
EXTRA VIEWS

Tunneling nanotubes: A possible highway in the spreading of tau and other prion-like proteins in neurodegenerative diseases

Saida Abounit^a, Jessica W. Wu^{b,*}, Karen Duff^b, Guiliana Soraya Victoria^a, and Chiara Zurzolo^a

^aUnité Trafic Membranaire et Pathogénèse, Institut Pasteur, Paris CEDEX 15, France;
^bTaub Institute, Columbia University Medical Center, New York, NY, USA

ABSTRACT. The mechanisms of intercellular spreading of amyloidogenic proteins involved in neurodegenerative diseases have yet to be fully elucidated. While secretion has been implicated in the transfer of many proteins, including prions and α -synuclein, tunneling nanotubes (TNTs) have also been demonstrated for prions and mutant Huntingtin. Here, we provide further evidence that Tau aggregates, which have been demonstrated to predominantly be transferred via secretion, can also be found in TNTs. Additionally, cells that have taken up Tau have increased TNT formation. Coupled with previous evidence that other amyloidogenic aggregates also induce TNT formation we propose that misfolded protein aggregates can, through a common mechanism, promote the formation of TNTs and thereby their own intercellular transfer, contributing to the propagation of pathology.

INTRODUCTION

The trans-cellular spread of amyloidogenic proteins like prions, α -synuclein and tau appears

to be a crucial event in the pathogenesis of neurodegenerative diseases as it could mediate the progressive spread of the pathology observed in the brain of patients.^{1,2} *In vitro* studies have shown

Correspondence to: Chiara Zurzolo; Email: chiara.zurzolo@pasteur.fr

*Current affiliation: Biological Engineering Department, Laboratory for Computational Biology & Biophysics, MIT, Cambridge, MA, USA.

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that protein aggregates may spread between cells via physical connections such as tunneling nanotubes^{3,4} as proposed for prions and Htt.^{5,6} Tunneling nanotubes (TNTs) are thin F-actin containing channels between remote cells which have recently been recognized as a mechanism for long-range intercellular communication in many cell types and contexts. Unlike other filamentous bridges (e.g., filopodia, cytonemes), TNTs directly connect cells, creating temporary cytoplasmic continuity between distant cells, of different types, and they do not attach to the substrate in culture. They have different diameters (from 100 nm to 800 nm) and can be very long (over 100 μm).^{3,5} These dynamic structures selectively transfer a wide variety of cellular materials, such as cytoplasmic molecules, plasma membrane components, vesicles and organelles. In addition TNTs can be hijacked by various pathogens, such as bacteria, viruses and prions⁵ and prion like proteins, including mutant Huntingtin (mHtt)⁶ and α -synuclein.⁷

Prions and prion-like proteins may also be released via secretory mechanisms, such as exosomes^{8,9} and internalized by neighboring cells. This has been proposed for superoxide dismutase (SOD-1),¹⁰ α -synuclein¹¹⁻¹⁴ and polyglutamine aggregates.¹⁵ In addition, earlier evidence had shown release and internalization of fibrillar aggregates of the Tau microtubule-binding region (MTBR).^{16,17} More recently we have shown that full length human tau readily aggregates into low molecular weight (LMW) aggregates and fibrils, which can enter HeLa cells and primary neurons through bulk endocytosis.¹⁸ Interestingly the uptake of Tau depends on both the conformation and size of the aggregates. In primary neurons internalization can occur not only at the somatodendritic compartment, followed by anterograde transport to axon terminals, but also at axonal terminals followed by retrograde transport to the cell body,¹⁸ supporting the hypothesis that tauopathy may spread trans-synaptically *in vivo*, via cell-to-cell transfer. Moreover, recent data indicate that endogenously formed tau released from primary neurons can transfer to acceptor neurons and seed the formation of tau aggregates suggesting that endogenous tau transfer is secretion dependent. Furthermore by increasing neuronal

activity the authors could demonstrate an increase of tau release *in vitro* and exacerbated tau pathology in murine hippocampus *in vivo*.¹⁹

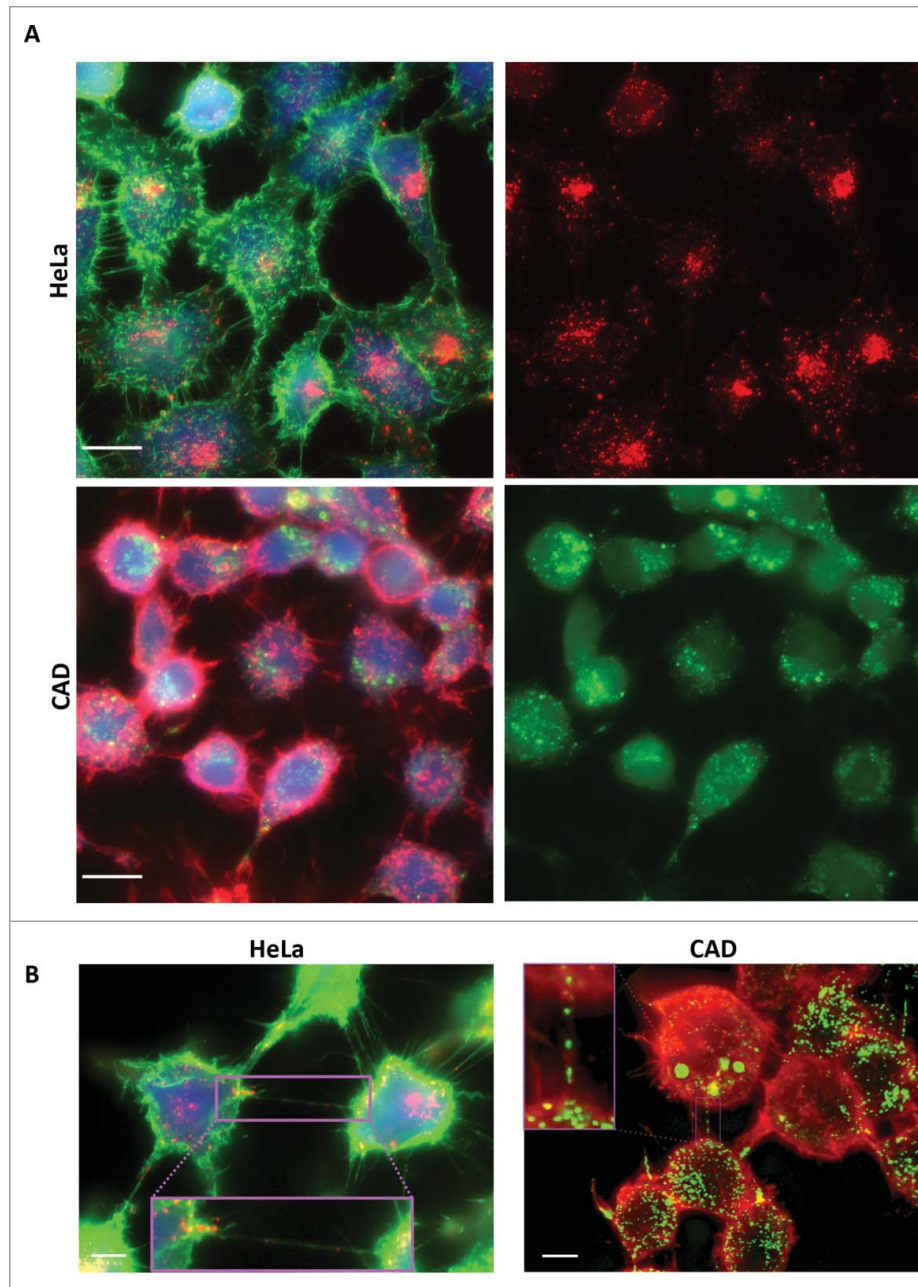
Here we have extended these findings by analyzing the potential role of tunneling nanotubes as an alternative pathway for intercellular spreading of exogenous recombinant human tau fibrils. We found that both in HeLa cells and neuronal CAD cells, internalized fibrillar tau is found in TNTs. Furthermore we show that tau aggregates increase TNT number supporting the hypothesis that this would facilitate its spreading. Coupled with previous evidence that misfolded protein aggregates such as prion, mHtt and α -synuclein increase TNT formation^{6,7,20} this suggests that TNTs can be induced by toxic protein aggregates and represent a general mechanism of spreading that exists alongside secretion.

RESULTS AND DISCUSSION

In order to determine whether recombinant human tau fibrils could be internalized in neuronal catecholaminergic murine CAD cells, which are an excellent model to study TNTs in culture,⁵ we used fluorescently labeled human tau fibrils, previously shown to be efficiently internalized in HeLa cells and primary neurons.¹⁸ We compared the rate of internalization after 16 hours incubation in HeLa and CAD cells in parallel cultures. By fluorescent microscopy we found that 100% of both cell type internalized tau fibrils. As shown in Fig. 1A, in HeLa cells tau fibrils were found to coalesce into several puncta within the cells and to accumulate in the peri-nuclear region. Similarly to HeLa cells, the totality of CADs cells (100%) have taken up fluorescent tau fibrils, however the distribution of tau fibrils appeared to be sparser than in HeLa cells (Fig. 1B).

Next, in order to investigate whether tau fibrils could use TNTs as a mechanism to spread between cells, we first analyzed whether tau fibrils could be found inside TNTs. To this end we incubated HeLa and CAD cells with Alexa-fluorophore-tau fibrils. After 16 hours, cells were labeled with fluorescent WGA to delineate the plasma membrane and TNT occurrence.²¹ By microscopic imaging we could identify

FIGURE 1. Tau fibrils are internalized in non-neuronal and neuronal cells. A: Representative picture of HeLa (top panel) and CAD (bottom panel) cells respectively incubated with fluorescent Alexa-555 or 488 (red and green) recombinant tau fibrils for 16 hours and stained for the cytosol (in blue) and plasma membrane (WGA, green and red, respectively). Left panels: Merge of WGA and Tau; Right panels: single channel showing fluorescent Tau. Scale bar: 15 and 10 μm for HeLa and CAD respectively. B: Representative deconvolved pictures of HeLa (left, labeled with green WGA to delineate the plasma membrane) and CAD cells (right, labeled with red WGA to delineate the plasma membrane and TNTs) containing fluorescent tau fibrils (red and green, respectively for HeLa and CAD) and connected by TNTs containing several tau fibrils puncta (inset). Scale bar is 15 and 10 μm for HeLa and CAD respectively. Boxed insets show close-ups of the TNTs containing punctate Tau aggregates.



several TNTs connecting cells in both HeLa and CAD cultures, respectively (Fig. 1B). Interestingly, among connected cells, we could identify TNTs that contained tau fibrils (Fig. 1B, inset). This suggests that internalized tau fibrils can be transported within TNTs.

Prions and prion-like proteins aggregates are known to share several similarities in terms of replication and mechanism of propagation.¹ Importantly, TNTs were demonstrated to act as a mechanism of intercellular transfer for prion and huntingtin aggregates in neuronal cells and primary neurons.^{5,6} Since prion, α -synuclein fibrils and huntingtin aggregates were found to increase the amount of TNTs in CAD cells,^{6,7,20} we investigated whether tau fibrils could similarly affect TNT formation in cultured cells. HeLa or CAD cells were incubated with fluorescent tau fibrils for 16 hours. Then, we analyzed the number of TNTs in the 2 cell populations as previously described.^{20,21} Interestingly cells exposed to tau appeared to form more TNTs than control cells (Fig. 2A). Based on this observation, we quantified the amount of TNTs based on 2 criteria: i) continuity of TNTs between 2 cells and ii) absence of contact of TNTs with the substratum.²¹ We found that the number of TNTs was increased for both HeLa and CAD cells containing tau fibrils compared to control cells (25% and 32% increase for HeLa and CAD cells, respectively, Fig. 2B).

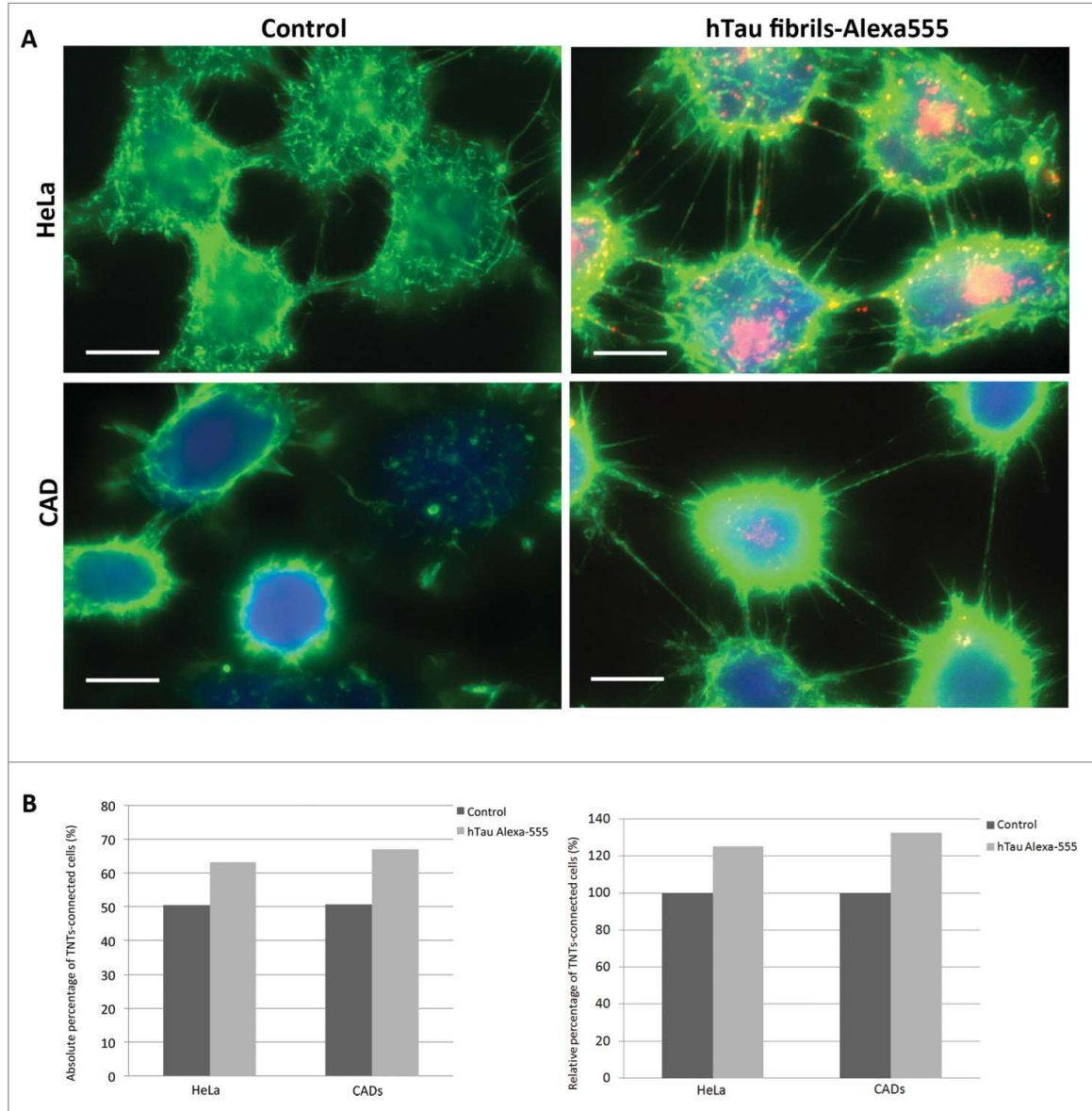
These data indicate that similarly to prion, α -synuclein and huntingtin aggregates, tau fibrils are able to enter cells and induce TNT formation. We therefore propose that prion-like proteins, when aggregated, are capable of inducing TNT formation via a common mechanism. While further studies will be necessary to address the specific mechanism leading to TNT increases, we speculate that this is linked to oxidative stress pathways. Indeed while oxidative stress has been shown to increase TNT formation in neuronal cells and primary neurons,^{5,22} oxidative stress is associated with the presence of aggregated proteins in neurodegeneration.²³ Therefore based on these findings, we propose a model where upon aggregation, prion-like proteins would create the formation of reactive oxygen species that would then stimulate TNT formation via an undeciphered

mechanism. One possibility could be the up-regulation of membrane biogenetic genes through the unfolded protein response (UPR).²⁴ Alternatively, stress mediated through lysosomes may be involved.²⁵ This hypothesis is especially attractive as prion aggregates²⁰ as well as synuclein aggregates⁷ are found in lysosomes within the soma and in TNTs and could cause lysosomal damage that may induce a stress-response pathway. The increased number of TNTs would in turn favor the transfer of prion-like aggregate from one cell to another and contribute to the spreading of the pathogenic aggregates (Fig. 3). The mechanism of aggregate-induced TNT formation is an area that requires substantial study in order to decipher the pathways and molecules involved in their formation.

Based on the bulk of our *in vitro* data we suggest that TNT-mediated transfer of prion-like proteins implicated in neurodegenerative diseases could be an efficient mechanism in addition to secretion, exosome release, trans-synaptic spreading, for the propagation of the pathology in the brain. It is possible that these events may occur in different phases of the disease or in propagation of the pathogenic seeds between different cell types during the diseases progression. Deciphering the occurrence and prevalence of these mechanisms will improve our understanding of the pathology progression of neurodegenerative diseases.

Recent studies have provided new insights into the existence of TNTs and TNT-like structures *in vivo*,²⁶⁻²⁹ especially in the case of cancer³⁰⁻³³ and more specifically in brain between human astroglia cells transplanted in mouse brain.³⁴ Nonetheless to assess the contribution of TNTs to disease spreading, it is imperative to find specific markers and use advanced imaging techniques in order to identify this structure *in vivo*. Therefore in-depth investigation regarding function and mechanism of formation of these conduits in control and diseased brain in model of neurodegenerative disease is required as it might have implications for developing novel therapeutic strategies to halt the progression of these incurable, devastating diseases.

FIGURE 2. TNT number is increased in cells containing tau fibrils. A: Representative pictures of HeLa (top) and CAD (bottom) cells labeled with WGA-Alexa 488 (green) in control untreated conditions (left) or incubated with Alexa-555 tau fibrils (red) for 16 h (right). Scale bar: 15 and 10 μm for HeLa and CAD respectively. B: Quantification of TNT connected cells from experiment done in A. Absolute and relative data are shown in left and right respectively.



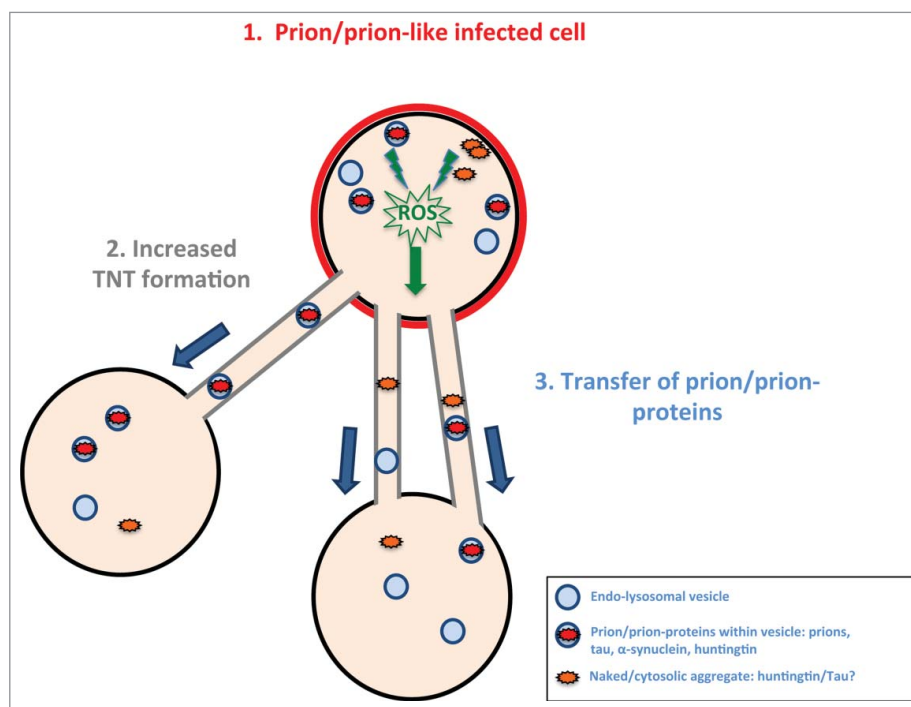
MATERIALS AND METHODS

Cell Lines

Mouse neuron-like CAD cells were a gift from Hubert Laude (Institut National de la Recherche Agronomique, Jouy-en-Josas,

France), they were cultured in Opti-MEM (Invitrogen) containing 1% Penicillin-Streptomycin and 10% fetal bovine serum (FBS). HeLa cells were cultured in DMEM (Invitrogen) containing 1% Penicillin-Streptomycin and 10% fetal bovine serum (FBS).

FIGURE 3. Model of prion/prion-like protein induced formation of TNTs and spreading. Prions or prion-like proteins such as tau fibrils after “infecting” a cell (in red) (1) induce via an unknown (possible common) mechanism (for example, induced via radical oxygen species (ROS) from oxidative stress) an increase in TNT number (2). The prion/prion-like aggregates would then be propagated via TNT-mediated intercellular trafficking from infected cells to naïve cells (3).



Preparation of Recombinant Tau Fibrils

Recombinant tau protein (tau-441, Protein Data Bank entry 2N4R) was expressed and purified by Dr. Rakez Kaye as previously described.^{18,19} For fibril assembly, tau solution (6 μ M) was incubated with DTT (Invitrogen), heparin (Invitrogen), and sodium azide (0.02%, Invitrogen) for hours up to several days at room temperature and centrifuged at 14,000 \times g. Short filaments were prepared by incubating tau with DTT and aracidonic acid (75 μ M) in 10 mM HEPES (pH 7.4), 100 mM NaCl as previously described.¹⁸ Formation of tau LMW aggregates and fibrils was monitored by electron microscopy.

Tau Fibrils Uptake in Cells

HeLa or CAD were plated in μ -Dish³⁵ mm, high Ibidi (Biovalley, France) 35 mm dishes at

a density that would allow sub-confluency to be reached after 10 h. Cells were then treated with 0,1 μ M of recombinant human tau fibrils labeled with Alexa-555 or Alexa-488 for 16 h. After the incubation time, cells were washed with PBS, fixed with PFA 4%, stained with WGA-Rhodamine or Alexa-488 (Life technologies), HCS CellMask Blue (ThermoFischer Scientific) (1/1000) and mounted with Aqua-Poly mount (Polysciences, Inc.). Images were acquired on an epifluorescence microscope AxioVision (Zeiss) with a 40x objective.

TNTs Imaging and Quantification

To image TNTs, HeLa and CAD cells were treated with 0,1 μ M of Alexa-labeled tau fibrils for 16 h as previously described. Then cells were gently washed several times with PBS. To preserve TNTs, cells were first fixed with fixative solution 1 for 20 min (2% PFA, 0.05%

glutaraldehyde and 0.2 M HEPES in PBS) then with fixative solution 2 for another 20 min (4% PFA and 0.2 M HEPES in PBS). Cells were then washed with PBS and incubated for 20 minutes with WGA-Alexa488 or Rhodamine (Life technologies) and mounted. Cells were imaged using AxioVision epifluorescence microscope and serial Z-stacks of 0.25 nm were acquired to image the whole cell volume. After imaging using a fluorescence microscope as described above, the TNT structures connecting remote cells and not touching the substratum were manually counted for both tau fibrils-loaded cells and the unloaded control cells. To better visualize tau fibrils inside TNTs, deconvolution was performed using the Huygens Professional software.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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