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Time- and dose-dependent seeding tendency of exogenous tau R2 and R3 aggregates in cells

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ABSTRACT

Tauopathies are a group of neurodegenerative diseases categorised into three types, 3R, 4R, or 3R+4R (mixed) tauopathies, based on the tau isoforms that make up the aberrant filaments. It is supposed that all six tau isoforms share functional characteristics. However, differences in the neuropathological features associated with different tauopathies offer the possibility that disease progression and tau accumulation may vary depending on the isoform composition. The presence or absence of repeat 2 (R2) in the microtubule-binding domain defines the type of isoform, which might influence tau pathology associated with a particular tau isoform. Therefore, our study aimed to identify the differences in the seeding propensities of R2 and repeat 3 (R3) aggregates using HEK293T biosensor cells. We show that the seeding induced by R2 was generally higher than by R3 aggregates, and lower concentrations of R2 aggregates are sufficient to induce seeding. Next, we found that both R2 and R3 aggregates dose-dependently increased triton-insoluble Ser262 phosphorylation of native tau, which is only visible in cells seeded with higher concentrations (12.5 nM or 100 nM) of R2 and R3 aggregates, despite the seeding by the lower concentrations of R2 aggregates after 72 h. However, the accumulation of triton-insoluble pSer262 tau was visible earlier in cells induced with R2 than in R3 aggregates. Our findings suggest that the R2 region may contribute to the early and enhanced induction of tau aggregation and define the difference in disease progression and neuropathology of 4R tauopathies.

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1. Introduction

Tauopathies are a group of neurodegenerative diseases characterised by the pathological inclusions or aggregates of microtubule-associated tau protein in neurons and glial cells. Cytoplasmic inclusions in primary tauopathies predominantly comprise either three-repeat (3R) or four-repeat (4R) tau isoforms. In contrast, inclusions in secondary tauopathy, like Alzheimer's disease, show the presence of both 3R and 4R isoforms. These inclusions also display prion-like properties and seed the aggregation of native proteins following intraneuronal spreading. The 3R isoform causes more severe axonal transport abnormalities and locomotor deficits, whereas the 4R isoform causes neurodegeneration and worsens learning and memory deficits [1]. Further, 4R tau isoforms are more effective than the 0N3R variant in causing oxidative stress [1].

The 3R and 4R tau isoforms differ by the absence or presence of Repeat 2 (R2), which harbours the PHF6* (275VQIINK280) amyloid sequence and cysteine-291. The other amyloid sequence PHF6 (306VQIVYK311) and cysteine-322 is present in Repeat 3 (R3) and, thus, in all tau isoforms. The remaining repeats, Repeat 1 and Repeat 4, show little to no tendency to aggregate in neutral solutions, except R4, which aggregates into filaments under acidic conditions [2]. We have recently shown that individual repeats, particularly R2 and R3, have different aggregation and seeding tendencies. Like R3, R2 forms seed-competent aggregates when assembled with heparin [3]. However, R3, but not R2, forms seed-

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competent aggregates without heparin, even though R2 and R3 have identical N-terminal hexapeptide and cysteine residue sequences. These indicate that R3 is possibly the 'initiator' minimal region for prion-seed production, with R2 needing polyanionic cofactors, contributing to the later stages of disease spreading. Whether the absence or presence of R2 in tau isoforms defines the difference in disease severity noted between 3R and 4R tauopathies remains unclear. Since R2 is absent in 3R tau isoforms, we decided to investigate the differences in the seeding tendencies of R2 and R3 using the HEK293T biosensor cells.

2. Materials and methods

2.1. Cell line and aggregates

Tau RD P301S HEK293T biosensor cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's growth medium (Lonza, #12–604F) supplemented with 10% fetal bovine serum and 1% antibiotics (streptomycin and penicillin) at 37 °C in a 5% CO_2/atmospheric air humidified incubator. Cells were passaged every 2–3 days when confluent and were tested for mycoplasma contamination and authenticated routinely.

Peptides of R2 and R3 were assembled into fibrillar aggregates in the presence of heparin for 48 h, as described elsewhere [3]. The aggregates were pooled from different wells of 384-well assay plates and stored at 4 °C for a maximum of 2 weeks if not immediately used.

2.2. Seeding quantification by microscopy

Biosensor cells were plated on a CellCarrier 96-well plate at a density of 5×10^4 cells/mL in 100 μL of medium and left in a 5% CO_2 incubator at 37 °C overnight. The next day, the cells were pre-stained with 20 ng/mL Hoechst 33342 and then transfected with aggregates using a jetPRIME® Polyplus transfection kit, as described elsewhere, with minor modifications [3]. Briefly, a two-fold serial dilution stock of 100 nM R2 or R3 aggregates in jetPRIME® buffer was prepared in a clear-bottom 96-well plate. After serial dilution, 0.5 μL jetPRIME® reagent was added per well of the transfection master plate, followed by brief vortexing and incubation for 10 min at room temperature. Next, Hoechst 33342-prestained cells in the 96-well plate were transfected with 5 μL/well of transfectants from the transfection master plate. Control wells were transfected with an equal volume of jetPRIME® buffer and reagent without aggregates. Cells were imaged on a Cell Voyager 7000S (Yokogawa) high-content imaging system with 20 × objective at 37 °C and 5% CO_2 in a live-cell chamber as described previously [3]. Cells were imaged every 3 h for 24 h and then after 48 h and 72 h post-transfection. Acquired images were analysed by Columbus Image Data Storage and Analysis System (Columbus 2.7.1.133403; Parkin Elmer), and the cell and aggregate numbers were quantified using pre-configured application solutions (scripts). The aggregates were detected as spots of P301S/CFP/YFP tau and normalised to the number of cells detected by Hoechst-stained nuclei, as described previously [4]. The ratio of the number of aggregates and cells per well is presented as seeding.

2.3. Seeding quantification by cell fractionation and western blotting

Cells were plated on Petri dishes at a density of 1.5×10^5 cells/ml and left to attach in a 5% CO_2 incubator at 37 °C overnight. Cells were then transfected with different concentrations of R2 and R3 aggregates premixed in jetPRIME® buffer and reagent. Control cells were transfected with only jetPRIME® buffer and reagent. After 72 h of transfection, cells were collected and lysed using an ice-cold buffer containing 0.05% Triton X-100 in 1 × Tris-buffered saline (TBS; pH 7.4) supplemented with cOmplete™ protease inhibitor (Cat. # 4693116001, Roche) and PhosSTOP™ phosphatase inhibitor (Cat. # 4906837001, Roche) by carefully pipetting the cells up and down using for 5 min on ice. Lysed cells were centrifuged at 500×*g* and 1000×*g* successively for 5 min at 4 °C. The supernatant was collected and transferred into a new ice-cold Eppendorf tube and centrifuged at 15 000 RPM for 60 min at 4 °C. The resulting supernatant was collected as the Triton-soluble fraction and stored on ice, while the pellet was washed with triton lysis buffer twice and centrifuged at 15 000 RPM at 4 °C for 1 min after each wash. After the second washing, the pellet was resuspended in 50 μL of RIPA buffer with protease and phosphatase inhibitors and sonicated for 1 min using a Branson Ultrasonic™ Sonifier Cup Horns (Marshall Scientific, Hampton, NH, USA) at 25% amplitude with a 15-sec pulse on/off cycle. The samples were centrifuged again at 65 000×*g* at 4 °C for 30 min to collect the supernatant as the Triton-insoluble fraction. Triton-soluble and Triton-insoluble fractions were either processed immediately or stored at −80 °C for later processing.

Equal volumes of Triton-soluble and insoluble fractions were mixed with SDS gel loading dye supplemented with 10% β-mercaptoethanol and heated at 95 °C for 5 min. The samples were then electrophoresed on 10% SDS-polyacrylamide gels and transferred onto a PVDF membrane (Cat. # IB24001, Invitrogen) using Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were later blocked in 5% BSA in 1 × TBS-Tween®20 (TBST) for 1 h at room temperature and stained with primary Tau-5 antibody (1:1000; Cat. #AHB0042, Invitrogen), Anti-Tau (4-repeat isoform RD4) Antibody, clone 1E1/A6 (1:1000; Cat. #05–804, Sigma-Aldrich) and phospho-Tau (Ser262) antibody (1:1000; Cat. #OPA1-03142, Invitrogen) overnight at 4 °C. Protein bands were developed using secondary anti-mouse (1:2000; Cat. #A1134) or anti-rabbit (1:2000; Cat. #A21202) Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) for 2 h at room temperature in the dark. The blots were imaged on a Gel Doc XR + Gel Documentation System (Bio-Rad) and analysed using NIH ImageJ (NIH, Bethesda, Maryland, USA).

2.4. Statistical analysis

All statistical analyses were performed using GraphPad Prism Software, and differences were considered significant at $P < 0.05$. Comparisons between two groups were performed by a two-tailed Student's t-test, whereas those between multiple groups were done by one-way analysis of variance (ANOVA) with Tukey's post hoc test. Data throughout the manuscript, unless otherwise mentioned, are presented as mean ± SEM of at least 2–3 independent experiments.

3. Results and discussion

Cells were treated with serially diluted concentrations of aggregates to calculate the EC_{50} values, established as aggregate concentrations needed to induce half of the maximal seeding (Fig. 1A–D). The highest concentration was always 100 nM, whereas the lowest concentration was either 0.0002 nM or 0.01 nM, depending on the number of serial dilutions. The EC_{50} values decreased over time and were similar for both aggregate types (Fig. 1D); however, the seeding induced by R2 was generally higher than R3 aggregates, as evident from the dose-response curves in Fig. 1A–C. Further, R2 as low at 0.01 nM resulted in significant intracellular seeding after 72 h compared to R3 (See the zoomed part of the graph in Fig. 1C; Fig. 1 E, F). This intracellular seeding at lower concentrations indicates that R2 aggregates may

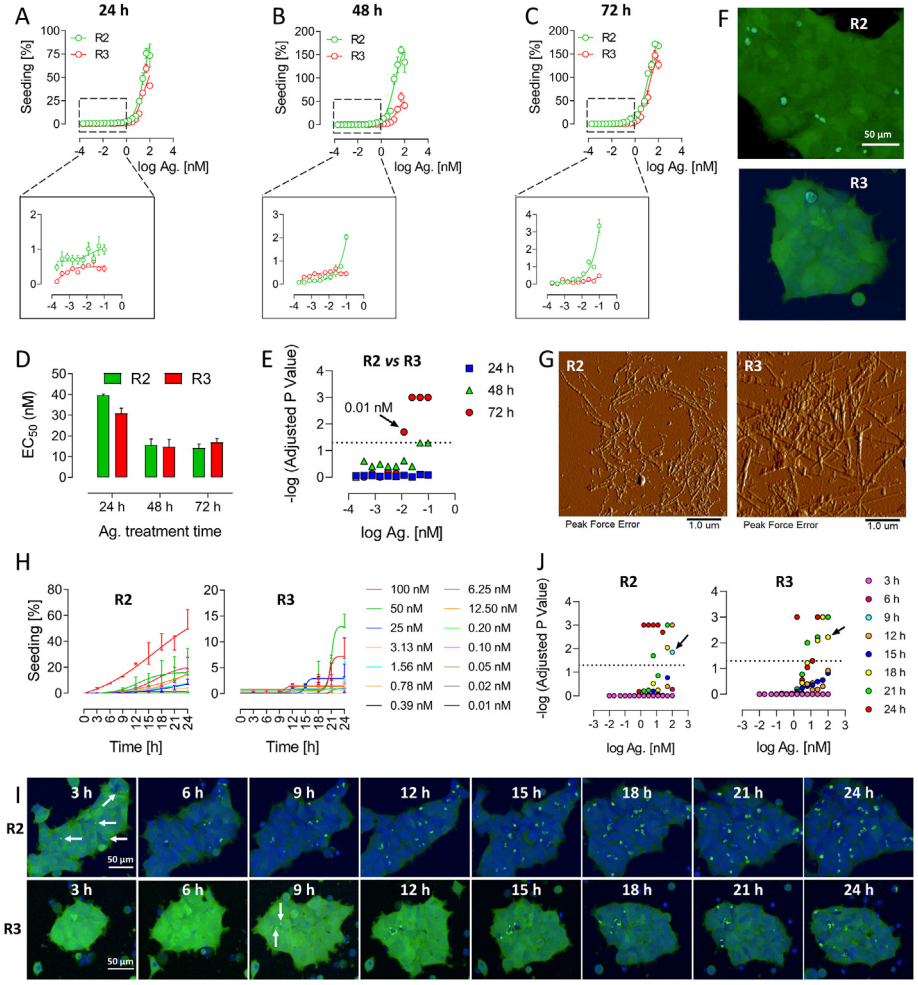

Fig. 1. Dose and time-dependent seeding of native tau. (A–C) Dose-dependent effect of R2 and R3 aggregates on tau seeding in biosensor cells after 24 h, 48 h and 72 h. Seeding at lower aggregate concentrations is shown in the magnified graphs. (D) EC_{50} of seeding following 24–72 h of aggregate treatment. (E) Plots of adjusted P-values of Student's t-test comparing the seeding induced by R2 and R3 aggregates at lower (0.002–0.1 nM) concentrations at 24–72 h. The dotted line denotes the significance level of P = 0.05. (F) R2 aggregates induce intracellular seeding in biosensor cells at a lower concentration of 0.01 nM. Green inclusions are the seeded tau. (G) R2 forms shorter fibrils compared to the larger fibrils of R3. (H) Time-dependent seeding at different concentrations of R2 and R3 aggregates. (I) Inclusions of intracellular seeded tau appear as early as 3 h following R2 aggregate treatment, whereas those in cells treated with R3 appear after 9 h. Cells were treated with 100 nM aggregates. Arrows show the first appearance of intracellular aggregates at the indicated times. (J) Plots of adjusted P-values of Dunnett's test in two-way ANOVA comparing seeding at different time points vs 0 h for each tested aggregate concentration. The dotted line denotes P = 0.05. Arrows point to the earliest timepoint at which a significant seeding was noted after cell treatment with 100 nM aggregates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

have a greater tendency to recruit, and misfold monomeric tau or R2 aggregates accumulate more efficiently intracellularly than R3 aggregates. The latter is partly supported by the fact that R2 forms a mixture of granular aggregates and shorter fibrils, compared to R3, which forms predominantly longer and larger fibrils of highly aggregated morphology (Figure G).

The difference in fibril size and aggregate type may facilitate better entry of R2 than R3 aggregates in cells, increasing the accumulation of R2 aggregates and inducing early seeding. This is further indicated by the appearance of seeded tau inclusions as early as 3 h following R2 treatment (Fig. 1H and I). However, the earliest timepoint at which R2 aggregates induced a statistically significant seeding was 9 h compared to t = 0 h (Fig. 1J, left). In contrast, detectable inclusions of seeded tau appear after 9 h following R3 treatment (Fig. 1I), with a statistically significant seeding noted only after 18 h (Fig. 1J, right). Overall, these findings show that although R2 and R3 have similar potencies in the later stages (24–72 h) of seeding, R2 potentially initiates the seeding of native tau due to its higher tendency to recruit monomeric tau.

We have previously shown that aggregates of R2 and R3 induce AD-specific phosphorylation of native tau in seeded cells [3]. Since we noted an early induction of seeding by R2 aggregates, we next checked whether this also caused solubility changes in the seeded tau. Cells were dose-dependently and time-dependently treated with aggregates and processed into Triton-soluble and Trion-insoluble fractions. For dose-dependent experiments, cells were induced with aggregates for 72 h at different aggregate concentrations, whereas for time-dependent experiments, cells were seeded with a single aggregate concentration of 100 nM for 3 h, 6 h, 9h and 24 h. Both R2 and R3 aggregates dose-dependently increased the accumulation of pSer262 tau in the Triton-insoluble fraction (Fig. 2A), indicating seeding. However, this seeding in R2 aggregate-treated cells was only evident at 12.50 nM and 100 nM concentrations, unlike those noted at lower R2 concentrations in imaging studies (Fig. 1C). When cells were induced with 100 nM aggregates, seeding of native tau and its accumulation in the triton-insoluble fraction was evident as early as 6 h following induction with R2 (Fig. 2B). Whereas insoluble bands of pSer262 appear post

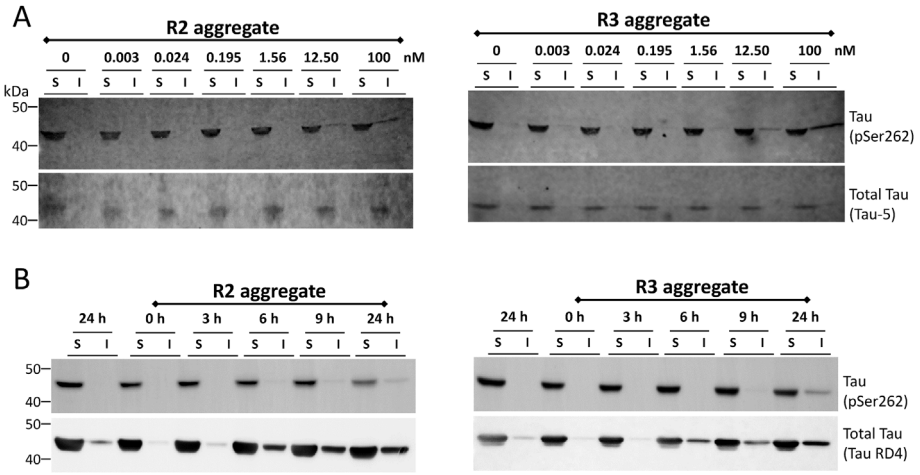

Fig. 2. Dose- and time-dependent effects on native tau solubility. Representative Western blots showing changes in phosphorylated pSer262 tau and total tau levels in triton-soluble (S) and triton-insoluble (I) fractions of biosensor cells induced with (A) the indicated concentrations of R2 and R3 aggregates for 72 h, and (B) R2 and R3 aggregates at a concentration of 100 nM for the indicated timepoints. Total tau bands in A were developed using Tau-5 antibody, whereas those in B were developed using Tau RD4 antibody. Images of uncropped blots are shown in SI.

9 h following induction with R3. This finding collaborates with the noted early induction of tau seeding by R2 aggregates in the study (Fig. 1). The minimal accumulation of triton-insoluble tau in the 24 h non-induced cells explains the nature of aggregation-prone P301S tau and the sensitivity of the Tau RD4 antibody, which was used to detect the total tau levels (Fig. 2B). Nevertheless, early time-dependent build-up of triton-insoluble tau aggregates is evident in cells that are induced with R2 aggregates and stained with Tau RD4 antibody (Fig. 2B).

In conclusion, we show that R2 and R3 aggregates seed intracellular tau aggregation in biosensor cells. However, the seeding potential of R2 aggregates is higher than R3 aggregates, requiring minimal concentrations and time to induce the seeding of native tau. These findings underline the importance of minimal regions of tau that define the nature of prion-like tau strains and affect their influence on the disease progression, accumulation and severity of the tauopathies. Thus, the regions contributing to the differences among tau strains highlight the importance of targeting these regions for treating tauopathies.

Data availability

The data generated during the current study are available from the corresponding author upon reasonable request.

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Author contributions

Narendran Annadurai: Investigation, Methodology, Writing; **Jiří Hrubý**: Investigation, Methodology; **Agáta Kubíčková**: Investigation, Methodology; **Lukáš Malina**: Investigation; **Marián Hajdúch**: Funding acquisition, Writing; **Viswanath Das**: Conceptualisation, Writing - Review & Editing, Funding acquisition, Supervision.

Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.02.057.

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