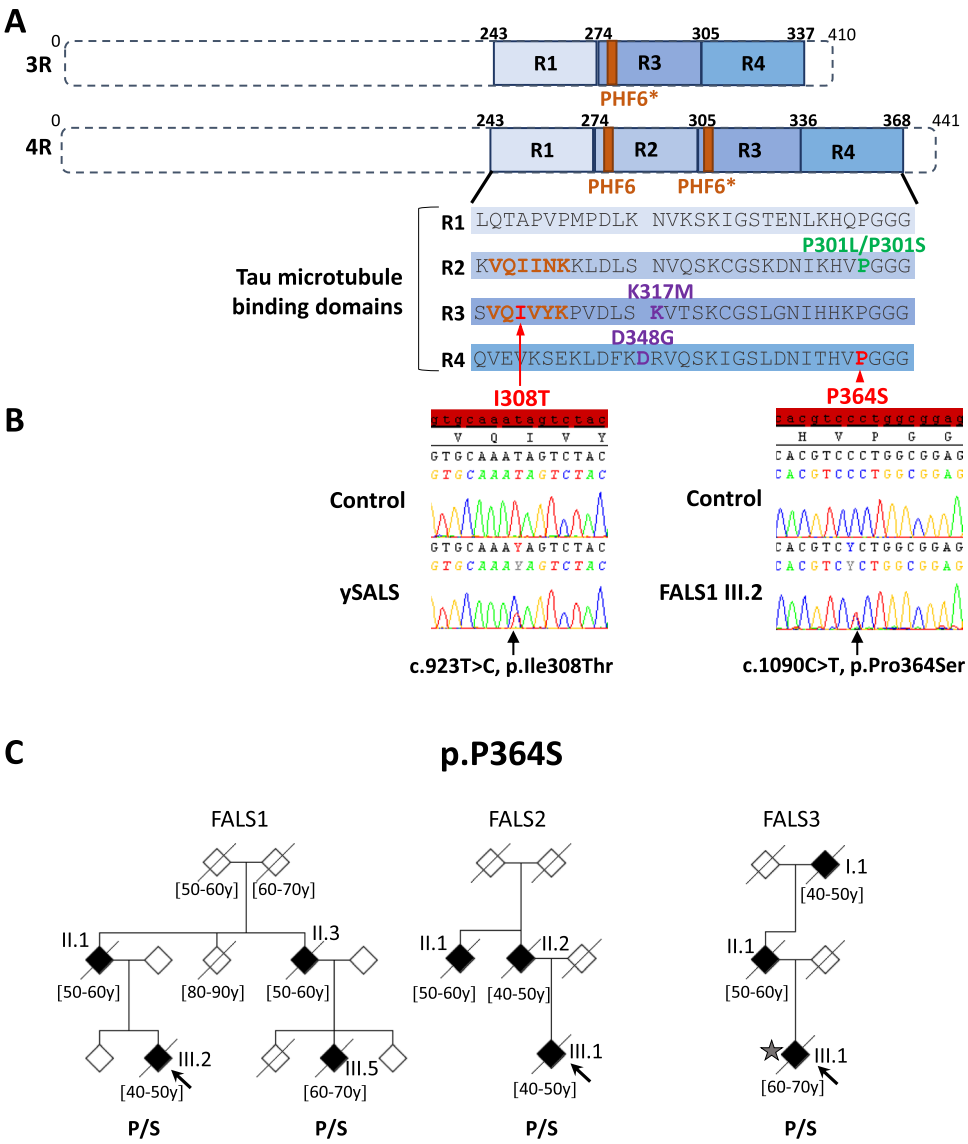

Fig. 1 Identification of two *MAPT* variants in ALS patients. **A** Schematic representation of the 3R and 4R isoform families of Tau protein according to NP_005901.2. Amino acid sequence is detailed for the 4 repeated microtubule-binding domains (blue) including the 2 hexapeptide domains (PHF6, PHF6*, brown). Mutations associated with motor neuron degeneration are indicated in purple and the main FTD associated mutations are in green. The positions of the 2 variants described in this study are pointed in red. **B** Part of fluorograms showing the position of the variants in mutated DNA sequences (arrow) compared to controls. **C** Pedigrees of the 3 FALS carrying the c.1090C>T, p.P364S variant. The genotype of patient's DNA (P/S) is indicated below the 4 patients with analyzed DNA. To ensure de-identification of the families, all individuals are represented with diamonds hiding any gender information. Black fill: ALS cases; brackets: range of age of death; arrows: index cases; star: patient with autopsy analysis

been identified in several autosomal dominant families with linkage to chromosome 17, clinically presenting fronto-temporal dementia (FTD) and parkinsonism [2]. These *MAPT* mutations are mainly missense substitutions, localized within the MBDs, or intronic variants regulating the splicing of exon 10 and producing an altered ratio of 3R- and 4R-Tau in the brain [2]. Some of these mutations have also been described in patients with both cognitive and motor neuron impairment, suggesting *MAPT* could also drive motor neuron degeneration [3–8]. The degeneration of both upper and lower motor neurons is characteristic of amyotrophic lateral sclerosis (ALS), the most common adult-onset motor neuron disease, leading to progressive paralysis and patient death in 3 to 5 years after symptom onset. Despite a genetic overlap spanning the FTD-ALS disease spectrum and although *MAPT* was proposed to be a susceptibility gene for ALS of sporadic occurrence in European and Asian population [9, 10], the number of *MAPT* mutations reported to be associated with ALS cases remains rare and insufficiently documented [11]. To define the contribution of *MAPT* mutations in ALS, we interrogated a whole-exome sequencing (WES) database including our large cohort of 470 French ALS patients, composed of familial (FALS) and sporadic (SALS) ALS cases, and further explored and compared the pathogenicity of the identified variants through familial, clinical, histological and cellular analyses.

Methods

Patient cohort and genetic analyses

The genetic study was conducted in accordance with the declaration of Helsinki and approved by the medical research ethics committee of "Assistance

Publique-Hôpitaux de Paris" (Authorization #A75, protocol code: DC-2013-2031-A75, date of approval: 2014/07/10, revised the 2019/10/11). The sponsor for this protocol was Inserm (Institut National de la Santé et de la Recherche Médicale), Paris, France. The human tissue collection was performed at the NEUROCEB biobank with "Authorization for the activity of conservation and preparation of elements of the human body for scientific use" (codes AC-2018-3290, AC-2024-6406 delivered on 2019/05/06 and 2024/08/26, respectively) by the French Ministry of Research and Universities. The declaration of a sampling protocol for scientific purposes and the protocol of "collections of biological samples for neurological research" (code PFS13-014) was approved the 2013/09/11 by the French Biomedecine Agency.

All patients signed a consent form for the genetic research. Blood samples were collected between years 1994 and 2024 in one of the 22 French ALS Reference Centers. DNAs were extracted and conserved by the Genethon DNA bank (Evry, France), registration number AC-2023-5465. The diagnosis of ALS and FTD was based on published criteria [12, 13]. This patient cohort of 470 French ALS patients includes 220 cases of FALS from 200 families, 250 SALS including 100 autopsied patients and 150 young patients (ySALS), with early-onset ALS, *i.e.,* before the age of 40 years (Table 1). This cohort of patients was devoid of mutations in *C9orf72, SOD1, TARDBP* and *FUS*, which are the 4 main ALS genes. Whole-Exome Sequencing (WES) analyses were performed using classical procedures as previously described [14]. These WES databases were interrogated to select MAPT variants with a minor allele frequency (MAF) < 0.005% in dbSNP, Hapmap, 1000genome, Exome Variant Server, and gnomAD databases. Variants were validated using Sanger analysis with BigDye chemistry as recommended by the supplier (Applied Biosystems) and fluorogram profiles were analyzed using SeqScape v2.5.

Post-mortem tissue analysis

For one of the ALS patients, muscle and brain paraffin-embedded post-mortem specimens were available at Limoges University Hospital. They were analyzed by the neuropathology department of the Pitié-Salpêtrière Hospital in Paris. Brain samples consisted of five paraffin-embedded coronal slices of left hemisphere. No spinal cord or brainstem was available. Slice 4, passing through the associative frontal, parietal, and temporal cortices, primary motor cortex, hippocampus, basal ganglia, and middle thalamus, was subdivided into seven blocks for analysis. After cutting to 3 µm thickness, the slides were stained with haematoxylin–eosin and immunohistochemistry was performed on a Benchmark UltraPlus Roche Diagnostics® automated system using the CC1 or CC2 proprietary processing protocols, and the Ultraview DAB revelation kit. Antibodies and experimental procedures are detailed in Table S1.

Predicted effect of the mutation on protein structure and function

Several in silico tools (Align GVGD, MutationTaster, PolyPhen2, SIFT, CADD phred score) to predict the pathogenic impact of the *MAPT* variants on protein function were provided through Alamut Visual Plus software. The precomputed Rare Exome Variant Ensemble Learner (REVEL) score was retrieved for each *MAPT* variant from the corresponding genome segment file https://sites.google.com/site/revelgenomics/downloads/revel-genome-segment-files

Table 1 Clinical and demographic characteristics of the studied cohorts

	FALS (index cases)	SALS (Autopsied)	ySALS
Number of patients	200	100	150
Men	124	60	113
Women	76	40	37
Sex ratio	1.6	1.5	3
Mean age of onset (years ± SD)	62 ± 0.84 [*n* = 200]	61 ± 1.2 [*n* = 84]	31.7 ± 0.5 [*n* = 150]
Range of years	33–90 years	35–82 years	16–40 years
Mean disease duration (months)	47.26 ± 3.55 [*n* = 177]	55 ± 4.48 [*n* = 84]	83 ± 8.3 [*n* = 90]
Range of months	6–263 months	8–194 months	6–348 months
Site of onset	[*n* = 192]	[*n* = 84]	[*n* = 143]
Bulbar	48 (25%)	25 (30%)	15 (10%)
Lower limbs	67 (35%)	28 (33.3%)	64 (45%)
Upper limbs	72 (37.5%)	28 (33.3%)	60 (42.3%)
Axial	2 (1%)	1 (1%)	3 (2%)
Respiratory	3 (1.5%)	2 (2.4%)	1 (0.7%)

The number of patients with available data is indicated between brackets [*n*] for each parameter

[15]. Multiple species protein alignments were performed using Ensembl Orthologues and Multalin interface [16]. The three-dimensional conformations of Tau with (p.P301L, p.I308T, p.P364S) or without amino acid substitution, were predicted using Phyre2 Protein Fold Recognition Server [17] and visualized by EzMol molecular display wizard [18].

Materials and methods for cell culture experiments are described in the supplementary files.

Results

Genetic studies: molecular analysis of MAPT in French ALS patients revealed two mutations in the repeated microtubule-binding domain of Tau

To clarify *MAPT* contribution to the ALS phenotype, we interrogated our WES databases including 470 ALS patients. Two variants in *MAPT* gene were identified in 4/220 FALS (3/200 FALS index cases) and 1/150 SALS with early-onset ALS (ySALS) corresponding to a frequency of 1.5% of FALS index cases devoid of mutation in any ALS genes and 0.7% of ySALS patients. No other variant in ALS-related genes [14] was found in these 5 patient's DNA. These *MAPT* variants were the Chr17(GRCh38) g.46014250T>C, c.923T>C, p.Ile308Thr (p.I308T) and the g.46018710C>T, c.1090C>T, p.Pro364Ser (p.P364S) on the National Center for Biotechnology Information (NCBI) transcript NM_005910.5 (Fig. 1A, B).

Both variants are localized in the Tau MBDs. The p.I308T is in R3 (Fig. 1A) and specifically affects one of the VQIxxK hexapeptide motifs, responsible for strong microtubule interactions and for the propensity of Tau protein to form β-sheet structures [19]. The p.P364S, which is in R4 (Fig. 1A), was recurrently identified in 3 unrelated families (Fig. 1C). Segregation of this variant with the disease could be confirmed in one pedigree on two first cousins (Fig. 1C).

Both amino acids concerned by these substitutions are highly conserved among species (Fig. S1A). Moreover, the proline residue position of p.P364S is also shared in the 3 other MBDs of Tau (Fig. 1A). Other mutations affecting these specific proline residues have been identified in some FTD patients including the p.P301S, p.P301L or p.P364T mutations [20, 21]. The isoleucine residue of p.I308T in R3 is also conserved in the second hexapeptide motif in R2 (Fig. 1A) but has never been concerned by any other amino acid substitution in diseases. These *MAPT* variants are absent from control (gnomAD v4.1.0) and ALS patients' database (project MinE data browser [22]), and are predicted to be deleterious by most of the in silico analyses grouped in Alamut Visual Plus software (Fig. S1B). They specifically have a Combined Annotation-Dependent Depletion (CADD) scores > 20 indicating they are among the 1% most deleterious variants in the genome [23], a high (REVEL) score (> 0.940) predicting their deleteriousness and disease-causing impact [15]. According to the American College of Medical Genetics and Genomics (ACMG) guidelines [24], the p.P364S variant is classified as likely pathogenic (class 4), whereas the p.I308T remains a class 3 variant of uncertain significance, requiring further investigations (Fig. S2). To further compare the possible modifications of the three-dimensional conformations of Tau with the p.I308T or p.P364S amino acid substitution, we used Phyre2 Protein Fold Recognition Server and EzMol molecular display wizard to recognize and visualize specific protein domains. Both p.I308T and p.P364S were predicted to modify Tau protein folding and led in particular to the appearance of a common C-terminal protein fibril domain of 47 amino acids (aa 377–341). Interestingly, this latter domain is also predicted by the Phyre2 interface for mutant Tau carrying the well-known p.P301L FTD-linked mutation (Fig. S1C, D).

Clinical features: patients with p.I308T or p.P364S MAPT variants meet the diagnostic criteria for ALS

Detailed clinical and paraclinical information was available for all five patients with *MAPT* mutations identified in the study. They were all diagnosed with ALS and followed up in one of the ALS Reference Centers in France. Clinical observations registered for patients carrying these *MAPT* variants are summarized in Table 2.

Case series

The p.P364S mutation was observed in 4 patients from 3 independent families (FALS1.III.2; FALS1.III.5; FALS2.III.1; FALS3.III.1). All patients had a familial history of ALS (Fig. 1C). They developed ALS between the ages of 40 and 70 years, with a mean age of onset of 54 years and a mean survival of 44.5 months. The site of onset varied, and notably, rare ALS presentations, such as respiratory onset and dropped head syndrome, were over-represented in this genotype. For these patients, despite the presence of clinical signs and symptoms of lower motor neuron dysfunction (cramps, fasciculations, amyotrophy), typical ALS electrophysiological profiles including chronic neurogenic changes and abnormal spontaneous activity (positive sharp waves and fasciculations) were not observed until later in the disease course (1 to 2 years after symptom onset). Therefore serial electromyograms (EMGs) during follow-up were necessary to confirm the involvement of lower motor neurons. However, in the case of patient FALS3.III.1, repeated EMGs failed to reveal a neurogenic pattern, necessitating histopathological muscular analysis (performed post-mortem) to document the lower motor neuron involvement. Symptomatic diaphragmatic dysfunction required

Table 2 Clinical features of patients harboring *MAPT* mutations

Variant/ Family	Subject	Age at onset (year range)	Site of onset	Predominant phenotype	Bulbar signs	Upper motor neuron dysfunction	Lower motor neuron dysfunction	Cognitive deficits	Age at death (year range)	Disease duration (months)
p.P364S/ FALS1	II.1[a]	NA	Spinal	NA	NA	NA	NA	NA	50–60	NA
	II.3[a]	NA	NA	NA	NA	NA	NA	NA	50–60	NA
	III.2	40–50	Spinal	Pyramidal	Yes	Brisk tendon reflexes and spasticity in the four limbs	Cramps, fasciculations; EMG: Chronic neurogenic changes + abnormal spontaneous activities	No	40–50	44 m
	III.5	60–70	Respiratory	Respiratory	No	Preserved tendon reflexes in amyotrophic regions	Amyotrophy, fasciculations; EMG: Mild chronic neurogenic changes + abnormal spontaneous activities	Apathy, executive dysfunction, and impairment in memory encoding	60–70	70 m
p.P364S/ FALS2	II.1[a]	NA	NA	NA	NA	NA	NA	NA	50–60	NA
	II.2[a]	NA	NA	NA	NA	NA	NA	NA	40–50	NA
	III.1	40–50	Spinal	Pyramidal	Yes	Brisk tendon reflexes and spasticity in the four limbs	Cramps, fasciculations; EMG: Chronic neurogenic changes	No	40–50	46 m
p.P364S/ FALS3	I.1[a]	NA	Axial	NA	NA	NA	NA	NA	40–50	NA
	II.1[a]	50–60	NA	NA	NA	NA	NA	NA	50–60	12 m
	III.1	50–60	Axial (dropped head syndrome) + respiratory	Pyramidal	No	Brisk tendon reflexes and spasticity in the four limbs	Amyotrophy; EMG: Inconclusive; Muscular pathology: Severe chronic neurogenic changes	Executive dysfunction and impairment in memory encoding	60–70	18 m
p.I308T	ySALS	≤35y	Spinal	Classical		Brisk tendon reflexes and spasticity in the lower limbs	EMG: Chronic neurogenic changes + abnormal spontaneous activities	No	≤40	54 m

NA: not available. NIV: non-invasive ventilations. M: male, F: female. [a]Subjects with no possible genotype and unavailable medical record: information on age, cause of death, and key clinical features was obtained from relatives

non-invasive ventilation in all patients. Cognitive deficits prompted extensive neuropsychological testing in two out of four patients (FALS1.III.5 and FALS3.III.1). Of note, these patients exhibited memory-encoding impairments but also other cognitive findings more typically associated with ALS such as executive dysfunction.

Single case report

The p.I308T mutation was identified in a young patient (≤35 years old) who presented with distal upper limb onset classical ALS, apparently sporadic. A neurogenic pattern on EMG was observed in the four limbs 11 months after the onset of clinical symptoms. The patient required respiratory non-invasive ventilation for respiratory support 3 years after disease onset and remained free of cognitive symptoms throughout the disease course.

The mean survival of ALS patients with *MAPT* mutations was 46.4 months, ranging from 18 months (FALS3.III.1) to 5 years (FALS1.III.5).

Neuropathological analysis: tissue with p.P364S MAPT variant showed accumulations of hyperphosphorylated Tau in brain and cleaved Tau-C3 form in muscle

Post-mortem brain samples of the FALS3.III.1 patient carrying the p.P364S *MAPT* mutation were processed to detect the hyperphosphorylated Tau using AT8 antibody. Hyperphosphorylated Tau was detected in numerous neurons including those of the dentate gyrus of the hippocampus (Fig. 2A), the substantia nigra (Fig. 2B) and the frontal and motor cortex (Fig. 2C–F). Comparing the two edges of Rolando's scissure, the density of anti-Tau labeling was much greater in the motor cortex than in the somatosensory cortex (Fig. S3A). This significant motor cortex distribution of hyperphosphorylated Tau was compared in various patients presenting Tauopathies including 2 FTD patients with a *MAPT* p.P301L mutation, 3 cases of PSP, 2 CBD, 2 Pick's disease and 2 AD. At low magnification, all patients showed diffuse Tau accumulation (Fig. S3). This staining predominated in the motor cortex in cases with *MAPT* mutations (Fig. S3A, B) and in Pick's disease (Fig. S3E), while this predominance was less marked or even non-existent in the motor cortex of PSP (Fig. S3C), CBD (Fig. S3D) and AD (Fig. S3F) cases. Moreover large Pick-like inclusions were observed in Betz cells with the *MAPT* p.P364S mutation (Fig. S3A, arrow), reminiscent of those found in Pick's disease (Fig. S3E, arrow). In other cases, Betz cells are devoid of inclusions or contain diffuse granular inclusions or neurofibrillary tangles. Thus, although hyperphosphorylated Tau accumulation in motor cortex neurons is not specific to the p.P364S *MAPT* mutation we report here, its distribution and shape are different from that observed for PSP, CBD and AD and suggests a specific vulnerability of the Betz cells to Tau aggregation for ALS and Pick's disease.

In the ALS case with p.P364S *MAPT* mutation brain, Tau protein accumulations were also stained with other anti-Tau antibodies including Tau-C3 (Fig. 2G–I), which is specific of C-terminally truncated Tau fragments produced in Tauopathies by the caspase-3 cleavage of full-length Tau at the carboxy-terminus residue aspartic acid (Asp421) [25], and Tau-5 antibodies (Fig. 2J–L) although lesion density and size were highest with AT8 (Fig. 2D–F). All three Tau antibodies revealed neurofibrillary tangles (Fig. 2, arrows) and pretangles (Fig. 2D, star), as well as Pick-like inclusions (Fig. 2, black arrowheads), and oligodendroglial inclusions (Fig. 2, empty arrowheads). Tau-5 and Tau-C3 positive stainings were also suggestive of glial plaques (Fig. 2H). RD3 and RD4 immunohistochemistries were negative on these tissue samples. No abnormal Aβ, Cystatin C, TDP-43 or α-synuclein accumulations were observed in this patient's brain (data not shown). Spinal cord sections were not available.

In muscle sections, small deposits positive for p62 (Fig. 3A, arrows) and TDP-43 (Fig. 3B, C, arrows) were evidenced. Moreover, numerous round structures were positively stained using Tau-C3 antibody (Fig. 3D–F, arrows). These structures remained negative when using the other anti-Tau antibodies (Fig. S4A, B). Also, they were not observed in muscles of other FALS patients (Fig. 3G–I) nor in control muscles presenting inclusion body myositis (IBM) (Fig. 3J, K) or motor denervation (Fig. 3L).

Cellular studies: both p.I308T and p.P364S MAPT variants showed similar cytoskeletal defects associated with impaired mitochondrial transport when expressed in a motor neuron cell line

As the analysis of biochemical properties of the p.P364S *MAPT* mutant previously revealed a reduced ability to promote the assembly of tubulin into microtubules in vitro [26], we aimed to study and compare the consequence of the two *MAPT* mutations we identified on the motoneuronal cytoskeleton organization. Thus, we expressed wild type ($MAPT^{WT}$) and mutant constructs ($MAPT^{I308T}$ and $MAPT^{P364S}$) in the NSC-34 motor neuron cell lines. A similar transfection efficiency was observed with the 3 constructs 48 h after the transfection (Fig. S5). The neurite organization was compared in these transfected cells 7 days after the transfection. Both mutants showed abnormal distribution of Tau (Fig. 4A, red), packed in successive "swelling structures" or "bulks" (Fig. 4A, arrows) along the tubulin network (Fig. 4A). These Tau bulk accumulations were also positive for the acetylated tubulin (Fig. S6, arrowheads), a marker of microtubule stability, resulting

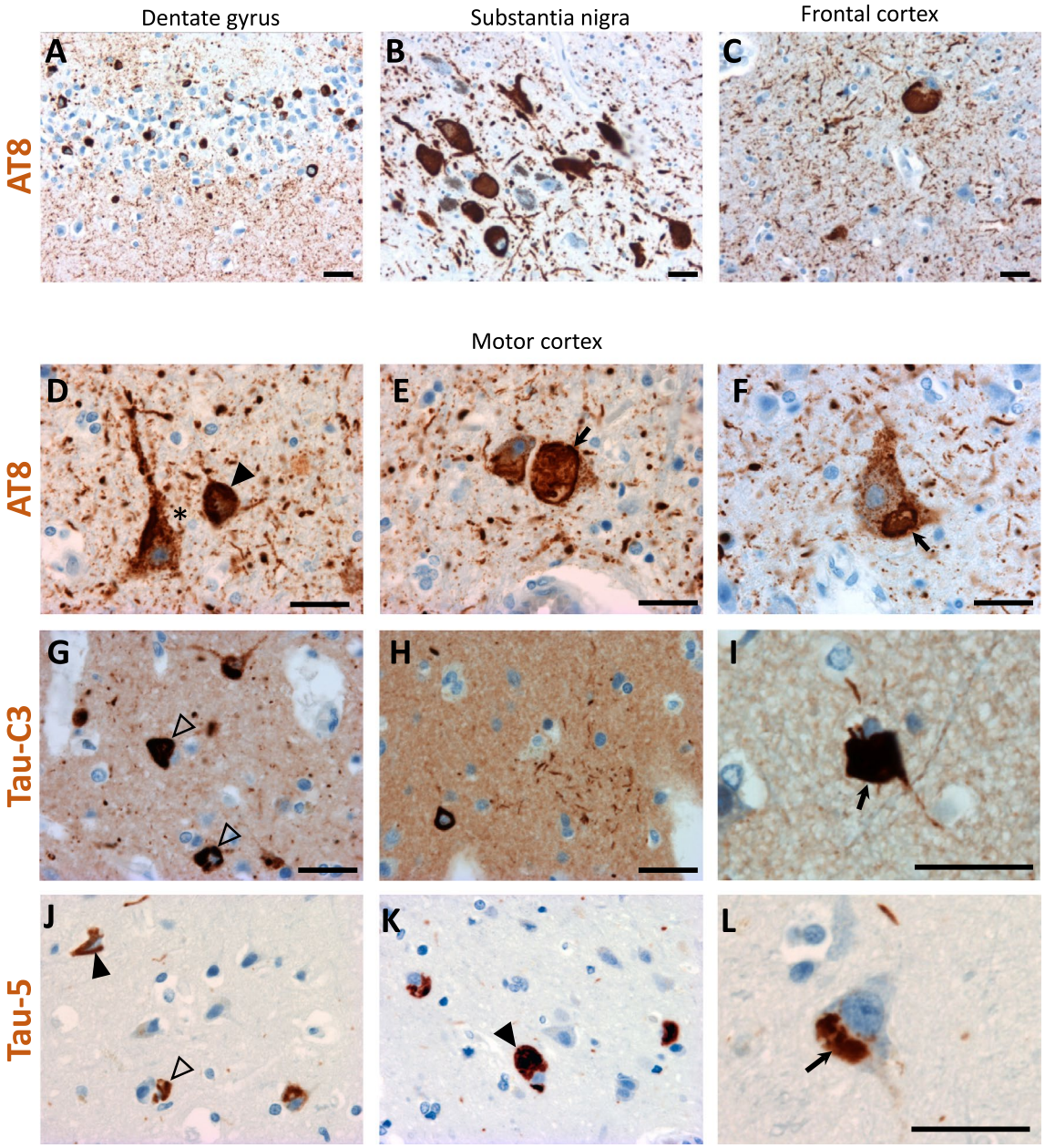

Fig. 2 Tau protein detection in patient brain with the MAPT^{P364S} mutation. Post-mortem histological analysis of brain tissues with *MAPT* p.P364S mutation using AT8 antibody in the dentate gyrus (**A**), the substantia nigra (**B**), the frontal cortex (**C**) and the motor cortex (**D**–**F**). These Tau accumulations were also stained with Tau-C3 (**G**–**I**) and Tau-5 (**J**–**L**) antibodies. Intraneuronal inclusions are tangles (black arrows) or pretangles (star) or Pick-like bodies (black arrowheads). Oligodendroglial inclusions are pointed by empty arrowheads. Scale bars: 20 µm

from the post-translational and reversible addition of an acetyl group to the alpha tubulin Lys40. The percentage of cells presenting those neuritic bulks was notably doubled after transfection of the mutant constructs (MAPT^{I308T} or MAPT^{P364S}) compared to the native MAPT^{WT} (Fig. 4B). As microtubule network disorganization can impair mitochondria trafficking, we used an antibody raised against cytochrome C to stain mitochondria in MAPT transfected cells and observed that mitochondria were clustered in these swelling structures (Fig. 5A, arrows). Metabolic mitochondrial activity was also compared in these transfected cells using the MTT colorimetric assay: it showed a decrease for the p.I308T mutant, 48 h after the transfection (Fig. 5B). To measure mitochondrial transport within MAPT expressing neurites, we performed additional co-transfection experiments in NSC-34 cells using the

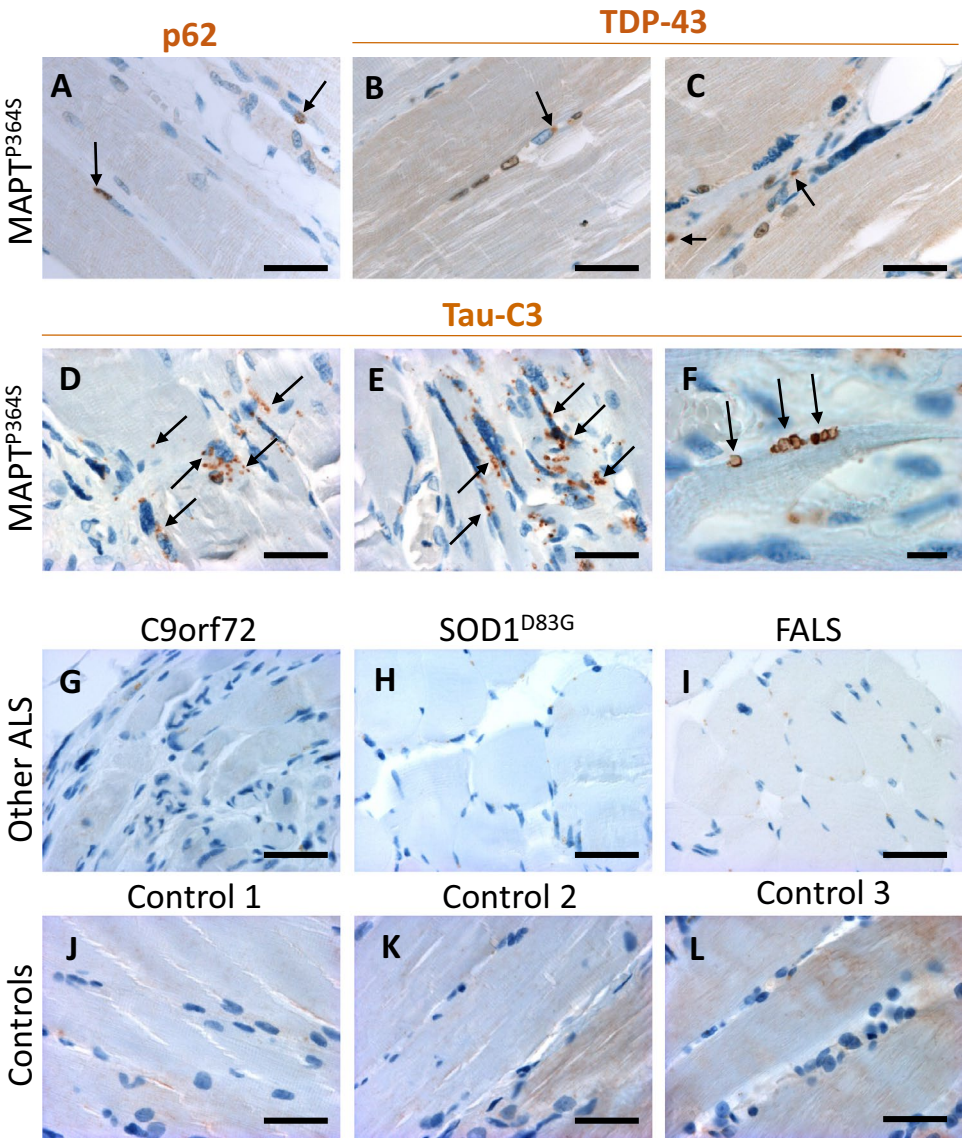

Fig. 3 Analysis of muscular tissues with the MAPT^{P364S} mutation. Immunostaining for p62 (**A**) and TDP-43 (**B**, **C**) in muscle fibers of the MAPT^{P364S} patient. Arrows point to cytoplasmic inclusions. Muscular histological analysis using Tau-C3 antibody in MAPT^{P364S} (**D**–**F**, arrows pointing to Tau-C3 positive round structures) or other FALS patients carrying C9orf72 repeat expansion (**G**), SOD1^{D83G} mutation (**H**), or no variant in any known ALS-related gene (**I**) and in 3 controls (**J**–**L**). Scale bars: 5 µm (F) and 20 µm (other panels)

mCherry-tagged MAPT constructs and a plasmid pCMV/myc/mito/GFP to stain mitochondria in green fluorescence. This approach allowed visualization and tracking of the movement of green mitochondria in red neurites expressing one form of Tau. After neurite outgrowth of NSC-34 by retinoic acid treatment, mitochondria trafficking was recorded in double transfected cells by live cell microscopy every 5 s during 5 min (Fig. 6A). Kymograph representations showed stationary mitochondria as straight vertical lines while moving mitochondria are deflected as diagonal lines (Fig. 6B). These experiments showed that mitochondria were stationary in Tau-positive bulk structures while motile ones were observed in the portion of the neurite devoid of any Tau swelling. Quantification of the number of motile mitochondria confirmed decrease in neurites expressing mutant MAPT, which was statistically significant for the p.P364S mutant (Fig. 6C).

Discussion

Tauopathies and ALS are treated as two distinct clinical and neuropathological entities. This prevailing paradigm is so strong that current biomarker development focuses on discriminating ALS from other neurodegenerative diseases by the absence of Tau pathology derivatives: indeed, a recent study aimed at identifying specific biomarkers to differentiate between various neurodegenerative disorders. The authors isolated plasma extracellular vesicles with distinct contents in ALS and Tau-linked FTD, which were largely specific to each disease and strongly correlated with the severity of degeneration [27]. Of note, one of the Tau-linked pathology patients of this study [27] carried the p.P364S *MAPT* mutation we identified in ALS patients.

Through systematic *MAPT* genetic screening of a large cohort of FALS and young-onset ALS patients, our study

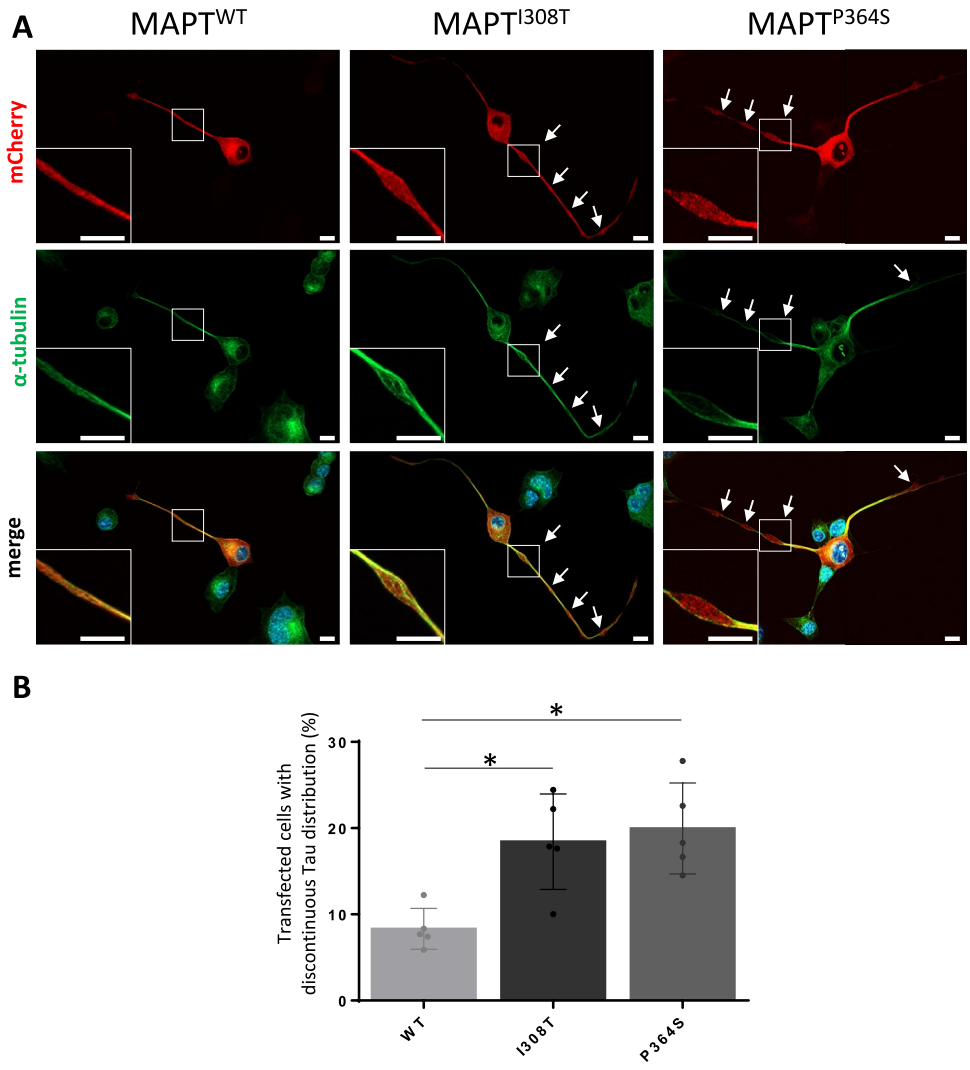

Fig. 4 Tau and cytoskeleton organization in motor neuron cells transfected with native (WT) or mutant (I308T, P364S) *MAPT* constructs. **A** Immunofluorescence staining of NSC-34 cells transfected with native $MAPT^{WT}$ or mutant $MAPT^{I308T}$ or $MAPT^{P364S}$ constructs and labeled with anti-mCherry (in red) and anti-α-tubulin (in green) antibodies to visualize MAPT and microtubule distribution, respectively. Bulks of Tau in NSC-34 neurites expressing mutant MAPT are pointed by arrows. DAPI-stained nuclei are in blue in merged pictures. Scale bars: 10 µm. **B** Comparison of the proportion of transfected cells presenting a discontinuous mCherry repartition along neurites between $MAPT^{WT}$ (light gray), $MAPT^{I308T}$ (black) and $MAPT^{P364S}$ (dark gray) constructs. Values are means ± standard deviation (SD) from 5 independent experiments. *$p < 0.05$

retrieves the p.P364S mutation in multiple FALS cases, along with a novel *MAPT* variant, p.I308T, in a case of early-onset ALS. For the p.P364S mutation, its recurrence in 3 unrelated French FALS, and familial segregation confirmed in one pedigree, provide strong evidence for its pathogenicity in the ALS phenotype. This p.P364S mutation has previously been described in various clinical presentations. It was reported in an Italian FTLD patient with disease onset in his late forties, without a familial history of dementia [26]. Two other studies described 4 relatives of a large Slovene family harboring this mutation and presenting with cognitive decline (3/4 patients), parkinsonism (2/4 patients) and/or associated motor neuron disease (3/4 patients), eventually leading to death from respiratory insufficiency after 18 months of disease, between the ages of 50 and 70 years [4, 5]. Although these clinical constellations are beyond classical ALS, some resemble those presented by our patients, suggesting a wide phenotype spectrum for this p.P364S *MAPT* mutation.

Indeed, increasing evidence further suggests a broad spectrum of neurological diseases associated with Tau mutations. It is now recognized that they are related to atypical parkinsonism [28]. Additionally, several publications have identified a lower motor neuron phenotype caused by the p.D348G *MAPT* mutation, with respiratory failure and a slow disease course [7, 8]. Consistently, our study expands the phenotypic spectrum to include the ALS phenotype. Encompassing upper motor and lower motor neuron involvement in more than two regions, our patients meet the diagnostic criteria for ALS. This diagnosis is comforted by the systematic diaphragmatic dysfunction in all patients. This motor phenotype is pure (without an association with FTD) in 3 out of the 5 extensively investigated patients, challenging the prevailing assumption that a cognitive–behavioral dysfunction is necessarily present in Tau-related disease. Interestingly, two of the 5 ALS patients we report here, presented with respiratory onset, suggesting the *MAPT* gene could be involved in this rare presentation of the disease.

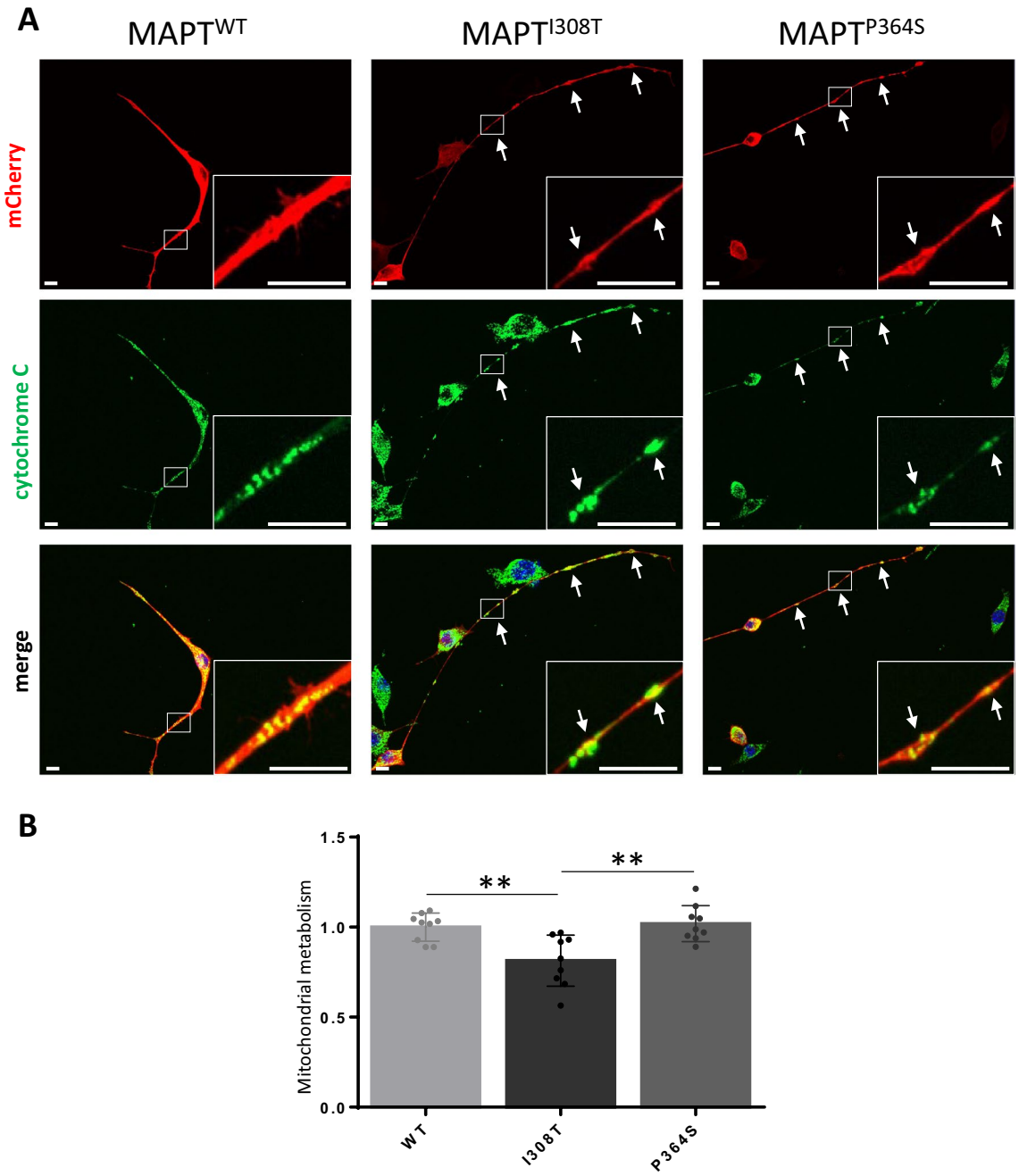

Fig. 5 *MAPT* mutations impact on mitochondrial distribution and metabolism in motor neuron cell line. **A** Double immunofluorescence staining of transfected NSC-34 cells with native $MAPT^{WT}$ or mutant $MAPT^{I308T}$ or $MAPT^{P364S}$ constructs and labeled with anti-mCherry (red) and anti-cytochrome C (green) antibodies to detect MAPT transgenes and mitochondria, respectively. Bulks are pointed by arrows. DAPI-stained nuclei are in blue in merge pictures. Scale bars: 10 µm. **B** Mitochondrial metabolism was measured in NSC-34 expressing $MAPT^{WT}$ (light gray), $MAPT^{I308T}$ (black) and $MAPT^{P364S}$ (dark gray) plasmid constructs using the MTT assay. MTT reduction ratio (570 nm–700 nm absorption values) was normalized to that of WT construct. Values are means ± SD of 9 values from 3 independent experiments. $^{**}p < 0.01$

Literature data regarding the prevalence of *MAPT* mutations in patients with motor neuron disease are lacking. In the much-selected population of this study, *MAPT* mutations account for 1.5% of FALS index cases for which the main ALS genes (*C9orf72, SOD1, TARDBP,* and *FUS*) have been excluded, and 0.7% of ySALS patients, also devoid of mutations in these genes. Since in our French population, mutations in one of the 4 main genes explain 61% of index FALS and 16% of ySALS [29], and to generalize these findings, we can estimate a frequency of 0.6% of *MAPT* mutations in

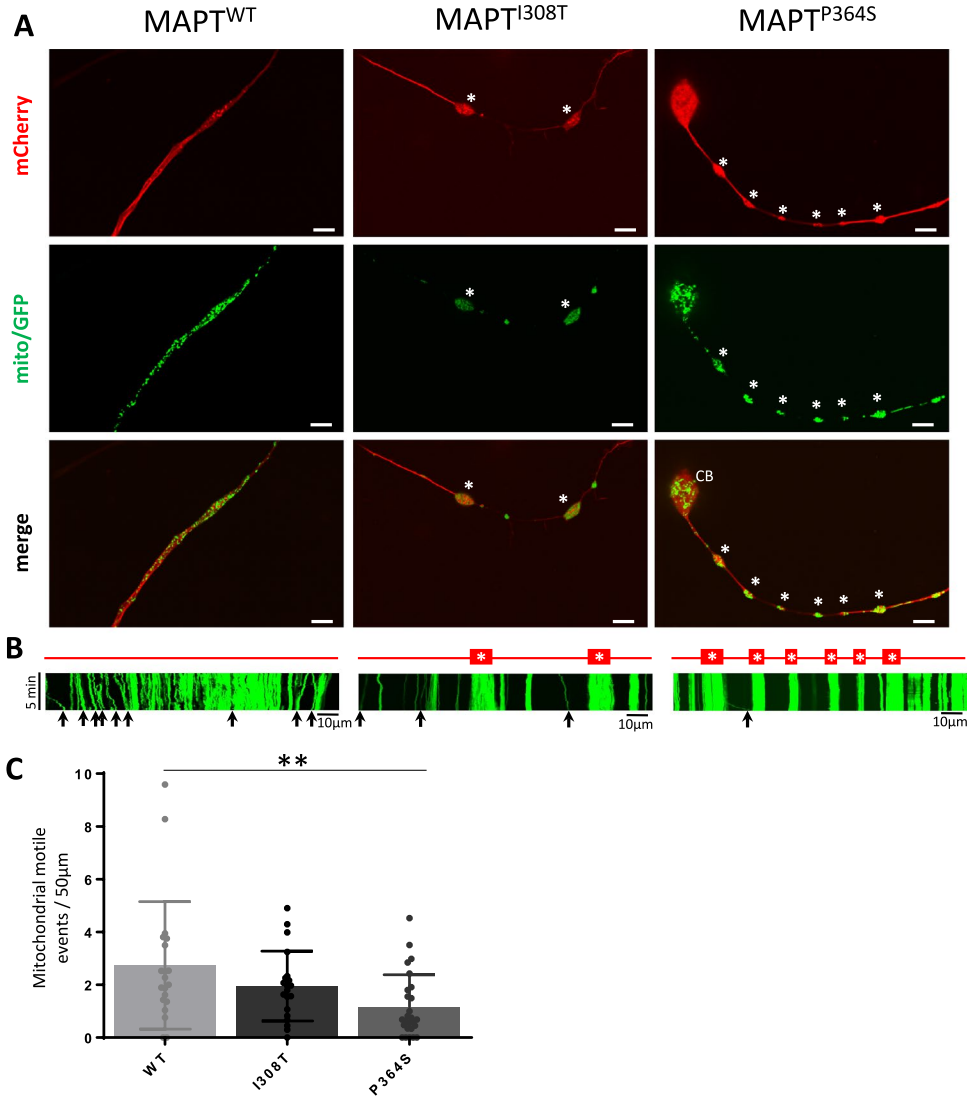

Fig. 6 *MAPT* mutations impact on mitochondrial dynamic in motor neuron cell line. **A** Representative images from live cell imaging of co-transfected NSC-34 cells with native MAPT^{WT} or mutant MAPT^{I308T} or MAPT^{P364S} constructs (mCherry, red) and pCMV/myc/mito/GFP (mito/GFP, green) to visualize distribution/dynamic of mitochondria in MAPT expressing neurites. **B** Kymographs (obtained with ImageJ) compiling positions of these mitoGFP-labeled mitochondria (*x-axis represents distance in µm)* during a total imaging time of 5 min (*y-axis is time)*. Note that in neurites expressing mutant MAPT constructions, stationary mitochondria (visible as straight vertical lines) are clustered into MAPT bulks (stars in **A**, red boxes with white stars in **B**). Mitochondria motile events are visualized by oblique trajectory lines (arrows, **B**). CB: cell body. Scale bars: 10 µm. **C** Quantification of the number of mitochondrial motile events in neurites was determined for each MAPT construct (MAPT^{WT}, light gray; MAPT^{I308T}, black; MAPT^{P364S}, dark gray) and normalized to neurite length. For each construct, values are means ± SD from 20 to 30 recorded neurites from 3 independent experiments. $^{**}p < 0.01$

unscreened FALS, and of 0.6% in unscreened ySALS, while the frequency in SALS with a classical age of onset cannot be retrieved from our study. Of the same order of magnitude, Origone et al. [7] found a frequency of *MAPT* mutations of 0.8% in a cohort of 120 Italian patients with ALS (FALS and SALS) without cognitive impairment. In our study, although *MAPT* mutation frequency is significantly lower than those of the 4 main related genes, it is high among the rare ALS genetic causes (Fig. S7), such as TBK1 [30]. This supports the inclusion of *MAPT* in the panel of genes routinely screened for FALS patients. The prevalence of *MAPT* mutations within a population of FTD patients (familial and sporadic) is, as expected, much higher, ranging from 2 to 23%, depending on the geographical origin of the patients [31]. *MAPT*-related FTD typically presents at a mean age of 49.5 years (range: 17–70) and has a mean disease duration of disease of 58.5 months (range: 24–93) [31]. In comparison, our Tau-related ALS cases had a slightly older mean age at the onset of 54 years, but the duration of the disease was shorter, with a mean of 44.5 months.

Other rare *MAPT* variants have already been identified in the repeat domains of the protein in patients with a complex phenotype including motor neuron dysfunction, such as p.K317M located in R3 (Fig. 1A). It was identified in two Spanish families including 14 patients who presented pyramidal and extrapyramidal syndrome, associated to frontal cognitive–behavioral impairment, and amyotrophy in half of them [6]. This mutation was also pointed out in a recent study, aiming to evaluate and discuss the pathogenic effect of all *MAPT* variants, retrieved from various large ALS sequencing databases [11]. This analysis revealed 8 pathogenic variants located at the C-terminal part of the protein, including 5 mutations already described in FTD/Tauopathies [32–35] (p.G201S, p.L266V, p.K317M, p.N410H and p.Q424K). Furthermore, they found a specifically altered Tau homeostasis in the *C9orf72*-linked ALS, indicating

that Tau might act as an ALS disease modifier, influencing disease onset and duration [11]. This study supports the probably underestimated role of *MAPT* mutations in ALS, particularly located in and around the repeat domain of the protein [11].

Beyond recurrence and familial segregation, our study provides strong evidence of the functional impact of the reported mutations. Both *MAPT* mutations were expected to modify Tau aggregation: indeed, using predicting tools, we highlighted the appearance of a similar C-terminal protein fibril domain in both the p.I308T and p.P364S Tau mutant forms. Their biochemical properties have been explored and reported by others. The p.P364S mutant shows an increased propensity to aggregate into filaments in vitro [26]. The p.I308T variant is within a hexapeptide motif with a high beta-sheet-forming propensity [36]. This property notably involves the interaction between I308 and Y310 residues of this motif, which are essential for initiating the molecular association of both MBDs leading to Tau filament formation [37] and for the stabilization of the β-strand conformation [38]. Amino acid substitutions at position 308 directly impacted the aggregation properties of Tau in heparin-induced fibrillization experiments [38, 39]. In these experiments, the p.I308T mutant (our patient mutation) leads to the formation of granular aggregates [40]. For the p.P364S mutation, our brain analysis revealed abnormal Tau aggregated deposits, distributed in many brain regions, in line with observations made for brains harboring the p.P364S [4, 5], p.K317M [6] or the p.D348G [8] MAPT mutation. Our analysis further revealed predominant Tau accumulation in motor cortex, with the specific presence of Pick-like inclusions in Betz cells, reminiscent of those observed in Pick's disease.

In the ALS muscle with the p.P364S mutation, we did not observe the hyperphosphorylated Tau deposits commonly observed in IBM or myopathies containing rimmed vacuole (either autosomal recessive inherited or induced by chloroquine exposure [41, 42]). We rather found unexpected round structures positive for anti-C3 Tau which could be a specific hallmark of ALS synapses carrying this *MAPT* mutation and deserves to be confirmed and further explored. This truncated Tau, particularly abundant in synaptosomes issued from parietal cortex of AD patients, is suggested to facilitate Tau secretion and propagation of Tau pathology [43]. An appealing hypothesis is that the positivities we observed in muscle could result from a presynaptic accumulation of Tau at the neuromuscular junction and/or at the denervated end plates. Interestingly, this truncated Tau at Asp421 was shown to be involved in mitochondrial damage and synaptic failure in various cellular and animal models of AD [44]. It promotes the accumulation of mitochondria in neuronal soma while decreasing the number of moving mitochondria in the axonal and dendritic processes [45, 46]. Therefore, its accumulation within motor neuron end plates could contribute to synaptic damage at the ALS neuromuscular junction.

Our cellular model analyses reveal similar cellular defects with both mutant MAPT transgenes compared to the WT one, including the presence of successive axonal swellings with accumulation of mutated Tau and mitochondria. Interestingly, the toxicity of such neuritic bulks is supported by a previous study showing similar structures, called "varicosities", stained by beta3-tubulin, in cortical neurons derived from induced pluripotent stem cells (iPSc) from an FTD patient with the p.P301L Tau mutation [47]. These varicosities colocalized with toxic protein accumulations including Tau (3R-, 4R-Tau, AT8) and α-synuclein. Interestingly, in these mutated iPSc-derived neurons, these morphological changes were also associated with defects in mitochondrial transport [47]. In line with these results, our observations suggest that the *MAPT* mutations we studied lead to cytoskeleton disorganization, disrupting mitochondrial dynamics, a common pathological signature of various motor neuron diseases. Indeed, mitochondrial accumulations in neurites have already been described and associated with Tau-positive swellings in spinal muscular amyotrophy (SMA) [48] or with acetylated tubulin positive bulks in spastic paraplegia culture models [49, 50]. Axonal mitochondrial transport was also shown to be defective in various mouse models of ALS. It is reduced in mouse-cultured neurons or NSC-34 cells expressing mutant SOD1 [51, 52] and in patient motor neurons (derived from iPSc) expressing mutant FUS or TDP-43 [53, 54]. For mutant TDP-43, this defect was consecutive to its insolubility, cleavage and phosphorylation [53]. For mutant FUS, this alteration is progressive and worsened with culture time [54]. Interestingly, HDAC6 inhibitor, which prevents deacetylation of alpha-tubulin and stabilizes microtubules, could restore the mitochondrial axonal transport defects in mutant FUS or TDP-43 patient-derived MNs. These observations suggest that impaired mitochondrial transport is part of the ALS disease process, and the stability of the microtubule network, for which MAPT plays a pivotal role, a possible therapeutic target to overcome this defect.

Our study presents some limitations. First, although clinical, electrophysiological, and pathological data attest to the involvement of the lower motor neuron in our patients, spinal cord samples of the neuropathologically studied patient were unavailable, and these data are missing. Nevertheless, pathological studies of the spinal cord from patients with FTD associated with motor neuron disease, carrying the p.K317M *MAPT* mutation, have previously been published by Zarranz et al. [6]. In his series of 8 autopsies, 7 out of the 8 spinal cords studied presented ALS typical neuropathological findings. Second, our study was conducted in a highly selected population at increased risk of a genetic cause of the disease: our cohort was strongly enriched with

familial and young-onset ALS patients, and we included only patients for whom the main ALS pathogenic mutations had been excluded. Consequently, the generalizability of our findings to unselected populations is limited. Third, the functional studies were conducted on NSC-34 cells, a hybrid mouse motor neuron-like cell line. Although these cells display several typical properties of motor neurons for cytoskeleton (expression of neurofilaments), neurotransmission (expression of choline acetyltransferase allowing acetylcholine synthesis, storage, and release), and generation of action potentials by electrical stimulation, and can induce acetylcholine receptor (AChR) clustering on co-cultured myotubes [55], express glutamate subunits in serum deprivation conditions [56] and be differentiated using retinoic acid treatment [57], they remain a limited model to study human motor neuron pathology. The differentiation of iPSc from patient fibroblasts (unfortunately not available in the present study) in motor neurons would have been a valuable model for studying these novel *MAPT* mutations in a human motor neuron context.

In conclusion, we detail a case series of patients with *MAPT* mutation who meet the full diagnosis criteria for ALS. To our knowledge, this is the first study to screen a large cohort of FALS and early-onset ALS patients for *MAPT* mutations. We provide clinical, neuropathological, and functional evidence supporting the association between ALS and two *MAPT* variants. One of these is the novel p.I308T *MAPT* variant, identified in a young ALS patient with a classical disease course. Our cellular investigations support the pathogenicity of both mutations, ascertained by the accumulations of Tau and mitochondria in neuritic swellings, the impaired mitochondrial transport, and the decreased mitochondrial metabolic activity, suggesting mutant forms could exert cellular toxicity through energetic breakdown. Overall, these results led to the ACMG re-classification of p.I308T as class 4 (likely pathogenic) and p.P364S as class 5 (pathogenic) (Fig. S2). This supports the inclusion of *MAPT* analysis in the genetic diagnostic screening of ALS patients with a higher probability of a genetic cause of the disease, including familial and young cases, as well as rarer presentations of the disease such as respiratory onset. Beyond the benefits of genetic counseling for patients and relatives, and considering the ongoing development of antisense oligonucleotides (ASOs) [58, 59], and immunotherapies [60–62] targeting *MAPT*, the identified *MAPT*-linked ALS patients may benefit from targeted therapies in future.

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Data availability All datasets generated or analyzed during the study are available upon reasonable request to the corresponding author.

Declarations

Conflict of interest The authors have no competing interests relevant to the content of this article to declare.

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Authors and Affiliations

Sibylle De Bertier[1] · Géraldine Lautrette[2] · Maria-del-Mar Amador[1,3] · Tomoko Miki[4] · Séverine Boillée[1] · Christian Stefan Lobsiger[1] · Delphine Bohl[1] · Frederic Darios[1] · Selma Machat[2] · Mathilde Duchesne[5] · Patrick Vourc'h[6,7] · Anne-Laure Fauret-Amsellem[8] · Philippe Corcia[6,9] · Nathalie Guy[10] · Philippe Couratier[2] · Danielle Seilhean[1,4] · Stéphanie Millecamps[1]

✉ Stéphanie Millecamps
stephanie.millecamps@icm-institute.org

Sibylle De Bertier
sibylle.debertier@icm-institute.org

Géraldine Lautrette
geraldine.lautrette@chu-limoges.fr

Maria-del-Mar Amador
mariadelmar.amador@aphp.fr

Tomoko Miki
tomoko.miki-ext@aphp.fr

Séverine Boillée
severine.boillee@sorbonne-universite.fr

Christian Stefan Lobsiger
christian.lobsiger@icm-institute.org

Delphine Bohl
delphine.bohl@icm-institute.org

Frederic Darios
frederic.darios@icm-institute.org

Selma Machat
selma.machat@chu-limoges.fr

Mathilde Duchesne
mathilde.duchesne@unilim.fr

Patrick Vourc'h
patrick.vourch@univ-tours.fr

Anne-Laure Fauret-Amsellem
anne-laure.fauret@aphp.fr

Philippe Corcia
philippe.corcia@univ-tours.fr

Nathalie Guy
nguy@chu-clermontferrand.fr

Philippe Couratier
philippe.couratier@chu-limoges.fr

Danielle Seilhean
danielle.seilhean@aphp.fr

[1] Institut du Cerveau - Paris Brain Institute - ICM, Inserm, CNRS, APHP, Hôpital de la Pitié-Salpêtrière, Sorbonne Université, 75013 Paris, France

[2] Service de Neurologie, Centre de Référence SLA et autres maladies du neurone moteur, CHU Dupuytren, 87000 Limoges, France

[3] Département de Neurologie, Assistance Publique Hôpitaux de Paris (APHP), Centre de Référence SLA Ile de France, Hôpital de la Pitié-Salpêtrière, DMU Neurosciences, 75013 Paris, France

[4] Département de Neuropathologie, APHP, Hôpital de la Pitié-Salpêtrière, DMU Neurosciences, 75013 Paris, France

[5] Service de Pathologie, CHU Dupuytren, 87000 Limoges, France

[6] UMR 1253, iBraiN, Université de Tours, INSERM, 37000 Tours, France

[7] Service de Biochimie et Biologie Moléculaire, CHU de Tours, 37000 Tours, France

[8] Unité Fonctionnelle de Neurogénétique Moléculaire et Cellulaire, Département de Génétique Médicale, APHP, Hôpital de la Pitié-Salpêtrière, Sorbonne Université, 75013 Paris, France

[9] Centre de référence SLA, CHU Tours, 37000 Tours, France

[10] Service de Neurologie, Centre de Ressources et de Compétences SLA, CHU Clermont-Ferrand, 63000 Clermont-Ferrand, France