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Fyn depletion ameliorates tau^{P301L}-induced neuropathology



Guanghao Liu¹, Kimberly L. Fiock², Yona Levites³, Todd E. Golde³, Marco M. Hefti² and Gloria Lee^{1,4*} 

Abstract

The Src family non-receptor tyrosine kinase Fyn has been implicated in neurodegeneration of Alzheimer's disease through interaction with amyloid β (A β). However, the role of Fyn in the pathogenesis of primary tauopathies such as FTDP-17, where A β plaques are absent, is poorly understood. In the current study, we used AAV2/8 vectors to deliver tau^{P301L} to the brains of WT and Fyn KO mice, generating somatic transgenic tauopathy models with the presence or absence of Fyn. Although both genotypes developed tau pathology, Fyn KO developed fewer neurofibrillary tangles on Bielschowsky and Thioflavin S stained sections and showed lower levels of phosphorylated tau. In addition, tau^{P301L}-induced behavior abnormalities and depletion of synaptic proteins were not observed in the Fyn KO model. Our work provides evidence for Fyn being a critical protein in the disease pathogenesis of FTDP-17.

Keywords: Fyn, Tau, Neurofibrillary tangles

Background

Alzheimer's disease (AD), along with a subset of disorders such as frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and Pick's disease, exhibit neurofibrillary tangles (NFTs) made up of aggregated hyperphosphorylated tau and are collectively known as tauopathies (reviewed by [78]). During the disease process, tau becomes abnormally hyperphosphorylated, detaches from microtubules, undergoes conformational changes, and ultimately aggregates to form NFTs (reviewed by [1, 21, 50, 52, 73]). AD also contains an additional neuropathological hallmark, amyloid β (A β) plaques. Although no mutations in the tau gene have been identified to cause AD, several mutations in tau, such as P301L, have been found to cause FTDP-17, where tau hyperphosphorylation,

neurofibrillary tangle formation, and neurodegeneration occur in the absence of A β plaques [12, 29, 60, 79].

In neurons, tau is enriched in axons to regulate microtubule assembly (reviewed by [17, 36, 81]). We have previously reported that tau can also associate with the cytoplasmic face of the plasma membrane [7] and interacts with the SH3 domain of Src family non-receptor tyrosine kinases (SFK) such as Fyn and Src [41]. We found that Fyn phosphorylates tau at tyr18 [42] and that the tau-Fyn interaction increased the auto-phosphorylation of Fyn as well as the enzymatic activity of Fyn [71]. In addition, we reported that tau^{P301L} and other FTDP-17 tau mutants had a higher binding affinity to the Fyn SH3 domain, when compared to WT tau [4]. Subsequently, it was discovered that tau is also involved in targeting Fyn to the postsynaptic space in dendrites; Fyn then affects synaptic plasticity by phosphorylating the *N*-methyl-d-aspartate receptor (NMDAR) which facilitates the interaction between NMDAR and postsynaptic density 95 protein (PSD-95) [32, 56, 63]. It has also been reported that tau^{P301L} is selectively enriched in dendritic spines whereas WT tau is not [27, 82].

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Initially, Fyn was linked to AD when it was discovered that neuritic plaques and dystrophic neurites in AD brain contained phospho-tyrosine and that Fyn was up-regulated in a subset of neurons and co-localized with NFTs in AD brain [26, 72, 90]. Subsequent studies using cell culture showed that A β treatment increased both Fyn activation and the level of tyrosine phosphorylated tau [48, 89]. In addition, genetic ablation of Fyn was protective against oligomeric A β -mediated neurotoxicity [39] while overexpressing Fyn in human amyloid precursor protein transgenic mice increased synaptic and cognitive impairments [10, 11]. Furthermore, inhibiting Fyn activity through a small molecule inhibitor in the triple transgenic AD mouse model also significantly rescued memory and cognitive impairments [33]. The mechanism by which A β activates Fyn involves the binding of soluble A β oligomers to cellular prion protein (PrPC) [85, 86]. The A β -PrPC complex then interacts with the metabotropic glutamate receptor (mGluR5) to activate intracellular Fyn kinase [84]. The effect of A β on Fyn activation has been linked to synaptic abnormality and behavioral deficits in APP mouse models (reviewed by [5, 6, 75]). Fyn has also been directly and indirectly implicated in the hyperphosphorylation of tau at disease-related sites. Firstly, Fyn has been reported to activate Cdk5 [67] and GSK-3 [43], two kinases that hyperphosphorylate tau on serine and threonine residues [24, 25, 47, 49, 58]. Secondly, we have found that phosphotyrl8-tau is present in neurofibrillary tangles in brains from AD patients [42] as well as brains from an FTDP-17 mouse model that expresses the tau^{P301L} mutation [2, 4]. In addition, pY18-tau also co-localized with activated Src family tyrosine kinases (SFK) in NFTs of FTDP-17 mouse brains [2]. In A β -mediated neurodegeneration, the importance of the tau-Fyn interaction has been supported by the finding that inhibition of the interaction ameliorated the toxic effects of A β oligomers [65].

In FTDP-17 mouse models, several changes induced by tau^{P301L} have been reported, such as behavioral deficits, tau hyperphosphorylation, electrophysiological changes, and structural atrophy of dendritic spines with reduction of surface NMDA and AMPA receptors [16, 27, 28, 37, 59, 62, 64, 66]. However, the role played by Fyn in the disease pathogenesis of FTDP-17, where A β is absent, is unknown. In this study, we have used a viral gene transduction method to facilitate somatic brain transgenesis and to create an FTDP-17 tauopathy model on a Fyn KO background. We found that depleting Fyn reduced tau-related pathology. In the absence of Fyn, tau^{P301L}-induced neurofibrillary tangle burden and tau hyperphosphorylation at disease related sites were decreased. Fyn depletion also reduced the neuroinflammatory cell activation caused by the mutant tau. Also, tau^{P301L}-induced behavior abnormalities and depletion

of synaptic proteins were not observed in the Fyn KO tauopathy model. Our results establish Fyn as a critical protein involved in the pathogenesis of FTDP-17.

Methods

Mice

Fyn KO (C57BL/6;S129) mice were obtained from Jackson Laboratories (strain 002385). WT mice were generated by crossing the Fyn KO and Tau KO (C57BL/6, strain 007251) mice, as described [46]. In this way, the strain of the WT would be closer to that of the Fyn KO, minimizing strain related differences. Litter mate controls were not used. All procedures and animal care were approved by the University of Iowa Institutional Animal Care and Use Committee and in compliance with the *NIH Guide for the Care and Use of Laboratory Mice*.

AAV-tau^{P301L} injection at postnatal day 0 (p0)

AAV2/8-tau^{P301L}, produced by the University of Iowa Viral Vector Core, was injected intracerebroventricularly (ICV) into WT and Fyn KO mouse pups on postnatal day 0 (4.2e12 viral particles/ μ l; 2 μ l/ventricle). ICV injections were performed as described [9], where newborn mice were anesthetized by placing on a cold pack and a 32-gauge needle was used to pierce the skull 2/5 way between eyes and bregma and inserted at 0.5 cm depth. Both right and left lateral ventricles were each injected with 2 μ l of AAV. At 6 months of age, behavioral testing was performed. Subsequently, mice were deeply anesthetized with isoflurane then perfused with phosphate-buffered saline. The brain was removed and bisected along the midline. Half was drop-fixed in 4% paraformaldehyde overnight at 4 °C for histology, whereas the other half was frozen in liquid N₂ after discarding the olfactory bulb, cerebellum, and midbrain. WT mice and Fyn KO mice injected with AAV-tau^{P301L} were designated WT-AAV and Fyn KO-AAV, respectively. In AAV2/8, the expression of tau^{P301L} was regulated by a cytomegalovirus enhancer/chicken beta actin (CBA) promoter, a woodchuck hepatitis virus post-transcriptional-regulatory element (WPRE) and the bovine growth hormone polyA [9].

Magnetic resonance imaging

Mice were imaged at 8 weeks of age. Varian Unity/Inova 4.7 T small-bore MRI system (Varian, Inc., Palo Alto, CA; Small Animal Imaging Facility, University of Iowa) with an in-plane resolution of 0.13 \times 0.25 mm² and 0.6 mm slice thickness was used. Coronal images were collected and lateral ventricle volume sizes were analyzed with ImageJ [8]. Quantification was carried out as previously described [46]. Fyn KO mice were divided by degree of hydrocephalus, with lateral ventricle volume of \leq 0.2 units considered normal, 0.2 < x \leq 2 units as

moderate, and > 2 units as severe hydrocephalus. The lateral ventricle volume (LLV) cut-offs used to define these groups were based on the total movement assay, where significant differences in movement occurred between the groups as defined by these LLV cut-offs.

Preparation of brain homogenates, crude synaptosomes, and western blot analysis

Mouse hemi-brains were homogenized in sucrose buffer (0.32 M sucrose, 1 mM NaHCO₃, 1 mM MgCl₂, 0.5 mM CaCl₂, 10 mM NaF, 1 mM NaVO₄, 1 mM AEBSF, 10 µg/ml pepstatin, 10 µg/ml leupeptin, 10 µg/ml aprotinin) and samples were centrifuged (1000 g, 10 min, 4 °C). The pellet, containing nucleus and cell debris, was discarded and the supernatant, containing cytosolic proteins, was retained as the crude brain lysate. An equal amount of 2X Laemmli sample buffer was added and heat-denatured for 5 min at 95 °C.

Western blotting of crude brain lysates has been previously described [41]. Primary antibodies used were: GAPDH (1: 20,000 mouse monoclonal, Chemicon); Fyn3 (1: 1000 rabbit polyclonal, Santa Cruz); tau5-HRP (1: 10,000, [44]); tau13 (1: 20,000 mouse monoclonal, gift from late Dr. Lester Binder); AT8 (1: 1000 mouse monoclonal, ThermoFisher); anti-pY18-tau (1:1000 rabbit polyclonal [42]); PSD95 (1:1000 rabbit polyclonal, Millipore Sigma); and NeuN (1:5000 mouse monoclonal, Millipore Sigma). Quantification of lightly exposed blots was done by densitometry using ImageJ. For Fig. 3, total tau levels from crude lysates, measured by tau5, were normalized to GAPDH levels; AT8 and pY18 signals were normalized to tau5 and protein level values from Fyn KO-AAV were normalized to those from WT-AAV. 19 WT-AAV and 7 Fyn KO-AAV mice were used for AT8 and tau5; 12 WT-AAV and 7 Fyn KO-AAV mice were used for anti-pY18-tau. For quantitating NeuN and PSD95 (Fig. 6), levels were first normalized to GAPDH and then normalized to WT-uninjected mice. At least 7 WT, 9 WT-AAV, 9 Fyn KO, and 9 Fyn KO-AAV mice were used.

Crude synaptosome preparations were prepared from the crude brain lysate as previously described [32]. Primary antibodies used to probe the crude synaptosome preparations were: Fyn3, tau5-HRP, and tau13 (as above); β-actin (JLA20 1:10,000 mouse monoclonal, Iowa DSHB); PSD-95 (EP2652Y, 1:1000 rabbit monoclonal, Millipore, Cat# 04–1066); and NR2B (1:1000 mouse monoclonal, One World Labs, StressMarq, Cat # SMC-33D). Quantification of lightly exposed blots was done by densitometry using ImageJ. PSD95, NR2B, and Fyn levels were normalized to β-actin levels. Protein level values from WT-AAV, Fyn KO, and Fyn KO-AAV were normalized to those from WT with the WT values represented as “1”. 7 WT, 10 WT-AAV, 6 Fyn KO, and 11

Fyn KO-AAV mice were used for synaptosome preparations.

Histology and immunohistochemistry

Hemi-brains fixed in 4% paraformaldehyde were embedded in paraffin, sectioned using a sagittal plane at 5-µm thickness, and mounted on glass slides. The tissue sections were deparaffinized in xylene and rehydrated in a graded series of alcohols. Antigen retrieval was performed by steaming in 1 M Tris pH 9 buffer for 30 min. Sections were stained using Bielschowsky silver stain or tau13 (1: 5000 mouse monoclonal, gift from late Dr. Lester Binder). Sections were also labeled with 0.05% Thioflavin S. Sections were examined with light or epifluorescence microscopy (Olympus BX61). 3 WT-AAV and 3 Fyn KO-AAV mice were examined. For quantitating Bielschowsky tangles, 3 random 400x fields containing the highest total tangle burden were used for each mouse and the total number of tangles was counted. 3 WT-AAV and 3 Fyn KO-AAV mice were examined.

9G3 (pY18, 1:1000 ms IgG_{2a}) and AT8 (pS199/S202, 1: 1000 ms IgG₁) or CP17 (pT231, 1:1000 ms IgG₃, gift from Dr. Peter Davies) were used for double labeling immunofluorescence experiments using sections from the same animals. Secondary antibodies used were Rhodamine goat anti-mouse IgG_{2a} (1: 250, SouthernBiotech) for 9G3, FITC goat anti-mouse IgG₁ (1:250, SouthernBiotech) for AT8, and Alexa 488 goat anti-mouse IgG (1:250, Molecular Probes). Sections were analyzed by epifluorescence microscopy (Nikon E800); to quantitate intensity, integrated optical density (IOD) was obtained from equally exposed images using ImageJ. Four random pictures taken from the hippocampus of 3 WT-AAV and 3 Fyn KO-AAV mice were examined.

Microglia or astrocytes were labeled with antibodies against Iba1 (1:300 goat polyclonal, Abcam) or GFAP (1: 400 mouse monoclonal, MilliporeSigma), respectively. The sections were also co-labeled with tau13 (1:5000 mouse monoclonal, gift from late Dr. Lester Binder) to show the expression of human tau^{P301L}. For analyzing microglia, the entire hippocampus was imaged and the total number of Iba1 positive cells was manually counted. For analyzing astrocytes, the entire hippocampus was imaged and the GFAP positive area was thresholded, then converted to a percent positive area as described previously [74]. Three animals from each of the four groups were analyzed. To determine the number of neurons, sections were labeled with anti-NeuN (1: 5000 mouse monoclonal, Millipore Sigma), and based on both NeuN positivity and morphology, neurons were counted from one 400X field in Ammon's Horn for each mouse. 3 WT, 3 WT-AAV, 3 Fyn KO, and 4 Fyn KO-AAV mice were examined.

Open field activity

The open field test was performed on 6 month old mice as described previously [15]. Briefly, mice were placed in a 40.6 × 40.6 × 36.8 cm open-field chamber (San Diego Instruments, San Diego, CA), 55 lx, for 20 min. Total activity was defined as total beam breaks throughout the entire box, and central activity was defined as beam breaks occurring in the center (15.2 × 15.2 cm). 8 WT, 21 WT-AAV, 10 Fyn KO, 8 Fyn KO-AAV (mod hydro) mice were used.

Elevated plus maze

Elevated plus maze was performed on 6 month old mice as described previously [87]. Briefly, a maze was constructed from stainless steel with a Plexiglas base (36 in. tall) and two pairs of arms (2 × 11⁵/₈ inches) intersecting at right angles. One pair of arms was closed and had six-inch walls on three sides. The two open arms lacked walls. A 2 × 2-in. intersection at the center of the maze connected the four arms. Naive mice were placed onto the center and allowed 5 min to roam freely. The time each animal spent in open arms was recorded. 15 WT, 18 WT-AAV, 10 Fyn KO, and 8 Fyn KO-AAV (mod hydro) mice were used.

Contextual fear conditioning

Contextual fear conditioning was performed on 6 month old mice as described previously [76], using a near-infrared video-equipped fear conditioning chamber (Med Associates, Inc., St. Albans, VT). On training day (day 1), acquisition of contextual fear conditioning was performed, where a mouse explored the chamber for 3 min and then received 5 shocks (1 s, 0.75 mA), administered 1 min apart through the grid flooring. Total training time was 8 min. On the testing day (day 2), context-evoked freezing was tested by returning the mouse to the conditioning chamber for 6 min without foot shocks. Freezing was defined as an absence of movement other than respiration and scored with VideoFreeze software (Med Associates, Inc.). 12 WT, 20 WT-AAV, 8 Fyn KO, and 9 Fyn KO-AAV (mod hydro) mice were used in Fig. 5. 8 Fyn KO (no hydro), 8 Fyn KO (mod hydro), 9 Fyn KO-AAV (mod hydro) and 6 Fyn KO-AAV (severe hydro) were used in SI Fig. 1B.

Statistical analysis

Statistical analysis was carried out with GraphPad Prism 7.0. Nonparametric t-test, unpaired parametric t-test, and two-way ANOVA with Tukey's post hoc multiple comparisons were used to analyze data when appropriate. Mean ± standard error of the mean was used in all figures. $p \leq 0.05$ was considered as statistically significant, with $p \leq 0.05$ denoted as *, $p \leq 0.01$ as **, $p \leq 0.001$ as ***, and $p < 0.0001$ as ****.

Results

Injecting AAV- tau^{P301L} at p0 caused widespread human tau expression

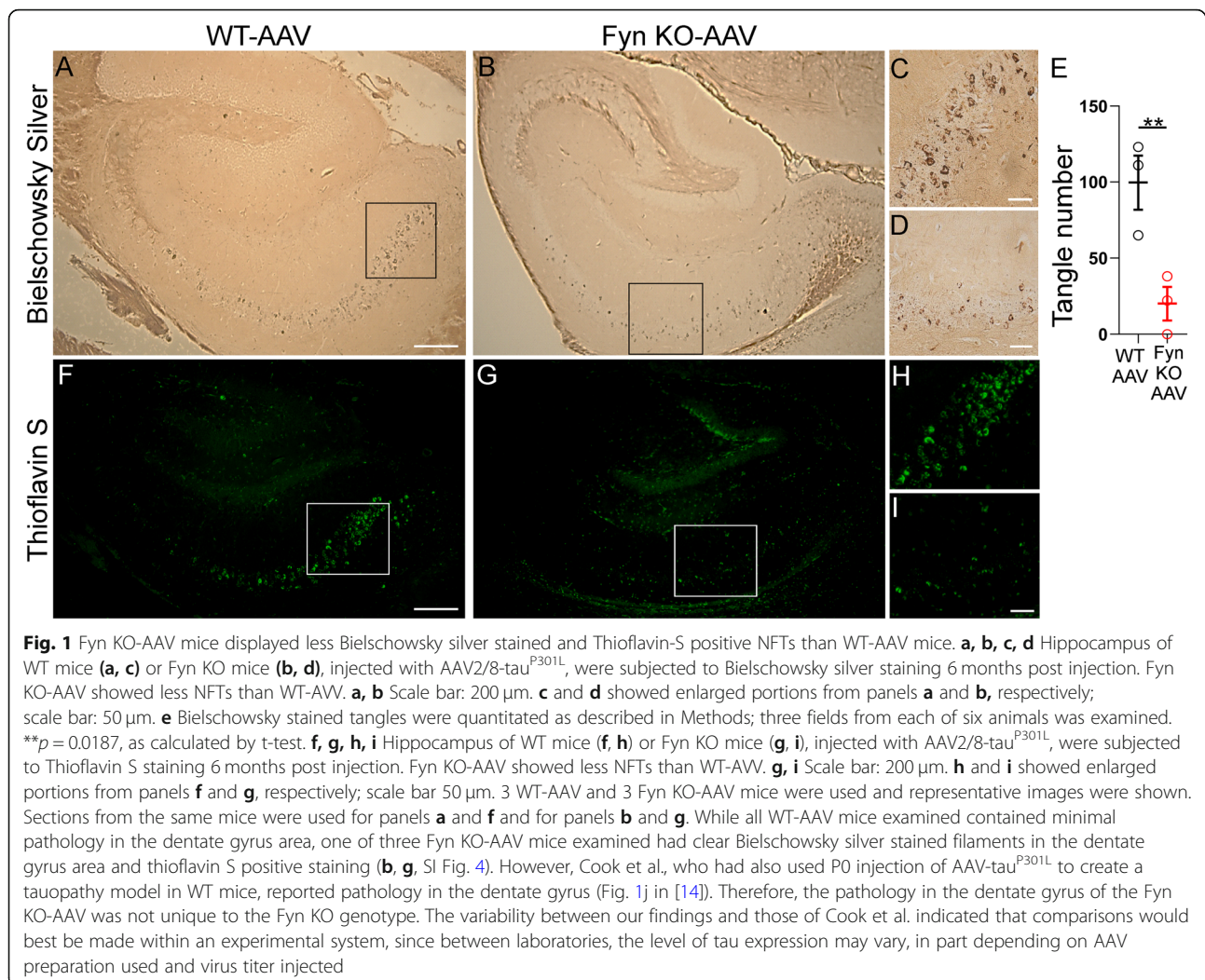
We used intracerebroventricular injection of AAV-tau^{P301L} in P0 mouse pups to create a P301L tauopathy model [14]. As described previously [22], depleting Fyn can predispose mice to develop non-obstructive hydrocephalus. Thus all AAV injected Fyn KO mice were screened with MRI as described in Methods, classifying animals as normal, or with moderate or severe hydrocephalus (examples of MRI images of mice with different levels of hydrocephalus were shown in SI Fig. 1A). All AAV injected Fyn KO mice were found to have some degree of hydrocephalus; of the 22 injected Fyn KO mice, 9 had moderate hydrocephalus and 13 had severe hydrocephalus. In contrast, when 22 AAV injected WT mice were examined, none developed hydrocephalus, indicating that injection of AAV-tau^{P301L} was not sufficient to induce hydrocephalus.

In order to control for the effects of hydrocephalus, Fyn KO-AAV mice with severe hydrocephalus, Fyn KO-AAV with moderate hydrocephalus, and uninjected Fyn KO mice, with moderate hydrocephalus or no hydrocephalus, were compared using behavioral tests. We found that Fyn KO mice with moderate hydrocephalus, either uninjected or injected with AAV, performed similarly to Fyn KO mice without hydrocephalus; only Fyn KO-AAV with severe hydrocephalus had abnormal behavior (SI Fig. 1B). We had also found that the Fyn KO-AAV with severe hydrocephalus had significantly higher levels of hyperphosphorylated tau than both WT-AAV and Fyn KO-AAV with moderate hydrocephalus (SI Fig. 2). Since our goal was to investigate the effects of Fyn KO, rather than the effects of hydrocephalus, Fyn KO-AAV mice with severe hydrocephalus were omitted from the study. Since Fyn KO-AAV with moderate hydrocephalus were similar in behavior to Fyn KO without hydrocephalus (SI Fig. 1B, C), such mice hereafter will simply be referred to as "Fyn KO-AAV".

Injected mice were harvested at 6 months and the distribution of human tau expression was evaluated histologically. Using immunohistochemistry, human-specific tau13 antibody was used to label human tau (SI Fig. 3). High levels of human tau were detected in the hippocampal regions of both WT-AAV (SI Fig. 3A, D) and Fyn KO-AAV (SI Fig. 3B, E) mice. As expected, uninjected WT mouse brain showed no tau13 labeling (SI Fig. 3C, F). In the previous study using intracerebroventricular injection of AAV-tau^{P301L} in P0 mouse pups, an AAV-GFP was injected as a control, showing that AAV injection alone was not sufficient to cause any of the tau^{P301L}-induced effects [14].

Fyn depletion reduced the effects of tau^{P301L} on NFT formation and abnormal tau hyperphosphorylation

At 6 months of age, WT-AAV mice displayed significant amounts of both Bielschowsky silver stained (Fig. 1a, c)

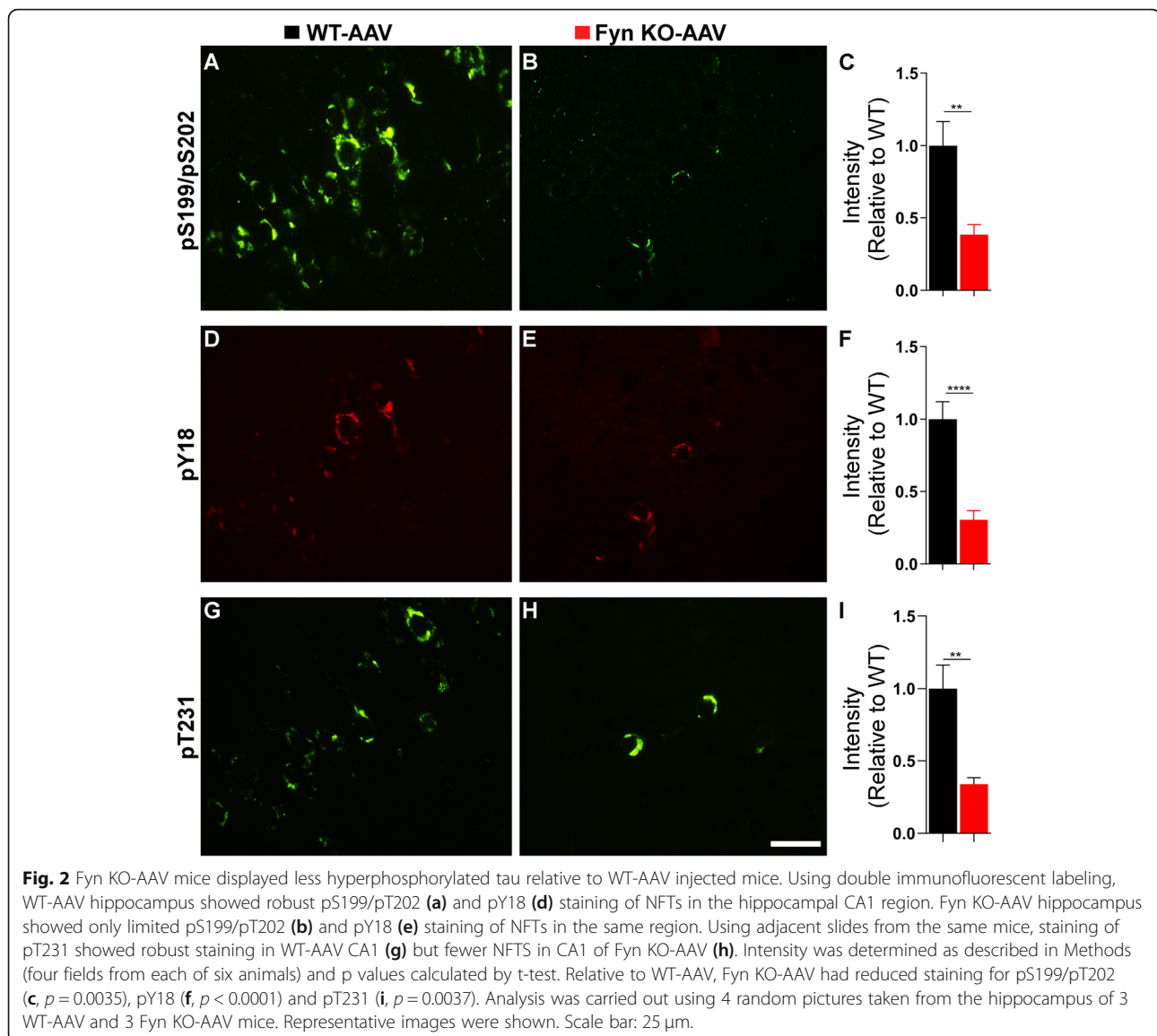


or Thioflavin S positive (Fig. 1f, h) tangles in the CA1–3 region of the hippocampus. In Fyn KO-AAV mice, a reduction in tangle pathology, relative to WT-AAV mice, was observed using Bielschowsky silver staining (Fig. 1b, d) or Thioflavin S (Fig. 1g, i). Upon quantitation of the Bielschowsky silver stain, we found that Fyn depletion resulted in a reduction of 79.6% in tangle pathology relative to WT-AAV ($p = 0.0187$). These results indicated that Fyn played an important role in NFT formation.

Given that NFT contain tau species that are abnormally phosphorylated, we examined the effect of Fyn depletion on tau hyperphosphorylation since Fyn is able to both directly and indirectly affect tau phosphorylation. WT-AAV mice displayed strong immunoreactivity to antibodies targeting pS199/pS202 (Fig. 2a), pY18 (Fig. 2d), and pT231 (Fig. 2g) in the hippocampus. In contrast, Fyn KO-AAV mice had a 61.4% reduction of pS199/pS202 (Fig. 2b, c, $p = 0.0035$), 69.5% reduction of pY18 (Fig. 2e, f, $p < 0.0001$), and 66.1% reduction of pT231 (Fig. 2h, i, $p = 0.0037$). Since pY18-tau persisted

in Fyn KO-AAV (Fig. 2e), another tyrosine kinase was still acting on tau in the absence of Fyn.

To assess the total level of phosphorylated tau species, soluble lysates from AAV injected WT and Fyn KO brains were subjected to western blot analysis using AT8 (pS199/S202) and pY18 antibodies. Since we had injected AAV-tau^{P301L} by hand, variability in tau expression might arise. To account for this possibility, we also probed the lysates with tau5, an antibody that detected both mouse and human tau, independent of phosphorylation. This allowed us to measure total tau levels for each injected mouse and to normalize pS199/S202 or pY18 levels to total tau levels; a scatter plot was used to display the normalized values. This analysis revealed that Fyn KO-AAV mice had a 72.4% decrease in pS199/pS202 ($p = 0.0106$) and a 44.0% decrease in pY18 levels ($p = 0.0168$) relative to WT-AAV (Fig. 3b, c). Uninjected mice were also examined, but pS199/pS202, pY18, and human tau were not detected (Fig. 3a). The total tau levels between Fyn KO-AAV and WT-AAV were not significantly different ($p = 0.0532$) (Fig. 3b, c).



Fyn depletion attenuated AAV-tau^{P301L} induced microgliosis

Several lines of evidence indicate that tau pathology is associated with inflammation in both human tissues [19, 20, 30] and animal models [3, 45, 88] and that increasing inflammation also worsens tau pathology [34, 38, 51]. Similar to results from Cook et al. [14], we also observed that AAV-tau^{P301L} expression in WT mice caused a significant increase in the number of Iba1 positive microglia in the hippocampus ($p = 0.0002$, Fig. 4a, c). AAV-tau^{P301L} expression also caused an elevated percent area of GFAP positive astrocytes in the same brain regions ($p = 0.0087$, Fig. 4b, d) compared to uninjected WT mice.

Interestingly, Fyn has also been shown to play a role in oligodendrocyte differentiation [77], astrocyte migration [18], and in the production of inflammatory cytokines in

natural killer cells [61]. In models of Alzheimer's disease, Parkinson's disease, and epilepsy, Fyn has also been implicated in modulating the neuroinflammatory response [55, 57, 70, 80]. We found that while tau^{P301L} increased microgliosis in WT mice, AAV injected Fyn KO mice did not experience tau^{P301L}-induced microgliosis when compared to Fyn KO uninjected mice ($p = 0.9027$; Fig. 4a, c). For astrocytosis, a similar result was obtained, where the Fyn KO and Fyn KO-AAV had a similar level of percent GFAP positive area ($p = 0.9473$; Fig. 4d).

Both Fyn depletion and AAV-tau^{P301L} caused behavioral abnormalities

To determine how Fyn depletion impacted tau^{P301L}-induced behavioral abnormalities, we evaluated performance on tasks designed to assess learning and memory as

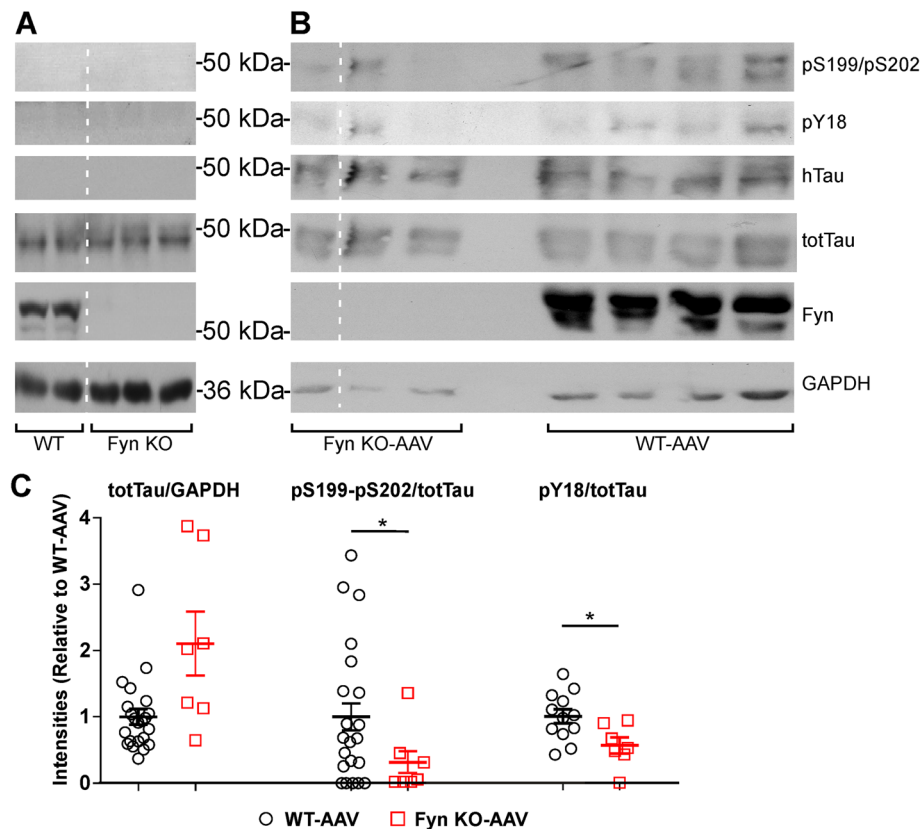


Fig. 3 Fyn depletion reduced tau^{P301L}-induced abnormal tau hyperphosphorylation. **a** Using soluble brain lysates, uninjected WT and Fyn KO mice did not show any abnormal tau hyperphosphorylation at the pS199/pS202 or pY18 epitopes. (Dotted white lines in A and B indicated places where noncontiguous lanes were brought together from the same blot.) **b** Using soluble brain lysates, WT-AAV and Fyn KO-AAV mice specifically showed the presence of human tau (hTau) as well as pS199/pS202 and pY18 epitopes. **c** Quantification for total tau normalized to GAPDH (left), pS199/pS202 normalized to total tau (center), and pY18 normalized to total tau [88]. Relative to WT-AAV, Fyn KO-AAV showed decreased tau hyperphosphorylation at pS199/pS202 (19 WT-AAV and 7 Fyn KO-AAV mice used, $p = 0.0106$) and pY18 epitopes (12 WT-AAV and 7 Fyn KO-AAV mice used, $p = 0.0168$) while total tau expression was not significantly different between Fyn KO-AAV and WT-AAV (22 WT-AAV and 7 Fyn KO-AAV mice used, $p = 0.0532$). To calculate p values, nonparametric t test was used for total tau and unpaired parametric t-test was used for pS199/pS202 and pY18

well as anxiety, which were all clinical manifestations of FTDP-17. In the open field assay, although none of the mice experienced significant differences in total movement (Fig. 5a), uninjected Fyn KO mice spent less time in the center of the apparatus compared to uninjected WT mice ($p = 0.0002$; Fig. 5b), indicating an anxiety phenotype. In the elevated plus maze, none of the mice experienced significant differences in time spent in the open arms (Fig. 5c). However, we did note that if the comparison was restricted to just the two groups, using parametric t test (SI Table 1), WT-AAV injected mice behaved significantly differently, relative to WT mice, in both the open field assay ($p = 0.0333$, Fig. 5b) and the elevated plus maze ($p = 0.0342$, Fig. 5c). Such a result agreed with data obtained by Cook and colleagues who used both the p0 AAV-tau^{P301L} approach [14], and the rTg4510 tauopathy mouse model [13]. In addition, we also noted that relative to uninjected WT mice, uninjected Fyn KO mice also behaved differently in elevated plus maze ($p = 0.0022$, SI

Table 1) if the comparison was restricted to just the two groups, using parametric t test.

In contextual fear conditioning, WT-AAV injected mice, relative to WT uninjected mice showed significant learning deficits during acquisition of shock on training day (day 1) (Fig. 5d) and significant memory deficit during the recall of shock in context on testing day (day 2) (Fig. 5e), as analyzed with two-way ANOVA. Interestingly, besides having a role in mediating anxiety behaviors (Fig. 5b), Fyn is also known to play a role in LTP and memory formation, which is consistent with our finding that uninjected Fyn KO mice displayed significant learning and memory deficits in contextual fear conditioning relative to WT uninjected mice (Fig. 5d, e). However, unlike WT-AAV, Fyn KO-AAV mice did not exhibit any learning (Fig. 5D) or memory deficits (Fig. 5e) relative to its uninjected mice. This agreed with our finding that Fyn KO-AAV mice did not experience any abnormalities in anxiety behaviors relative to Fyn KO uninjected mice (Fig. 5b, c). Lastly, we noted that the

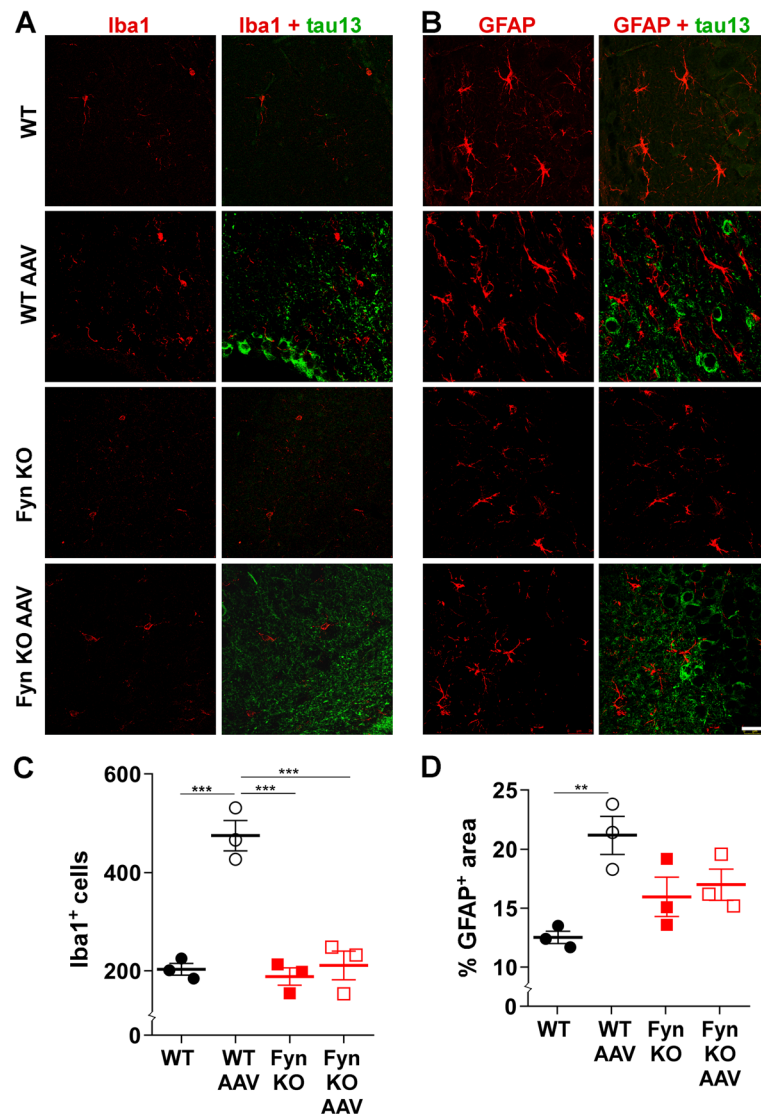


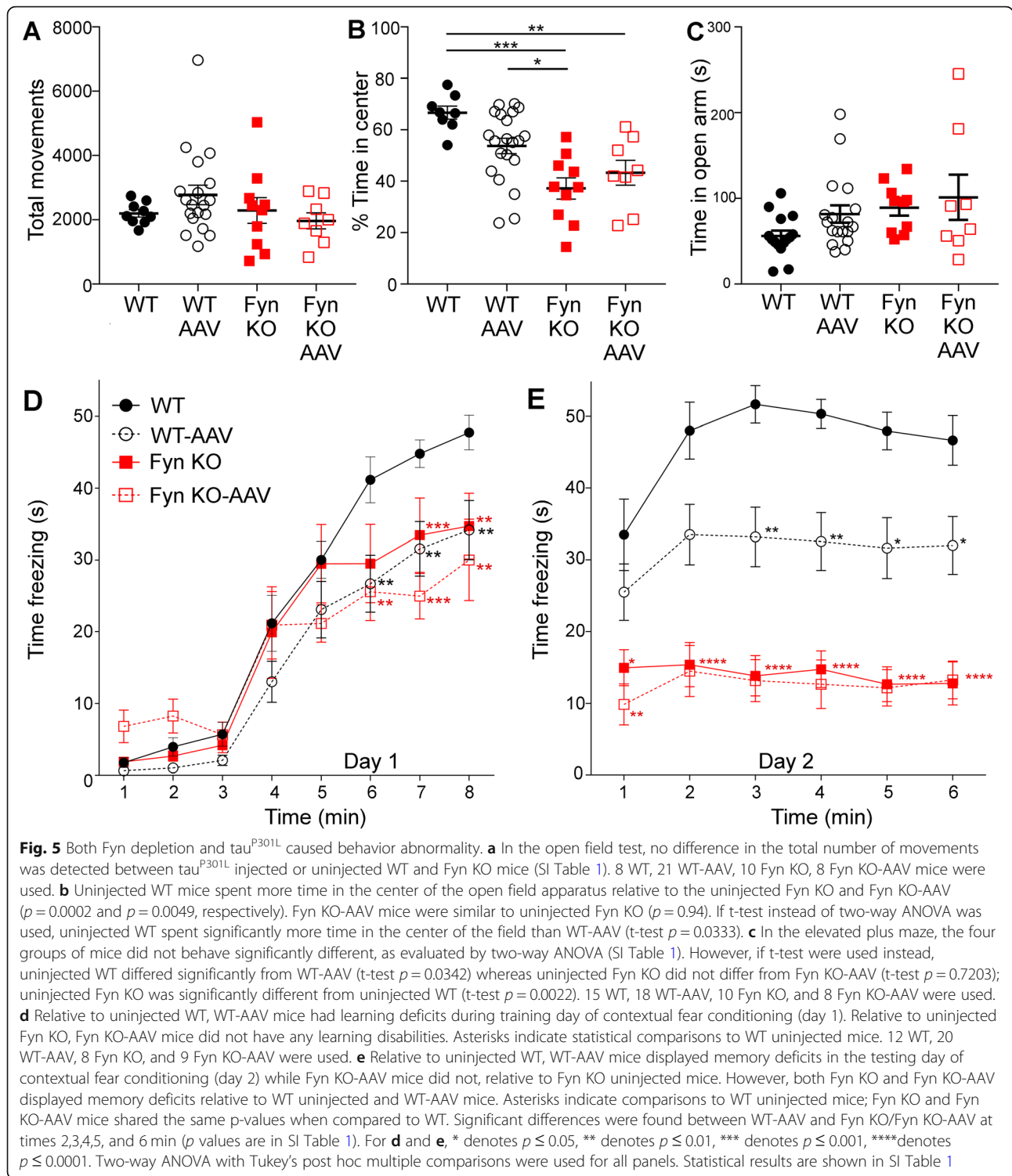
Fig. 4 Fyn depletion modulated tau^{P301L}-induced microgliosis. **a** WT-AAV had increased total number of Iba1 positive cells in the hippocampus relative to WT uninjected; no such change was found when comparing Fyn KO uninjected and Fyn KO-AAV mice. **b** WT-AAV had increased total area of GFAP positive staining in the hippocampus relative to WT uninjected; no such change was found when comparing Fyn KO uninjected and Fyn KO-AAV mice. **c** In the hippocampus, WT-AAV showed an increase in total number of Iba1 positive cells relative to WT uninjected ($p = 0.0002$) while Fyn KO-AAV did not show any increase relative to Fyn KO uninjected ($p = 0.9027$) or to WT uninjected ($p = 0.9954$). Fyn KO-AAV was significantly less relative to WT-AAV ($p = 0.0002$). **d** In the hippocampus, WT-AAV showed an increase in percent GFAP positive area relative to WT uninjected ($p = 0.0087$) while Fyn KO-AAV did not show any increase relative to Fyn KO uninjected ($p = 0.9473$), WT uninjected ($p = 0.1712$), or WT-AAV ($p = 0.2115$). 3 mice from each group was examined. Scale bar: 25 μ m. Two-way ANOVA with Tukey's post hoc multiple comparisons was used for panels C and D. Statistical results are shown in SI Table 1

deficits exhibited by the uninjected Fyn KO mice were either similar to (Fig. 5c, d) or larger (Fig. 5b, e) than those exhibited by WT-AAV.

Fyn depletion prevented tau^{P301L} induced synaptic protein degradation while no neuronal cell loss was detected in AAV injected mice

To determine whether the behavioral deficits experienced by the WT-AAV mice were due to overt neuronal

loss or more subtle synaptic abnormalities, we first probed whole brain crude lysates for NeuN, as a marker for neurons, and for PSD95, as a synaptic marker, in WT, WT-AAV, Fyn KO, and Fyn KO-AAV mice. In the crude brain lysates, there was no statistical difference in NeuN for all four groups of mice (Fig. 6 A, B left, SI Table 1). However, we found that in PSD95, WT-AAV had a 39.1% reduction relative to WT uninjected mice ($p = 0.0049$), a 39.6% reduction relative to Fyn KO



uninjected ($p = 0.0021$), and a 43.3% reduction relative to Fyn KO-AAV ($p = 0.00005$; Fig. 6a, b right). There was no difference between WT uninjected and Fyn KO uninjected mice ($p = 0.9999$), between WT uninjected and Fyn KO-AAV ($p = 0.9073$), or between Fyn KO and Fyn KO-AAV ($p = 0.9162$; Fig. 6a, b right). We also examined

brain slices stained with anti-NeuN and found no differences between the different groups in the total number of neurons in the Ammon's horn of the hippocampus (Fig. 6c, SI Table 1).

To further characterize the synaptic integrity of the mice, we obtained crude synaptosome preparations from

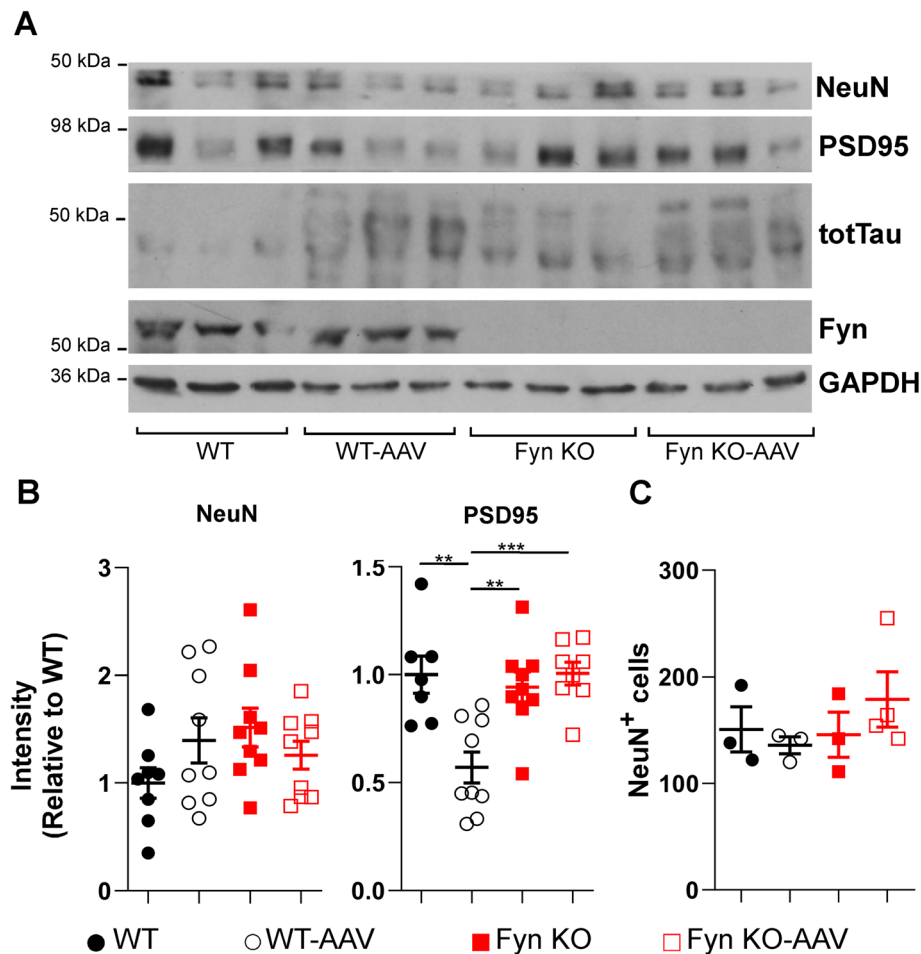


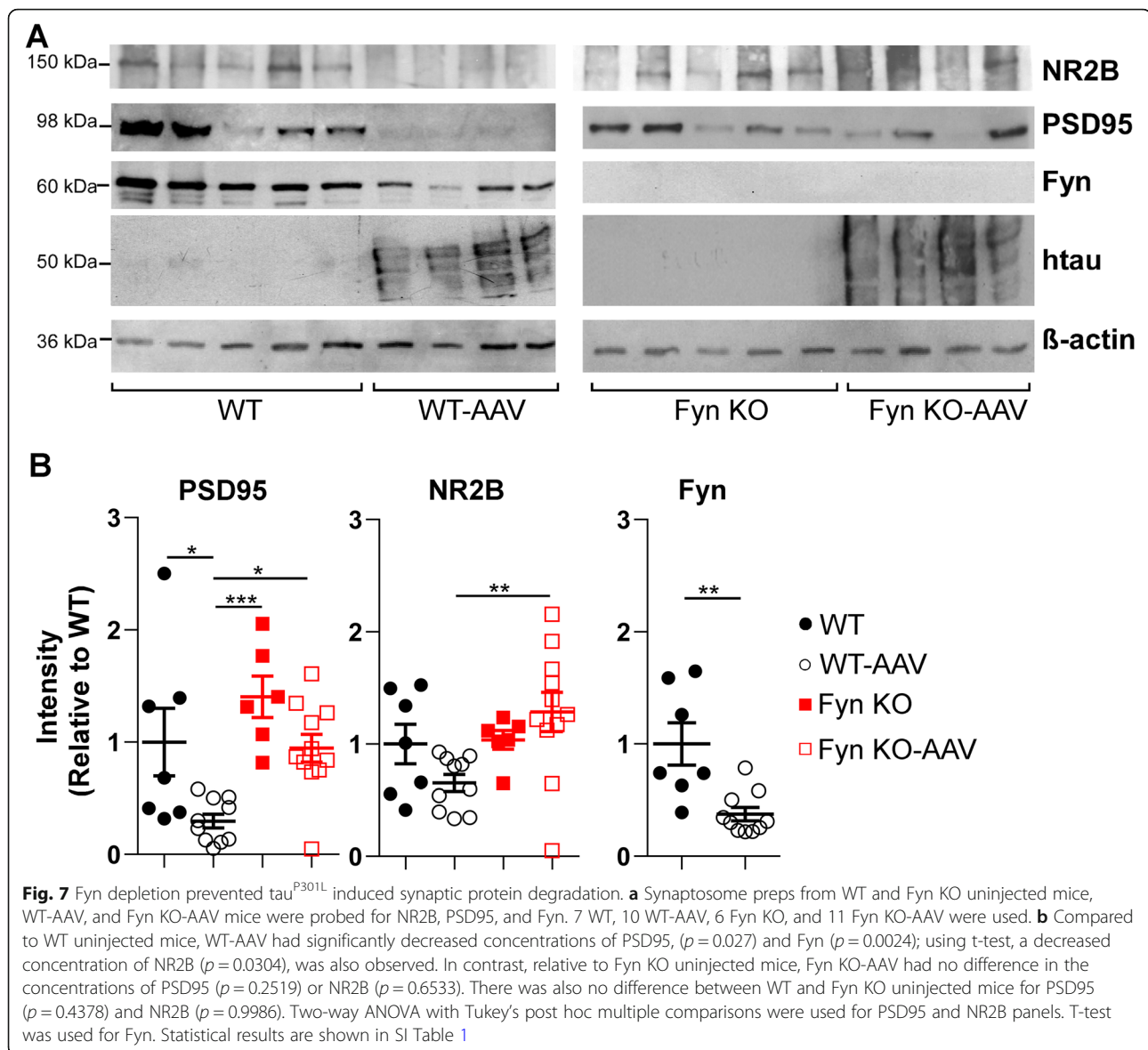
Fig. 6 AAV-tau^{P301L} did not cause overt neuronal cell loss at 6 months of age. **a** Crude brain lysates, probed by western blot, showed no change in NeuN between all mice groups. However, PSD95 was significantly decreased in WT-AAV mice. **b** Left: Quantification of NeuN showed no difference between the 4 groups (SI Table 1). Right: Quantification of PSD95 showed a 39.1% decrease in WT-AAV relative to WT uninjected ($p = 0.0049$). There was no difference between WT uninjected and Fyn KO uninjected mice ($p = 0.9999$) nor between WT uninjected and Fyn KO-AAV ($p = 0.9073$). For NeuN, 8 WT, 9 WT-AAV, 9 Fyn KO, and 9 Fyn KO-AAV were used. For PSD95, 7 WT, 9 WT-AAV, 9 Fyn KO, and 8 Fyn KO-AAV were used. **c** Quantification of NeuN positive cells showed no difference between the four groups in Ammon's horn ($p = 0.5214$). 3 WT, 3 WT-AAV, 3 Fyn KO, and 4 Fyn KO-AAV mice were examined. Two-way ANOVA with Tukey's post hoc multiple comparisons were used for all panels. Statistical results are shown in SI Table 1

WT, WT-AAV, Fyn KO, and Fyn KO-AAV brains and probed for postsynaptic markers such as PSD 95 and NR2B. Again, we found that relative to WT uninjected mice, WT-AAV mice had a 70.3% decrease in PSD95 ($p = 0.027$) (Fig. 7a, b). The 34.3% decrease in NR2B levels was not significant by two-way ANOVA ($p = 0.3607$) but was significant if only WT and WT-AAV were compared using the unpaired t-test ($p = 0.0304$, SI Table 1). Similar to our previous findings [46], Fyn KO uninjected animals had no changes in PSD95 ($p = 0.4378$) or NR2B ($p = 0.9986$) when compared to WT uninjected animals (Fig. 7a,b; SI Table 1), indicating that Fyn depletion itself did not cause alterations in synaptic integrity at 6 months of age. When examining the Fyn KO-AAV mice, we found that the levels of PSD95 and NR2B in Fyn KO-AAV mice were comparable to their

levels in either WT uninjected mice or Fyn KO uninjected mice (Fig. 7b, 5B; SI Table 1). Therefore, our data showed that Fyn depletion was able to reverse tau^{P301L} induced synaptic loss. Interestingly, the WT-AAV synaptosome preps also had a 62.5% decrease in Fyn when compared to uninjected WT mice (Fig. 7a, b) ($p = 0.0024$), an effect that may be due to the overall loss of PSD protein. Our data supports findings by other investigators where mice expressing tau^{P301L} at 6 months of age displayed synaptic loss without overt neuronal loss [14, 16, 64, 69].

Discussion

In this study, we reproduced the P301L tauopathy mouse model using injection of AAV-tau^{P301L} into P0 WT mouse brain, as previously described [14], then used



the same strategy to investigate the role of Fyn by injecting Fyn KO P0 brains. In the WT mice, we reproduced the results of Cook et al., where widespread expression of human tau was reported, together with tau hyperphosphorylation, neurofibrillary tangles, inflammation, behavioral abnormalities, and loss of postsynaptic markers [14]. In working with homozygous Fyn KO mice, one caveat was their tendency to develop non-obstructive hydrocephalus [22]. In our study, given that WT-AAV injected mice did not develop hydrocephalus, AAV-tau^{P301L} injection did not correlate with the occurrence of hydrocephalus. However, the combination of the P0 brain injection and the homozygous Fyn KO trait resulted in a higher incidence of hydrocephalus. Nevertheless, by eliminating mice with severe hydrocephalus, we were able to show that depleting Fyn (1) reduced

tau^{P301L}-induced abnormal tau hyperphosphorylation and NFT formation and (2) prevented tau^{P301L}-induced microgliosis and synaptic loss. These data provide evidence for Fyn as an essential protein involved in the disease pathogenesis of tauopathies.

In terms of regulating tau phosphorylation at Y18, Fyn depletion reduced but did not eliminate pY18. Since our lab has found that both Fyn and Src can phosphorylate this residue [42], Src may phosphorylate Y18 in a Fyn KO. Moreover, other tyrosine kinases such as Syk [40] and Lck [68] have also been reported to phosphorylate Y18. This suggests that the phosphorylation of Y18 is sufficiently important such that in spite of the absence of Fyn, the residue is phosphorylated. The importance of pY18-tau for glutamate-induced calcium influx and excitotoxicity in neurons has been shown [54]. As for ser/thr

phosphorylation, we found that depleting Fyn caused a significant reduction in phosphorylation of tau at a Cdk5 site (S199/S202) and a GSK3 β site (T231), suggesting that depletion of Fyn may have resulted in the reduction of Cdk5 and GSK3 β activities. In the Fyn KO, the reduction in tau hyperphosphorylation correlated with a reduction in Bielschowsky silver stained and thioflavin S positive NFT. While our findings did not rule out effects caused by potential strain differences, our data suggested that Fyn is critical for NFT formation.

In our somatic transgenic model, we were also able to replicate the neuroinflammatory changes, such as the prominent microgliosis and astrocytosis associated with tau^{P301L} expression [14, 28, 45, 51]. Our data showed that Fyn depletion was able to eliminate the effects of AAV-tau^{P301L} injection on microgliosis and astrocytosis, providing evidence that Fyn plays an important role in the microglial inflammatory response in tauopathies.

Lastly, the impact of Fyn depletion on tau^{P301L}-induced behavioral deficits was investigated. Our WT-AAV injected mice developed aberrant exploratory behavior and cognitive deficits that were also found in other transgenic FTDP-17 tauopathy mouse models [62]. At 6 months of age, these deficits have been attributed to tau^{P301L}-induced changes in dendritic spine architecture, synaptic dysfunction and loss, and abnormalities in electrophysiology, all preceding NFT deposition and neuronal loss in the rTg4510 transgenic FTDP tauopathy mouse model [14, 16, 64]. Our findings are consistent, as our data also showed losses in post-synaptic proteins without neuronal cell loss in WT-AAV.

In analyzing the behavioral deficits of Fyn KO-AAV, we found that the introduction of tau^{P301L} was not able to induce further deficits in the behaviors of Fyn KO mice (Fig. 5). However, Fyn KO mice were already known to have abnormal exploratory behavior and cognitive deficits relative to WT mice [31, 46, 53], stemming from Fyn's role in mediating LTP and memory formation through the phosphorylation of NMDARs [23, 35, 56, 83]. Therefore, the Fyn KO mice may already be performing at such a low level that further decreases might not be possible no matter the insult, such as tau^{P301L} (also known as the "floor effect"). Moreover, it was difficult to determine whether tau^{P301L} required Fyn to induce behavior abnormalities or that the deficits caused by Fyn depletion alone superseded those induced by tau^{P301L}. The latter possibility (the "floor effect") was supported by our finding that in two tests, the Fyn KO mice had significantly more deficits than the WT-AAV (Fig. 5b, e, SI Table 1). The fact that Fyn KO and Fyn KO-AAV both had normal levels of PSD95 and NR2B (Figs. 6 and 7) suggested that Fyn KO had no synapse loss and that tau^{P301L} could not induce synapse loss in the absence of Fyn. These findings suggest

that synapse loss cannot be used to explain the behavioral deficits of the Fyn KO and Fyn KO-AAV mice.

In summary, using AAV to create a P301L tauopathy mouse model on a Fyn KO background, we have found that Fyn was critical for neurofibrillary tangle formation and tau hyperphosphorylation. Our data suggests that reducing Fyn activity might be an effective therapy for tauopathies. However, given the importance of Fyn in normal physiological processes, such therapy would be difficult, especially since dementia patients usually have reduced cognitive reserves. Thus, more specific therapies aimed at targeting either disease related processes involving Fyn or Fyn interaction partners may yield a more desirable therapeutic result; the recent report of a peptide inhibitor of the tau-Fyn interaction [65] is a first step in this direction. In closing, as A β oligomers had no role in these findings, Fyn alone has an important role in tauopathy.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s40478-020-00979-6>.

Additional file 1. Supplementary Information

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Authors' contributions

G. Liu and G. Lee made substantial contributions to the conception and design of the work and to the acquisition, analysis, and interpretation of the data. G. Liu and G. Lee also drafted the work or substantially revised it. KF, YL and TEG made substantial contributions to the acquisition of data. MMH made substantial contributions to the acquisition, analysis and interpretation of data. G. Liu and G. Lee approved the submitted version. The author(s) read and approved the final manuscript

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Availability of data and materials

All data used/analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

Animal care complied with National Institutes of Health guidelines for the care and use of laboratory animals and all animal procedures were approved by the University of Iowa Institutional Animal Care and Use Committee.

Consent for publication

Not applicable.

Competing interests

TE Golde is a cofounder of Lacerta Therapeutics Inc. and a member of their Scientific Advisory Board. The other authors declare no competing financial interests.

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