HIV Tat protein and amyloid-β peptide form multifibrillar structures that cause neurotoxicity

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Deposition of amyloid-β plaques is increased in the brains of HIV-infected individuals, and the HIV transactivator of transcription (Tat) protein affects amyloidogenesis through several indirect mechanisms. Here, we investigated direct interactions between Tat and amyloid-β peptide. Our in vitro studies showed that in the presence of Tat, uniform amyloid fibrils become double twisted fibrils and further form populations of thick unstructured filaments and aggregates. Specifically, Tat binding to the exterior surfaces of the Aβ fibrils increases β-sheet formation and lateral aggregation into thick multifibrillar structures, thus producing fibers with increased rigidity and mechanical resistance. Furthermore, Tat and Aβ aggregates in complex synergistically induced neurotoxicity both in vitro and in animal models. Increased rigidity and mechanical resistance of the amyloid-β–Tat complexes coupled with stronger adhesion due to the presence of Tat in the fibrils may account for increased damage, potentially through pore formation in membranes.

Despite antiretroviral therapy, neurocognitive dysfunction is detected in nearly 30% of HIV-infected individuals1, and the incidence is higher in older people2,3. HIV-infected individuals have increased deposition of amyloid-β plaques in the brain4,5. Amyloid plaques are a hallmark of Alzheimer’s disease, and their role in disease pathogenesis is an area of intense investigation. Another factor contributing to neuronal injury in HIV-infected individuals may be the presence of an HIV reservoir in the brain. Even when viral replication is suppressed with brain-penetrant antiretroviral drugs, Tat protein can still be produced from proviral DNA6. HIV-infected cells release Tat into the extracellular space, where it interacts with amyloid-β peptide. Tat also affects amyloid levels by inhibiting amyloid breakdown7,8, be produced from proviral DNA6. HIV-infected cells release Tat into the extracellular space, where it interacts with amyloid-β peptide. Tat also affects amyloid levels by inhibiting amyloid breakdown7,8, and directly interacting with amyloid precursor protein (APP) and stimulating amyloid-β-peptide production9. Here, we explored whether Tat directly forms a complex with amyloid-β peptide and affects amyloid-β polymerization and neurotoxic properties.

Tat is a small protein composed of 86 to 101 amino acids10. It is the first protein expressed after HIV enters the cell and is a key activator of HIV transcription11. Exon 1 encodes the first 72 amino acids, which constitute the most active part of the protein. The second exon comprises residues 73–101; this exon has high sequence heterogeneity, and its complete biological function is unclear. Structural studies of Tat in solution through NMR performed at pH 4.1 or pH 6.5 have predicted an unstructured protein15,16 with a tendency to fold at pH 6.5 (ref. 15). The absence of a fixed conformation and the observation of fast dynamics are consistent with the ability of Tat to interact with a variety of molecules, thereby supporting the possibility that it is a natively unfolded protein. A common mechanism of action for natively unfolded proteins involves partial or complete folding after interaction with a binding partner17. CD studies of Tat have shown a lack of secondary structure of the protein; however, these tests were performed under denaturing conditions (10 mM acetate buffer at pH 4.7 (ref. 16) or at pH 4.5 (ref. 18)). The crystal structure of Tat in complex with pTEFb shows that, under milder crystallization conditions, the protein presents a fold or dramatically changes conformation in the bound state. This active Tat bound to its target shows a well-folded portion of 42 amino acids, which is held together by two Zn2+ ions and is coordinated by most of the cysteine residues in the cysteine-rich region19.

Amyloid β(1–40) (Aβ) is found in amyloid plaques and is the most abundantly secreted amyloid peptide from the cells. The structure of Aβ fibrils has been extensively studied20, and their molecular structure, as determined by solution NMR, electron microscopy and atomic force microscopy (AFM)22–24, is largely dependent on the polymerization conditions. Moreover, fibrils formed under quiescent or agitated conditions exhibit substantial differences22,25.

Tat affects amyloidogenesis through several mechanisms, including increased amyloid production through disruption of the endolysosome26 and decreased amyloid degradation via binding to neprolysin. Tat also affects Aβ transport across endothelial cells through...
interactions with low-density lipoprotein-1 (ref. 27). Here, we analyzed the direct interaction of Tat with Aβ peptide and, because both Tat and Aβ aggregates have been shown to be independently neurotoxic, we determined the role of this interaction in the neurotoxicity of these complexes. We used a combination of techniques including AFM, thioflavin T (ThT) bulk and single-fibril fluorescence, CD and molecular modeling to study the Tat-Aβ interaction, and we propose a model to explain the increased neurotoxic properties.

RESULTS

Tat increases β-sheet formation and aggregation of Aβ fibrils

Our bulk measurements showed that Tat protein increased β-sheet formation, aggregation of Aβ fibrils and their adherence to surfaces. CD measurements of Aβ samples showed that the predominant structure in Aβ fibrils was the β-sheet, which increased in the presence of Tat (Fig. 1a). Up to a 20% increase in β-sheet formation occurred with 1.8 µM Tat, and an increase was observed at all concentrations. ThT fluorescence measurements similarly showed an increase in the aggregation of Aβ peptide in the presence of Tat (Fig. 1b). In contrast, bovine serum albumin inhibited Aβ aggregation at the same concentrations (Supplementary Fig. 1). The Tat-Aβ complexes, compared with Aβ, presented greater adherence to the surfaces of the measuring wells, as evidenced by a weaker ThT signal compared with that for nonbinding wells (Fig. 1b). Adhesion of these complexes to the neuronal cells was also higher when Tat was present within the aggregates: larger aggregates were attached to the surfaces of neurons (Fig. 1c) and were not removed by standard washing of the cells.

Tat structure and aggregation

The Tat segment comprising amino acids 1–72 activates transcription with the same proficiency as that of the full-length protein and has a relatively conserved sequence13,28,29. We used this segment in our experiments. An AFM topography image of Tat(1–72) as adsorbed from a PBS solution, pH 7.4, onto a mica surface showed that Tat exists as multidisperse aggregates ranging from monomers to large oligomers (Fig. 2a). The monomer appeared as a globule, with a volume of ~12 nm³, a value in agreement with the theoretical calculation of the volume on the basis of molecular mass (Supplementary Note) and with a computer simulation of the dimensions of the hydrated molecule (Supplementary Fig. 2). Analysis of the distribution of sizes of Tat aggregates at pH 7.4 (Fig. 2b) showed that the structures ranged from monomers, dimers and small oligomers to 40- or 50-mers and larger. There appeared to be two coexistent populations: one comprising small oligomers and the other comprising more dispersed large aggregates. Even though the monomers and dimers were more frequent than larger aggregates (for example, the sample presented here showed 11 monomers and only four 50-mers), less than 10% of the sample was in monomeric form (Fig. 2b, inset) and therefore was

Figure 1 Tat protein increases aggregation and adherence of Aβ fibrils. (a) Typical far-UV CD spectra shown for 200 µM Aβ (red trace), 200 µM Aβ/0.4 µM Tat (blue trace) and 200 µM Aβ/1.8 µM Tat (black trace) (each an average of 3 traces). CDPro/CONTIN software was used to calculate the predominant β-sheet content for these traces. (b) Spectrofluorimetry used to determine the degree of Aβ aggregation on the basis of ThT bulk fluorescence. The nonbinding wells used prevented attachment of material to the wells. Data represent mean ± s.e.m. from two independent experiments with five technical replicates (first experiment) and seven technical replicates (second experiment). *P < 0.05 (two tailed) by unpaired Student’s t test. (c) ThT-labeled Aβ and Aβ-Tat structures (cyan) adhered to neuronal cells in culture. Neurites and axons appear red, owing to labeling of tubulin fibers with tdTomato. Scale bars, 30 µm. The graph shows the fold increase in the size of the aggregates attached to the cells. Data represent mean ± s.e.m. of 49 (Aβ with 0.08 µM Tat), 31 (Aβ with 0.4 µM Tat) and 25 (Aβ with 1.8 µM Tat) complexes, and results are representative of three independent experiments. *P < 0.0001 (two tailed) by ANOVA with Fisher’s means comparison test. Source data for a and c are available online.

Figure 2 Structure of Tat protein. (a) AFM topography image of Tat protein adsorbed from a PBS solution, pH 7.4, onto a clean atomic flat mica surface. Scale bar, 50 nm. Inset, experimental zoom-in view showing a Tat monomer. Scale bar, 20 nm. (b) Tat size distribution. The data are derived from 150 particles analyzed and are representative of 3 technical replicates. Inset, relative percentage of Tat monomers, dimers and trimers (mean ± s.e.m.) from three technical replicates with 139, 150 and 543 particles analyzed, respectively. (c) Far-UV CD spectra of 10 µM Tat in PBS solution, pH 7.4, showing the presence of α-helical structure. The CD spectrum presented is an average of 3 traces and is similar in shape to spectra from three independent experiments at 20 µM, 1 µM and 0.1 µM Tat concentrations. Source data for b are available online.
functionally active. Evaluation of the Tat secondary structure in PBS, pH 7.4, by CD was performed for Tat samples at 0.1 µM, 1 µM, 10 µM and 20 µM. The spectra exhibited three characteristic bands (208 nm, 220 nm and 194 nm), which are typical in the presence of α-helical structure (Fig. 2c, 10 µM sample). Analysis of the Tat CD data showed ~20% α-helical structure. It is likely that the α-helical structure represented Tat oligomers, because more than 90% of the population was in an oligomeric state at pH 7.4.

Changes in physical properties of Aβ fibrils in the presence of Tat
AFM topography imaging showed that Aβ fibrils formed at a 200 µM concentration were typically uniform along their length (Fig. 3a). These fibrils were the main structures observed. At a 2,500:1 ratio of Aβ peptide to Tat, twisted fibrils were the predominant structures (Fig. 3b). At a ratio of 500:1, thick irregular (along length and width) fibrils were the most frequent (Fig. 3c), and at an ~100:1 ratio, large aggregated patches were present (Fig. 3d).

AFM topography imaging of a typical twisted fibrillar structure (Fig. 4a) showed that the height at the top of the twist was approximately twice the height in the groove of the twist, whereas the groove of the twist had approximately the same height as that of a typical single fibril (Fig. 4b,c), thus indicating that the twisted fibrillar structure was formed from two single fibrils twisted together. Whereas the two twisted fibrils remained at their initial thickness, the distance between twists increased with Tat concentration (Fig. 4d), by up to 38% at a concentration of 1.8 µM Tat. This untwisting was indicative of the mechanism responsible for the lateral interaction between fibrils. The lateral aggregation of amyloid fibrils is associated with hydrophobic interaction. The untwisting of the fibrils within the twisted structure may have been due to electrostatic screening of the amyloid fibrils. Here, the positive charge of Tat may have screened the interaction between the fibrils, thereby leading to their untwisting.

The fibrils attached to mica were rapidly dried with a compressed N₂ flow. AFM topography images indicated that Aβ fibrils ruptured under N₂ flow during the drying procedure (single fibril shown in Fig. 4e). Hence, these fibrils were both rigid and brittle, unlike other biological fibers. With increasing Tat concentration, the distribution of the rupture lengths of single fibrils shifted toward larger values, thus indicating increased mechanical resistance of the fibrils. The average values for the rupture lengths grew substantially with increasing Tat concentration, up to 42% at 1.8 µM Tat (Fig. 4f).

Tat protein binds to the external surfaces of Aβ fibrils
The interaction between Aβ and Tat at the single-fiber level was determined by fluorescence microscopy. Tat conjugated to a Venus fluorescent tag was incubated with Aβ to form the fibrils. The fibrils were labeled with ThT and immediately imaged (Aβ fibril labeled with ThT in Fig. 5a). The amount of fluorescent Tat incorporated into the amyloid fibrils increased in a dose-dependent manner (Fig. 5b). We used a computer simulation to visualize Tat binding to the fibrils. We modeled the structure of Tat(1–72), which was calculated by ab initio threading, starting with the crystallographic structure of Tat(1–48) as a template (resultant model of Tat B(1–72) in Fig. 5c). The height of single Aβ fibrils obtained from high-resolution AFM topography images was in agreement with the dimensions obtained by NMR for a triangular cross-section of a ‘triple hairpin’ structure of Aβ fibrils formed under quiescent conditions. The predicted model of Tat with the highest score (C score of −0.9) was docked to the fibril, and the first three independent solutions of the docking were superimposed (Fig. 5d). The simulation suggested that Tat binds to the external side of the hairpin, through an interaction between a negatively charged region from the terminal regions and the hairpin turn of the Aβ molecules, on the one hand, and positively charged residues mostly from the basic region of Tat, on the other hand (Supplementary Fig. 3a–c). The surface positive charge of the Aβ–Tat system increased after the binding (Supplementary Fig. 3d,e). When we used other tridimensional structures for Tat that were obtained from solution NMR studies (PDB 1TIV, PDB 1TBC, PDB 1JFW and PDB 1K5K) and therefore were obtained under conditions that would affect Tat folding, the most probable binding region remained the basic region (Supplementary Fig. 4). Because Tat is a very small protein, the Aβ amino acid sequence appeared to be more relevant than its variation in the three-dimensional structure. Binding of a fluorescently labeled anti-Tat antibody to Aβ–Tat fibrils showed that Tat was present on the surfaces of the fibrils (Supplementary Fig. 5), in agreement with the binding mode suggested by the simulation.

Importantly, we found that Tat aggregates were bound to the surfaces of Aβ fibrils and were incorporated in the thick fibrils, as visualized by AFM (Supplementary Fig. 6). The computational analysis was restricted to the Tat monomer, although the interaction of Tat oligomer might use a mechanism similar to that of the monomer.
Aβ–Tat fibrils have increased neurotoxicity

Cultures of fluorescently labeled rat hippocampal neurons were exposed to Aβ–Tat fibrils and compared with regular Aβ fibrils, and images of neurons after 48 h exposure were obtained (Fig. 6a). Increasing Tat concentration in the Aβ–Tat complexes resulted in increased neuronal damage (Fig. 6b), as evidenced by decreasing neuronal cell count, decreased mean neurite lengths in the remaining neurons and the appearance of puncta along neuronal processes. When neurons were exposed to only Tat and incubated in PBS similarly for 7 days at room temperature, no substantial toxicity was observed (Supplementary Fig. 7). Further, freshly prepared Tat at 180 nM did not induce significant neuronal damage (Supplementary Fig. 7). Tat neurotoxicity at these concentrations and incubation times was much lower than the neuronal damage induced by Aβ or Aβ–Tat complexes (APP-presenilin1 (APP-PS1)). To determine whether Aβ–Tat complexes occurred in vivo, we initially prepared Tat brain injection in a well-characterized transgenic mouse model of Alzheimer’s disease known to form amyloid plaques. We found that the injected Tat protein localized to the amyloid plaques (Fig. 7a). We next generated APP/Tat double-transgenic mice and found that Tat colocalized with APP in the brains. The Tat–amyloid complexes were present both inside the neurons, mainly in the granular structures (Fig. 7b), and outside of the neuronal cells (Fig. 7c). These data confirmed that the Tat protein has specific avidity for Aβ in the brain, and animals with Aβ–Tat complexes had increased evidence of neurodegeneration. Additional pathological features of the transgenic mice are presented in Supplementary Figure 8.

DISCUSSION

Because Tat is released into the extracellular space by HIV-infected cells, and amyloid plaques are extracellularly present in increased amounts in the brains of HIV-infected individuals, we investigated whether the two proteins might interact with each other. We found...
that Tat protein localizes to the amyloid plaques within the brains of APP-PS1 transgenic mice. Hence, we investigated the biophysical properties of Tat-Aβ interaction through bulk methods and then used techniques with fibrillar and molecular resolution. Our main finding was that, at pH 7.4, Tat exhibited substantial molecular interaction with Aβ, thus resulting in major changes in its physical and functional properties.

We studied the Tat structure at pH 7.4 in PBS, by using AFM high-resolution imaging to characterize the sizes and distribution of the monomers and oligomers. AFM showed that Tat has a compact globular structure. Tat has a remarkable ability to form oligomers, and we found that more than 90% of the protein was in an oligomerized state. Some oligomers were large, comprising more than 50 molecules of Tat. The monomeric Tat assumed a globular structure, as expected for a small unstructured protein, and the multimeric forms of Tat maintained a globular shape. The spherical shape allows Tat to assume the least possible volume, at the lowest free-energy state. The oligomerized state is believed to be functionally inactive30, and thus Tat

Figure 6 Aβ–Tat complexes show synergistic neurotoxicity in cultured neurons. (a) Fluorescence images showing rat neuronal cell cultures with tubulin fluorescently labeled with a tdTomato fusion protein. Cultures were exposed to Aβ–Tat complexes, and images were taken after 48 h of exposure. Aβ–Tat complexes were formed with 200 µM Aβ and varying concentrations of Tat (0.08 µM, 0.4 µM and 1.8 µM) and diluted ten-fold by incubation with the neuronal cell culture medium. Final concentrations are presented in the graphs. Scale bars, 300 µm. (b) Neuronal cell counts, mean neurite lengths and numbers of cell puncta after exposure of cells to Aβ–Tat complexes for 48 h. Data represent mean ± s.e.m. for 12 images for media control sample and 6 images for each dose, from four independent experiments: *P < 0.01; **P < 0.001; ***P < 1 × 10−6 (two tailed) by ANOVA with Fisher’s means comparison test. Source data for b are available online.

Figure 7 Formation of Aβ–Tat complexes in vivo. (a) Immunofluorescence of amyloid precursor protein (APP, green) on brain slices from APP-PS1 transgenic (tg) mice injected with Tat (red). (b,c) Colocalization of Tat (red) and APP (green) in brains of APP/Tat double-transgenic mice, inside neurons, mainly in granular structures (b) and outside of neuronal cells (c). Free-floating vibratome sections from nontransgenic (n = 8), Tat transgenic (n = 4), APP transgenic (n = 4) and APP/Tat transgenic mice (n = 8). In nontransgenic mice, no reactivity was detected; in the Tat transgenic mice, immunopositive punctae were detected in association with glial cells; in the APP transgenic mice, no Tat reactivity was detected, but Aβ plaques were identified in the neocortex and hippocampus. DAPI (blue) was used to stain cell nuclei. Scale bars, 20 µm.
may be nearly 10-fold more potent as a neurotoxin than suggested by the in vitro assays. The role of oligomers in the neuropathogenesis of HIV infection remains unclear; however, oligomerized Tat has been shown to be taken up by lymphocytes and to consequently activate these cells. Here, we found that Tat oligomers bind amyloid fibrils (Supplementary Fig. 6) and add substantial electrostatic charge to the Aβ, thus providing another functional role for the oligomers.

Our CD measurements showed that, in an aggregated state, Tat's conformation changed to a more ordered α-helical state. NMR studies have suggested that the most likely region to fold into a secondary structure is the cysteine-rich region. Cysteine-cysteine interactions are also involved in oligomerization, thus suggesting that the α-helix structure revealed by CD might be due to the cysteine region of the protein.

We found that the growth of Aβ fibrils was enhanced in the presence of Tat. Both β-sheet formation, leading to elongation of fibrils, and lateral aggregation of elongated fibrils increased substantially. One of the structures observed was the double twisted fibril (Fig. 4). Amyloid fibrils of denatured β-lactoglobulin show similar structures involving two, three and four fibrils twisted together. In contrast, twisted fibrils of Aβ and Aβ–Tat contained only two fibrils. Aβ fibrils are rigid structures (2–4 GPa). Aβ fibrils may induce pore formation. Alternatively, in the case of small Aβ fibrils, Aβ and Tat may come close to each other and attach because of Tat and twist around each other. Top, AFM three-dimensional topography images. Scale bars, 50 nm. (c) Structures that are more rigid and have increased adherence because of Tat bind more strongly to the neuronal-cell membrane and may induce pore formation. Alternatively, in the case of small Aβ–Tat aggregates, Tat, a transmembrane-penetrating molecule, might enter the cells along with its Aβ cargo and induce intracellular damage.

Figure 8 Proposed model of Aβ–Tat interaction and their increased neurotoxicity. (a) Tat attaches to the surfaces of typical amyloid-β fibrils. (b) At low concentrations, (Tat/Aβ) ratio of 1:2,500, fibrils that randomly come close to each other attach because of Tat and twist around each other, thus forming double fibrils. (c) At a 1:500 molar ratio, more fibrils, both long and short, attach to one another and form irregular fibrils. (d) At a 1:110 molar ratio, large patches appear as many fibrils attach to each other. Top, AFM three-dimensional topography images. Scale bars, 50 nm. (e) Structures that are more rigid and have increased adherence because of Tat bind more strongly to the neuronal-cell membrane and may induce pore formation. Alternatively, in the case of small Aβ–Tat aggregates, Tat, a transmembrane-penetrating molecule, might enter the cells along with its Aβ cargo and induce intracellular damage.
On the basis of our data, we propose a model that may account for the increased neurotoxicity of the Aβ–Tat complexes. Although Tat induces high aggregation of Aβ peptide, a limited number of small Aβ–Tat aggregates might also contribute to neurotoxicity. Tat is able to penetrate membranes; therefore, Tat attached to small aggregates might enter cells along with the Aβ ‘cargo’ and induce damage. In particular, the segment between Tyr47 and Arg57 of Tat has been used to transport a variety of materials across cell and nuclear membranes. However, as demonstrated here, the predominant species in Aβ–Tat complexes are large aggregates. Therefore, the major pathway that we propose involves these large Aβ–Tat complexes (Fig. 8). Specifically, Tat attaches to the surface of a typical Aβ fibril (Fig. 8a). When present at low concentrations, Tat binds to the surface of the fibrils, which randomly come close to each other in solution and then twist around each other, forming double fibrils (Fig. 8b). At higher concentrations of Tat, more fibrils, both long and short, attach to one another and form irregular thick fibrils (Fig. 8c). Further increases in Tat concentrations lead to aggregation of these fibrils, which form large patches (Fig. 8d). These thick structures are rigid and show increased adherence to neurons, owing to the known binding capacity of Tat to membranes. In the contact region, at the edge of the attached plaque or fibril, shear forces occur because of the normal dynamic fluctuations of the membrane near a rigid surface, and these forces can induce pore formation and eventually rupture of the cell membrane. Tat is a highly positively charged molecule. Although the majority of these positive charges are predicted by computer simulation to participate in the interaction with the Aβ–Tat aggregates, a number of them are predicted to remain at negatively charged patch, a number of them are predicted to remain unbound and subsequently enter other cells. The observed colocalization of Tat and Aβ in extracellular aggregates and in some neurons of double-transgenic mice (Fig. 7) was consistent with the possibility that small Aβ–Tat aggregates can enter neurons from the extracellular compartment, as presented in our model (Fig. 8). Previous studies have shown that Aβ aggregation is enhanced at lower pH and is more cytotoxic than at pH 7.4. The production of Aβ is further enhanced by exposure of neurons to Tat and results in disruption of the endolysosome. Events at the acidic pH in the endolysosome might therefore also be relevant in cell malfunction and death. Nevertheless, we suggest that a substantial amount of cellular damage might arise from extracellular aggregates that form at pH 7.4.

In conclusion, we propose a model explaining the increased neurotoxicity of the Aβ–Tat complexes, based on the possible penetration of a few small aggregates through the cell membrane and predominantly on increased adherence of the mainly large positively charged Aβ–Tat complexes to membranes. These complexes may induce mechanical disruption of membranes, both because of the large difference in stiffness between the neurons and the rigid Aβ–Tat complexes and because of Tat-induced local reorganization of the membrane.

METHODS
Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS
We thank M. Bachani for preparing the Tat protein stocks, K. Mather for preparing the neuronal cell cultures used in the adhesion imaging experiments and A. Savonenko (Johns Hopkins University School of Medicine) for providing the APP-PS1 mice. This work was supported by intramural funds from NINDS, NIH Z01-NS003130 to A.N.; E.M. was supported by NIH grant R01-AG05131; N.H. was supported by NIH grant R01-MH096636; and E.K.D. was supported by NIH Z01-EB00083.

AUTHOR CONTRIBUTIONS
A.H. and A.N. conceived and designed the study. A.H. performed AFM, ThT bulk fluorescence and single-fibril and cell-adhesion experiments, and analyzed and interpreted data. M.A.B. performed the computer simulations. E.K. performed the CD measurements. J.S. performed neurotoxicity experiments. E.M. and A.F. performed the transgenic mouse experiments and immunohistochemistry analysis of brain samples. M.-H.L. performed immunohistochemistry of the Tat-injected mouse brain samples. A.M.D. and N.H. performed the experiments on Tat-injected mice. E.K.D. contributed to and supervised AFM data acquisition. A.H., A.N. and M.A.B. wrote the paper, and all authors edited the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Materials. Aβ peptide was purchased from Bachem (cat. no. H 194), ThT was obtained from Sigma-Aldrich (cat. no. T3516-ST), FITC anti-Tat antibody was purchased from Abcam (cat. no. ab3016), TatVenus (Tat tagged with a mutagenic form of GFP called Venus) was produced in our laboratory58. OTESPA AFM probes were purchased from Bruker. For the Tat-injected experiment, mouse anti-HIV Tat antibody was purchased from BioLegend (cat. no. 919001), and rabbit anti-APP antibody was purchased from Abcam (cat. no. ab2072). Secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG (cat. no. A11029) and Alexa Fluor 594 goat anti-mouse IgG (cat. no. A11012) were obtained from Invitrogen. For the second mouse experiment, the antibody against Aβ (4G8) was purchased from Sigma-Aldrich (cat. no. A1349). The mouse monoclonal antibody against HIV Tat was from the NIH AIDS reagent program (cat. no. 1974). The secondary antibodies used were FITC-tagged anti-mouse antibodies. Validation for each of the primary antibodies is provided on the manufacturers’ websites. The tyramide red amplification system (Thermo-Fisher Scientific, cat. no. B49057) was used for Tat detection. The superfrost slides used were from Fisher (cat. no. 12-550-15). The Wizard genomic DNA isolation kit was from Promega. Doxycycline was purchased from Sigma-Aldrich.

Preparation of Tat. Recombinant Tat(1–72) was prepared with column chromatography, as previously described59. Protein was produced in an endotoxin-free manner and was more than 95% pure, as determined by gel electrophoresis and western blot analysis. Stocks of 0.2 µg/µl were stored at −80 °C until use.

Preparation of Aβ fibrils. The lyophilized Aβ powder was dissolved in ultrapure water to a 1 mM concentration and was immediately vortexed for 1 min before further dilution in PBS to a 200 µM concentration, which was used for growth of fibrils in quiescent conditions. The samples were incubated at room temperature for 7 d in the presence of various concentrations of Tat. As observed by both AFM and ThT fluorescence imaging, most of the fibrils were relatively short, in the range of up to 1–2 µm. Therefore, for single-fibril fluorescence imaging, the method of Ban et al.60 was used to prepare long fibrils through seeded growth. Briefly, Aβ monomers60 at 50 µM were mixed with 25 µM seeds (Aβ aggregates sonicated for 1 min) and incubated for 7 d at room temperature.

AFM imaging. AFM imaging was performed with a Multimode Atomic Force Microscope (Bruker). The stock Tat solution was diluted 100 times in PBS, centrifuged at 16,000g for 30 min to eliminate the very large aggregates, then diluted further in PBS to a 5 nM concentration. Aliquots of 10 µl of which were allowed to adsorb for 5 min on clean freshly cleaved mica surfaces. Each mica surface was subsequently washed with PBS (2 × 200 µl) and pure water (5 × 100 µl) to avoid salt-crystal formation on the surface, and the samples were dried under N2 flow. To image the Aβ–Tat samples, 10 µl of the incubated fibrils was allowed to adsorb for 5 min on mica surface, and then the samples were washed as in the case of Tat samples and dried under N2 flow. Imaging was performed in air in tapping mode with a Multimode Atomic Force Microscope. The images were flattened before the height measurements. The lengths of Aβ fibrils were determined at half height. At least two independent experiments were used in the study of Aβ and Aβ–Tat complexes.

Circular dichroism. Far-UV CD spectra were recorded with a Jasco J-815 spectropolarimeter at a temperature of 25 ± 0.2 °C, which was maintained by a Peltier temperature controller (Jasco). The CD spectra were recorded in triplicate in 0.05 cm-path-length quartz cuvettes (Starna) from 300 nm to 180 nm with a scan speed of 100 nm/min, a bandwidth of 1.0 nm and a resolution of 0.2 nm. All samples were prepared and measured in PBS, pH 7.4. PBS was run as a blank before the sample solutions, and the baseline values were subtracted from the sample measurements. For time-course CD measurements of Aβ–Tat complexes, the samples were incubated for 7 d at room temperature in the measuring cuvettes to avoid possible effects of pipetting on ongoing structural alteration. The ellipticity of CD spectra was expressed in millidegrees (mdeg).

Fluorescence bulk ThT measurements. Fluorescence bulk ThT measurements were performed spectrophotometrically with a FlexStation3 plate reader (Molecular Devices) equipped with SoftMaxPro software. ThT solution at a concentration of 5 µM in PBS was filtered with a Miller-GS 0.22-µm syringe filter before use. For the measurement, 9 µl of each Aβ or Aβ–Tat sample was added to 375 µl of ThT solution, and 125 µl of this mixture was introduced in each of the plate wells for measurements. The excitation wavelength was used was 440 nm, and the emission was measured at 485 nm with an automatic-cutoff filter set at 475 nm. The standard plates (wells) used were Costar 3631, the low-binding plates (wells) were Corning 3651, and the experiments were performed in parallel. The Corning 3651 low-binding plates had a modified polymer surface that resulted in a nonionic hydrophilic surface that minimized molecular interactions. Several independent experiments were performed for incubation of Aβ–Tat complexes in vials and plates.

Single-fibril imaging. Single-fibril imaging was performed with a Nikon Eclipse Ti total internal reflection fluorescence (TIRF) microscope equipped with a 488/561/647/1444A Lasers System and an Andor iXon3 EMCCD camera with an APO TIRF 100×/1.49-NA objective. Images were acquired with NIS-Elements AR software. We incubated TatVenus with Aβ for 7 d, according to a standard procedure of forming fibrils60, and then observed the localization of Tat at the fibril level. The exposure time for the TIRF images was 20 ms at 2% 488-nm laser power. The Aβ fibrils were identified on the basis of ThT fluorescence through simultaneous imaging with the same system, with an exposure time of 10 ms under an X-cite fluorescence lamp at 75% power (Lumen Dynamics). ThT was added to the formed fibrils before imaging, because its hydrophobic binding to the fibril β-sheets makes the fibrils visible through fluorescence microscopy60. The TatVenus signals are reported relative to the ThT signal for each fibril to quantify the increase in TatVenus per fibril unit area. Two independent experiments were performed, and both yielded similar results.

Neurotoxicity experiments. All experiments were approved by the NIH Institutional Animal Care and Use Committee (protocol number 1330-14). To generate Sprague Dawley rat hippocampal neuronal cell cultures, rat embryos were euthanized at gestation day 18, and their hippocampi and cortices were harvested and used to generate the neuronal cellular cultures. The cultures tested negative for mycoplasma. The cells were plated in 96-well plates at a density of 4 × 10^5 cells per ml. This mixed rat neuronal culture consisted of 40–45% β-III tubulin–expressing neurons, 50–55% GFAP-expressing astrocytes and ~1% microglia61. Rat neuronal cultures were transfected with β-III tubulin–tdTomato fusion protein driven by the CamKII promoter and were plated at 40,000 cells/well on a 96-well plate. These neuronal cultures were allowed to differentiate for 12–17 d before treatment with Aβ or Aβ–Tat complexes, which were prepared as described. After application of the Aβ and Aβ–Tat complexes to the wells, the final concentrations in the wells (therefore on the neurons) were ten times lower (to 8 nM, 40 nM or 180 nM for Tat in complexes and to 20 µM Aβ); however, the fibrils and complexes were already formed and active; although these were not the ‘fibril formation’ concentrations, they reflect the amount of material in the wells. Live-cell imaging of the neuronal cultures was performed after 24, 48 and 72 h of exposure with 10× magnification on a GE INCell Analyzer 2000 imager, and images of four fields per well were acquired at each time point. GE Developer Toolbox was used to quantify the neuronal cell number, neurite length and neuronal degradation puncta from neuronal cultures that expressed β-III tubulin–tdTomato. Quantification was fully automated. These fluorescence images were segmented and analyzed at a medium sensitivity setting of 60. Visual inspection of 10–12 random images showed that we had captured and measured more than 95% of the fluorescently labeled cells in these fields. At least four independent experiments were performed for neurotoxicity tests.

Cell-adhesion experiments. Rat neuronal cell cultures prepared as described above were allowed to attach to the polylsine-coated glass of MatTek Petri dishes, to form a uniform layer of cells. The neurons were treated with Aβ or Aβ–Tat complexes, which were prepared as described. The complexes were allowed to attach for 2 h, and then the unbound structures were washed away twice with cell
medium. Medium containing 2 μM ThT was added to cells to indicate the presence of Aβ complexes. After 2 min, the cells were washed with PBS and imaged with the system described in the ‘single-fibril imaging’ section. Exposure times with an X-cite fluorescence lamp at 75% power were 10 ms for the ThT (CFP) channel and 50 ms for the tdTomato (TRITC channel). The exposure times and display ranges in the color channels of the merged CFP/TRITC images were the same for both images presented in Figure 1c. Three independent experiments were performed for the adhesion experiments.

Immunohistochemistry and confocal microscopy of transgenic-mouse brain samples. Two independent experiments were performed, in two different laboratories. Animals were not randomized to groups.

In the first experiment, six-month-old transgenic C57BL/6 mice bearing mutations in human amyloid precursor protein (APPsw;K670M/N671L) and human presenilin 1 (PS1ΔE9) were used. APP-PS1 mice express APP and PS1 under the control of prion promoters. 2 They were maintained as a heterozygous genotype. Mice were housed in an animal facility on a 12 h light/dark cycle and given ad libitum access to food and water. All procedures were conducted in accordance with the NIH guidelines for the Use of Animals and Humans in Neuroscience Research and were approved by the Institutional Animal Care and Use Committee (Johns Hopkins University School of Medicine, protocol nos. M015 and M268). Recombinant Tat(1–72) protein was stereotactically injected (0.5 μl/μg in saline) into the frontal cortex (bregma, AP +1.5 mm, ML −1 mm, DV +1 mm)63 and the hippocampus (AP −2 mm, ML −1.2 mm, DV +1 mm, +2 mm)63. Mice were sacrificed one week (n = 2) or two weeks (n = 2) after Tat injections. Animals were deeply anesthetized with isoflurane and were transcardially perfused with saline followed by 4% (v/v) paraformaldehyde. After being postfixed in paraformaldehyde overnight, brains were immersed in a 30% (v/v) sucrose solution. On the following day, brains were cryoprotected and cut in the coronal or horizontal plane into 40-μm-thick sections on a sliding microtome. Sections were washed in Tris-buffered saline (TBS) (10 mM Tris-HCl and 150 mM NaCl, pH 7.5). Tissues were incubated in blocking solution (TBS with 0.5% (v/v) Triton-X-100 and 2.5% (v/v) donkey serum). Primary antibodies were diluted in blocking solution before use, as follows: mouse anti-HIV Tat, 1:500; rabbit anti-APP, 1:500. Secondary antibodies (conjugates of Alexa Fluor 488 or Alexa Fluor 594) were diluted 1:250 before use, and the tissues were then washed and counterstained with DAPI to label the nuclei. The images were obtained with an LSM 510 META laser-scanning confocal microscope (Carl Zeiss) equipped with a 63× Plan-Apo/1.4-NA oil objective. Tat imaging was performed by using the 488-nm channel with 20% laser power, and Aβ imaging was performed by using the 561-nm channel at 20% laser power. Investigators were not blinded to the group allocation during the experiment and when assessing the outcome.

In the second experiment, mice expressing human APP751 cDNA containing the London (V717I) and Swedish-(K670M/N671L) mutations were deeply anesthetized with isoflurane and were transcardially perfused with saline followed by 4% (v/v) paraformaldehyde. After being postfixed in paraformaldehyde overnight, brains were immersed in a 30% (v/v) sucrose solution. On the following day, brains were cryoprotected and cut in the coronal or horizontal plane into 40-μm-thick sections on a sliding microtome. Sections were washed in Tris-buffered saline (TBS) (10 mM Tris-HCl and 150 mM NaCl, pH 7.5). Tissues were incubated in blocking solution (TBS with 0.5% (v/v) Triton-X-100 and 2.5% (v/v) donkey serum). Primary antibodies were diluted in blocking solution before use, as follows: mouse anti-HIV Tat, 1:500; rabbit anti-APP, 1:500. Secondary antibodies (conjugates of Alexa Fluor 488 or Alexa Fluor 594) were diluted 1:250 before use, and the tissues were then washed and counterstained with DAPI to label the nuclei. The images were obtained with an LSM 510 META laser-scanning confocal microscope (Carl Zeiss) equipped with a 63× Plan-Apo/1.4-NA oil objective. Tat imaging was performed by using the 488-nm channel with 20% laser power, and Aβ imaging was performed by using the 561-nm channel at 20% laser power. Investigators were not blinded to the group allocation during the experiment and when assessing the outcome.

Briefly, sections were incubated overnight at 4 °C with antibodies against Aβ (4G8) and Tat (mouse monoclonal antibody against HIV Tat). Sections were then reacted with secondary antibodies tagged with FITC to detect Aβ and with the tyramide red amplification system to detect HIV Tat protein. Sections were then reacted with secondary antibodies tagged with FITC to detect Aβ and with the tyramide red amplification system to detect HIV Tat protein. Sections were then reacted with secondary antibodies tagged with FITC to detect Aβ and with the tyramide red amplification system to detect HIV Tat protein.

Computer simulation. To perform the docking of Tat to the Aβ fibril, first we created an Aβ fiber model consisting of 12 triple hairpins spanning at least 1.5 times the longest linear dimension of the Tat structure (which was used as the docking target in the simulation). This model was made by elongating, in the direction of the fiber main axis, the triangular structure of the Aβ fibrils grown under quiescent conditions (PDB 2LMQ, model 1)67. Briefly, to produce this elongated model, the mathematical transformation between two consecutive triple hairpins in the fiber was applied iteratively to the fiber-end hairpin to generate a new triple-hairpin element growing the fiber along its main axis. The transformation and coordinates of the new hairpin element were obtained with the molecular modeling program O68. We did not attempt to model the highly flexible Aβ N-terminal region (amino acids 1–8) in our docking simulation because of the large nonfavorable entropic contribution expected from the participation of highly flexible regions in protein-protein interactions. Even though this small region was expected to contribute to stronger binding, it was expected to have a small favorable free-energy contribution, owing to its high flexibility. We chose not to use the PDB 2M4J structure of an Aβ(1–40) fibril determined from a single patient69, because more research into the variability in structures among patients is needed before one or the other structure can be presumed to be the most probable structure formed in vivo69, and because that structure in particular has been found to be unstable by computer simulation70. We chose the PDB 2LMQ structure for the fibrils formed in vitro to characterize the fibrils that we used experimentally to interact with Tat, because they were prepared in the same way.

The unobserved C-terminal region of Tat in the crystallographic structure contains most of the positively charged residues. To include this relevant region and to complete the partial crystallographic structure of Tat (PDB 3M9, model 1), an ab initio threading of the unobserved C-terminal region (amino acids 49–72) was performed with the I-tasser server71 with default parameters (Fig. 5c). We found the crystallographic structure of Tat to be the closest representation of our active Tat, because our force spectroscopy experiments indicated a β-helical structure within the Tat monomer at pH 7.4 (data not shown), in a 44–amino acid region on average, a result in good agreement with the crystallographic structure. Additionally, the crystallographic structure was obtained under milder conditions than those for the other structures available in the Protein Data Bank (which were NMR derived, at low pH and in an extremely highly reducing environment). The resulting hybrid model of Tat with the highest score (C score of −0.9), ranked on the basis of the strength of the interaction with the target. We used fast Fourier transform–based rigid docking, solution clustering and, to remove clashes, minimization with a CHARMM force field, as implemented through the ClusPro server. The first three docking solutions are presented (Fig. 5d and Supplementary Fig. 3). The solutions obtained by displacement along the fibril axis and the three-fold symmetry around the axis were discarded by inspection and were considered not to be independent. The solution NMR structures of Tat (obtained at low pH and under denaturing reducing conditions known to decrease folding) were docked as well, to determine whether there would be changes in the binding. All the simulations using different Tat structures obtained from the Protein Data Bank (the first model of each of PDB 1K5K, PDB 1JFW, PDB 1TIV and PDB 1TBC) clustered almost all of the higher-score docking solutions in the junction region of the triple hairpin, whereas there were few docking solutions at the external side of the triple hairpin (Supplementary Fig. 4). All the programs used in the computer simulation are freely available online, as reported here, in the Supplementary Note and in the references.
Data availability. Source data for Figures 1b,c, 2b, 4b.d.f, 5b and 6b are available with the paper online. Other data are available from the corresponding author upon request.

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