Mark4 ablation attenuates the pathological phenotype in tauopathy model mice

Grigorii Sultanakhmetov
Tokyo Metropolitan University Graduate School and Faculty of Science: Tokyo Toritsu Daigaku
Rigakubu Daigakuin Rigaku Kenkyuka

Sophia Jobien M. Limlingan
Tokyo Metropolitan University Graduate School and Faculty of Science: Tokyo Toritsu Daigaku
Rigakubu Daigakuin Rigaku Kenkyuka

Aoi Fukuchi
Tokyo Metropolitan University Graduate School and Faculty of Science: Tokyo Toritsu Daigaku
Rigakubu Daigakuin Rigaku Kenkyuka

Keisuke Tsuda
Tokyo Metropolitan University Graduate School and Faculty of Science: Tokyo Toritsu Daigaku
Rigakubu Daigakuin Rigaku Kenkyuka

Hirokazu Suzuki
Tokyo Metropolitan University Graduate School and Faculty of Science: Tokyo Toritsu Daigaku
Rigakubu Daigakuin Rigaku Kenkyuka

Taro Saito
Tokyo Metropolitan University Graduate School and Faculty of Science: Tokyo Toritsu Daigaku
Rigakubu Daigakuin Rigaku Kenkyuka

Adam Z. Weitemier
Tokyo Metropolitan University Graduate School and Faculty of Science: Tokyo Toritsu Daigaku
Rigakubu Daigakuin Rigaku Kenkyuka

Kanae Ando (✉️ k_ando@tmu.ac.jp)
Tokyo Metropolitan University  https://orcid.org/0000-0002-3956-276X

Research Article

Keywords: Mark4, Alzheimer's disease, tauopathy, neurodegeneration, phosphorylation, aggregation, astrogliosis

Posted Date: October 5th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3382904/v1
Abstract

Background

Accumulation of abnormally phosphorylated tau proteins is linked to various neurodegenerative diseases, including Alzheimer's disease and frontotemporal dementia. However, what modifications of tau proteins cause disease phenotypes have not been fully understood. Microtubule affinity-regulating kinase 4 (MARK4) has been genetically and pathologically associated with Alzheimer's disease and reported to enhance tau phosphorylation and toxicity in Drosophila and mouse traumatic brain-injury models but not in mammalian tauopathy models.

Methods

To investigate the role of MARK4 in tau-mediated neuropathology, we crossed P301S tauopathy model (PS19) and Mark4 knockout mice. Following standard behavior, biochemical, and histology analyses were performed to evaluate changes in PS19 pathological phenotype with and without Mark4.

Results

In this study, we demonstrated that Mark4 deletion ameliorated the tau pathology in a mouse model of tauopathy. In particular, we found that PS19 with Mark4 knockout showed reduced mortality and memory loss compared with those bearing an intact Mark4 gene. These phenotypes were accompanied by reduced neurodegeneration and astrogliosis in response to the reduction of pathological forms of tau, such as those phosphorylated at Ser356, AT8-positive tau, and thioflavin S-positive tau.

Conclusion

Our data indicate that Mark4 critically contributes to tau-mediated neuropathology, suggesting that MARK4 inhibition may serve as a therapeutic avenue for tauopathies.

Background

The abnormally phosphorylated form of the microtubule-associated protein tau has been identified as a major component of paired helical filaments (PHFs) in neurofibrillary tangles (NFTs) and plaque neurites in the brains of patients with Alzheimer's disease (AD) [1–3]. NFT depositions have been associated with cognitive decline and pathology severity in AD [4, 5], which are the most prevalent causes of aging-associated dementia [6]. Under physiological conditions, tau regulates microtubule stability in the axon, whereas in disease, it is hyperphosphorylated, present in oligomers and NFTs [7].

Among its many phosphorylation sites, those located in the tau microtubule-binding repeats, such as Ser262 and Ser356, regulate its physiological and pathological functions [8]. High Ser262 phosphorylation has been observed from early-stage AD disease in pre-NFT neurons [9] and correlates with the propagation of tau pathology [10]. Tau phosphorylation at Ser262 and Ser356 was previously
shown to promote phosphorylation at other AD-associated phospho-epitopes such as AT100 (phosphorylation at Thr212 and Ser214) and AT8 (phosphorylation at Ser202 and Thr205) \[11\]. Tau phosphorylation at Ser262 and Ser356 affects its interactions with chaperone complexes and degradation \[12, 13\], intracellular distribution \[14\], and liquid–liquid phase separation \[15\]. Substitution of these sites by non-phosphorylatable alanines dramatically reduces tau toxicity in \textit{Drosophila} models \[11, 16, 17\], suggesting that phosphorylation at these sites is critical for tau toxicity.

\textbf{MARK4} belongs to the Par-1/microtubule affinity-regulating kinase (MARK) family, which constitutes evolutionarily conserved Ser/Thr kinases that phosphorylate microtubule-associated proteins, including tau, to regulate microtubule-dependent transport and stability \[18–21\]. Previous studies reported that MARK3 and MARK4 are sequestered to granulovacuolar degeneration bodies along with tau phosphorylated at Ser262, which is present in patients with AD \[22\], and elevation of the MARK4-tau interaction correlates with Braak stages \[23\]. Moreover, genomic studies showed that a \textit{Mark4} de novo genetic variant was linked to sporadic early-onset AD \[24\], and AD-linked single-nucleotide polymorphisms were identified within the \textit{Mark4} gene by a Bayesian genome-wide association study \[25\]. In \textit{Drosophila}, Par-1 overexpression enhances human tau toxicity, whereas Par-1 suppression mitigates it \[11, 26\]. Previously, we showed that expression of human \textit{MARK4} also enhances tau toxicity in a \textit{Drosophila} model \[27, 28\], suggesting mediation of tau abnormality by MARK4. Other studies have uncovered additional \textit{Mark4} functions in mouse models of obesity \[29\] and ischemic brain injury \[30\]. Nevertheless, the role of \textit{Mark4} in a mammalian tauopathy model has not been investigated.

Here, we examined \textit{Mark4} involvement in the disease pathogenesis of a tauopathy mouse model (PS19). By combining PS19 with a \textit{Mark4} knockout genetic background, we demonstrated that \textit{Mark4} deficiency significantly improved lifespan and memory of the PS19 model. We found that although \textit{Mark4} knockout did not affect tau phosphorylation at Ser262, it decreased Ser356 phosphorylation and reduced the abundance of AT8 phospho-epitopes and thioflavin S-positive aggregates. Interestingly, \textit{Mark4} deletion mitigated astrogliosis in brains of both PS19 and aged non-transgenic mice. Our results demonstrate that \textit{Mark4} ablation is sufficient to ameliorate the tauopathy phenotype in a mouse model, suggesting its critical involvement in neurodegenerative pathology.

\section*{Materials and methods}

\subsection*{Animals}

\textit{Mark4} knockout-mouse cryo-preserved spermatozoa (strain name: C57BL/6NCrl-\textit{Mark4}\textsuperscript{em1(IMPC)}Mbp/Mmucd, RRID: MRRRC\textsubscript{043405-UCD}) were purchased from the Mutant Mouse Resource and Research Center at the University of California at Davis. In this strain, exons 3, 4, and the flanking splicing regions of the \textit{Mark4} gene have been deleted using CRISPR/Cas9 gene editing. Litters were recovered by RIKEN BioResource Research Center, (Tsukuba, Ibaraki, Japan). Mice expressing the human 1N4R tau protein bearing the frontotemporal dementia-associated P301S mutation (PS19; strain name: B6;C3-Tg (Pmp-MAPT*P301S) PS19Vle/J, RRID: IMSR\_JAX:100010) under the prion promoter

Page 4/30
were purchased from Jackson Laboratories (Bar Harbor, Maine, the United States). We used heterozygous PS19 animals in this study.

All mice were bred in the Tokyo Metropolitan University animal facility in a special pathogen-free area with a 12:12 h light/dark cycle and free access to food (Picolab mouse diet 20, 5058) and water. *Mark4* knockout and PS19 mice were crossed to obtain littermates *Mark4*+/+ (wild type [WT]), *Mark4*+/-, *Mark4*−/−, PS19, PS19:*Mark4*+/-, and PS19:*Mark4*−/−. The first generation was obtained by crossing PS19 with *Mark4*−/− and the second was obtained by crossing PS19:*Mark4*+/- with *Mark4*+/. We used littermates of the first generation for the survival assay and the second generation for the behavioral, histological, and biochemical assays (breeding scheme: Fig. 1A). Genotypes were confirmed by PCR of tail DNA according to the manufacturer's genotyping protocols using the primers listed in Table 1. Ataxia or reaching the age of 12 months was considered an endpoint in survival experiments. Mice which developed ataxia were not used in any experiments. The study was approved by the Research Ethics Committee of Tokyo Metropolitan University (approval numbers: A5-5, A5-6, A4-6, A4-23, and A3-11). All animal experiments were performed according to the Tokyo Metropolitan University animal experimentation guidelines (following Science Council of Japan guidelines).

**Behavioral tests**

PS19 mice are known to manifest cognitive impairments at the age of 6 months [31]. Mice of different genotypes were randomly assessed on different experimental days. For behavioral assays, mice habituated the experimental space at least 30 min before assay initiation. Experiments were performed during the light cycle in a space with dim illumination and were blinded to mice genotypes on the experimental day and during analysis for all the behavioral assays.

**Open field assay**

Mice were allowed to explore a 40×40×40 cm box for 10 min. The box was wiped with 70% EtOH prior to usage by each individual mouse. An open field test was performed one day before novel object recognition and considered a habituation phase. We excluded mice from experiments (approximately 1–3 per genotype) that showed abnormal behavior such as intensive jumping, tail rattling, and/or sitting at a corner for more than half of the experimental time. A video was recorded on a 720p web camera. We used tracking software to evaluate the time spent in the middle area of the arena (20×20 cm) as well as the traveled distance (ToxTrack).

**Novel object recognition assay**

Experiments were performed according to a previously published protocol [32]. Briefly, the day after the open field assay, mice were allowed to explore two identical objects for 20 s within a 10-min session with a subsequent 24-h intersession interval before the test phase, during which mice were allowed to explore familiar and novel objects for 20 s within a 10-min session. Mice were considered to explore objects when
their nose was headed to the object’s direction at a 2-cm distance. Then, the time spent exploring each object was analyzed. The discrimination index (DI) was calculated using the following equation:

\[
DI = \frac{T_{\text{novel}} - T_{\text{old}}}{T_{\text{novel}} + T_{\text{old}}}
\]

where \(T_{\text{novel}}\) and \(T_{\text{old}}\) are the times spent observing novel and old objects, respectively. Mice that did not show any interest in objects, i.e., they explored all objects for less than 20 s within a 10-min session, were excluded from the experimental analysis.

**Y-maze assay**

We observed mouse behavior in the Y-maze to evaluate spatial working memory in our transgenic mice, as previously described [31]. Briefly, we placed a mouse into the center of the Y-maze and allowed it to observe the maze for 10 min. Video was recorded for every mouse, to analyze their behavior. The Y-maze has 35-cm arms with 10-cm height and 5-cm width. The successive alternation between arms is desired when mice do not return to previously explored arms, and the percentage of successive alternation (%SA) was calculated using the following equation:

\[
\%SA = \frac{A_{\text{successive}}}{A_{\text{total}} - 2}
\]

where \(A_{\text{successive}}\) and \(A_{\text{total}}\) represent the amounts of successive and total alternations, respectively. We subtracted the first two alternations because they could not be successful or wrong. Mice that climbed onto walls or jumped out from the maze were excluded from the analysis.

**Histology**

Mice were deeply anesthetized with a double dose of 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol tartare [33]. Then, mice were perfused with ice-cold phosphate-buffered saline (PBS) following 4% paraformaldehyde (PFA)/PBS. Mouse brains were extracted and fixed in 4% PFA/PBS solution for 24 h. Next, brains were immersed in 30% sucrose in PBS until the brain sunk to the bottom of the 15-mL tube. Brains were sliced in 40-µm sections using a Leica cryostat CM 1510 S (Wetzlar, Germany). The sections were immersed in cryoprotectant solution (30% ethylene glycol and 20% glycerol in PBS) and kept at -20°C until further use. Mouse brain slices were observed using a Keyence BZ-X710 (Osaka, Japan) epifluorescence microscope.

**Immunofluorescence**

We performed standard free-floating mouse brain section staining to examine the effect of Mark4 ablation on P301S tau levels, gliosis, and neurodegeneration in PS19 mice [34]. Briefly, mouse brain
sections were washed in PBS followed by permeabilization with 0.1% Triton X-100. Samples were blocked in 5% normal goat or donkey serum, depending on the host in which the secondary antibodies used were raised, for 1 h at room temperature. Then, sections were incubated with primary antibodies overnight at 4°C, followed by incubation for 2 h with secondary antibodies at room temperature and counterstaining with DAPI. Samples were stored in the dark at 4°C prior to being imaged using a Keyence BZ-X710 epifluorescence microscope. The primary and secondary antibodies used are listed in Table 2.

**Thioflavin S staining**

Thioflavin S staining was performed to analyze tau tangles in the brains of tauopathy model mice upon MARK4 protein ablation. Briefly, after washing, slices were mounted on a glass slide and allowed to completely dry on a heat plate at 42°C, followed by incubation with 0.5 mM thioflavin S (Merck, T1892) in 50% ethanol for 7 min [29, 30]. The number of thioflavin S-positive puncta was quantified using default thresholding segmentation and measure-particle functions in ImageJ (NIH).

**Biochemical analysis**

At 9 months of age, mice were sacrificed by cervical dislocation, and their brains were collected, snap-frozen in liquid nitrogen, and kept until further use at -80°C.

**In vitro kinase assay**

*In vitro* kinase assay was performed to validate kinase activity levels in our *Mark4* knockout mice. Mouse whole brains were homogenized in 10 vol (1:10 weight/volume ratio) of 3-morpholino-propane-sulfonic acid (MOPS) buffer (20 mM MOPS, pH 6.8, 1 mM EGTA, 0.1 mM EDTA, 0.3 M NaCl, 1 mM MgCl₂, 0.5% Nonidet P-40, and 1 mM DTT + proteinase inhibitors: 0.2 mM Pefabloc SC, and 1 mg/mL leupeptin, and phosphatase inhibitors: 10 mM b-glycerophosphate and 5 mM NaF) with a Teflon pestle homogenizer and centrifuged at 10,000×g for 15 min at 4°C to collect the extract as a supernatant. MARK4 was immunoprecipitated from brain lysates with an anti-MARK4 antibody (Novus Biologicals, NB100-1013) and protein-G Dynabeads (Thermo Fisher Scientific). Its kinase activity was measured using Chktide (SignalChem) and [γ-³²P]ATP as substrates. The incorporation of ³²P into Chktide was quantified using a liquid scintillation counter (Beckman Coulter).

**Western blotting**

Western blots were performed to analyze the relative protein levels of total and fractionated tau. One of the two brain hemispheres was homogenized in 10 vol of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris base, pH 8.0, 0.15 M NaCl, 1% Nonidet P-40, 0.5% Na-deoxycholate, 5 mM EDTA, 1 mM EGTA, 0.1% SDS + proteinase inhibitors and phosphatase inhibitors) using a Teflon pestle homogenizer and centrifuged at 15,800×g for 20 min at 4°C to collect the extract as a supernatant. Protein concentration was measured by Bradford protein assay (Pierce, 23200) and adjusted to 2 µg/µL. Then, protein homogenates were mixed with 2× loading buffer (124.8 mM Tris base pH 6.8, 4% SDS, 20% glycerol, 0.2 mg/mL bromophenol blue, 10% 2-mercaptoethanol + proteinase inhibitors and phosphatase inhibitors) to a final concentration of 1 µg/µL, and 10 µg of protein from each sample was loaded to a 10% gel for
SDS-PAGE. After gel electrophoresis, proteins were transferred to PVDF membranes, which were blocked in 5% bovine serum albumin/TBST (0.1 M Tris base pH 7.6, 0.15 M NaCl, 0.05% Tween 20) and then incubated with the designated primary and secondary antibodies described in Table 2. The band signals were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore) in Fusion SL (Vilber, France).

**Tau protein extraction**

Sequential extraction of tau protein was performed to test the effect of MARK4 on tau protein solubility. We performed fractionation analysis as previously described for PS19 mice [34, 35]. Briefly, one brain hemisphere was homogenized in 5 vol high-salt reassemble buffer (HS-RAB: 100 mM MES, pH 7.0, 1 mM EGTA, 0.5 mM MgSO$_4$, 0.75 M NaCl, 0.1 mM EDTA + proteinase inhibitors and phosphatase inhibitors) using a Teflon pestle homogenizer, and the homogenate was centrifuged at 50,000×g in an Optima MAX-TL ultracentrifuge (Beckman Coulter, Brea, CA, the United States) for 40 min at 4°C to collect the supernatant as an HS-RAB soluble fraction. The pellet was homogenized in 1 M sucrose/RAB buffer and the solution was centrifuged at 50,000×g for 20 min, followed by pellet homogenization in 1 vol RIPA buffer and centrifugation at 50,000×g for 20 min at 4°C to collect the supernatant as a RIPA soluble fraction. Next, we extracted a RIPA-insoluble pellet with 1 vol of cold 70% formic acid solution and centrifuged it at 15,800×g for 20 min at 4°C to collect the supernatant as an FA soluble fraction. The FA fraction was diluted in 1:10 (v/v) neutralization buffer (1 M Tris base, 0.5 M Na$_2$HPO$_4$), and the pH was checked using pH strips (Merck). All fractions were processed for western blotting as described above.

**Analysis**

For the open field test, the traveled distance and time spent in the middle area (20×20 cm) were quantified using ToxTrack version 2.96 [36, 37]. Western blot and microscopy data were analyzed using ImageJ version 1.53c [38, 39]. Band intensities were quantified using gel-selection and plot-line functions followed by band peak underline area measurements. In immunohistochemistry experiments, the area covered by astroglia and microglia was quantified using default thresholding segmentation and measured-particle functions. For other signals, integral intensities were evaluated using the measurement function. To analyze human tau-level changes, we subtracted the background intensities of WT animals. We normalized signals to GAPDH and DAPI intensities in western blot and immunofluorescence microscopy experiments, respectively. Data are represented as fold changes, normalized to average levels of PS19 or WT groups. Statistical analysis was performed in GraphPad Prism 9. The Mantel–Cox test was used to compare survival curves between PS19 and PS19:Mark4$^{+/−}$ animals. A two-way analysis of variance (ANOVA) test was used for the behavioral assay, where Mark4 deficiency was analyzed in WT and PS19 mice followed by a Fisher’s least significant difference (LSD) multiple comparisons test of the means between each group. For other tests, in which the effect of Mark4 deficiency was analyzed in PS19 mice, and WT was used as a negative control, one-way ANOVA was implemented followed by Fisher’s LSD or Dunnett’s multiple comparison tests among the means in each group or between the means of the PS19 and the PS19:Mark4$^{+/−}$ or PS19:Mark4$^{-/-}$ groups, respectively. For thioflavin S staining analysis, we performed a Kruskal–Wallis test followed by a Dunn’s multiple comparisons test.
Statistical tests for each experiment are specified in the figure legends. Differences were considered statistically significant when $p < 0.05$.

Results

**Mark4 knockout prolonged lifespan and rescued memory deficits in PS19 mice**

To test whether MARK4 suppression affects the abnormal behavioral phenotype and mortality of PS19 mice, we crossed them with *Mark4* knockout mice (Fig. 1A). Homozygous *Mark4* knockout mice (hereafter referred to as *Mark4*−/−) are fertile and develop to adulthood without apparent abnormalities [40, 41]. In addition to tail PCR genotyping, we confirmed the absence of MARK4 in *Mark4*−/− mice by western blotting and *in vitro* kinase assays (Fig. 1B, C). PS19 mice have a shorter lifespan [34], and in our colony, they had a median lifespan of 10.9 months, while all WT and *Mark4*+/− mice survived until 12 months of age. We found that PS19:*Mark4*+/− mice lived significantly longer than their PS19 counterparts ($p < 0.001$, Fig. 1D): at 12 months, the survival rates of PS19 and PS19:*Mark4*+/− were 16% vs. 65%, respectively.

Hyperactivity of PS19 mice, such as enhanced locomotion and more frequent alterations in the Y-maze [31], as well as enhanced locomotor activity in the open field test [31, 42], has been previously reported. In agreement with previous studies, 8-month-old PS19 mice showed enhanced locomotor activity compared with WT mice in the open field test. PS19:*Mark4*−/− mice showed a shorter, albeit not significantly, traveled distance than PS19 mice ($p = 0.24$, Figure 1E) and spent a similar amount of time in the middle area of the arena as PS19 mice (Figure S1A, B).

To assess the memory performance of our transgenic mice, we used the Y-maze spontaneous alteration (SAT) and novel object recognition (NOR) tests. The Y-maze SAT is designed to evaluate spatial working memory in mice [43]. As previously reported [31, 42], PS19 mice showed a lower number of correct alterations. Interestingly, *Mark4* copy number negatively correlated with increased performance of PS19 mice in the Y-maze SAT (57%, 63%, 71% of correct alterations in PS19, PS19:*Mark4*+/−, and PS19:*Mark4*−/− mice, respectively), with a significant difference between PS19 and PS19:*Mark4*−/− mice ($p < 0.01$, Figure 2F). However, the number of alterations in the Y-maze was not significantly different between experimental groups (Figure S1C).

To assay recognition memory in our transgenic mice [32], we performed the NOR test and observed that PS19 mice performed more poorly than WT, and PS19:*Mark4*+/− or PS19:*Mark4*−/− performed better than PS19 ([31, 42] and Fig. 1G). The NOR memory performance scores of PS19:*Mark4*+/− and PS19:*Mark4*−/− were significantly higher than those of PS19 ($p = 0.025$ and $p = 0.017$, respectively) and similar to those of WT ($p = 0.97$ and $p = 0.70$, respectively, Fig. 1F).
Our findings corroborate previous studies which did not identify any pathological abnormalities in \textit{Mark4} deficient mice [29, 40, 41]. We did not observe abnormalities in the survival, open field activity, or memory functions of our \textit{Mark4} knockout mice (Fig. 1D–G; WT, \textit{Mark4}^{+/−}, and \textit{Mark4}^{−/−} groups).

\textbf{Mark4 knockout ameliorated the loss of synapses and dendrites in PS19 mice}

We were then interested to address whether \textit{Mark4} knockout affects neurodegeneration in PS19 mice. To this end, we analyzed neurons involved in memory functions in the hippocampus, amygdala, and piriform cortex, where abnormalities were previously reported for PS19 mice [34, 44, 45]. We performed immunostaining using an antibody against the postsynaptic marker PSD-95 to identify the relative synapse signals. We observed that synapse density was lower in all tested brain regions of PS19 mice (\(p < 0.05\), Figure 2A, B; compare WT and PS19). PS19: \textit{Mark4}^{−/−} brains displayed significantly higher PSD-95 signal in the amygdala and piriform cortex than PS19 brains (\(p < 0.05\)), and the signals were as high as those in WT (\(p = 0.63\) and \(p = 0.51\), respectively, Fig. 2A, B). The average PSD95 staining intensity in PS19: \textit{Mark4}^{−/−} mice was also higher in the hippocampus than in PS19 mice, albeit not significantly (\(p = 0.056\)). PS19: \textit{Mark4}^{+/−} brains exhibited only moderate changes in PSD-95 immunoreactivity compared with PS19 animals (\(p > 0.1\), Fig. 2A, B).

Immunostaining with MAP2 antibody was analyzed to detect dendritic loss [46]. Among the tested regions, MAP2 immunoreactivity was similar in the hippocampus and amygdala between all experimental groups (\(p > 0.5\), Fig. 2A, C). MAP2 significantly reduced only in the piriform cortex of PS19 mice compared with the WT (\(p < 0.01\)), whereas it was recovered in PS19: \textit{Mark4}^{−/−} animals compared to PS19 (\(p < 0.05\)).

\textbf{Mark4 knockout reduced Ser356 phosphorylation levels in PS19 mice}

\textit{MARK4} phosphorylates the KXGS motifs of the tau microtubule-binding repeats, such as at Ser262 and Ser356 [18]. Therefore, we sought to identify whether \textit{Mark4} ablation affects tau phosphorylation at Ser262 and Ser356 (pSer262 and pSer356) in PS19 mice. Immunostaining with a tau anti-pSer262 antibody showed no differences among PS19, PS19: \textit{Mark4}^{+/−}, and PS19: \textit{Mark4}^{−/−} genotypes in all tested regions (\(p > 0.1\), Fig. 3A, B). pSer356 immunolabeling was weak in CA1 and prevalent in CA3 and the dentate gyrus in the hippocampus of PS19 mice (Fig. 3C, insets). By contrast to pSer262, pSer356 signal intensity was reduced by more than two-fold in PS19: \textit{Mark4}^{+/−} and more than four-fold in PS19: \textit{Mark4}^{−/−} compared with PS19 in all tested regions (Fig. 3C, D). The reduction in pSer356 was significant for hippocampus and piriform cortex regions in PS19: \textit{Mark4}^{−/−} and PS19: \textit{Mark4}^{+/−} compared to PS19 (\(p < 0.05\), Fig. 3D).

Additionally, we analyzed human tau expression with the human tau-specific antibody HT7. Immunostaining with HT7 exhibited significantly higher signal intensity in the hippocampus of...
PS19: *Mark4*<sup>−/−</sup> mice than in PS19 animals (p < 0.05), whereas it displayed similar intensity in the amygdala and piriform cortex (p > 0.1, Fig. 3E, F). PS19: *Mark4*<sup>+/−</sup> mice showed similar levels of HT7 tau as PS19 mice (p > 0.1, Fig. 3E, F).

**Mark4 knockout reduced AT8-positive tau levels in PS19 mice**

Immunostaining intensity using an AT8 antibody (pSer202, pThr205) correlates with disease severity in tauopathy [9]. PS19 mice start to exhibit an AT8 signal above baseline around 5 months of age in the hippocampus [47]. We conducted immunohistochemistry and western blotting to evaluate the effect of *Mark4* knockout on the abundance of the AT8 phospho-epitope in PS19 mice. We observed lower AT8 staining intensity in PS19: *Mark4*<sup>+/−</sup> animals and even lower AT8 signal in PS19: *Mark4*<sup>−/−</sup> than in PS19 mice (Fig. 4A-C). The reduction in AT8 was significant for all tested regions in PS19: *Mark4*<sup>−/−</sup> (p < 0.05), and the difference between PS19: *Mark4*<sup>+/−</sup> and PS19 was significant in the amygdala region (p < 0.01, Fig. 4B). Next, we compared the AT8-positive total tau levels in brain lysates. In line with immunohistochemistry experiments, a four-fold reduction in AT8 signal in PS19: *Mark4*<sup>−/−</sup> mice was observed by western blotting (p < 0.05, Fig. 4C). AT8-positive tau levels appeared to be on average two-fold reduced in PS19: *Mark4*<sup>+/−</sup> compared with PS19 mice; however, this difference was not significant (p > 0.1, Fig. 4C). Additionally, we confirmed the presence of *Mark4* knockout in our tested groups (Fig. 4C).

**Mark4 knockout reduced thioflavin S-positive tau aggregates and increased tau solubility in PS19 mice**

Thioflavin S-positive tau depositions have been observed in the neocortex, hippocampus, and amygdala at 6 months of age in PS19 mice [34]. Thus, we sought to evaluate the effect of MARK4 suppression on NFT pathology in PS19 mice by performing thioflavin S staining. Thioflavin S staining of tau appeared as puncta-like inclusions (Fig. 5A, white arrows). Thioflavin S puncta were significantly fewer in PS19: *Mark4*<sup>+/−</sup> than in PS19 mice in all brain regions tested (p < 0.05, Fig. 5B). A reduction in Thioflavin S-positive puncta was observed in PS19: *Mark4*<sup>+/−</sup> animals, but the difference was not significant due to variations among individuals (p > 0.1). In particular, PS19: *Mark4*<sup>+/−</sup> mice showed almost two-fold reduction compared to PS19, and PS19: *Mark4*<sup>−/−</sup> showed further reduction: whereas PS19 had more than hundred of puncta, PS19: *Mark4*<sup>−/−</sup> animals displayed only approximately a dozen puncta (Fig. 5B).

Next, we analyzed the effect of *Mark4* knockout on tau solubility by conducting sequential protein extraction from brain lysates using buffers with different extraction strengths (HS-RAB→RIPA→formic acid, Fig. 5C), followed by western blotting with the anti-human tau antibody HT7 [34, 35]. Tau in brain homogenates of PS19: *Mark4*<sup>+/−</sup> and PS19: *Mark4*<sup>−/−</sup> mice tended to be fractionated more in HS-RAB and less in RIPA or formic acid fractions compared with PS19. Tau proteins in the HS-RAB fraction from PS19: *Mark4*<sup>+/−</sup> and PS19: *Mark4*<sup>−/−</sup> mice were 40% and 28% more, respectively, whereas those in the RIPA
fraction were 52% and 37% less, than in PS19 samples, respectively (Fig. 5C, D). Tau proteins from PS19:Mark4−/− samples fractionated to the formic acid fraction and were 33% less than those in PS19 brain lysates (Fig. 5C, D). However, the difference was insignificant due to the large variation among individuals (p > 0.1).

**Mark4 knockout reduced astrogliosis in WT and PS19 mice**

We also sought to analyze the effect of Mark4 ablation on the activation of microglia and astroglia, which are signs of neuroinflammation. PS19 mice show neuroinflammation detected by gliosis at 6 months of age [34]. Immunostaining with the microglial marker Iba1 revealed that although the number of microglia was increased in PS19 compared with WT mice, it was not reduced upon Mark4 ablation (p > 0.1 among PS19, PS19:Mark4+/−, and PS19:Mark4−/− mice, Fig. 6A, B). Glial fibrillary acidic protein (GFAP), a marker of reactive astroglia, was increased in the PS19 amygdala and piriform cortex, indicating astrogliosis. In contrast to microgliosis, Mark4 knockout displayed a prominent effect on astrogliosis, as GFAP signals were lower in PS19:Mark4+/− and PS19:Mark4−/− than those in PS19 mice (Fig. 6C, D). Astrogliosis reduction was significant in both the PS19:Mark4+/− and PS19:Mark4−/− amygdala (p < 0.05) but only in the PS19:Mark4−/− piriform cortex (p < 0.05) compared with PS19 mice. In the hippocampus, GFAP staining was similar among WT, PS19, PS19:Mark4+/−, and PS19:Mark4−/− mice, and no difference was detected (p > 0.1, Fig. 6B, D). We also analyzed the levels of GFAP in brain extracts by western blotting. GFAP levels were significantly reduced in PS19:Mark4−/− compared with PS19 mice (p < 0.001, Fig. 6E).

Since MARK4 is directly involved in inflammation processes [48], we were motivated to test whether Mark4 ablation suppresses astrogliosis in the absence of human tau expression. We compared GFAP staining of reactive astroglia in the hippocampus of 9-month-old WT, Mark4+/−, and Mark4−/− mice (Fig. 7A), where GFAP-positive astroglia was more abundant than in other tested regions of WT mice (Fig. 6C, D). Mark4+/− mice showed the same reactive astroglia levels as their WT counterparts (p > 0.5, Fig. 7B). Importantly, the GFAP area was more than two-fold smaller in Mark4−/− mice than in WT mice (p < 0.01, Fig. 7B), indicating that MARK4 functions independently of tau toxicity in the activation of astroglia.

**Discussion**

In this study, we analyzed the role of MARK4 in tau-induced neurodegeneration by crossing a tauopathy model (PS19) [31, 34, 42, 49] with Mark4 knockout mice. We showed that Mark4 deletion could decrease the mortality rate, ameliorate memory deficits, and reduce synapse and dendritic loss in PS19 mice (Figs. 1 and 2). Mark4 deficiency decreased the levels of tau phosphorylation at Ser356 and AT8 while increasing its solubility (Figs. 3–5). We also demonstrated that Mark4 knockout suppressed astrogliosis in the PS19 model (Fig. 6) and in the hippocampus of mice that did not express human tau protein (Fig. 7), thus revealing a novel role of MARK4 in astrogliosis.
Previous reports suggest that a reduction in tau phosphorylation correlates with lower accumulation of pathological aggregates [42, 45, 49–51]. As a Par-1/MARK protein family member, MARK4 phosphorylates tau at the serines of KXGS motifs of microtubule-binding repeats, including Ser262 and Ser356 [20]. Tau phosphorylation at Ser262 was detected in pre-NFT neurons when prominent staining of NFT was observed using the 12E8 antibody, which recognizes both pSer262 and pSer356 tau [9]. This finding suggests that tau phosphorylation at these sites promotes tau aggregation. However, tau phosphorylation at Ser262 has been reported to prevent tau aggregation [52], and phosphorylation sites that drive tau aggregation do not include pSer262 [53]. Interestingly, the effect of Ser356 phosphorylation on tau aggregation has only been investigated in the presence of pSer262. Here, we found that Mark4 knockout decreased the pSer356 without affecting pSer262 levels in PS19 animals (Fig. 3). pSer356 reduction was correlated with increased tau solubility, less AT8-positive tau, and fewer thioavin S-positive tau aggregates (Figs. 4 and 5). These results suggest that reduced tau phosphorylation at Ser356 is sufficient to block its aggregation. They are also in agreement with a previous study which demonstrated that the NUAK Family Kinase 1 (NUAK1) exclusively phosphorylated Ser356, and its ablation reduced NFT formation and rescued memory deficits and synaptic plasticity in PS19 mice [49]. Interestingly, both MARK4 and NUAK1 ubiquitination appears to be controlled by the ubiquitin-specific protease-9 [54], and they are both associated with AD [22, 23, 49].

We found that Mark4 ablation caused a dramatic reduction in AT8-positive and insoluble tau (Figs. 4 and 5). Although the AT8 sites, Ser202 and Thr205, are not direct MARK4 phosphorylation targets [55], MARK4 may affect their phosphorylation via other kinases. GSK3β can phosphorylate more than 15 tau phosphorylation sites, including AT8 sites [8, 56–59], and is believed to be essential for tau toxicity in vivo [60]. In Drosophila, it was shown that tau phosphorylation at Ser262 and Ser356 by Par-1, the fly homolog of MARK, primed tau hyperphosphorylation at GSK3β sites [11]. This was further confirmed in mammalian primary neurons [61]. We observed a four-fold reduction AT8 signal reduction in PS19 mice upon Mark4 ablation (Fig. 4C), which may in part be due to the effects of a lower level of priming phosphorylation caused by decreased tau phosphorylation at Ser356. AT8 sites are also targets of Cdk5 [8, 62], and MARK4 can enhance tau phosphorylation mediated by Cdk5 [27]. Tau phosphorylation at Ser356 can affect its interactions with molecular chaperones which enhances tau aggregation [63], suggesting that altered interactions with molecular chaperones may contribute to decreasing the insoluble levels of tau in a Mark4 knockout background. Our results highlight a critical role of tau phosphorylation at Ser356 upstream of tau aggregation in vivo.

Finally, we found that Mark4 knockout reduced astrogliosis to WT levels in the PS19 mouse model (Fig. 6), which may also contribute to ameliorating tau toxicity. Previous reports indicate that a reduction in microgliosis and astrogliosis attenuates brain atrophy without changing tau pathogenic phosphorylation [44], suggesting that gliosis affects the disease phenotype downstream of tau pathological perturbations. Interestingly, microgliosis was not significantly affected by Mark4 knockout in PS19 mice (Fig. 6), and Mark4 deletion reduced the number of active astroglia in the hippocampus of 9-month-old mice that did not express human tau (Fig. 7). These findings point towards a physiological role of MARK4 in astrogliosis activation, independent of tau lesions. MARK4 has been reported to mediate the
activation of NLPR3 inflammasomes, which constitute critical signaling platforms in bone marrow-derived macrophages of the innate immune system [48]. *Mark4* ablation may ameliorate tau-induced neurodegeneration by not only reducing pathological tau modifications but also by suppressing astrogliosis. Further investigation of glial changes in response to MARK4 suppression in other neurodegenerative disease models may reveal novel aspects of the mechanisms underlying astroglial activation in disease pathogenesis.

**Conclusion**

In this study, we demonstrated the critical role of MARK4 in tau-induced neuropathology and indicated that reducing MARK4 activity is sufficient to ameliorate tau pathology. In particular, *Mark4* knockout in PS19 mice prolonged survival and restored memory to WT levels, which was accompanied by reduced synapses and dendritic loss, disease-associated tau phosphorylation, tau aggregation, and astrogliosis. Our results suggest that MARK4 is a reasonable target for the identification of novel tauopathy treatments.

**Abbreviations**

AD, Alzheimer’s disease

MARK, Microtubule affinity-regulating kinase

PHF, paired helical filament

NFT, neurofibrillary tangle

WT, wild type

DI, discrimination index

%SA, percentage of successive alternation

PBS, phosphate-buffered saline

PFA, paraformaldehyde

RIPA, radioimmunoprecipitation assay

HS-RAB, high-salt reassemble buffer

ANOVA, analysis of variance

LSD, least significant difference

SAT, spontaneous alteration test
**Declarations**

**Ethics approval**

The study was approved by the Research Ethics Committee of Tokyo Metropolitan University (approval numbers: A5-5, A5-6, A4-6, A4-23, and A3-11).

**Consent for publication**

Not applicable

**Data availability**

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

This work was supported by research grant from Japan Science and Technology Agency, grant number JPMJFS2139 (to GS), the Takeda Science Foundation (to KA), a research award from the Japan Foundation for Aging and Health (to KA), the Novartis Foundation (Japan) for the promotion of Science (to KA), a Grant-in-Aid for Scientific Research on Challenging Research (Exploratory) [JSPS KAKENHI Grant number 19K21593] (to KA), and TMU strategic research fund (to KA).

**Authors’ contributions**

KA conceptualized and supervised the study. GS, TS, AZW, and KA designed the experiments. TS supervised transgenic mouse husbandry. GS, SJML, and AF performed immunohistochemistry experiments. GS analyzed immunohistochemistry data. GS, KT, and HS performed and analyzed the behavioral experiments. GS and TS carried out and analyzed the biochemical experiments. GS, AZW, and KA wrote the manuscript.

**Acknowledgements**

We thank Drs. Naruhiko Sahara (National Institutes for Quantum Science and Technology, Chiba, Japan) for valuable advice on the tauopathy mouse model, Akiko Asada (Department of Biological Sciences,
Tokyo Metropolitan University) for technical helps and comments, and Shin-Ichi Hisanaga (Department of Biological Sciences, Tokyo Metropolitan University) for critical comments on the manuscript.

References


14. Zempel H, Thies E, Mandelkow E, Mandelkow EM. Aβ oligomers cause localized Ca2+ elevation, missorting of endogenous Tau into dendrites, Tau phosphorylation, and destruction of microtubules


45. Sarah L. DeVos1, Rebecca L. Miller1,†, Kathleen M. Schoch1,†, Brandon B. Holmes1, Carey S. Kebodeaux1, Amy J. Wegener1, Guo Chen1, Tao Shen1, Hien Tran2, Brandon Nichols2, Tom A. Zanardi2, Holly B. Kordasiewicz2, Eric E. Swayze2, C. Frank Bennett2, Marc I and TMM. Tau Reduction Prevents Neuronal Loss and Reverses Pathological Tau Deposition and Seeding in Mice with Tauopathy. Physiol Behav. 2017;176:139–48.


Tables

Tables 1 to 2 are available in the Supplementary Files section

Figures
MARK4 knockout prolonged lifespan and rescued memory deficits in PS19 mice. (A) Transgenic mouse breeding scheme and experimental timeline. (B) Western blot of MARK4 from total brain lysates of WT (Mark4+/+), Mark4+/-, and Mark4-/- mice. (C) MARK4 kinase activity immunoprecipitated from WT, Mark4+/-, and Mark4-/- mouse brain. The activity was measured as counts per minute of [g-32P]ATP incorporation into substrate peptides. Data represent the mean ± standard deviation (SD). (D) Kaplan–
Meier survival curve of PS19 compared with PS19:Mark4+/-. The endpoint for survival experiments was 12 months of age: 16% of the PS19 and 65% of PS19:Mark4+/− mice survived by this time. PS19 and PS19:Mark4+/− curves were compared by a Mantel-Cox test (***p=0.0008). N=15 (WT), N=15 (Mark4+/−), N=19 (PS19), and N=20 (PS19:Mark4+/−). (E) Total distance traveled within 10 min in the open field test. Data represent the mean ± SD. N=8 to N=13 mice/group. Two-way ANOVA was used to investigate the effect of the PS19 genotype (p=0.0041). *p<0.05; Fisher's least significant difference (LSD) multiple comparisons test. (F) Percentage of correct alteration in the Y-maze spontaneous alteration test. Data represent the mean ± SD. N=8 to N=11 mice/group. Two-way ANOVA revealed the interaction effect between PS19 and Mark4 knockout mice (p=0.0413). *p<0.05, **p<0.01; Fisher's LSD multiple comparisons test. (G) The discrimination index was quantified to assess recognition memory in WT and PS19 mice in the presence or absence of Mark4. Data represent the mean ± SD. N=5 to N=10 mice/group. *p<0.05; two-way ANOVA with Fisher’s LSD multiple comparisons test.
Figure 2

Mark4 knockout ameliorated the loss of synapses and dendrites in PS19 mice. (A) Representative images of double immunostaining of the hippocampus, amygdala, and piriform cortex regions for the postsynaptic marker PSD-95 and dendritic marker MAP2. The dashed line highlights the hippocampus (H), piriform cortex (PC) and amygdala (A). Insets represent magnified CA1, CA3, amygdala (A), and piriform cortex (PC) regions. Scale bars, 500 μm and 50 μm (insets). (B) Quantification of PSD-95 integral...
intensity in the hippocampus, amygdala, and piriform cortex normalized to region area size. Fold changes are represented relative to WT levels. Data represent the mean ± SD. N=4 to N=5 mice/group. *p<0.05, **p<0.01; one-way ANOVA with Fisher's LSD multiple comparisons test. (C) Quantification of MAP2 integral intensity in the hippocampus, amygdala, and piriform cortex normalized to region area size. Fold changes are represented relative to WT levels. Data represent the mean ± SD. N=4 to N=5 mice/group. ns, nonsignificant; *p<0.05; **p<0.01; one-way ANOVA with Fisher’s LSD multiple comparisons test. Mice were 9 months old.

Figure 3
Mark4 knockout decreased tau phosphorylation at Ser356. (A) Representative images of pSer262 tau immunostaining in the hippocampus, amygdala, and piriform cortex. (B) Graphs show the integral intensity quantification, normalized to the DAPI signal, from which the average background signal from non-transgenic mice (WT) was subtracted. Fold changes are represented relative to levels in PS19 mice. Data represent the mean ± SD. N=4 to N=5 mice/group. ns, nonsignificant; one-way ANOVA with Dunnett's multiple comparisons test. Insets represent the CA1, CA3, amygdala (A), and piriform cortex (PC) regions. Scale bars, 500 μm and 50 μm (insets). (C) Representative images of pSer356 tau immunostaining with (D) its integral intensity quantification in the hippocampus, amygdala, and piriform cortex. Data represent the mean ± SD. N=4 to N=5 mice/group. ns, nonsignificant; *p<0.05; **p<0.01; one-way ANOVA with Dunnett's multiple comparisons test. Insets represent the CA1, CA3, amygdala (A), and piriform cortex (PC) regions. Scale bars, 500 μm and 50 μm (insets). (E) Representative images of total human tau (HT7) protein immunostaining with (F) its integral intensity quantification in the hippocampus, amygdala, and piriform cortex. Data represent the mean ± SD. N=4 to N=5 mice/group. ns, nonsignificant; *p<0.05; one-way ANOVA with Dunnett's multiple comparisons test. Insets represent the CA1, CA3, amygdala (A), and piriform cortex (PC) regions. Scale bars, 500 μm and 50 μm (insets).
Figure 4

Mark4 knockout reduced tau phosphorylation at AT8 sites in PS19 mice. (A) Representative images of AT8 (pSer202/pThr205) immunostaining, with (B) its integral intensity quantified after normalization to DAPI fluorescence in the hippocampus, amygdala, and piriform cortex. The average background signal from WT was subtracted. Fold changes are represented relative to levels in PS19. Data represent the mean ± SD. N=4 to N=5 mice/group. ns, nonsignificant; *p<0.05; **p<0.01; ***p<0.001; one-way ANOVA
with Dunnett's multiple comparisons test. Insets represent the CA1, CA3, amygdala (A), and piriform cortex (PC) regions. Scale bars, 500 μm and 50 μm (insets). (C) Immunoblot of AT8 and total tau (Tau5) from brain lysates of WT, PS19, PS19:MARK4+/-, PS19:MARK4-/- with AT8 band integral intensity quantification (brackets) after normalization to GAPDH. The average background signal from WT mice was subtracted. Fold changes are represented relative to levels in PS19 samples. Data represent the mean ± SD. N=3 mice/group. ns, nonsignificant; *p<0.05; one-way ANOVA with Dunnett’s multiple comparisons test.

**Figure 5**

Reduction of thioflavin S-positive tau aggregates and increasing tau solubility as a result of Mark4 knockout in PS19 mice. (A) Representative images of thioflavin S staining of the hippocampus, amygdala, and piriform cortex of the mouse brain. Examples of thioflavin S-positive (ThioS+) puncta are labeled with arrows in the magnified panels. Panels represent CA1, CA3, amygdala (A), and piriform cortex (PC) regions. Scale bars, 500 μm and 50 μm (panels). (B) Quantification of the amount of ThioS+ puncta in the hippocampus, amygdala, and piriform cortex. Data represent the mean ± SD. N=4 to N=5 mice/group. ns, nonsignificant; *p<0.05; Kruskal–Wallis test with Dunn's multiple comparison test. (C) Western blot of total human tau (HT7) from high-salt reassembly buffer (HS-RAB), radioimmunoprecipitation assay (RIPA) buffer, and formic acid extraction of brain lysates of WT, PS19,
PS19:Mark4+/-, PS19:Mark4/- mice with (D) band integral intensity quantification normalized to GAPDH levels for HS-RAB lysate. Fold changes are represented relative to levels in PS19 samples. Data represent the mean ± SD. N=3 mice/group. No significant differences between groups were found (p>0.05). One-way ANOVA with Dunnett’s multiple comparisons test.

Figure 6

Mark4 knockout reduced astrogliosis in PS19 mice. (A) Representative images of Iba1 immunostaining with (B) quantification of the percentage of Iba1-positive area in the hippocampus, amygdala, and piriform cortex. Data represent the mean ± SD. N=4 to N=5 mice/group. ns, nonsignificant (p>0.05); ***p<0.001, ****p<0.0001; one-way ANOVA with Fisher’s LSD multiple comparisons test. Insets represent the CA1, CA3, amygdala (A), and piriform cortex (PC) regions. Scale bars, 500 μm and 50 μm (insets). (C) Representative images of GFAP immunostaining with (D) quantification of the percentage of GFAP-positive area in the hippocampus, amygdala, and piriform cortex. Data represent the mean ± SD. N=4 to N=5 mice/group. ns, not significant (p>0.05); *p<0.05; **p<0.01; one-way ANOVA with Fisher’s LSD multiple comparisons test. Insets represent the CA1, CA3, amygdala (A), and piriform cortex (PC) regions. Scale bars, 500 μm and 50 μm (insets).

(E) Immunoblot of GFAP from total brain lysate of WT, PS19, PS19:Mark4+/-, PS19:Mark4/- mice with GFAP bands integral intensity quantification normalized by GAPDH. Data are mean ± SD. N=3
mice/group. ns – non-significant, ***p<0.001; One-way ANOVA with Fisher's LSD multiple comparisons test.

**Figure 7**

Mark4 knockout reduced astrogliosis in the hippocampus of wild-type (WT) mice. (A) Representative images of GFAP immunostaining with (B) quantification of the percentage of GFAP-covered area in the hippocampus of WT, Mark4+/–, and Mark4–/– mice. Data represent the mean ± SD. to mice/group. ns, nonsignificant; **; one-way ANOVA with Dunnett’s multiple comparisons test. Insets represent the CA1, CA3, amygdala (A), and piriform cortex (PC) regions. Scale bars, 500 μm and 50 μm (insets).

**Figure S1.** Mark4 knockout did not affect the activity of wild-type and PS19 mice. (A) Trajectories of 9-month-old mice, running for 10 min in the open field test. (B) Time spent in the 20×20 cm middle zone (red square) of the arena for over 10 min during the open field test. Data represent the mean ± SD. to mice/group. Two-way ANOVA with Fisher's LSD multiple comparisons test. (C) Number of alterations in the Y-maze spontaneous alteration test. Data represent the mean ± SD. to mice/group. ns, nonsignificant; two-way ANOVA with Fisher's LSD multiple comparisons test.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.
• FigureS1.pdf
• Table1.csv
• Table2.csv