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A 31-residue peptide induces aggregation of tau's microtubule-binding region in cells

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Abstract

The self-propagation of misfolded conformations of tau underlies neurodegenerative diseases, including Alzheimer's disease. There is considerable interest in discovering the minimal sequence and active conformational nucleus that defines this self-propagating event. The microtubule-binding region, spanning residues 244-372, reproduces much of the aggregation behavior of tau in cells and animal models. Further dissection of the amyloid-forming region to a hexapeptide from the third microtubule-binding repeat resulted in a peptide that rapidly forms fibrils *in vitro*. We show here that this peptide lacks the ability to seed aggregation of tau₂₄₄₋₃₇₂ in cells. However, as the hexapeptide is gradually extended to 31 residues, the peptides aggregate more slowly and gain

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COMPETING INTERESTS

UCSF is conducting research sponsored by Daiichi Sankyo (Tokyo, Japan) to develop diagnostics and therapeutics for tau. Currently, there is no direct relationship between these agreements and the present manuscript.

Author contributions

J.S., W.F.D. & S.B.P. conceived and designed the experiments; J.S., M.N., H.W., M.B., C.C., N.J., J.R., T.L., S.A., J.B. M.J.S.K. and K.R. performed the experiments; J.S., M.N., H.W. Y.W., M.B. and C.C analyzed data; J.S., W.F.D. and S.B.P. co-wrote the paper. J.S., M.N. and H.W. contributed equally to this work.

DATA AVAILABILITY STATEMENT

All relevant data are available from the authors.

potent activity to induce aggregation of tau₂₄₄₋₃₇₂ in cells. X-ray fiber diffraction, hydrogen-deuterium exchange and solids NMR studies map the beta-forming region to a 25-residue sequence. Thus, the nucleus for self-propagating aggregation of tau₂₄₄₋₃₇₂ in cells is packaged in a remarkably small peptide.

INTRODUCTION

Misfolding of the tau protein is a molecular event that accompanies a wide range of devastating neurodegenerative diseases, including Alzheimer's disease (AD) and frontotemporal dementia (FTD). It is believed that the progressive nature of tauopathies is based on the propagation of misfolded tau isoforms throughout the brain in an autocatalytic process¹⁻³, which bear the hallmarks of prion formation. Indeed, injection of patient-derived brain extract containing misfolded tau into transgenic (Tg) mouse models causes endogenous tau to aggregate and spread throughout the brain^{4,5}.

Elucidating the conformational basis of tau misfolding and aggregation has been hampered by the protein's large size, variable splicing isoforms, and highly heterogeneous post-translational modifications^{6,7}. Variable splicing of tau leads to six different isoforms^{6,8} containing either three or four microtubule-binding repeat domains. The microtubule-binding region is essential for maintenance of microtubules by normal tau, but this same region also is the locus of self-propagating misfolding during disease⁹⁻¹¹. All tau isoforms share the third repeat domain (R3, residues 306-336). Mutagenesis showed the importance of the R3 domain in inducing aggregation and further identified a hexapeptide near the domain's N-terminus as an essential element for amyloid formation¹¹. Thus, it appeared that this hexapeptide contributed significantly to the formation of the critical self-propagating core, in much the same way that active site residues are critical for catalysis in enzymes, or a set of core residues are critical for productive normal folding of proteins. However, in each case the critical residues work in concert with the remainder of the protein to effect folding or catalysis.

There was thus considerable interest raised by the observation that a hexapeptide from the R3 domain very rapidly forms amyloid and the structure of this peptide was determined by X-ray crystallography^{12,13}. This groundbreaking work provided some of the first high-resolution insights into motifs such as steric zippers that underlie assembly of amyloids. However, it seemed unlikely that the tau hexapeptide alone would represent the full, self-propagation competent core of tau¹⁴. Longer synthetic R3 domain peptides are also capable of forming amyloid aggregates^{15,16} in cell-free systems, but the biological activities of fibrils formed from these peptides has not been investigated in a cellular context. In summary, essential residues for aggregation of tau have been delineated, but the minimal fragment required for sequence- and conformation-specific seeding of the self-propagating aggregation of the full tau repeat domain region in cells remain to be determined.

We report here that a peptide spanning the R3 domain forms fibrils that induce aggregation of the four repeat microtubule binding and amyloid-forming region of tau (tau₂₄₄₋₃₇₂ designated here as tau-RD) *in vitro* and in mammalian cells. Hydrogen-deuterium exchange, Fourier transform infrared spectroscopy (FTIR), X-ray fiber diffraction, and solid-state

NMR (ssNMR) indicate that the active R3 peptide forms a parallel 3-stranded cross-beta structure, most likely with in-register hydrogen bonding between the individual strands. Conservative changes to the sequence of R3 lead to amyloid with altered physical properties and greatly differing ability to induce tau aggregation in cells, demonstrating the specificity of the system.

RESULTS & DISCUSSION

Systematic C-terminal truncation of the R3 domain impacts aggregation propensities and self-propagation

We measured the amyloid-forming tendency of a set of peptides containing successive 5-residue truncations from the C-terminus of the R3 domain (residues 306-336), and compared this propensity with their ability to induce aggregation of tau's repeat domain region in cells. Each peptide begins at residue 306 and the position of the C-terminal truncation is designated as a superscript in Fig. 1a (e.g., the widely studied R3 hexapeptide spanning residues 306-311 is designated as R3³¹¹). The peptides formed amyloid as monitored by thioflavin T (ThT) binding¹⁷, even in the absence of aggregation inducers such as heparin^{18,19}, which are required for aggregation of full-length tau and tau-RD (Fig. 1b). The short hexapeptide R3³¹¹ formed amyloid most rapidly; as the chain was extended, the lag time increased significantly (Fig. 1b, Supplementary Fig. 1). Electron microscopy (EM) confirmed the formation of amyloid aggregates (Fig. 1c) with significant differences in fibril morphology between the peptides. In summary, each peptide formed fibrils and amyloids, but at widely differing rates and markedly different fiber morphology.

The peptides showed even larger differences in their abilities to induce aggregation of tau-RD in mammalian cells. We used a cell culture model sensitive to the propagation of tau derived from recombinant tau amyloids, Tg mouse models, and, most importantly, patients affected by primary or secondary tauopathies² (Fig. 1d). We combined this cell assay with a high content confocal analysis system to enable high-throughput and quantitative measurement of the induced cell infections²⁰. Human embryonic kidney (HEK) cells express the tau-RD (residues 244-372) with the disease-associated mutations P301L/V337M fused to the yellow fluorescent protein (YFP) (denoted HEK(tau-RD(LM)-YFP)), which allows automated measurement of aggregation by quantitative confocal microscopy. Natural or synthetic tau prions induce the aggregation of the tau-RD(LM)-YFP construct, creating YFP-positive inclusions (Fig. 1d). Because the externally applied aggregates are not fluorescent, the YFP-positive granules are unambiguously the result of externally induced propagation of the endogenous substrate. Amyloids formed by tau-RD (residues 244-372, lacking a fluorescent protein tag) induced tau-RD(LM)-YFP aggregates in ~45% of the imaged cells (Fig. 1f; Supplementary Fig. 2).

Remarkably, amyloids formed from the 31-residue synthetic R3 protein displayed the same ability to induce tau-RD(LM)-YFP aggregation as the fibrillized tau-RD (Fig. 1e/f) despite the ~100 residue difference in their length. Importantly, further C-terminal truncation of the R3 peptide by five or ten residues led to an approximately tenfold reduction in intracellular tau aggregation (Fig. 1e/f). Even shorter constructs showed no templating activity, and the widely studied^{12,13} hexapeptide R3³¹¹ was completely inactive despite being taken up into

the cytosol (Supplementary Fig. 4). Thus, the nucleus for prion-propagation of tau lies within a remarkably small core of approximately 30 residues. The biological inactivity of peptides shorter than 20 residues speaks to the specificity of the process.

Modification of Cys322 of R3 modulates amyloid formation *in vitro* and tau-RD aggregation in cells

Oxidation and other post-translational modifications of Cys322 in the R3 domain of tau alters its tendency to aggregate^{21–23}. Also, the methods routinely used to form tau-RD fibrils results in a mixture of reduced monomers and disulfide-dimers²⁴. Thus, we examined the cellular activity of fibrils formed from: 1) R3 peptide under ambient oxygen (fibrils from this treatment are designated as R3 fibrils); 2) R3 under reducing conditions (R3_{SH} fibrils); 3) the preformed disulfide dimer of R3 (R3_{S-S} fibrils); 4) and a monomeric mutant in which Cys322 is replaced by Ser (R3_{C322S} fibrils) (Fig. 2a/b; Supplementary Fig. 1). We first examined the rates of fibril formation using the ThT fluorescence assay as in Fig. 1. The half time ($t_{1/2}$) for aggregation of R3 peptide under reducing conditions is similar to that seen for the R3_{C322S}, while the disulfide-bonded dimer, R3_{SS}, formed amyloid much more rapidly. Interestingly, $t_{1/2}$ for aggregation of initially reduced R3 peptide under ambient oxygen was intermediate between the value seen for R3_{S-S} and R3 under reducing conditions (Supplementary Fig. 1). Also, SDS-PAGE showed the R3 fibrils formed from R3 under aerobic conditions was a mixture of reduced and oxidized peptides (Supplementary Fig. 1). Once formed the R3 fibrils appear resistant to further oxidation, indicating that air oxidation of R3 occurred during the process of amyloid formation.

All peptides formed similar amounts of fibrils, although there were marked differences in the morphology, ranging from highly aggregated morphologies for R3 and R3_{S-S} to homogeneous fibrils with sharp contours and no observable twists for R3_{SH} and R3_{C322S}¹⁶ (Fig. 2b). Also, the fibrils formed by each species were resistant to digestion by proteinase K (PK), which is a characteristic of proteins in amyloid and prion states (Supplementary Fig. 6). We also examined the ability of the peptide fibrils to induce aggregation and amyloid formation of the tau's full 4R repeat domain region (WT tau_{244–372} lacking any disease promoting mutations or a fused fluorescent protein) in cell-free medium. In the absence of inducers such as heparin, this protein is completely resistant to aggregation. Fibrils from the three Cys-containing variants, R3, R3_{SH} and R3_{S-S}, each seeded amyloid formation of tau_{244–372} within a 90 hr incubation period in each of four replicates. By contrast, the variant R3_{C322S} showed significantly poorer seeding, resulting in amyloid in only half of the replicates over this time period. Also we observed longer lag-times for the two runs that showed some aggregation.

The differences between the ability of R3_{C322S} and the other Cys-containing R3 peptides were greatly amplified when measured against tau-RD(LM)YFP in cells. R3 and R3_{SH} fibrils induced aggregation of tau-RD(LM)YFP expressed in HEK cells, resulting in 45% and 33% infected cells, respectively (Fig. 2c/d). In comparison R3_{S-S} fibrils had a lower titer, transmitting to only ~5% of cells under the same conditions. Surprisingly, fibril preparations of R3_{C322S} were completely inactive, showing <1% infectivity. Thus, subtle changes to Cys322 induce major changes in infectivity (Fig. 2c/d). Furthermore, the cellular

milieu provides a more discriminating and less promiscuous environment for templating. Taken together, the *in vitro* and in-cell templating studies show that the mere ability to form amyloid is insufficient to induce a self-reproducing prion structure into tau-RD endogenously expressed in a cellular milieu.

Significant differences of R3 variants revealed by fluorescence microscopy and stability against denaturation by guanidine hydrochloride

We next sought to determine whether differences in the physical and conformational properties of the R3 variants might underlie their differing activities in the cellular assay.

The final ThT fluorescence intensity varied for each of the peptide fibrils, despite the fact that the extent of fibril formation was similar for each peptide (Supplementary Fig. 1 and Fig. 4 a). This finding suggests that the variations in fluorescence intensity for different peptides with the same primary structure reflect conformational differences. Given the large differences in fluorescence intensity apparent for ThT between the different amyloid species, we asked whether they might induce changes in the shape and wavelength of the maximal emission of the amyloid-staining dyes ThT, BF-188, and FSB^{25,26}. We developed a microplate-based method to visualize polymerized aggregates *in vitro* by confocal microscopy (Fig. 3 and Supplementary Fig. 5). Fibrils (formed in the absence of any dye) embedded in low melting point agarose and were treated with one of the three dyes. Using confocal microscopy (Fig. 3a, staining with BF-188 is shown) we analyzed the microscopic appearance and fluorescence emission spectra of 1000–1500 aggregates. Comparing intensity-normalized fluorescence emission spectra for individual peptides elucidates differences in their structure, homogeneity, and conformation. The shape of the ThT emission spectra showed no discriminatory power for all R3 preparations (Fig. 3b), despite the marked differences in intensity seen in Fig. 2. In contrast, FSB showed overlapping spectra for R3 and R3_{S-S} ($\lambda_{\text{max}} = 527$ nm) but discriminated these species from R3_{SH} and R3_{C322S} (R3_{SH} $\lambda_{\text{max}} = 490$ nm and R3_{C322S} $\lambda_{\text{max}} = 475$ nm). BF-188 clearly distinguished R3 from R3_{S-S} (R3 $\lambda_{\text{max}} = 527$ nm and R3_{S-S} $\lambda_{\text{max}} = 512$ nm), but it had less ability to distinguish R3 from R3_{SH}. Together with the large differences in ThT fluorescence intensity seen for these samples (Fig. 3b), the environment-sensitive fluorescence emission spectra of the absorbed dyes revealed significant differences in the structures of fibrillar aggregates formed by R3 and its analogs. Interestingly, the spectra of R3 are not well described by a linear combination of R3_{SH} and R3_{S-S}, indicating that R3 amyloids do not contain large segregated amyloids of each species.

We also determined the differences in the thermodynamic stability of the fibrils formed from different peptides by comparing their guanidine hydrochloride (GdnHCl) denaturation profiles (Fig. 4a). Fibrils were mixed with varying concentrations of GdnHCl, and the samples mixed until equilibrium between soluble and insoluble peptides was reached after 20 hr. The single major difference between the peptides was that the disulfide dimer, R3_{S-S}, was markedly more resistant to denaturation than the other peptides.

FTIR and fiber diffraction show that the R3 variants form cross- β structure

The fibrils appear to consist of parallel cross- β secondary structures, but there were apparent differences in the packing and degree of order seen for the different peptides. The infrared (IR) spectra (Fig. 4b) of the peptides are highly similar and predict a parallel orientation for the β -strands (Fig. 4c). The amide I (carbonyl stretch) region of fibrils formed from each peptide is dominated by a sharp feature centered near 1630 cm^{-1} , consistent with beta-structure. The lack of a second exciton-coupled band near 1690 cm^{-1} suggests that the strands run parallel to one another. Fiber diffraction provided additional confirmation of the cross- β structure (Fig. 4c), and revealed significant differences in the ordered packing within fibrils formed from the different peptides. For amyloid fibrils, diffraction is usually dominated by a $\sim 4.8\text{ \AA}$ meridional reflection, which corresponds to the separation of beta-strands along the fibril axis, and an equatorial intensity near $\sim 10\text{--}12\text{ \AA}$, which corresponds to the spacing between beta-sheets perpendicular to the fibril axis. Diffraction patterns from all four peptides are dominated by strong cross-beta diffraction (arrow), characteristic of amyloid structures, and equatorial diffraction at $12\text{--}13\text{ \AA}$ (arrowhead), suggesting that the amyloids share a stacked-sheet architecture^{27–31}, with beta-strands running perpendicular to the fiber axes and beta-sheets stacked $12\text{--}13\text{ \AA}$ apart. The monomeric R3_{SH} and R3_{C322S} samples displayed the highest degree of order, as also shown by the well-aligned fibrils in the electron micrographs (Fig. 2b). This observation matches the high degree of homogeneity seen in overall morphology of the fibrils seen by electron microscopy. In R3_{SH} and R3_{C322S}, numerous equatorial intensities can be seen, suggesting that the diffraction is from ordered filaments perhaps $50\text{--}150\text{ \AA}$ in diameter. Although these samples both exhibit relatively high-frequency diffraction ripples, there are distinct differences in the details of their underlying molecular structures.

In summary, although each of the Cys322 variants form fibrils with very similar secondary structure, they display significant differences in their fiber morphology and underlying higher order structure – and these nuanced differences give rise to large scale differences in their ability to template tau prions in cells. The use of multiple fluorescent dyes to probe structural differences between the fibrils demonstrated significant differences for each peptide. For example, this method demonstrated that the structure of R3 fibrils is fundamentally different from a mixture of R3_{SH} and R3_{S-S} fibrils. This conclusion matches the observation that R3 fibrils are significantly more active than either R3_{SH} or R3_{S-S} fibrils. Thus, the reduced and oxidized forms of the peptide mix in the R3 fibrils to create a different conformation and greater cellular activity than seen in either peptide alone.

It is interesting to speculate on the origins of the very low-activity of some of the fibrils. R3_{S-S} fibrils, in particular, had low templating potency and high thermodynamic stability. Previous studies with different conformational strains of PrP have shown that high thermodynamic stability can lead to low prion titers, possibly because of the difficulty of breaking the large amyloid into smaller highly infectious seeds *in vivo*³². It is also interesting that the mutant peptide peptide, R3_{C322S} is entirely inactive, which reflects exquisite sequence and/or conformational specificity of the prion templating process.

Hydrogen-deuterium exchange (HDX) and solids-state NMR (ssNMR) reveal residue-specific differences in the conformation of R3_{SH} and R3_{S-S}

To obtain residue-specific, structural information for the fibrils, we performed quenched H/D exchange (Fig. 4d)³³, focusing on the chemically homogenous R3_{SH} and R3_{S-S} fibrils. Strong hydrogen-bonding between backbone amides and low accessibility to water (and hydrated hydroxide ions) in beta-structures leads to a low rate of exchange of amide protons with deuterons in deuterium oxide (D₂O) relative to unstructured amides. To identify the position of strongly protected backbone amides, fibrils were allowed to exchange in D₂O for 20 h, and exchange was quenched by rapid freezing and lyophilizing individual samples. Each sample was then dissolved in an organic solvent system (DMSO-D₆/D₂O/DCA-D₂, 95:4.5:0.5 (v/v), pH 5.5³³) that strongly inhibited further exchange, and the extent of exchange that had occurred during the aqueous D₂O incubation period was determined by solution NMR. H/D exchange data show strong protection for residues 308-311 and 315-322, which defines the most stable amyloid-core region (Fig. 4d). Residues 323-329 also showed intermediate levels of protection, indicative of a third, less stable strand. The strong protection near the N-terminus is consistent with the tight steric-zipper motif found in the crystal structure¹² of the hexapeptide, ³⁰⁶VQIVYK³¹¹. However, Gln307 was not protected, suggesting a potential structural difference in the context of amyloid versus crystalline packing. The most pronounced differences in HDX between R3_{SH} and R3_{S-S} occurred at Cys322 and Leu325 near the location of the disulfide. These subtle distinctions in an otherwise similar global structural organization might also contribute to the major differences in biological activity.

Finally, we used MAS ssNMR to further examine the secondary structure and conformational homogeneity of R3_{SH} (Fig. 5), chosen for its high biological activity and apparent structural homogeneity, as shown by TEM and fiber diffraction. Two peptides were synthesized with uniform ¹³C labeling of a total of eight residues, and 2D ¹³C-¹³C correlation spectra were acquired at 12 KHz MAS using dipole-assisted rotary resonance recoupling (DARR)³⁴. Single peaks with narrow ¹³C line widths (100–130 Hz without apodization) were observed for all of the labeled residues in the fibril core defined above by H/D exchange (Fig. 4d), with the exception of L315, which is at the first position of a highly protected region spanning residues 315-322. Essentially complete ¹³C backbone and side-chain assignments were possible using the two specifically labeled samples (Supplementary Fig. 7). Secondary chemical shift analysis^{35,36} showed that all labeled residues, except L315 and G326, have a beta-sheet conformation (Fig. 5b). The major conformers of L315 are predicted to be in the β -conformation, although a minor conformer of L315 is predicted to have a non-beta conformation (Fig. 5c), possibly induced by the proximal Pro312. Gly334 and 335 were not detectable, likely because they are dynamic and cross-polarization acts as a dynamic filter. These observations are in excellent agreement with H/D exchange data, which also indicates increased plasticity around P312 and at the C-terminus of R3.

In summary, the biologically active R3_{SH} fibrils consist of three parallel (FTIR), stacked beta-sheets with a regular inter-strand spacing of ~4.8 Å (fiber diffraction). The sequence-specific assignment of secondary structure obtained by integrating information from fiber diffraction, FTIR, H/D exchange NMR, proteolysis, and ssNMR (Fig. 5d) reveals that

residues 308-330 are generally ordered and form the core of the cross-beta structure. Breaks in the strands occur near 313-314 and 326-327. The C-terminus is much less structured but as an intrinsically disordered region still plays a crucial role in the kinetics of aggregation and biological activity of the peptide.

CONCLUSIONS

Here, we explore the relationship between amyloid versus prion formation for peptides from the R3 region of tau. Amyloid refers to a cross- β conformation, classically assessed by fiber diffraction and the ability to be stained by dyes, such as ThT. The word prion, refers to a non-native conformational state of a given protein, which is able to induce misfolding of the same or closely related proteins in a self-propagating manner *when in an appropriate biological milieu* (e.g., within a cell for tau). The native states of prion-forming proteins can be globular folded proteins (e.g., PrP), membrane associated helices (e.g., A β), or intrinsically disordered (e.g., monomeric tau). Prions can spread within a cell, between neighboring cells, or even between individuals or species in rare human disorders such as mad cow disease. When the word was first coined, the only prion-forming protein known was, PrP, which at the time was known to cause disease spontaneously in sporadic CJD, or by infection between individuals, as in Kuru. More recently, the list of prion-forming proteins has rapidly expanded to include proteins that serve functions that are beneficial to organisms (e.g., yeast prions), as well as a large number of neurodegenerative diseases caused misfolding of tau and synuclein (which do not act as infectious agents in animals or humans).

Given the frequent accumulation of insoluble proteins in the cross-beta conformation in most prion diseases it is generally hypothesized that prions are themselves amyloid fibrils, or smaller more soluble versions of amyloids. However, this has not been rigorously proven with the possible exception of fungal prions. For example, fragments of PrP readily forms a variety of amyloid conformations in cell-free systems but only a minority of these preparations are infectious. Thus, great interest was generated by the finding that purified proteins spanning the microtubule-binding region of tau could be misfolded in cell-free systems to form amyloid fibrils that act as sequence-specific prions capable of entering cells and spreading throughout the brain.

Previous studies have shown that pathogenic tau fibrils have a protease-resistant core, which was localized to the vicinity of the R3 domain. Furthermore, solid state NMR studies on a protein spanning the three microtubule-binding repeat domains present in certain splice isoforms of tau localized the amyloid-forming domain to approximately the first 25 residues of R3 and additionally found that the remaining chain was dynamically disordered. Thus, we hypothesized that a peptide spanning the full R3 domain be sufficient to form amyloid fibrils that induce template misfolding of the full-length micro-tubule repeat domain, tau₂₄₄₋₃₇₂. Templating might occur through end-on addition of the longer tau proteins to the peptide fibrils to the exposed mainchain amides that are exposed along the long axis of the fiber, or the templating could occur along the fibril surface as discussed previously. Conversely, we expected that shorter peptides, such as the widely studied N-terminal hexapeptide of the R3 repeat, would not misfold into the same structure as longer R3 constructs, and hence would

be unable to induce aggregation of the tau protein in cells. Our experimental findings strongly support these underlying hypotheses, but also unveil a number of significant subtleties for further investigation.

Yet to be explored are the detailed conformational differences between the fibrils formed from Cys322 variants, and their differing abilities to template aggregation of different tau splice isoforms *in vitro* and *in vivo*. Here, confocal microscopy using multiple amyloid-staining fluorescent dyes – each sensitive to environment by virtue of their quantum yield and/or Stokes shift – has emerged as a powerful beacon to differentiate conformations and assess the degree of heterogeneity within a population of aggregates. The discovery of distinct conformational forms for the various C322 R3 variants is reminiscent of recent results with A β peptides, in which seemingly minute changes in sequence or even aggregation conditions *in vitro* can lead to markedly different structures as revealed by solid state NMR. Differing conformational forms also occur *in vivo* in distinct familial mutants, disease states, and even regions of the brain. The R3 series of peptides provide a small but biologically relevant system for addressing detailed biophysical related questions concerning the relationship between structure and prion formation in tau.

METHODS

Formation of fibrils from R3 and C-terminally truncated analogs

Lyophilized peptides were dissolved at 145 μ M in PBS(–/–). Samples were incubated at 37 °C for 72 h in 1.5-mL centrifugation tubes under constant agitation at 900 rpm.

Transmission electron microscopy

Following fibril formation in the absence of ThT, samples were adsorbed onto freshly glow-discharged, 200-mesh formvar/carbon-coated copper grids (Ted Pella) and negative-stained with a 2% (wt/vol) uranyl acetate solution. Images were taken on an FEI Tecnai F20 electron microscope at 80 kV.

HEK(tau-RD(LM)-YFP) cell infection assay

HEK(tau-RD(LM)-YFP) cells were a generous gift from Marc I. Diamond. One thousand cells per well (including 16 ng/mL Hoechst 33342) were plated in 384-well cell culture plates (Greiner). Cells were allowed to adhere to the wells for 4–6 h before exposure to ~30 nmol/well aggregates premixed in 3% (volume/total volume) Lipofectamine2000 (Life Technologies) and 77% (volume/total volume) Opti-MEM media (Life Technologies). Cell infections were incubated for 72 h before quantification using the GE INCell 6000 with custom analysis protocols for aggregate detection developed by GE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Amyloid formation R3-derived peptides and Transmission to HEK(tau-RD(LM)-YFP) cells

(a) Sequential truncation of five residues starting with R3 generated six different peptides used in this study. (b) Time courses of aggregation reported by thioflavin T (ThT) fluorescence (normalized) showed that all peptides form ThT-positive aggregates with distinct kinetic profiles (three replicates, average $\pm$ s.e.m.). (c) Negative-stain transmission electron microscopy (TEM) confirmed the presence of amyloid fibrils with distinct aggregate morphologies (scale bar represents 100 nm). (d) Schematic of the assay format using HEK cells stably transfected with tau-RD(LM)-YFP. Naïve HEK(RD(LM)-YFP) cells do not develop tau-RD(LM)-YFP aggregates and therefore show only diffuse YFP fluorescence. Exogenous exposure to self-propagating tau aggregates induces intracellular aggregation of tau-RD(LM)-YFP, resulting in focal puncta with high YFP fluorescence. (e) Representative confocal microscopy images for cell infections quantified in (f) are shown. (f) Quantification of the percentage of HEK(tau-RD(LM)-YFP) cells displaying aggregates after exposure to the various R3-derived peptides as well as the full tau-RD aggregates (gray bars represent samples that induced aggregation; white bars represent samples without any aggregates present after visual inspection of all images used for quantification by high content analysis; PBS control values were subtracted from all samples; all values are expressed as average $\pm$ s.e.m.).

Figure 2. Amyloid formation and self-propagation of covalent variation of R3

(a) Kinetics of fibril formation show that amyloid formation was completed within 6 h for all conditions and constructs. R3 variants show distinct fluorescence amplitudes suggestive of the formation of distinct amyloid conformations (six replicates, average $\pm$ s.e.m.). (b) Representative transmission electron microscopy (TEM) images of R3-derived amyloid fibrils (scale bar represents 100 nm). (c) Quantification of transmission efficiency of R3 amyloids to cell cultures following 72 h exposure (all values are expressed as average $\pm$ s.e.m.). (d) Representative images of HEK(RD(LM)-YFP) showing cell nuclei in blue (Hoechst stain) and YFP-positive tau-RD(LM) aggregates in yellow.

Figure 3. Fluorescence emission fingerprinting of R3 amyloids

(A) (a) Representative confocal images of the four different R3 preparations stained with BF-188, revealing a unique aggregate structure for R3_{C322S} compared with the other R3 amyloid preparations. (b) Fluorescence emission spectra of ThT (left), FSB (middle), and BF-188 (right) bound to R3 amyloids. Each spectra represents an average of ~1000–1500 spectral measurements of individual aggregates (average $\pm$ s.e.m.).

Figure 4. Structural investigations reveal a common parallel, stacked beta-sheet architecture in R3 amyloids

(a) Chemical denaturation of R3-derived amyloid samples using guanidine hydrochloride reveals a higher structural stability for the R3_{S-S} amyloids compared with the R3, R3_{SH}, and R3_{C322S} amyloids (average $\pm$ s.e.m.). (b) ATR-FTIR spectra of the amide I region of the R3 peptide preparations. In all four, the sharp feature occurring near 1630 cm⁻¹ is consistent with beta secondary structure, and the lack of an additional band near 1690 cm⁻¹ suggests that the parallel beta-sheet orientation is adopted. Contributions are seen from carbonyl-containing side chains and unstructured amide backbone vibrations in a broad band near 1660 cm⁻¹. (c) X-ray fiber diffraction images for the four different R3 amyloid preparations suggest a stacked beta-sheet conformation for all of the samples. (d) Comparison of H/D exchange rates between R3_{SH} and R3_{S-S} amyloids expressed as a ratio of relative intensities of residue-specific backbone amide protons at t = 20 h and t = 0 h. Values close to 1.0 correspond to highly protected backbone amide protons, whereas values close to 0 suggest nearly complete deuterium exchange after 20 h of incubation in D₂O (average $\pm$ s.e.m.).

Figure 5. Solid-state NMR spectra of R3SH fibrils show a homogenous, predominantly beta-strand structure

(a) ^{13}C - ^{13}C correlation spectra (with 50 μs DARR mixing) show narrow line widths and well-resolved peaks. (b) Secondary shift analysis indicates beta-strand structure with breaks at Leu315 and Gly326. $S = (\delta(^{13}\text{C}\alpha)) - (\delta(^{13}\text{C}\beta))$, where $(\delta(^{13}\text{C}\alpha))$ and $(\delta(^{13}\text{C}\beta))$ are the difference between experimental shifts and the corresponding random-coil chemical shifts for each residue. (c) Temperature-dependent spectra show that Leu315 populates three different conformations (#1, #2, and #3) that are in exchange. (d) Representation of R3SH subunits in an amyloid state combining structural information from H/D exchange NMR (magenta underlining), fiber diffraction (interstrand stacking), and MAS-ssNMR (green underlining) (cyan residues = beta structure, gray residues = non-beta structure). Peptide strands of R3SH in the amyloid conformation adopt a parallel beta-sheet conformation with 4.8-Å interstrand distance, with most residues participating in beta-strand formation.