

Amyloid beta exacerbates Tau-seeded pathology and drives tangle formation in human neuronal xenografts

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Abstract

The pathological cascade of Alzheimer's disease (AD) begins decades before clinical symptoms, yet modelling this slow and progressive process in experimental systems has remained challenging. Current models largely depend on Tau mutations associated with primary Tauopathies and fail to reproduce AD-relevant neurofibrillary tangle formation, even when human Tau isoforms and amyloid pathology are combined. Here, we xenotransplanted human neurons into *Rag2*^{-/-} and *Rag2*^{-/-} *App*^{NL-G-F} mice and exposed them to AD-derived Tau seeds via parenchymal brain injections, providing both the pathological nucleation event and the amyloid-rich environment needed to compress decades of human disease into the lifespan of a mouse. Whilst AD-Tau seeding alone was sufficient to induce Tau misfolding in human neurons, amyloid exposure markedly accelerated Tau aggregation, driving neurofibrillary tangle formation and elevated insoluble pTau181 and pTau231 in aged xenografts. This effect was abolished in human Tau-knockout neurons, although LAMP1 positive neuritic dystrophy persisted. Late-stage Tau pathology was absent in non-grafted *App*^{NL-G-F} mice, indicating a unique human neuronal susceptibility to Tau aggregation. Together, these findings demonstrate that the human neuronal environment is a critical determinant of neurofibrillary tangle formation, and that amyloid causally accelerates this process, thereby establishing the Tau seeded xenograft model as a platform to dissect the interaction between amyloid and Tau in a human-relevant system.

Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disorder characterized by two hallmark pathologies: amyloid- β (A β) plaques and neurofibrillary Tau tangles (NFTs). Moreover, a strong temporal component underlies disease progression, as A β pathology advances considerably before the robust induction of Tau pathology. Existing *in vivo* model systems have not yet succeeded in fully replicating the comprehensive and mature neuropathological features of AD, particularly the progression of AD-Tau pathology leading to neurofibrillary tangle formation¹⁻³.

While amyloid pathology has been more evident to model, given the familial AD mutations in PSEN1/PSEN2/APP and its upstream role in the pathogenic cascade, modelling of Tau pathology in AD-relevant experimental systems has faced more fundamental challenges. Notably, mouse neurons do not develop robust Tau pathology in response to amyloid plaques, underscoring a human neuron-specific vulnerability to Tau aggregation^{4,5}. To compensate for this limitation, murine models have relied on the overexpression of frontotemporal dementia (FTD)-associated *MAPT* mutations to model Tau pathology. While effective at inducing Tau aggregation, the resulting pathology does not fully reflect AD, as such mutations are absent in AD and the Tau filaments differ structurally and biochemically from those observed in human AD brain⁶⁻¹⁰.

To more faithfully reproduce AD-like Tau pathology *in vivo*, Tau seeding models have been developed where pathological Tau, extracted from AD brains, is injected into amyloid-expressing mice^{11,12}. This approach induces the accumulation of neuritic plaque-associated Tau and, at later stages, intracellular

hyperphosphorylated (AT8) and conformational altered Tau (MC1) in amyloid-overexpression mice (5xFAD). Thioflavin S-positive Tau aggregates appear, however they lack the morphology of neurofibrillary tangles (NFT) observed in AD patient brains, suggesting additional factors may be needed to progress into NFT pathology¹².

A further limitation of conventional mouse models lies in Tau isoform composition. Adult mouse neurons express the 4R Tau isoform, whereas NFTs in human AD patients are composed of both 3R and 4R Tau, limiting the interpretability of Tau aggregates in conventional mouse models⁷. To address this species-specific difference, human MAPT knock-in mice were generated, enabling expression of all human Tau isoforms⁵. Tau seeding in humanized *MAPT* (*hMAPT*) mice resulted in increased Tau hyperphosphorylation compared to mice expressing endogenous mouse Tau, demonstrating enhanced susceptibility to Tau aggregation^{5,13}. Tau pathology was further exacerbated when *hMAPT* mice were crossed with *App*^{NL-G-F} mice. However, even by 24 months of age, conformationally altered Tau (MC1-positive labelling) or Gallyas-silver positive NFTs did not develop, indicating humanisation of Tau isoform composition alone is insufficient to drive progression into tangle formation^{5,13}. In addition, when *hMAPT* mice were crossed to a “milder” amyloid model, the *App*^{NL-F}, those mice exhibited no increase in Tau pathology¹³. These discrepancies likely reflect differences in the extent of amyloid deposition: Thioflavin-positive Tau aggregates were only detected in 5xFAD mice, which strongly overproduce amyloid¹⁴. In *App*^{NL-G-F} mice, which generate endogenous levels of APP, amyloid provided a modest but insufficient amplification of Tau aggregation, whereas *App*^{NL-F} mice have even lower amyloid plaque burden and showed no measurable impact^{5,13}. Together, these findings underscore that neither amyloid burden nor Tau isoform humanisation is sufficient to recapitulate NFT formation, pointing to the human neuronal environment as a key missing element.

There is a need for human-relevant systems capable of capturing the intrinsic susceptibility of human neurons to Tau aggregation and the temporal relation between amyloid and Tau in AD³. Human model systems have shown that amyloid plaques can directly induce Tau hyperphosphorylation and aggregation in 3D *in vitro* cultures^{15–18}, whereas 2D cultures fail to reproduce this^{19,20}. Xenotransplantation of human neurons into amyloid plaque producing mice provides a complementary approach, to study human neurons in an *in vivo* environment^{21,22}. Notably, amyloid deposition in these chimeric models triggers Tau hyperphosphorylation in human neurons and increases plasma levels of pTau181 and pTau231, changes not observed in non-grafted amyloid mice²². These findings demonstrate early amyloid-induced alterations in Tau phosphorylation. However, widespread late-stage Tau pathology, marked by conformationally altered Tau (MC1+), and neurofibrillary tangle formation remain limited. This may partly reflect an inherent temporal limitation of experimental modelling, as the decades-long disease trajectory of AD is difficult to recapitulate within a mouse lifespan. Providing the primary pathological nucleation through injection of exogenous AD-derived Tau seeds could circumvent this constraint, the amyloid-rich xenograft environment then determines the progression to tangle formation.

Here, we directly tested this hypothesis by combining AD brain-derived Tau seeding with the xenograft model across defined temporal windows, asking whether the human neuronal environment, either in the presence or absence of amyloid pathology, is sufficient to drive Tau pathology to completion. AD-Tau seeding induced MC1-positive conformationally altered Tau, exclusively in transplanted human neurons and not in the surrounding murine tissue, demonstrating a striking species-specific vulnerability. Combining Tau seeding with amyloid exposure in *App^{NL-G-F}* mice revealed that extracellular amyloid markedly exacerbates this process, accelerating progression to neurofibrillary tangle formation in a manner not observed in non-amyloid conditions. Tau aggregation was entirely absent in MAPT-knockout human neurons, confirming that AD-Tau seeds act through templated misfolding of endogenous Tau rather than passive accumulation. Together, these findings demonstrate that neurofibrillary tangle pathology requires both the human neuronal environment and amyloid exposure, establishing the xenograft model as a platform to study their causal interaction in a human-relevant context

Results

AD-Tau seeding induces rapid conformational Tau pathology in human xenografted neurons

To determine whether pathological Tau seeding induces Tau pathology in human neurons, we transplanted human neural progenitor cells (NPCs derived from H9-GFP embryonic stem cells) into immunodeficient amyloid-producing (*Rag2^{-/-} App^{NL-G-F}*) mice. Next, we stereotactically injected Sarkosyl-insoluble (SI) fractions from AD patient brains (AD-Tau seeds) (Fig. 1A; Supplementary Fig. 1) in proximity to the original graft site at 1 month post-transplantation (1 MPT)^{14,23}. We measured Tau pathology three months later (4 MPT; Fig. 1A).

As xenografted human neurons follow their endogenous developmental timeline²⁴, they express developmentally regulated phospho-Tau at this stage²⁵, limiting the utility of phosphorylation-based markers as specific readouts of pathological Tau. We therefore focussed on conformationally altered Tau as detected by MC1 immunoreactivity, which is specific to pathological Tau species²⁶. Strong MC1-positive labelling was detected at 4 MPT in AD-Tau-injected mice (Fig. 1B).

Quantification of MC1 burden revealed a significant increase in grafted regions of AD-Tau-injected mice compared to grafted regions in non-injected controls (Fig. 1C, 1D; see Methods). Host neurons (non-grafted regions) of the same brain did not show an increase in MC1 signal after AD-Tau injection (Fig. 1C, 1D), suggesting that human neurons are markedly more susceptible than surrounding mouse neurons to developing conformational Tau pathology following Tau seeding.

Moreover, a subset of MC1 + Tau co-labelled with X34, which labels β -sheet-rich fibrillar structures, indicating the presence of aggregated neuropil structures (Fig. 1E'; white arrow). Whilst we observe early somatic MC1 + Tau labelling in human neurons (Fig. 1E"; white arrow), we do not detect tangle pathology

(defined here as profound X34-positive somatic Tau pathology) at 4 MPT in human neurons. Together, these findings indicate that human neurons rapidly develop conformational and early fibrillar Tau pathology, but not full tangle pathology, at 4 MPT in the *App*^{NL-G-F} context.

AD-Tau induces insoluble pTau accumulation in human grafts, with amyloid-specific emergence of early tangle pathology

Next, we performed this experimental paradigm at a more advanced stage of amyloid pathology, to determine whether increasing amyloid burden would further exacerbate Tau pathology, and compared Tau load in amyloid-exposed grafts (in *Rag2*^{-/-} *APP*^{NL-G-F} mice) to that in control grafts (in *Rag2*^{-/-} mice). Similarly to the previous experiment, human neurons were exposed to AD-Tau seeds for 3 months; AD-Tau or PBS was injected at 4 months of age and mice were sacrificed at 7 MPT (Fig. 2A).

MC1 pathology was detected at 7 MPT following AD-Tau seeding in both amyloid-exposed and control human grafts (Fig. 2B), demonstrating that human neurons are intrinsically susceptible to AD-Tau-induced misfolding, even in the absence of amyloid pathology.

Tau aggregation coincides with increased levels of Sarkosyl-Insoluble pTau181 and pTau231, further described as insoluble pTau²⁷. We therefore used ELISA to assess the levels of these pTau species in xenotransplanted animals. A human-specific Tau detection antibody was used to measure human-derived pTau (ADx204). Whole brain ELISA showed significantly increased insoluble pTau181 and pTau231 in xenografted AD-Tau-injected *Rag2*^{-/-} and *Rag2*^{-/-} *App*^{NL-G-F} mice, compared to PBS-injected animals (Fig. 2C, 2D). We observed a non-significant trend towards higher levels of insoluble pTau181 and pTau231 in amyloid-producing mice compared to control mice (Fig. 2C, 2D). Soluble pTau181 and pTau231 were comparable across all groups regardless of genotype or Tau seeding (Fig. S2A, S2B), indicating a basal level of developmentally regulated Tau phosphorylation in xenografted human neurons. To determine the relative contribution of insoluble pTau to the total pTau pool, we expressed insoluble pTau as a fraction of total pTau (insoluble + soluble), confirming a significant increase following AD-Tau seeding in both *Rag2*^{-/-} and *Rag2*^{-/-} *App*^{NL-G-F} mice (Fig. S2C, S2D), consistent with the findings in Fig. 2C and 2D.

Importantly, qualitative differences in pathology emerged at this stage. MC1 + dystrophic neurite pathology was solely detected in *Rag2*^{-/-} *App*^{NL-G-F} mice (Fig. 2E). Moreover, and although still sparse, the first tangle shaped inclusions (positive for MC1 and X34 labelling) appeared at 7 MPT in *Rag2*^{-/-} *App*^{NL-G-F} mice (present in 2/4 mice) but not in the *Rag2*^{-/-} mice (0/3 mice), for which Tau pathology remained limited to neuropil-like structures (Fig. 2F).

Thus, while overall Tau burden is comparable between conditions, amyloid exposure is associated with distinct features of Tau pathology at 7 MPT, including neuritic dystrophies and early tangle formation, that are absent in control grafts.

Amyloid pathology exacerbates Tau pathology at 18 months post transplantation

To examine whether the amyloid-dependent acceleration of Tau pathology observed at 7 MPT would progress further with time, we extended the paradigm to 18 months post-transplantation. We injected AD-Tau seeds at 9 months post transplantation and aged the human xenotransplanted grafts up to 18 months in *Rag2^{-/-}* and *Rag2^{-/-}App^{NL-G-F}* mice (Fig. 3A). Consistent with earlier time points, Tau seeding induced robust Tau pathology in human neurons, as indicated by AT8 + labelling (AT8 + labelling pTau Ser202 and Thr205) with human-specific cytoplasmic marker (STEM121), even in the absence of amyloid plaques (Fig. 3B).

At this late stage, a pronounced amyloid-dependent acceleration of Tau aggregation became evident, with significantly higher levels of insoluble pTau181 and pTau231 in amyloid-exposed mice compared to Tau-seeded controls and non-seeded animals (Fig. 3C, 3D), demonstrating that the influence of amyloid on Tau progression strengthens considerably over time. Assessment of soluble pTau revealed reduced levels in grafts residing in *Rag2^{-/-}App^{NL-G-F}* mice compared to *Rag2^{-/-}* controls, which may reflect reduced graft size in amyloid-exposed mice. We detected no effect of Tau seeding on soluble pTau181 and pTau231 (Fig. S3A, S3B). Expressing insoluble pTau as a fraction of total pTau confirmed a significant effect of both Tau seeding and genotype (Fig. S3C, S3D), supporting the biochemical shift toward insoluble pTau species in amyloid-exposed, Tau-seeded conditions.

In amyloid-producing mice, we observed advanced neuritic plaque-associated Tau pathology positive for AT8 and human-specific LAMP1 (Fig. 3E). Tau pathology was not confined to the neuritic compartment as individual human neurons (HUNU+) had developed somatic neurofibrillary tangle-like inclusions strongly positive for AT8 and MC1, which co-stained for X34 (Fig. 3F, 3G). Gallyas-silver staining provided further evidence that the labelled structures were neurofibrillary tangles (NFTs) (Fig. 3H' and H''). In addition, neuropil threads and dystrophic neurites were positive for Gallyas-silver staining (Fig. 3H').

Together, these data demonstrate that while Tau seeding alone initiates pathology, prolonged amyloid exposure amplifies Tau aggregation, driving progression toward neurofibrillary tangles that stain positive for Gallyas-silver staining.

Endogenous Tau is required for Tau-seed induced aggregation

We next asked a simple but mechanistically critical question: whether AD-Tau seeds are passively retained by human neurons or actively induce misfolding and aggregation of endogenous Tau. To address this, we leveraged the xenotransplantation model to engraft CRISPR-Cas9-generated H9 *MAPT* knockout (KO) human neuronal progenitors (Fig. 4A, 4B; Supplementary Fig. 4) into an amyloid-rich *in vivo* environment, allowing us to expose human neurons completely devoid of endogenous Tau to AD-Tau seeds (Fig. 4C). At 6 MPT, mice were bilaterally injected with AD-Tau or PBS, and analysed three months later. Tau KO neurons matured and integrated successfully (STEM121 and HUNU labelling of

human neurons) (Fig. 4D). Importantly, grafted Tau KO neurons remained devoid of human Tau protein (hTau/SP70-negative). We found no evidence of pathological Tau accumulation following AD-Tau seeding in human neurons (MC1-negative; Fig. 4E, 4G). Moreover, pTau181 and pTau231 in KO grafts were indistinguishable between AD-Tau and PBS groups (Fig. 4F) and below levels detected in wild-type PBS-injected animals (Fig. 2C, 2D). These findings demonstrate that the insoluble pTau signal observed in Tau-expressing xenografts reflects templated misfolding of endogenous human Tau rather than retention or accumulation of the injected AD-Tau seeds themselves, establishing that endogenous Tau expression is a prerequisite for seed-induced aggregation in human neurons.

To determine whether other AD associated features persisted independently of neuronal Tau expression, we assessed additional markers in Tau KO grafts. Notably, LAMP1-positive dystrophic neurites persisted around amyloid plaques in the absence of human Tau, demonstrating that amyloid-driven lysosomal stress and structural neuritic degeneration occur through Tau-independent mechanisms (Fig. 4G).

Finally, AT8-positive dystrophic neurites of murine origin (STEM121-negative, hLAMP1-negative) were observed in close proximity to human Tau KO dystrophic neurites (STEM121-positive, hLAMP1-positive; Fig. 4H), indicating that host mouse neurons expressing endogenous murine Tau remain responsive to AD-Tau seeding.

Mouse neurons are less vulnerable to Tau aggregation than human neurons

Whether this initial phosphorylation response in mouse neurons can progress toward Tau aggregation remained an open question. To address this directly, and to assess the murine response to AD-Tau seeding in isolation from any influence of the human neuronal environment, we administered AD-derived seeds into non-xenotransplanted *Rag2*^{-/-} *App*^{NL-G-F} mice. We administered AD-derived seeds at six months of age, when amyloid pathology was already advanced, and analysed brains three months later (Fig. 5A). In these mice, we observed increased AT8-positive phosphorylated Tau localized to dystrophic neurites surrounding amyloid plaques (Fig. 5B), confirming that mouse Tau can undergo phosphorylation in response to seeding. However, consistent with previous reports^{5,28}, conformationally altered Tau was absent at this timepoint, as indicated by the lack of MC1 immunoreactivity (Fig. 5C).

These results demonstrate that, although mouse neurons exhibit an initial response to Tau seeding, they fail to progress toward aggregated, conformationally altered Tau species within the observed timeframe. In contrast to human neurons, which rapidly develop MC1-positive pathology and fibrillar tau inclusions (X34+) within 3 months post Tau seeding, mouse neurons display a relative resistance to Tau aggregation, suggesting a slower or less efficient progression rather than a complete inability to form advanced Tau pathology.

Discussion

Human neurons xenotransplanted into amyloid mice spontaneously develop Tau pathology by 18 months post-transplantation²². Building on these findings, we now show that AD-derived Tau seeds induce robust and accelerated Tau aggregation, leading to AD relevant pathological features as early as 4 months post-transplantation. At this stage, human neurons display conformationally altered Tau but do not yet form NFTs. Tangle formation becomes evident by 7 months and progresses further by 18 months, but only in the presence of amyloid pathology, demonstrating that amyloid accelerates the temporal progression of Tau aggregation. This temporal relationship recapitulates findings from human longitudinal studies, where A β accumulation consistently precedes and predicts Tau pathology progression^{29,30}.

Rodent neurons, in contrast, fail to develop fibrillar tau inclusions even after Tau-seed injection in the presence of amyloid plaques^{5,13}. Our results therefore confirm a striking human-mouse difference in susceptibility to Tau aggregation: human neurons, but not murine ones, rapidly develop conformationally altered Tau and over time progress toward NFT formation under otherwise identical extracellular conditions. Whilst conformational Tau alterations emerge in human neurons independently of amyloid, progression to NFT formation within the experimental timeframe appears to require amyloid exposure, suggesting that amyloid acts as an accelerant rather than solely a prerequisite for Tau aggregation. This apparently primate-specific vulnerability is consistent with findings in macaque brains, where Tau seeding induced NFTs only when co-injected with A β ³¹. Moreover, a vascularized human AD organoid model has recently recapitulated tangle formation³². Together, these studies collectively suggest that NFT formation in primate neurons is facilitated by both pathological Tau and an amyloid-rich environment, and that this combination is sufficient to drive tangle formation within experimentally tractable timeframes.

The aggregation propensity in primate neurons points to cell-intrinsic determinants beyond Tau isoform composition alone^{5,13,33}. This distinction is most directly illustrated by comparison with the *App*^{NL-G-F} *MAPT* double knock-in model, in which replacement of the entire murine *Mapt* locus with the human ortholog, thereby restoring physiological expression of all six human Tau isoforms, enhances Tau phosphorylation relative to single *MAPT* knock-in mice. Yet even in this model, MC1-positive conformationally altered Tau and neurofibrillary tangles fail to develop after Tau seeding, even by 24 months of age and in the presence of robust amyloid pathology⁵. The failure of this model to recapitulate tangle pathology, despite carrying human Tau isoforms in an amyloid-rich environment, is consistent with the findings presented here, and collectively argues that a lower aggregation threshold in human neurons is not fully explained by the 3R/4R Tau isoform composition alone, but rather points to additional properties of the human neuronal environment.

What those properties might be remains an important open question. That the murine neuronal environment is not intrinsically incapable of tangle formation is demonstrated by mouse models overexpressing FTD-associated *MAPT* mutations such as P301S or P301L, which develop Gallyas silver-positive neurofibrillary tangles^{34,35}. However, this requires overexpression of disease-causing mutant Tau

at levels substantially exceeding physiological range, indicating that the aggregation threshold in mouse neurons is present, but elevated compared to human neurons. This is further supported by the finding that knockout of the PP2A regulatory subunit PR61/B'δ, which elevates GSK3β activity and drives Tau hyperphosphorylation, produces MC1-positive conformationally altered Tau in mouse neurons yet fails to drive tangle formation, an outcome attributed to possible compensatory HSP70/HSP90 upregulation in the affected brain regions³⁶. This suggests that an effective murine chaperone response may act as a brake on Tau aggregation that is less efficiently engaged in the human neuronal context³⁷. Direct paired comparisons of kinase activity, phosphatase regulation, and proteostasis capacity between human and mouse neurons and across ageing are currently absent from the literature, making it difficult to pinpoint a single mechanism responsible for this species-specific vulnerability. It is most likely that multiple cell-intrinsic pathways converge to lower the effective threshold for pathological Tau conversion specifically in human neurons^{38–40}.

The persistence of LAMP1-positive dystrophic neurites around amyloid plaques in Tau KO grafts demonstrates that amyloid drives lysosomal stress and axonal structural disruption in human neurons independently of intracellular Tau expression. Previous work shows that extracellular amyloid disrupts axonal microtubule integrity and impairs lysosomal trafficking in surrounding neurites, leading to the accumulation of protease-deficient, LAMP1-positive lysosomes that are functionally distinct from healthy axonal lysosomes^{41,42}. Moreover, it was shown that the progressive enlargement of these plaque-associated axonal spheroids, driven by endolysosomal vesicle accumulation and mechanistically linked to the lysosomal protein PLD3, acts as an electric current sink that disrupts long-range axonal connectivity independently of Tau pathology⁴³. Whether these Tau-independent neuronal responses to amyloid contribute to the cognitive consequences of amyloid pathology, and how they interact with Tau-dependent mechanisms, remains an open and important question.

The causal role of amyloid to Tau pathology progression established here and by others^{11,12,44,45} raises the question of how this acceleration is mechanistically achieved. Transplanted neurons are devoid of disease-causing mutations and are solely exposed to Aβ species produced by the host. This demonstrates that extracellular Aβ alone is sufficient to drive Tau acceleration in human neurons, independently of intracellular amyloidogenic APP processing. It is important to note that the induction of Aβ to pTau is not modelled in this experimental paradigm, rather what is modelled is the aggravation of pathology after the initial Tau seeds are present. Recent work shows that the combination of both Aβ and pTau result in a synergistic effect on NMDA receptor localization, resulting in NMDAR hypofunction and causing network dysfunction⁴⁶. Interestingly, presence of Aβ only results in network hyperfunction^{47,48} and seems to prime neurons such that when pTau arrives the network switches to a state of hypofunction⁴⁶. Tau phosphorylation is associated with a hypometabolic state⁴⁹ and upregulated in hibernating animals^{50,51}. A positive feedback loop between energy preservation and Tau phosphorylation might result in enhancement of Tau pathology, mitochondrial dysfunction and ultimately synaptic loss^{40,52}.

Xenotransplantation models are highly relevant methods to model human disease, but also pose challenges. Variability in xenotransplantation outcomes stemming from NPC differentiation, graft integration, and the proximity of Tau-seed injections, introduces noise that can obscure small but biologically relevant differences between conditions. While trends toward higher Tau pathology in amyloid mice are already evident at 7 MPT, these differences become statistically robust only at 18 MPT. Ongoing efforts to standardize graft quality and seed delivery will help improve reproducibility across studies. In addition, human ESC and iPSC-derived neurons remain relatively immature²⁴ compared to aged neurons that develop AD pathology in the human brain. Therefore future work is important to improve modelling of ageing in human-derived neurons⁵³.

In summary, this work establishes several principles with relevance to the understanding and modelling of Alzheimer's disease. First, human neurons possess an intrinsic vulnerability to Tau aggregation that cannot be replicated in mouse neurons under otherwise identical extracellular conditions, confirming that species-specific neuronal factors, beyond Tau isoform composition alone, are critical determinants of whether pathology progresses to neurofibrillary tangles. Second, amyloid pathology causally accelerates Tau progression, indicating a critical function of amyloid after initial nucleation of Tau has occurred. Third, this vulnerability is dependent on endogenous human Tau expression, as demonstrated by the complete absence of pathology in MAPT-knockout neurons, establishing that AD-Tau seeds do not passively accumulate but require an endogenous Tau as a template. Fourth, amyloid exerts Tau-independent neuronal stress, suggesting that amyloid toxicity is broader in scope than its role in enhancing Tau pathology alone. Together, these findings position the xenograft model as a unique platform for studying the amyloid-Tau interaction in a human neuronal context and supports the hypothesis that A β pathology reduces the threshold for Tau aggregation in neurons.

Methods

Mice

Rag2^{-/-} (*Rag2*^{tm1.1cgn}; Jackson Laboratory, strain 008309) carrying the wild-type murine App allele used as control and *Rag2*^{-/-}/*App*^{NL-G-F} (*Rag2*^{tm1.1cgn}; *App*^{tm3.1Tcs}) mice on a C57BL/6 background were maintained in our specific Opportunistic pathogen-free (SOPF) animal facility. Colonies were bred and both sexes were used for experiments. Animals were housed in groups of two-four per cage, with ad libitum access to food and water, under a 14-hour light/10-hour dark cycle at 21°C. All procedures were approved by the KU Leuven ethics committee and performed in accordance with institutional guidelines for animal research. Xenotransplanted mice that received no AD-Tau injection at 18MPT in Fig. 1C and 1D were previously generated in Balusu et al. (2023). Spare brain material from the contralateral hemisphere, which was not used in the previous study, was utilized for protein isolation and further quantifications.

Generation of Homozygous MAPT Knockout hESCs

Homozygous *MAPT* knockout human embryonic stem cells (hESCs; H9 line) were generated using a dual-guide CRISPR-Cas9 strategy. Optimal guide sequences were designed via CRISPOR (Haeussler et al., 2016). Two custom crRNAs targeting *MAPT* exon 1 and intron 7/8 were synthesized (#Custom, IDT, Leuven, Belgium): gRNA1: CAAGAGGGTGACACGGACGC and gRNA2: CACTGAGAATGAATAACCCC. Ribonucleoprotein (RNP) complexes were assembled using Alt-R™ S.p. HiFi Cas9 Nuclease V3 (IDT, #1081060), tracrRNA (IDT, #1072534), and the respective crRNAs. H9 cells were maintained in the Cellartis DEF-CS 500 culture system (Takara Bio, #Y30010/12), dissociated with TrypLE™ Express (ThermoFisher Scientific, #12604013), and counted using the LUNA-FL™ cell counter (Westburg, EU). Electroporation was performed with the NEPA21 system (Nepagene) using 250,000 cells resuspended in 100 µL Opti-MEM™ Reduced Serum Medium (ThermoFisher Scientific, #31985070) with both RNP complexes and 0.5 µg of the pmaxFP-Green-N plasmid to allow for monitoring of transfection efficiency (Amara, Lonza). Electroporation parameters were: 125 V, 7.5 ms (2× pore pulse); 20 V, 50 ms (5× transfer pulse). Cells were plated in Coat-1-coated 12-well plates (Cellstar®) and cultured in DEF-CS 500 medium with Growth Factor 3 (Takara Bio) for 24 h. At 72 h, cells were split: one half for PCR-based editing efficiency assessment and the other half was seeded in 96 well dishes at a density of 0.8 cells/well. Single cells were expanded, homozygous deletion of the *MAPT* locus was confirmed by PCR and Sanger sequencing, and loss of MAPT protein was validated by immunohistochemistry and western blot in neurons. Off-target editing was assessed by Sanger sequencing of the top five predicted sites per gRNA. Chromosomal integrity was verified by CGH array (UZ Leuven). Pluripotency was confirmed via qPCR and immunostaining for OCT4, KIA67, and TRA-1-81.

Pluripotent stem cell culture and neuronal differentiation

All experiments were performed with H9 stem cell line. The H9-GFP cells stably expressed GFP under chicken b-actin promotor (CAG). H9-GFP and H9-Tau KO cells were cultured as previously described in Balusu et al. 2023. In brief, stem cells were maintained in E8-flex growth media (Thermo; #A2858501) in a humidified chamber at 37°C with 5% CO₂. Stem cells were passaged using 0.5mM EDTA. For neuronal differentiation, we followed a slightly adapted protocol based on Shi et al. 2012. On day *in vitro* (DIV) – 2 of neural induction, human ESCs were enzymatically dissociated into single cells using StemPro™ Accutase™ Cell Dissociation Reagent (Sigma, #A1110501). Single cells were plated at a density of 2×10^6 cells per well onto Matrigel-coated 6-well plates in mTeSR1 medium (STEMCELL Technologies) supplemented with 10 µM Y-27632 ROCK inhibitor (Calbiochem). At DIV 0, medium was replaced with neural maintenance medium (NMM) supplemented with 1 µM StemMACS™ LDN-193189 (Miltenyi Biotec, #130-106-540) and 10 µM SB431542 (STEMCELL Technologies, #72234), refreshed daily until DIV12. Between DIV12 and DIV24, the cells were passaged three times via mechanical dissociation to enrich for neural rosettes. Around DIV27, neural rosettes were enzymatically dissociated into single cells using StemPro™ Accutase. Neural progenitor cells (NPCs) were collected on DIV30 and cryopreserved for future use. NPCs were stained for NPC markers to confirm successful differentiation.

Neural progenitor cell preparation for grafting

Xenotransplantation of H9-derived NPCs was performed as previously described (Espuny-Camacho et al. 2017; Balusu et al. 2023). In brief, time-controlled mating of females was performed. Five days prior to transplantations, NPCs were thawed in NMM supplemented with 10 μ M Y-27632 ROCK inhibitor (Calbiochem, #688000). Growth medium was refreshed every 48 hours. On the day of transplantation, Revitacel (Thermo Fisher, #A2644501) was added to the cells 30 minutes prior to dissociation. Cells were enzymatically dissociated into single-cell suspension using StemPro™ Accutase™ (Sigma, #A1110501). Cell viability was determined via trypan blue exclusion assay. Cells were resuspended in Leibovitz's L-15 Medium (Thermo Fisher, #11415064) supplemented with 34 mM glucose to 50.000 cells/ μ L.

Grafting

Grafting was performed as previously described by Balusu et al. (2023). In brief, P1-P2 pups were cryo anesthetized briefly prior to transplantation. A small incision was made at the injection site using a surgical blade with coordinates relative to bregma: anteroposterior – 1 mm, lateral $\pm$ 1 mm. Mice were injected unilaterally or bilaterally with 1 μ L of a 50,000 cells/ μ L suspension per hemisphere using a 26G Hamilton syringe. Xenografted mice used at the 4 MPT and 18 MPT time points were grafted unilaterally. Mice used at the 7 MPT and 9 MPT time points were grafted bilaterally, allowing one hemisphere to be used for ICC while the other hemisphere was used for SI-protein isolation. Following injection, pups were placed under a 37°C heat lamp to recover. After full recovery, the pups were placed back in the cage and monitored daily for a week.

Sarkosyl-insoluble Tau Extraction from Human AD Brain Tissue

Frozen post-mortem superior occipital gyrus from AD patients were thawed on ice, and wet tissue weight was measured. Tissue was homogenized at 500 rpm (20 strokes) using a glass Dounce homogenizer in 9 volumes (v/w) of ice-cold high-salt buffer (HSB) (10 mM Tris-HCl pH 7.4, 0.8 M NaCl, 1 mM EDTA, 10% sucrose, 1M DTT, 0.1% sarkosyl), supplemented with protease inhibitors (PI) (1 tablet/50 mL, Roche cOmplete™, EDTA-free), PhosSTOP™ (1 tablet/10 mL), and PMSF (1mM final concentration). Homogenates were centrifuged at 10,000 $\times$ g for 10 min at 4°C, and the supernatant (S1) was collected. The pellet (P1) was re-homogenized two more times in the same buffer, generating pellets and supernatants P2, S2 and P3, S3. P3 pellet was stored at -80°C and all supernatants (S1 + S2 + S3) were pooled. Sarkosyl was added to a final concentration of 1%. Samples were incubated 1 hr at RT on a rotating shaker (200 rpm). Sarkosyl-treated lysates were ultracentrifuged at 50,000 rpm ($\sim$ 150,000 $\times$ g) for 3 hr at 4°C in a Ti50.2 rotor (Beckman Coulter). Supernatants (S4) were collected and stored at -20°C. Pellets (P4) were washed with PBS and resuspended in PBS ($\sim$ 600 μ L PBS per gram of initial tissue). Pellets were mechanically dissociated and sonicated in a water bath sonicator (90 pulses, 1 s on / 2 s off, 20% amplitude). Following sonication, samples were ultracentrifuged at 85,000 rpm ($\sim$ 300,000 $\times$ g) for 1 hr at 4°C (TLA-110 rotor). The supernatant (S5) containing soluble contaminants was removed, and the pellet (P5), enriched in insoluble Tau, was resuspended in PBS ($\sim$ 100 μ L/g tissue). A second sonication (60 pulses, 1s on / 2s off, 50% amplitude) was performed to aid pellet dispersion. Final debris

was removed by low-speed centrifugation (10,000 × g, 30 min, RT), and the clarified supernatant (S6), enriched for sarkosyl-insoluble Tau, was collected and stored at – 80°C. All used materials were decontaminated in 1% SDS for 24 hr, followed by 70% ethanol. Quality and concentration of the sarkosyl-insoluble Tau fractions were assessed by western blotting.

Electron microscopy on AD-derived SI-Tau fraction

Sarkosyl-insoluble Tau fractions were analysed by immunogold labelling and electron microscopy. Samples were diluted 1:10 and adsorbed for 5 min onto 400-mesh formvar/carbon-coated grids (EM Sciences, #CF400-Cu) that had been glow-discharged using a Leica EM AC600. Grids were washed three times and blocked for 10 min in 5% acetylated BSA (Aurion). Primary antibodies against PHF1 and total Tau (DAKO) were applied and incubated for 3 h at room temperature. After three washes, secondary goat anti-mouse and anti-rabbit HRP antibodies were added and incubated for 2–3 h at room temperature. Grids were then washed, negatively stained with 2% uranyl acetate for 1 min, air-dried, and imaged using a JEM-1400 transmission electron microscope (JEOL) equipped with an 11-megapixel Olympus SIS Quemesa camera.

Aβ-MSD on AD-derived SI-Tau fraction

Aβ40, and Aβ42 concentrations were quantified using a single spot Meso Scale Discovery (MSD) 96-well ELISA platform. Monoclonal capture antibodies selective for the C-terminus of Aβ species ending at amino acids 40 (LTDA_40) or 42 (LDA_42) are neoepitope house made antibodies⁵⁴. For detection, human-specific sulfo-TAG-labelled antibodies (JRF/AβN/25) were used. Plates were analysed on an MSD plate reader.

Dot blot on AD-derived SI-Tau fraction

α-Synuclein concentrations in isolated AD-Tau fractions were measured using a dot blot analysis. Samples were diluted 1:100 in PBS or PBS containing 1% SDS. A standard curve was generated using serial dilutions of recombinant human α-Syn (AlexoTech). For each sample, 1 μL and 2 μL were spotted onto a 0.2 μm nitrocellulose membrane, alongside 1 μL of each standard dilution. Membranes were air-dried and blocked for 1 h at room temperature in 5% non-fat milk prepared in TBS-T. Primary antibody against α-Syn was added directly to the blocking solution and incubated overnight at 4°C. The following day, membranes were washed three times in TBS-T and incubated for 1 h at room temperature with the secondary antibody. Unbound antibodies were removed by washing three times in PBS-T followed by two washes in TBS. Signal was developed using Western Lightning Plus-ECL substrate (PerkinElmer) and imaged on an ImageQuant LAS4000 (GE Healthcare). For quantification, band intensities were analysed using Aida Image Analyzer v5.0. A standard curve was generated from α-Synuclein dilutions, and mean values were calculated for each sample condition.

Tau seeding with stereotaxic injection

Tau seeds were thawed and resuspended to a concentration of 0.4 μg/μl. The seeds were sonicated with 60% amplitude for 40 pulses (1s on/ 2s off). Mice received 1 μg Tau seeds per hemisphere. Mice were

anesthetized with 4% isoflurane in 2.5% O₂ and maintained at 2.5% isoflurane during surgery. Buprenorphine (0.075 mg/kg) was administered subcutaneously prior to the procedure. Animals were placed on a pre-warmed heating pad (37°C) and secured in a stereotaxic frame using ear-bars. Terramycin ointment was applied to the eyes using cotton swabs. The scalp was shaved, followed by a midline incision. A hole was drilled at the injection sites (bregma: -0.05 mm; lateral: +1.4 mm and - 0.05 mm; lateral: -1.4 mm). The Hamilton syringe filled with Tau seeds or PBS was mounted onto the stereotaxic holder. 0.5µg of seeds or similar volume of PBS was injected at -2.2mm and - 1.4mm depth. A total of 2.5µl was injected per hemisphere. After injection, the wound was sealed with cement and the skin was sutured. Iso Betadine was applied to the suture and the animals were placed under a heat lamp for recovery. Animals were monitored closely for the next two weeks.

Sample processing

Mice received an overdose of Pentobarbital and were perfused with 20ml PBS. The brain was removed from the mouse. The brain was sliced in two hemispheres. The cerebellum was discarded from the left hemisphere and then snap-frozen in liquid nitrogen. The samples were stored at -80°C until further processing. The right hemisphere was stored in 4% PFA o/n at 4°C. The next day the brain was washed in PBS and cut in 40µm-thick coronal free floating sections using the Vibratome Leica VT1000S. Brain sections were stored for two weeks in PBS in 24-well plates and then frozen in cryo-protectant solution (30% ethylene glycol, 30% glycerol, 40% PBS) for long-term storage at -20°C.

Sarkosyl-insoluble Tau extraction from mouse brain samples

Frozen mouse brain tissue was thawed on ice, weighed and homogenized in 9 volumes (v/w) of freshly prepared high-salt buffer (HSB: 10 mM Tris-HCl pH 7.4, 0.8 M NaCl, 1 mM EDTA, 2 mM DTT, 0.1% sarkosyl, 10% sucrose) supplemented with PI, PhosSTOP™ and PMSF 1mM. 300 µL of HSB was first added and the sample was homogenised using a motorized hand pestle, followed by syringes (10 strokes with 18G and 22G needles). The homogenized sample together with the remaining HSB was added to a 5ml Eppendorf and 15% homogenate in total was removed for further analysis. The homogenate was centrifuged (10.000g for 10min at 4°C), the supernatant was collected and the pellet was re-suspended in 7.65 (85% of 9 volumes) volumes of HSB and re-homogenized (2x). The pooled supernatants (S1-S3) were incubated with sarkosyl to a final concentration of 1% and were let shaking for 1hr (200 rpm) at RT. The samples were loaded in Sarstedt high speed Eppendorf tubes and centrifuged at 300.000 g for 1hr at 4°C. Pellets were washed with PBS and resuspended in PBS with pipette tips (P1000 wide tip, P1000 small tip, P200 tip and 27G needle). After resuspension, samples were sonicated in water bath sonicator, 40 pulses (1 sec on – 2 sec off) at 20% amplitude. Then samples were centrifuged for 30min at 100000g using Beckman TLA-110 rotor. The P5 pellet was resuspended in 50µl PBS and sample was sonicated for 60 cycles (1 s on/ 2 s off, 50% amplitude). The samples were centrifuged for 30 min at 10.000g at 4°C. The supernatant (S6) is enriched for sarkosyl-insoluble Tau.

Tau Elisa

The sarkosyl-insoluble (S6) Tau fractions were analysed using a Meso Scale Discovery (MSD) immunoassay. MSD 96-well plates were coated overnight at 4°C with capture antibodies for pTau181 (ADx252, 2µg/ml in PBS) and pTau231 (ADx253, 2µg/ml in PBS). The next day, the plates were blocked with PBS + 0,1% casein. Prior to the Elisa, the material used from the S6 fraction was sonicated (50% amplitude, 60pulses with 1s on/ 2s off). The samples were diluted 1:5 in PBS with 0.1% casein in PBS. Standard curve was prepared using synthetic pT229 peptide standard. The detection antibody ADx204-sulfo tag (1:2000 diluted in PBS/casein) was added to the sample and standard curve 1:1. The samples were shaken at 600rpm for 3 min prior to transferring them to the coated plate. The next day, the wells were washed, read buffer A was added and the plate was read using the MSD plate reader. Raw counts were displayed normalized to mg tissue.

Gallyas silver staining

Free-floating sections were mounted and dried onto positively charged microscope slides (ThermoFisher Scientific, #6776214). Once sections were dried, the slide was washed in water for 1min and transferred to alkali silver iodide solution (4 g sodium hydroxide, 10 g potassium iodide, 3,5 mL 1% silver nitrate in 100 mL distilled water) for 1 min, followed by 3x 1 min washes in 0.5% acetic acid. Then the slides were incubated in freshly prepared physical developer solution. The developer solution consists of three components mixed in 10:4:6 ratio. Solution A: 50 g sodium carbonate in 1 L distilled water, solution B: 2 g ammonium nitrate, 2 g silver nitrate, and 10 g silicotungstic acid in 1 L distilled water, solution C: 2 g ammonium nitrate, 2 g silver nitrate, 10 g silicotungstic acid, and 7.3 mL of 35% formaldehyde in 1 L distilled water. The slides were incubated for 15–30 min. The development was halted by a 5 min wash in 0.5% acetic acid and 1% sodium thiosulfate. Then the silver deposits were stabilized by immersing the slides in 1% sodium thiosulfate for 5 min, rinsed in distilled water for 5 min followed by a 5min wash in 0.1% gold chloride. Then slides were again washed in water for 5 min followed by another incubation in 1% sodium thiosulfate for 5 min. Slides were washed and mounted using permount solution (ThermoFisher Scientific, #15820100). Sections were imaged using a brightfield microscope.

Immunostaining and confocal microscopy

Brain sections were washed with PBS for 5 min. Sections for X34 (Sigma #SML1954) staining were permeabilized for 20 min in PBS + 0.2% Triton X-100 (PBS/T) and then incubated with X34 solution in 40% Ethanol for 20 min. After washing, sections were blocked in PBS/T supplemented with 5% Normal Goat/Donkey Serum, (2% Bovin Serum Albumin) for 2 h at RT on an orbital shaker. Brain sections were incubated with primary antibody solution o/n at 4°C.

Image acquisition was performed using the Nikon AX microscope at ×20 (NA 0,75) and x60 (NA 1.42), the Nikon NiE upright A1R microscope x60(1.2 NA), and for super resolution microscopy we used the ZEISS LSM900 in Airyscan mode with a 63x Plan Apochromat (1.40) oil objective. Images were processed in the FIJI/Image J software (version 1.54f with Java 1.8.0_322 (64-bit), Imaris (10.2) and NIS Elements Analysis software (6.02).

Antibodies

The following antibodies were used for immunofluorescence staining, ELISA, WB, Dotplot and EM: Oct4 1:100 (POU5F1, Abcam, #ab19857), Nanog 1:200 (R&D Systems, #AF1997), Nestin 1:200 (R&D Systems, #MAB1259), Sox (Sox family, R&D Systems, #AF3369), NeuN 1:500 (NeuN/RBFOX3, Synaptic Systems, #266004), Ki67 1:250 (Abcam, #ab16667), FoxG1 1:100 (Abcam, #ab18259), SatB2 1:200 (Abcam, #ab34735), TUBB 1:500 (β -Tubulin, BioLegend, #801202), TBR1 1:250 (Abcam, #ab183032), MAP2 1:500 (Abcam, #ab5392), CTIP2 1:500 (Abcam, #ab18465), X34 1:1000 (amyloid- β / β -sheet fibrillar aggregates, Sigma, #SML1954), AT8 1:200 (phospho-Tau Ser202, Thr205, Thermo Scientific, #MN1020), Stem121 1:500 (human cytoplasmic marker, Takara, #Y40410), GFP 1:1000 (Abcam, #ab13970), PHF1 1:500 (PHF-Tau Ser396/Ser404, from Davis), MC1 1:500 (conformational PHF-Tau, from Davis), HuNu 1:200 (human nuclei, Millipore, #MAB1281), hTau SP70 1:500 (Tau N-terminal, Abcam, #ab93726), hLamp1 1:100 (LAMP1, Abcam, #ab278043), ADx252 1:1000 (pTau Thr181, ADx), ADx253 1:1000 (pTau Thr231, ADx), ADx204 1:1000 (N-terminal epitope of Human Tau, ADx), Tau Dako 1:1000 (Tau AA243-441, Dako, #A0024), anti-alpha-synuclein 1:1000 (alpha-synuclein, BD, #610787), Tau-5 1:50 (Human Tau AA218-225, Abcam, #ab80579), HT7 1:50 (Human Tau AA159-163, Invitrogen, #MN1000) A β 40 (in-house generated), A β 42 (in-house generated).

Data Analysis and Statistics

MC1 load through immunohistochemistry analysis was measured using NIS elements software 6.02. Around 12 images were taken for each mouse of human graft-rich areas from > 3 brain sections. Bright spot detection was used to quantify MC1 pathology. Based on the bright spot detection values the images were scored from 1–4 to generate a semi-quantitative MC1 score. 1 point for values between 1000–1500, 2 points for 1500–2000, 3 points for 2000–3500 and 4 points for > 3500. Detection for images between 0-1000 received 0 points as these often comprised unspecific staining/artefacts. The sum of all images per mouse was used as semi-quantitative proxy for Tau load and normalized to GFP area.

Results are displayed as mean $\pm$ standard error of the mean (SEM) and statistical significance was set at $\alpha = 0.05$. Two-way analysis of variance (ANOVA) was used when comparing multiple groups, when needed data was log-transformed to meet assumptions of normality and equal variances. Main effects included genotype (*Rag2*^{-/-} *App*^{NL-G-F} to *Rag2*^{-/-} mice) and Tau seeding (Tau-PBS injection effect) and their relevant interactions. Upon a significant interaction effect, a post-hoc test for multiple comparisons was run (Tukey's p-value adjustment). Unpaired t-test was used when two groups were compared. GraphPad Prism and R were used for statistical analysis. All details on the analyses per figure can be found in Supplementary Table 1.

Declarations

Competing interests

B.D.S. has been a consultant for Eli Lilly, Biogen, Janssen Pharmaceutica, Eisai, AbbVie and other companies and is now consultant to Muna Therapeutics. B.D.S is a scientific founder of Augustine Therapeutics and a scientific founder and stockholder of Muna Therapeutics. P.V. is the scientific founder of Jay Therapeutics. DRT collaborated with Novartis Pharma AG (Switzerland), and GE-Healthcare (UK), Fujirebio (“ADx NeuroSciences”; Belgium) and received consultant honoraria from Muna Therapeutics (Belgium). All other authors declare no conflicts of interests.

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Authors' contribution

Conceptualization: J.V.V., S.B., D.R.T., B.D.S.

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Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

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Figures

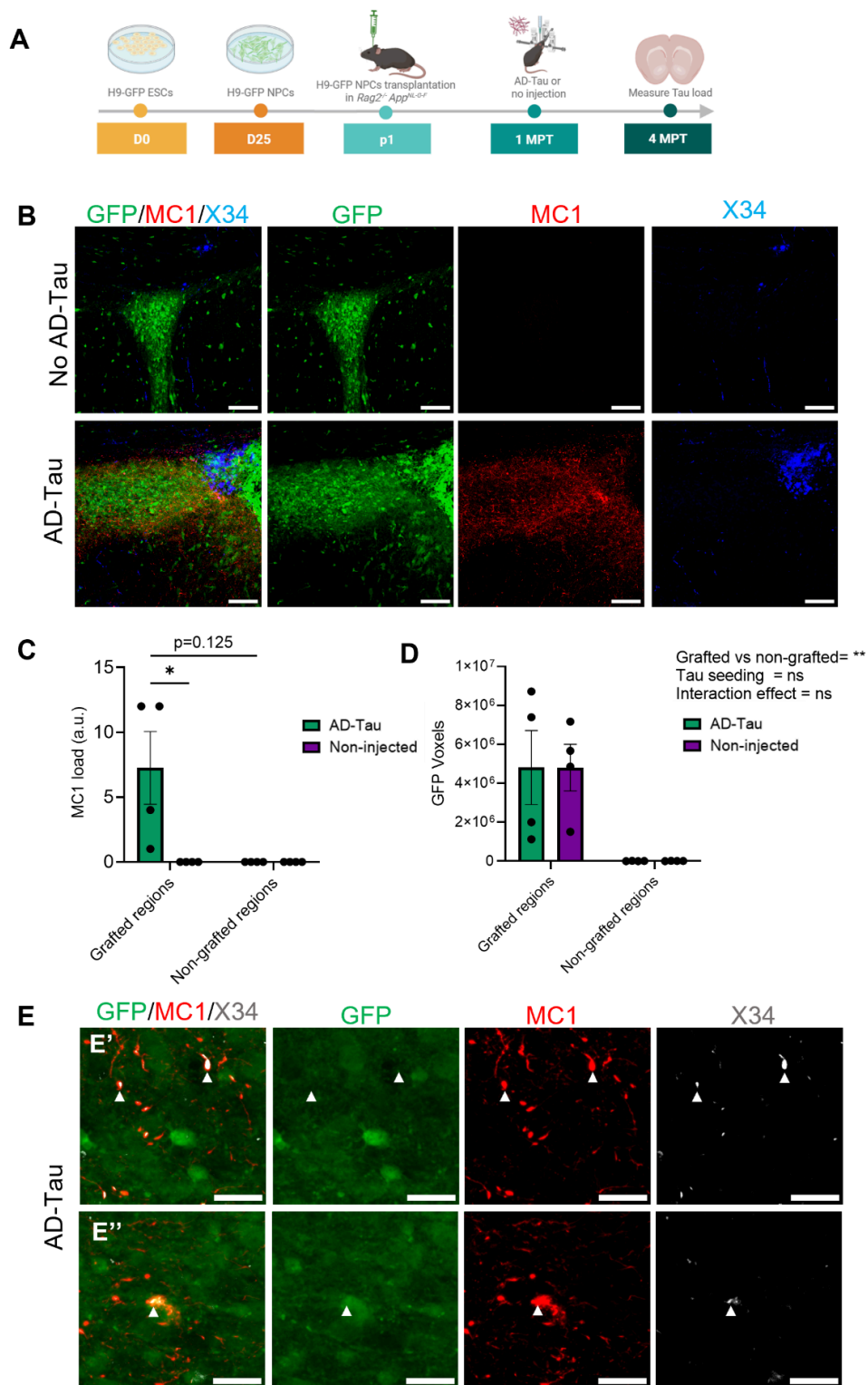


Figure 1

Rapid progression into conformationally altered Tau pathology in human grafts at 4 MPT after Tau seeding

A. Experimental timeline. H9-derived Neural progenitor cells (NPCs) were generated using the DUAL-SMAD protocol. *Rag2^{-/-} App^{NL-GF}* mice were xenografted with GFP labelled human NPCs at postnatal day 1 (P1) and received AD-Tau injections at 1 MPT (Months post transplantation). Mice were sacrificed at 3

MPI (Months post injection) (corresponding to 4 MPT) to assess Tau pathology.

B. Conformational changes in Tau, detected by MC1 (red) immunolabelling are seen within grafted regions containing GFP-positive human neurons at 4 MPT in mice that received AD-Tau injections at 1 MPT. X34 (blue) marks β -sheet structures in A β -dense core plaques and occasionally co-labelled with MC1 indicating β -sheet rich Tau structures (scale bar: 100 μ m).

C-D. Quantification of MC1-positive signal/burden in grafted regions of AD-Tau injected and non-injected (no AD-Tau) *Rag2^{-/-} App^{NL-GF}* mice using bright-spot detection in NIS Elements Analysis Software v6.02, converted to semi-quantitative values per mouse. Data presented as mean $\pm$ SEM, $n = 4$ mice per group. Statistical analysis was performed using a two-tailed exact Mann–Whitney test to compare AD-Tau vs. non-injected (grafted regions) ($p=0.0286$), and a Wilcoxon signed-rank test to compare grafted vs. non-grafted regions within the AD-Tau condition ($p=0.125$). Two-way ANOVA was performed for **(D)**, data was log-transformed to meet assumptions. A significant effect of grafted vs. non grafted was detected ($p=0.0011$). * $p < 0.05$, ** $p < 0.01$ (More info in Supplementary Table 1).

E. MC1-positive cell bodies are detected within xenografted neurons (D'), but tangle formation is not yet evident at this stage, as indicated by the absence of profound X34 staining. In contrast, neuropil threads occasionally display both MC1 and X34 positivity (D'') (scale bar: 50 μ m)

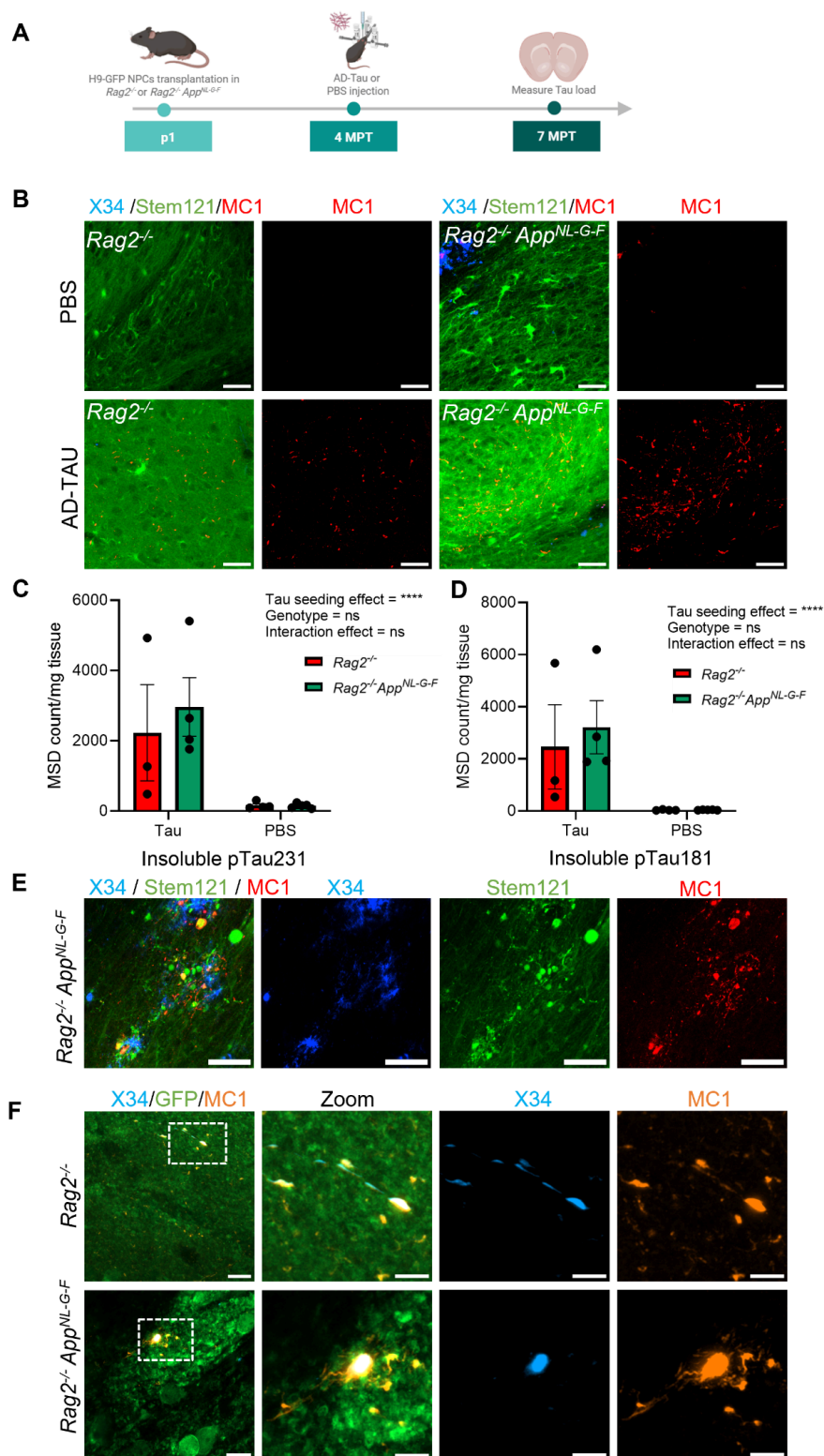


Figure 2

Human xenografts develop insoluble pTau pathology following Tau seeding, with amyloid-specific emergence of dystrophic neurites and tangle formation.

A. Schematic overview: $Rag2^{-/-}$ and $Rag2^{-/-} App^{NL-G-F}$ mice were xenografted with H9 neural progenitor cells. At 4 MPT, mice were injected with AD-Tau or PBS and sacrificed 3MPT (corresponding to 7MPT).

B. Representative images showing Tau pathology (MC1-immunolabelling) in $Rag2^{-/-}$ and $Rag2^{-/-} App^{NL-G-F}$

mice following AD-Tau or PBS injection (scale bar: 30µm).

C-D. Human-specific Tau-detection antibody (ADx204) was used and MSD counts of Sarkosyl-Insoluble (SI) pTau levels are displayed. Statistical analysis was performed using two-way ANOVA, data was log-transformed to meet assumptions of normality and equal variances. A significant main effect was detected for Tau seeding for **C.** pTau181 ($p < 0.0001$) and **D.** pTau231 ($p < 0.0001$). For both pTau sites, the interaction effect was not significant.

E. Dystrophic neurites positive for MC1 and Stem121 surround Amyloid-beta plaques in 7 month old *Rag2^{-/-} App^{NL-G-F}* mice.

F. NFT pathology, defined by co-labelling of somatic X34 and MC1, is present at 7 MPT in *Rag2^{-/-} App^{NL-G-F}* mice but absent in *Rag2^{-/-}* mice, which display X34+ MC1+ neuropil pathology (scale bar: 10µm and inset: 5µm).

For statistical analysis: *Rag2^{-/-}* mice n=3 (AD-Tau) and n=4 (PBS); *Rag2^{-/-} App^{NL-G-F}* mice n=4 (AD-Tau) and n=5 (PBS). Data are presented as mean $\pm$ SEM. ** $p < 0.01$, **** $p < 0.0001$ (For more details see Fig legend 1C-D and Supplementary Table 1).

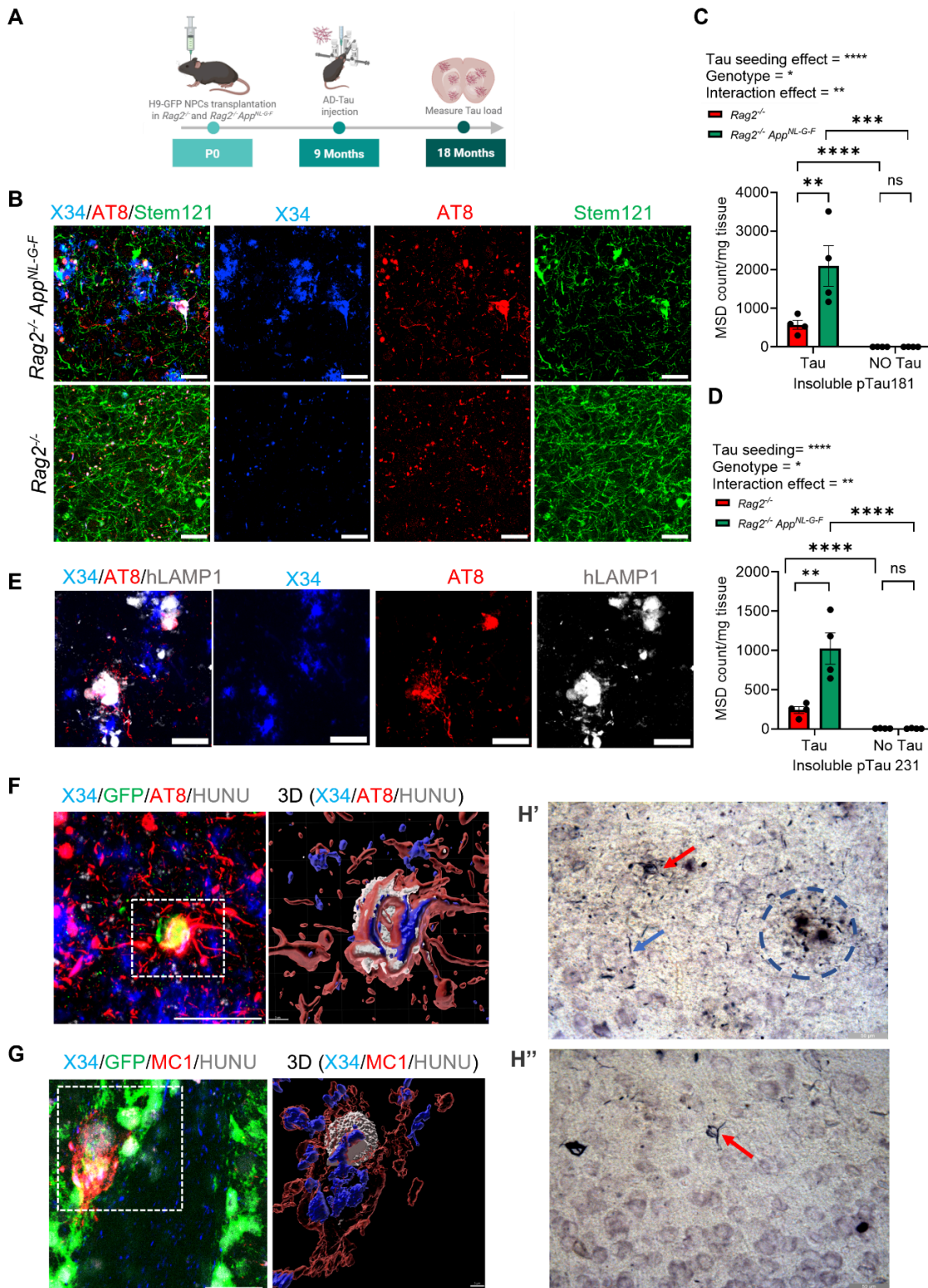


Figure 3

Amyloid pathology exacerbates Tau pathology in Tau seeded xenografted human neurons at 18 MPT.

A. Experimental timeline. H9-GFP derived NPCs were xenografted in *Rag2^{-/-}* and *Rag2^{-/-} App^{NL-G-F}* mice at postnatal day 1 (P1) and received AD-Tau injections at 9 MPT. Mice were sacrificed at 18 MPT.

B. STEM121⁺ xenotransplanted human neurons exhibited severe AT8⁺ Tau pathology following AD-Tau injection in both *Rag2^{-/-}* and *Rag2^{-/-} App^{NL-G-F}* mice (scale bar: 20µm).

C-D Insoluble pTau181 and pTau231 levels (MSD Counts) are significantly elevated in AD-Tau-injected *Rag2^{-/-} App^{NL-G-F}* mice vs. Tau-injected *Rag2^{-/-}* mice and non-injected *Rag2^{-/-} App^{NL-G-F}* mice. The non-injected xenotransplanted mice shown in Fig. 3C and 3D originated from the cohort generated for Balusu et al. (2023), but these animals were not analysed in that study. Data are mean $\pm$ SEM; n = 4 mice/group. Data were log-transformed prior to two-way ANOVA to meet assumptions. Significant main effects were observed for genotype (pTau181 p = 0.0286; pTau231 p = 0.0415) and Tau seeding (pTau181 p < 0.0001; pTau231 p < 0.0001). A significant genotype $\times$ Tau seeding interaction was detected (pTau181 p = 0.0033; pTau231 p = 0.0021); Tukey's post hoc test: AD-Tau *Rag2^{-/-} App^{NL-G-F}* vs. AD-Tau *Rag2^{-/-}* (pTau181 p < 0.01; pTau231 p < 0.01); AD-Tau *Rag2^{-/-} App^{NL-G-F}* vs. PBS *Rag2^{-/-} App^{NL-G-F}* (p < 0.0001 both); AD-Tau *Rag2^{-/-}* vs. PBS *Rag2^{-/-}* (p < 0.0001 both); PBS *Rag2^{-/-} App^{NL-G-F}* vs. PBS *Rag2^{-/-}* (ns both). *p < 0.05, **p < 0.01, ****p < 0.0001. See Supplementary Table 1.

E. Dystrophic neurite pathology in AD-Tau injected *Rag2^{-/-} App^{NL-G-F}* mice are AT8, and human LAMP1 positive (scale bar: 25 μ m).

F-G. NFT pathology develops in xenografted human neurons (GFP+/ human nuclear marker HUNU+) in *Rag2^{-/-} App^{NL-G-F}* mice, confirmed by co-labelling with X34, and Tau markers. **F.** AT8, **G.** MC1, 3D Imaris renderings highlight intracellular X34+ structures within Tau+ neurons (HUNU+) (scale bar: 30 μ m and insets: 3 μ m (F), 5 μ m (G)).

H. Gallyas-silver staining reveals NFTs (red arrows), neuropil threads (blue arrows), and dystrophic neurites surrounding amyloid plaques (blue circle) in AD-Tau *Rag2^{-/-} App^{NL-G-F}* mice (scale bar: 50 μ m).

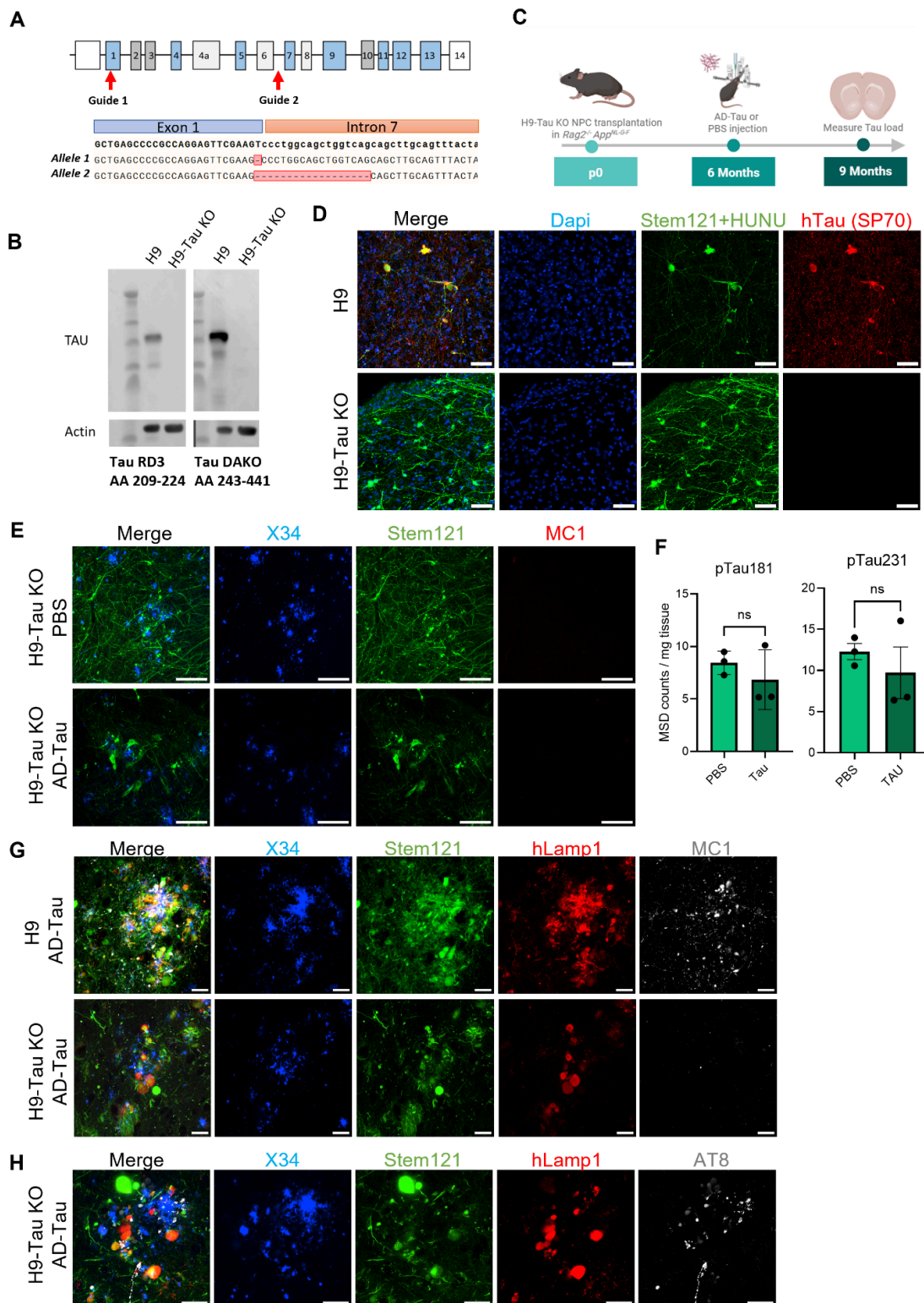


Figure 4

Human endogenous Tau is required for seed-based templating of Tau pathology but not for amyloid-induced dystrophic neurite formation.

A. CRISPR/Cas9-mediated knockout strategy targeting exons 1 and 7 of the *MAPT* locus in H9 human embryonic stem cells to generate a Tau knockout (H9-Tau KO) line. Sanger sequencing confirmed a deletion spanning exons 1-7 of the *MAPT* locus.

- B.** *In vitro* validation shows absence of Tau protein in H9-Tau KO cells as determined by western blot with RD3 and DAKO Tau antibodies.
- C.** Experimental design: *Rag2*^{-/-} *App*^{NL-G-F} mice were grafted with H9-Tau KO NPCs at P1. At 6 MPT, mice were injected with AD-Tau or PBS and sacrificed 3 MPI (corresponding to 9 MPT).
- D.** H9-Tau KO neurons integrate into the mouse brain and remain devoid of Tau protein (SP70-negative) at 9 MPT. H9-WT grafts at 7 MPT are shown as a positive control for SP70 immunolabelling (scale bar: 50µm).
- E.** H9-Tau KO neurons do not develop MC1-positive pathology following AD-Tau injection at 6 MPT. H9-Tau KO neurons in PBS injected *Rag2*^{-/-} *App*^{NL-G-F} mice serve as controls. Mice were sacrificed at 9 MPT (scale bar: 100µm).
- F.** Sarkosyl-insoluble protein extracts from xenografted mouse brains show minimal MSD signal for pTau181 and pTau231 in H9-Tau KO grafts with no difference between PBS and AD-Tau injected groups, confirming the absence of Tau pathology. Data are presented as mean ± SEM (*n* = 3 mice per group for PBS and AD-Tau injection in *Rag2*^{-/-} *App*^{NL-G-F} background). Statistical analysis was performed using unpaired t-test (*p* = ns for pTau181 and pTau231) (see Supplementary Table 1).
- G.** Both H9-WT (7 MPT) and H9-Tau KO (9 MPT) xenografts display robust dystrophic neurite pathology in response to extracellular amyloid, indicated by hLAMP1 labelling. MC1-positive pathology is present around H9-WT neurons but absent in H9-Tau KO cells following AD-Tau injection (scale bar: 20µm).
- H.** Dystrophic neurites of human H9-Tau KO neurons are negative for AT8-labelling, but mouse dystrophic neurites are present and develop AT8-positive dystrophic neurites (scale bar: 25µm).

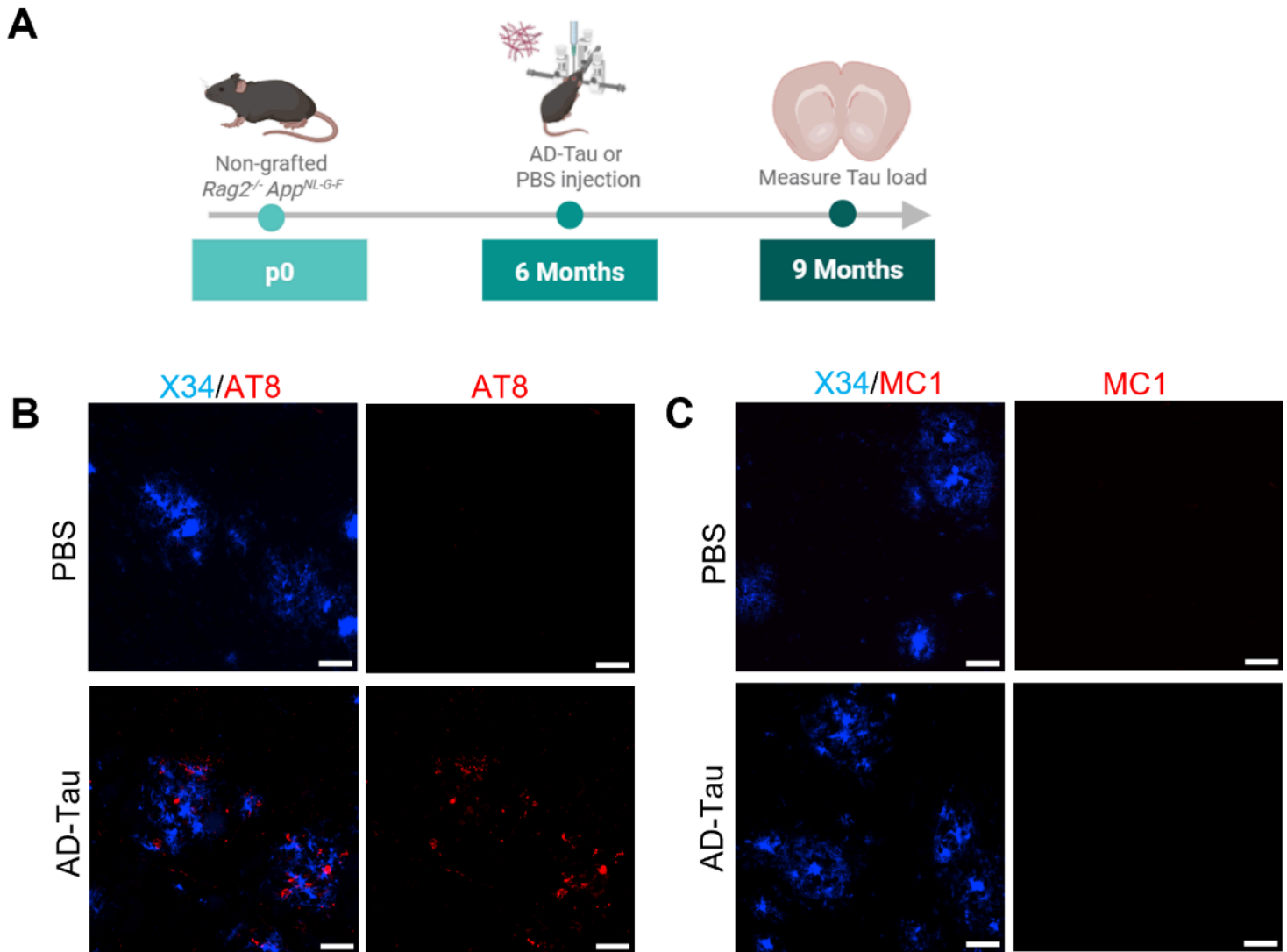


Figure 5

Mouse neurons develop limited Tau pathology following AD-Tau seeding.

- A.** Experimental design. Non-grafted *Rag2^{-/-} App^{NL-G-F}* mice received intracerebral injections of AD-Tau or PBS at 6 months of age and were sacrificed 3 MPI (9 months of age).
- B.** AD-Tau-injected mice exhibited an increase in dystrophic neurites labelled with AT8 around the amyloid plaques, indicating the accumulation of hyperphosphorylated Tau (scale bar: 25µm).
- C.** Mouse neurons did not develop conformationally altered Tau pathology within 3 MPI as shown by the absence of MC1 immunoreactivity (scale bar: 25µm). Data are representative of *n* = 4 mice per group (AD-Tau and PBS) in *Rag2^{-/-} App^{NL-G-F}* background.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [VanVierbergenetal.SupplementaryTable1.xlsx](#)

- [VanVierbergentetal.Supplementary.docx](#)