Title
Spreading the Seeds of Neurodegeneration: Tau Fibrils Enter Cells by Macroendocytosis

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Publication Date
2010

Peer reviewed|Thesis/dissertation

SPREADING THE SEEDS OF NEURODEGENERATION: TAU FIBRILS ENTER CELLS BY MACROENDOCYTOSIS

by

Rachel Lauren Jacks

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOMEDICAL SCIENCES

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

[redacted]

Acknowledgements

I joined the Diamond lab because of the friendly, helpful environment and Marc Diamond's seemingly endless enthusiasm for his work. My hope for graduate school was that I would grow as a scientist, and thanks to Marc's encouragement, I know I have. On a day-to-day basis, however, my labmates provided huge amounts of help and support. My fellow graduate students, Bess Frost and Suzanne Angeli, were a constant source of commiseration, advice, and friendship. Technician Kiriko Masuno made the lab more fun, and I'm happy to count her as a good friend. (Super) post-doc Mei Li was an essential collaborator on this project and always willing to share her extensive knowledge of lab techniques. Post-docs Jieya Shao, Tony Gerber, and Jeremy Jones, and lab manager Aye Aye Ma, were all essential to creating the lab's collegiate environment.

Thank you to my committee members, Dr. Robert Edwards and Dr. Frances Brodsky, who both offered valuable suggestions and advice. I especially appreciate the Edwards lab for welcoming me to their lab meetings after Marc left UCSF. Special thanks to Keith Yamamoto, for making sure that the leftover Diamond lab members had space to finish our work after the lab moved. Thank you also to Dr. Brodsky for supplying me with Rab plasmids and clathrin antibodies and protocols.

Finally, my parents, Ann and Edwin Jacks, have supported me in many ways throughout my numerous years of education, and for this I thank them from

the bottom of my heart. They instilled a deep appreciation for the value of knowledge and learning in me and I know that my pursuit of an advanced degree can be traced back to this. But without the support of my best friend and partner of ten years, Steven Gaynor, I might not have made it through graduate school. He took care of me when I was hurt, encouraged me when I doubted, congratulated me when I achieved, and stood by me even when I was stressed out, work-obsessed, and no fun to be around. Thank you, Steven, for all of the love and support.

Abstract

The deposition of tau protein characterizes over 20 neurodegenerative disorders, among them Alzheimer's disease and fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). Neurodegeneration and dementia have been shown to be caused by mutations in the tau gene [1-3]. The presence of tau is necessary for β-amyloid-induced neurotoxicity in cultured neurons and extracellular tau aggregates are taken up by cells and can induce the misfolding of intracellular tau [4, 5]. In order to determine the mechanism of cellular entry, we used the mouse undifferentiated neuronal precursor C17.2 line to study the uptake of recombinant extracellular tau fibrils using fluorescence and EM imaging. We found that tau fibrils are internalized in a saturable, actin-, energy- and temperature-dependent manner that does not require clathrin. This internalization can be blocked by the macropinocytosis inhibitors rottlerin and amiloride. Degradation of internalized tau can be blocked by the lysosomal inhibitors bafilomycin A and ammonium chloride. EM imaging indicates that fibrils are engulfed by the membrane in a manner consistent with a macroendocytic process, and are then contained within membrane-bound structures. This provides a mechanism to understand how extracellular tau aggregates can gain entry to cells, and could potentially lead to the development of drugs to block this internalization.

Contributions

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Dr. Mei Li and Dr. John Heuser performed the electron microscopy.

Aside from these items, the work presented in this thesis was conducted by its author, Rachel Lauren Jacks, under the supervision of Dr. Marc Diamond.

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Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 1: Introduction

Neurofibrillary tangles occur in the brains of victims of a diverse range of neurodegenerative disorders. Tau protein is the primary component of these tangles in a class of diseases known as tauopathies. Neurofibrillary tangles composed of hyperphosphorylated tau are found in more than 20 different neurodegenerative disorders, among them Alzheimer's disease (AD) and fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). The discovery of mutations in the tau (or microtubule-associated protein tau, MAPT) gene responsible for FTDP-17 established that dysfunctional tau protein can cause neurodegeneration and dementia [1-3]. Structurally diverse tau tangles accumulate in intraneuronal lesions and are associated with varying degrees of neurofibrillary degeneration. Electron microscopy has shown that the tangles are composed of paired-helical filaments (PHFs) in AD, straight filaments in PSP [6, 7], and random filaments in PiD [8]. Extracellular "ghost" tangles (GTs) are found in the brains of tauopathy patients, and are believed to be remnants from dead neurons. Chemical analysis of GTs has revealed that, unlike the intracellular NFTs, they are composed of truncated tau protein that lacks much of the carboxyl terminal sequence and part of the amino half [9]. It has been estimated that if the intraneuronal concentration of tau is approximately 2 μM and the soma of a neuron is modeled as a sphere of radius 10 μm, then upon lysis of the cell and diffusion of its contents the concentration of extracellular tau will be around 130 nM at a distance of 15 μm (which is several times the distance between two

neurons in the hippocampus) [10]. According to many studies, elevated levels of tau are also found in the cerebrospinal fluid of patients afflicted with tauopathies, although the range varies widely, from 40 to 820 pg/ml in AD patients, and from 27 to 380 pg/ml in control cases (reviewed in [11]). Although tau's normal function is thought to be restricted to the intracellular space, there is clear evidence that it exists in an aggregated form in the extracellular space.

Structure and Conformation

Tau is involved in the assembly and stabilization of microtubules. In neurons, microtubules are particularly important in the three-dimensional organization of the axoplasm and the transport of cargo-vesicles from the cell body towards nerve endings. The microtubule-binding region (MTBR) is required for tau's ability to bind microtubules. Tau's phosphorylation and isoform size modulate its ability to interact with tubulin, and it has an N-terminal projection domain that allows it to interact with the neural plasma membrane [12]. The tau primary transcript contains 16 exons, and in normal adult human brain, there are six isoforms due to alternative splicing of exons 2, 3, and 10 (Fig 1) [13-17].

Non-pathogenic tau is highly soluble and contains little secondary structure, existing mainly as random coil [18, 19]. Upon aggregation, tau polymerizes into fibrillar amyloid structures thought to contain extensive cross β-sheets [20-23]. Tau's MTBR alone has been shown to be capable of polymerization *in vitro* and forms the β-sheet core that composes the backbone of filaments *in vivo* [20, 24]. Because the MTBR alone is thought to be the functional unit in tauopathies, and

it is widely used to study the structure and function of tau, we used the four repeat MTBR fragment in our experiments.

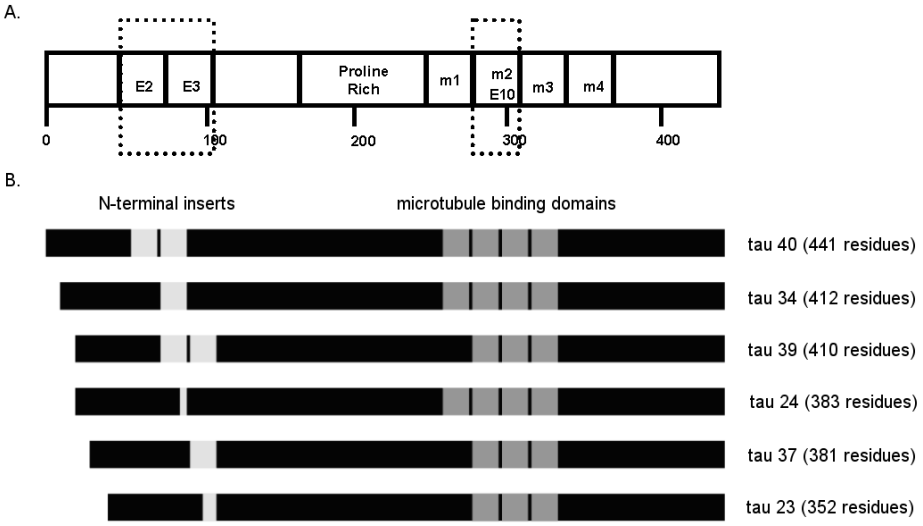

Figure 1. The primary transcript and six isoforms of tau. (a) Exons 2, 3, and 10 are alternatively spliced. **(b)** Tau isoforms have either three or four microtubule binding domains and N-terminal inserts of varying sizes.

Anatomical spreading

In AD, neurofibrillary changes spread within the brain along neuron-to-neuron connections in a stereotyped fashion, from the limbic structures toward the neocortical association areas [25-27]. The first cells to exhibit tauopathy are the glutamatergic projection cells of the transentorhinal region, with pathology then growing to encompass the hippocampal formation and the amygdala. Degeneration next extends to the higher order multimodal association areas of the neocortex, followed by the primary motor area and primary sensory fields. From the neocortex, lesions spread superolaterally from the inferior temporal

areas [27]. Similar spreading based on anatomical connections also appears in other sporadic tauopathies, such as PSP.

Hypotheses to explain the spatio-temporal spread of pathology have mainly focused on the idea of uniquely vulnerable neuronal populations or the existence of diffusible cytotoxic factors. In the case of a cell autonomous explanation for amyloid spread, specific populations of cells, especially pyramidal neurons giving rise to long cortico-cortical connections [28, 29], are selectively susceptible to the formation of tau aggregates and/or the toxic effects of these aggregates. This vulnerability could be due to the cellular environment, the tau isoforms expressed in that cell type, or both. Spared neurons either do not form the aggregates in the first place, are able to clear them before significant damage occurs (likely through proteasomal degradation [30]), or form aggregates but can simply work around them without interference with normal cellular function. The cholinergic hypothesis proposes that dysfunction of acetylcholine-containing neurons in the basal forebrain leads to the decrease in cholinergic innervation in the cortex and hippocampus, causing the vulnerability of these neurons seen in AD [31]. Another possibility is that damaged or dying neurons release excitotoxic or cytotoxic factors (such as cytokines or chemokines) to their connecting neurons that increase the vulnerability of these cells [32]. While all of these factors possibly play a part, however, the above hypotheses largely fail to provide both an initial cause of neuronal damage and an explanation for the highly stereotyped (but disease-specific) spread of tau pathology in sporadic and genetic tauopathies.

The cortical disconnection/cortical connectivity model of Alzheimer's disease pathogenesis (discussed in [28]) supposes that AD is a disconnection syndrome with spread resulting from the transport of a toxin or the lack of transport of a trophic factor. We hypothesize that tau is the toxic factor that can spread from cell-to-cell, causing progressive degeneration via trans-cellular uptake. Thus, tauopathies may begin in a specific vulnerable neuronal population, spreading and propagating throughout the brain between functionally and anatomically connected cells [33]. The mechanism of the spread of pathology between cells is unknown, but one possibility is that degenerating neurons release tau into the extracellular space, where it is then internalized by anatomically connected neurons. Polymerization of endogenous tau within these cells might then be seeded by the exogenous tau fibrils, leading to degeneration and the release of additional pathogenic tau.

Friedhoff et al. showed that tau can seed fibrillization and that the formation of PHFs occurs in a nucleation-dependent manner [34]. Seeding refers to the ability of fibrillar protein to induce the polymerization of monomer. Tau's multiple fibrillar morphologies, coupled with the diverse range of neurodegenerative diseases in which it is involved, leads to the question of how one protein can have so many different disease profiles. One possibility is that, like prion proteins ([35], reviewed in [36, 37]), misfolded tau could accomplish templated conformational change *in vivo*. Various disease-causing mutations in tau have recently been shown to have different abilities to nucleate and promote filament formation [38, 39]. Conformationally distinct tau might spread between cells, seeding the

formation of a consistent type of fibril within each subsequent cell and causing a distinct disease profile.

Endocytosis and processing

In order for pathology to spread between cells, tau must be able to enter cells from either the extracellular space or directly through some type of connection between neighboring cells. Because it is known that tau can be found in the extracellular space, we chose to study the endocytosis of extracellular tau. Endocytosis is categorized based on the size of the particles engulfed and proteins involved in uptake. The generally accepted categories are clathrin-mediated endocytosis (CME), caveolar endocytosis, clathrin-independent carriers (dynamin-dependent or –independent), and macropinocytosis. Phagocytosis, the cellular uptake of large pathogens and debris, is defined as a process that is only performed by specialized cells such as monocytes, neutrophils, and macrophages. Besides sharing common molecular machinery, it appears that these mechanisms may also interact in other ways. The same ligand/receptor complexes can be internalized through different uptake mechanisms, with different signaling outcomes [40]. CME is the most intensively studied and best understood of the endocytosis pathways, but our data indicates that it is not the primary mechanism of tau internalization. According to the data I will present here, tau is internalized by a non-clathrin mediated, energy- and actin-dependent pathway that could most accurately be called macroendocytosis, although it could also be considered consistent with macropinocytosis.

Macropinocytosis is believed to be the least specific form of endocytosis, allowing cells to accomplish bulk uptake of the extracellular fluid in response to stimulation. Membrane protrusions are generated by membrane ruffles, which fuse together to create large (>1 μm) vesicles called macropinosomes. Various drugs, including the protein kinase inhibitor rottlerin [41], the actin-disruptor cytochalasin D [42], the phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 [43, 44], the Rho GTPase inhibitor Toxin B [45], and the Na^+/H^+ exchange inhibitor amiloride [46], have all been reported to block macropinocytosis in cells. Relative to CME, macropinocytosis is poorly characterized, with much of the classification depending on morphological distinctions. Macropinosomes are recognized primarily by their large size, formation from membrane protrusions as observed by EM, and actin-bound structure, and these are some of the criteria on which we relied to classify the internalization of tau fibrils. However, because technically macropinocytosis refers to the uptake of fluids, and tau fibrils are solids, their entry can perhaps best be described as a process of macroendocytosis.

In an initial set of experiments designed to show that tau gains entry to the cell in an active process, cells were incubated with AF488-labeled tau fibrils and 50 μg/ml rhodamine-dextran (MW=10,000 KD). Dextran is a marker of so-called “fluid-phase” endocytosis, which refers to the nonspecific macropinocytic uptake of bulk fluids and nutrients. 24% of tau aggregates co-localized with dextran (*n* = 3, 200 aggregates counted per experiment) (Fig 2). This result suggests that tau uptake is a matter of membrane engulfment, rather than direct penetration or

permeabilization of the membrane. The next chapters provide a confirmation of this result and a more detailed study of the mechanism behind the uptake of tau fibrils.

Exogenous tau, once internalized, interacts with and can induce the misfolding of endogenous tau [5]. After tau is internalized it has many potential fates. It is unknown what endocytic compartments the cell uses to process tau that it takes up, and if/how the protein is degraded. Classically, the endocytic pathways converge at early endosomes, where molecules are sorted and either recycled to the plasma membrane, or delivered elsewhere in the cell. Molecules that are to be degraded are sent to multivesicular bodies (MVBs), which transport them via microtubules to late endosomes. Additional sorting occurs in the late endosomes, where the molecules can be sent to the *trans*-Golgi network (TGN) for packaging for secretion/exocytosis, or to the lysosomes for degradation (reviewed in [47-50]). The low lysosomal pH, accomplished by a proton pump, aids the hydrolytic cleavage and digestion of proteins sent to this compartment. Whether internalized exogenous tau fibrils are lysosomally digested is unknown, but previous work from our lab has shown that some of these fibrils are able to come into contact with endogenous tau and induce it to misfold [5]. This suggests that even if some tau fibrils are subject to lysosomal degradation, at least some percentage must somehow be able to escape lysosomes to come into contact with native tau.

Summary

While much is known about the structure and aggregation of misfolded tau protein, until now little work has been done to understand how extracellular tau fibrils can enter cells. Upon entering the cell the exogenous tau fibrils may affect the endogenous protein, but how they are trafficked, degraded, and/or potentially released into the cytoplasm has formerly not been explored. Using what is known about endocytic processes, in this study we attempted to elucidate the mechanism of tau fibril entry into the cell and the eventual fate of these fibrils.

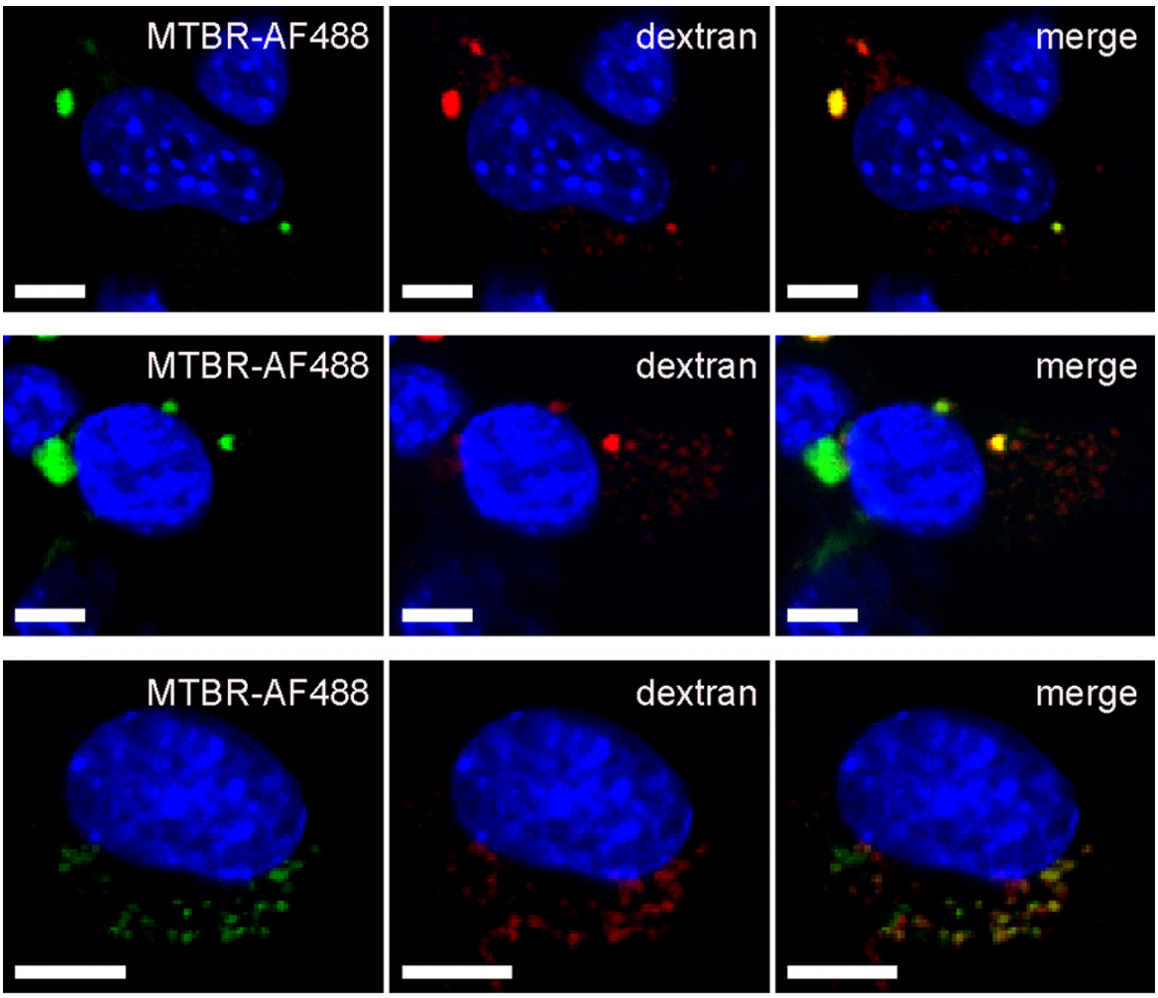

Figure 2: Multiple images of C17.2 cells treated with MTBR-AF488 aggregates and rhodamine-dextran contain co-localizing and non-co-localizing aggregates. *Scale bars*, 5 μm.

Chapter 2

Note: At this time the manuscript is not yet submitted.

Tau fibrils enter cells by macroendocytosis

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Tauopathies are a class of over 20 neurodegenerative disorders that are characterized by the deposition of tau protein. Neurodegeneration and dementia have been shown to be caused by mutations in the tau gene [1-3]. The presence of tau is necessary for β-amyloid-induced neurotoxicity in cultured neurons and extracellular tau aggregates are taken up by cells and can induce the misfolding of intracellular tau [4, 5]. In order to determine the mechanism of cellular entry, we used the mouse undifferentiated neuronal precursor C17.2 line to study the uptake of recombinant extracellular tau fibrils using fluorescence and EM imaging. We found that tau fibrils are internalized in a saturable, actin-, energy- and temperature-dependent manner that does not require clathrin. This internalization can be blocked by the macropinocytosis inhibitors rottlerin and amiloride. Tau degradation can be blocked by the lysosomal inhibitors bafilomycin A and ammonium chloride. EM imaging indicates that the uptake of tau fibrils is accomplished by membrane protrusions, consistent with a macroendocytic process, and fibrils are trafficked to large membrane-bound vesicles. This provides a mechanism to understand how an extracellular aggregate can gain entry to cells.

Amyloidopathies are characterized by the presence of protein aggregates composed of β-sheet-rich filamentous amyloid structures. It is well established that extracellular amyloid protein can penetrate cells and induce cytotoxicity. The specific mechanisms of amyloid protein cellular uptake are unclear. Even the

well-studied prion protein has been reported to enter cells multiple pathways. Like prion protein, α-synuclein has been shown to spread from neuron-to-neuron in cultured cells and animal models [51, 52], but the mechanism is unknown. Similarly, fibrillar polyglutamine aggregates can be internalized by cultured cells and gain entry to the cytoplasmic compartment where they seed the misfolding of intracellular polyglutamine [53], but the cellular machinery involved in this process is unknown.

To characterize the internalization of tau fibrils, we purified a fragment of tau consisting of the four-repeat microtubule-binding region (MTBR) from the 441-residue tau isoform and an N-terminal HA tag (MTBR-HA). The MTBR is capable of polymerization *in vitro* and forms the β-sheet core that composes the backbone of filaments *in vivo* [20, 24]. We have shown in previous work that fibrillar MTBR-HA, but not monomeric protein, is internalized by C17.2 cells [5].

It has been proposed that amyloid fibrils can directly permeabilize membranes, producing cellular toxicity, and potentially gaining entry into the cell. To test this possibility, we included propidium iodide (PI) as we incubated cells with preformed MTBR-HA fibrils or monomer (at 0.1µM final concentration), or buffer. PI readily penetrates cells with membrane defects and stains DNA. We directly evaluated the cells using fluorescence microscopy. As a positive control, we permeabilized cells with 5% ethanol. Under these conditions, 72.2%+/-6.4% of ethanol-treated cells scored PI-positive. Under experimental conditions, we saw no effect of tau fibril incubation, as 0.5%+/-0.6%, 0.5%+/-0.6%, and 1.5%+/-1.7% of cells were PI-positive when treated with fibril, monomer, or buffer,

respectively (Fig 1a,b). Thus, it is unlikely that tau fibrils are directly permeabilizing the cell membrane at the concentrations used here.

We next tested the saturability of tau internalization by incubating cells with increasing molar concentrations of MTBR-HA fibrils for 30min, harvesting the cells, and performing a western blot. Maximal MTBR-HA fibril internalization occurred between 0.67 and 1.33 μM (Fig 1c), which is well-within estimates of physiological extracellular tau concentrations [10].

We have previously developed techniques to measure tau uptake using fluor [5]. We previously observed that the trypsin treatment used in this method completely digests all extracellular fibrils. To further test for the involvement of cell metabolism in tau uptake (as opposed to direct membrane penetration), we compared uptake following incubation for 30min at 37℃ vs. 4℃. We used both flow cytometry and western blot to measure uptake. At 4˚C MTBR-HA fibril internalization was virtually abolished (Fig 2a,b). As an additional test, we depleted cellular ATP stores by co-treatment with sodium azide (a respiratory chain inhibitor) and deoxyglucose (an inhibitor of glycolysis). These agents dose-dependently decreased MTBR-HA fibril uptake as measured by western blot (Fig 2f).

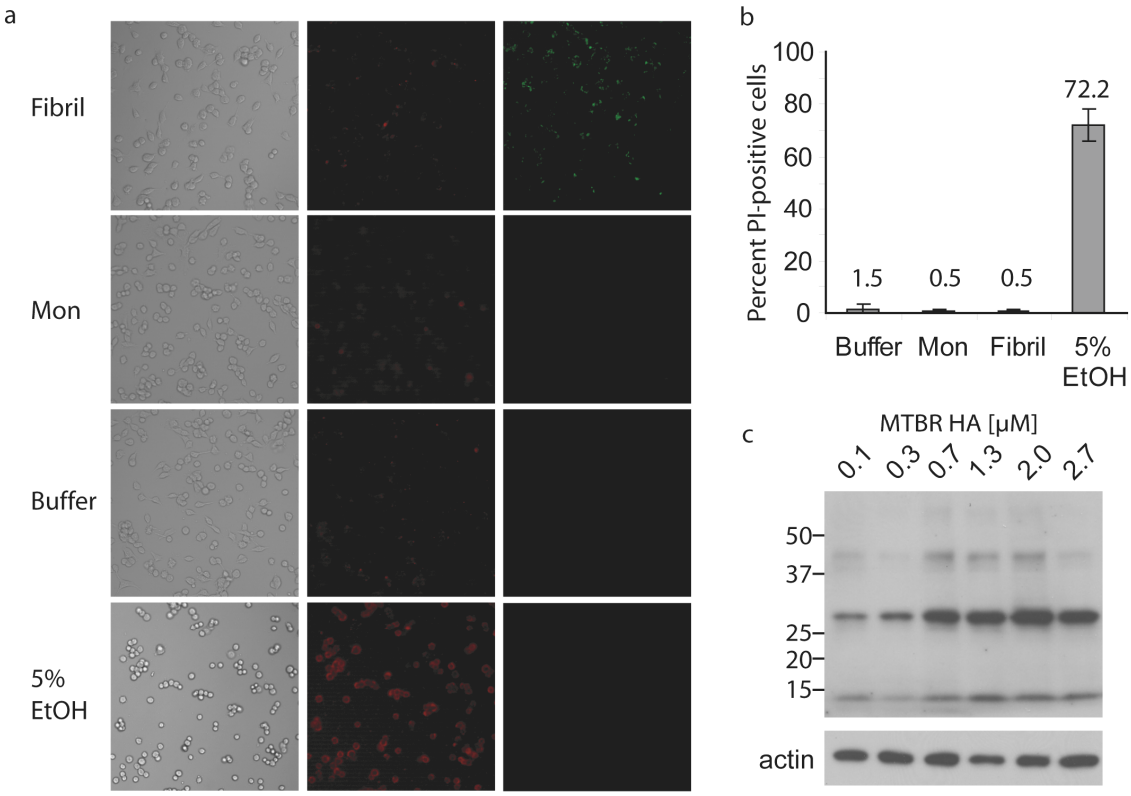

Figure 1. MTBR-HA fibril internalization is active and saturable.
(**a**) Cells were incubated with 150 nM AF488 WT-HA fibrils, monomer or buffer and PI for 3 hours. Cells were incubated for the same time period with 5% ethanol as a positive control for PI staining.
(**b**) The percentage of PI-positive cells was quantified by counting the number of PI-positive cells (n=4, 100 cells per treatment).
(**c**) To determine whether fibril uptake is saturable, cells were incubated with MTBR-HA fibrils at the indicated μM concentration for 30 minutes. After incubation, cells were trypsinized, then washed, lysed, and run on a 4-15% gradient gel before being probed with anti-HA and anti-actin antibodies.

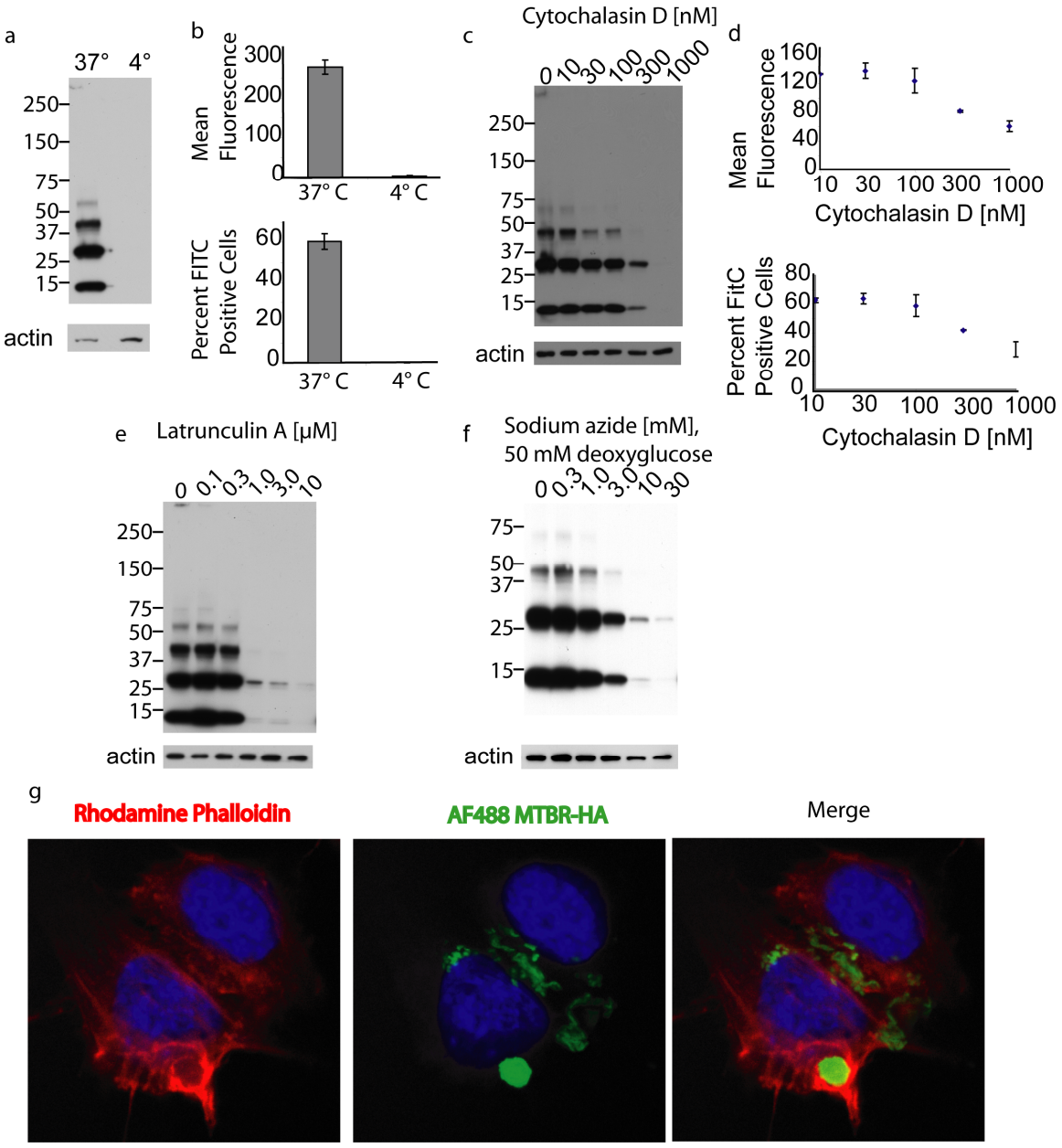

Figure 2. MTBR-HA fibril internalization is actin-, energy-, and temperature-dependent.

Western blots for **a**, **c**, **e** and **f** were performed as in [5], as follows: after incubation, cells were trypsinized with 0.25% trypsin for 5 minutes, then washed with media, spun down, rinsed with PBS, lysed with 0.1% Triton, and run on a 4-15% gradient gel before being probed with anti-HA and anti-actin antibodies. (**a**) MTBR-HA fibrils were added to C17 cells at 150 nM and the cells were incubated at either 4˚ or 37˚ C for 3 hours. Westerns were performed as described above.

(**b**) AF488 MTBR-HA fibrils were added to C17 cells as in **a** and the mean FITC fluorescence and percent FITC positive cells measured by flow cytometry. Samples were measured in triplicate.
(**c**) Cells were pretreated with the indicated nM concentration of cytochalasin D for 30 minutes before a 3-hour incubation with MTBR-HA fibrils, then the cells were harvested and processed as described above for western blot.
(**d**) Cells were pre-treated with cytochalasin D as in **c** and incubated with AF488 MTBR-HA fibrils, then harvested with 0.25% trypsin for 5 minutes, washed with media, fixed, resuspended in PBS and the mean FITC fluorescence and percent FITC positive cells were measured by flow cytometry.
(**e**) Cells were pretreated with the indicated μM concentration of Latrunculin A for 30 minutes before a 3 hour incubation with MTBR-HA, then the cells were harvested and processed as described above for western blot.
(**f**) After a 30 minute pre-treatment at the indicated concentration of sodium azide and 50 mM deoxyglucose, C17 cells were incubated with MTBR-HA fibrils for 1 hour before westerns were performed as described above.
(**g**) Cells were grown overnight on glass coverslips, then 30 nM (final concentration) AF488 MTBR-HA fibrils were incubated with cells for 1 hour. Cells were washed with PBS, fixed, then stained with Rhodamine phalloidin (33 nM).

We next determined whether clathrin mediated endocytosis (CME) is involved in tau uptake. We blocked CME by knocking down clathrin heavy chain (CHC) using siRNA, as done by Huang et al [54]. After separately transfecting cells with either CHC siRNA or negative control siRNA, cells that received each treatment were plated on glass coverslips and incubated with either transferrin conjugated to AlexaFluor 488 (Tfn488), which is internalized via CME, or AF488 MTBR-HA fibrils. Tfn488 typically produces a speckled appearance within cells in which it has been taken up [54]. CHC protein depletion was initially confirmed by western blot of CHC siRNA-treated cells and negative control siRNA-treated cells (Fig 3b). These blots were quantified to reveal a CHC/actin ratio of 2.26+/-0.81 and 0.26+/-0.26 for the negative control siRNA-treated and CHC siRNA-treated, respectively, an almost 9-fold difference (Fig 3b). We counter-stained cells with antibody to CHC to determine which cells had a significant reduction. Tfn488 formed rings around the perimeter of cells in which successful knockdown occurred, but retained its normal speckled appearance in cells that still expressed CHC (Fig 3a). Tau internalization was not affected by CHC depletion. We observed no difference in either the percentage of cells with AF488 MTBR-HA aggregates or the number of aggregates per cell between negative control-treated and cells in which CHC was knocked down (Fig. 3c). Thus, the bulk of tau uptake does not involve CME. We additionally tested for the involvement of caveolin-mediated endocytosis by using antibodies against caveolin to test for

colocalization with tau fibrils. We did not observe any colocalization (Fig. 3d), suggesting that this mechanism does not play a role.

Actin rearrangements mediate a variety of cellular uptake mechanisms, including macropinocytosis. To test the role of the actin cytoskeleton in aggregate uptake, we treated cells with cytochalasin D or latrunculin A. Both drugs dose-dependently decreased the cellular uptake of MTBR-HA fibrils, measured by flow cytometry or western blot (Fig 2c-e). To further characterize the role of actin in fibril uptake, we incubated cells with AF488-labed MTBR-HA and stained them with rhodamine-tagged phalloidin, which binds to filamentous actin. Large MTBR-HA inclusions appeared to be surrounded by actin (Fig 2g). Such actin-surrounded structures are typical of macropinosomes.

Macropinocytosis allows cells to accomplish bulk uptake of the extracellular fluid in response to stimulation. It involves the generation of membrane protrusions (through membrane ruffling), which fuse together to create large (>1 μm) vesicles called macropinosomes. These vesicles are believed to contain bulk amounts of heterogeneous fluids and macromolecules. The protein kinase inhibitor rottlerin has been shown to selectively inhibit macropinocytosis [41]. Rottlerin treatment dose-dependently inhibited fibril internalization while having no effect on transferrin uptake as indicated by microscopy and flow cytometry (Fig 4a,b). The Na^+/H^+ exchange inhibitor amiloride (an EIPA analogue) has also been reported to block macropinocycotis in cells [46]. Treatment of cells with amiloride dose-dependently decreased AF488 MTBR-HA fibril uptake as demonstrated by flow cytometry (Fig 4c).

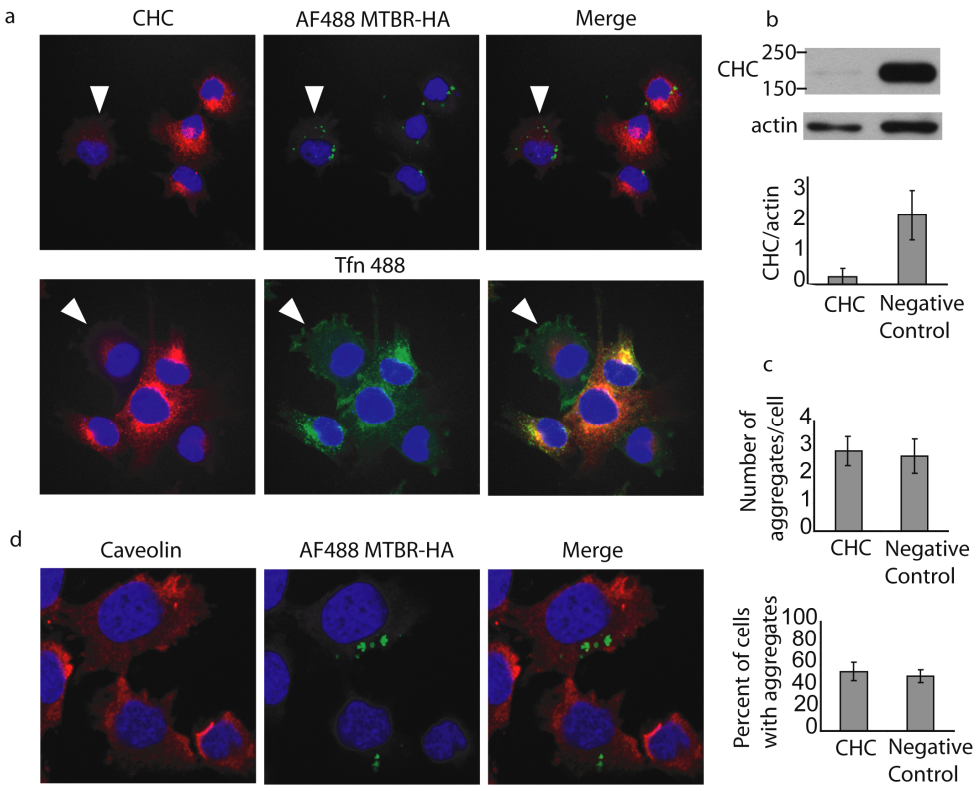

Figure 3. Clathrin and caveolae are not involved in MTBR-HA fibril internalization.

For siRNA transfections, C17 cells were separately transfected with anti-CHC siRNA or Allstars negative control siRNA for 24 hours, then they were trypsinized and the negative control-treated cells and CHC siRNA-treated cells were plated together on glass coverslips for immunostaining and microscopy.

(**a**) CHC-depleted cells are indicated with arrows. Cells were treated with as follows: Media was removed and replaced with serum free media containing 0.1% BSA for 30 minutes at 37°C and either 150 nM A F488 MTBR-HA fibrils and the cells were incubated for 1 hour, or Tfn488 (5 µg/ml) was added and cells were incubated for 15 minutes on ice.

(**b**) Cells were transfected with CHC or negative control siRNA and incubated with AF488 MTBR-HA fibrils or Tfn488 as above but the two populations were plated in separate wells and cells were harvested and blotted with mouse anti-CHC antibody and rabbit anti-actin antibody, followed by HRP secondary antibody. Quantification of the CHC: actin ratio was performed with ImageJ (n=3).

(**c**) Cells were transfected with CHC or negative control siRNA as in **b**. Cells were counted and scored for presence or absence of aggregates and number of aggregates per cell.

(**d**) Cells were incubated with 30 nM AF488 MTBR-HA fibrils or AF488 buffer for 1 hour and then washed with PBS, fixed, and stained for caveolin-1.

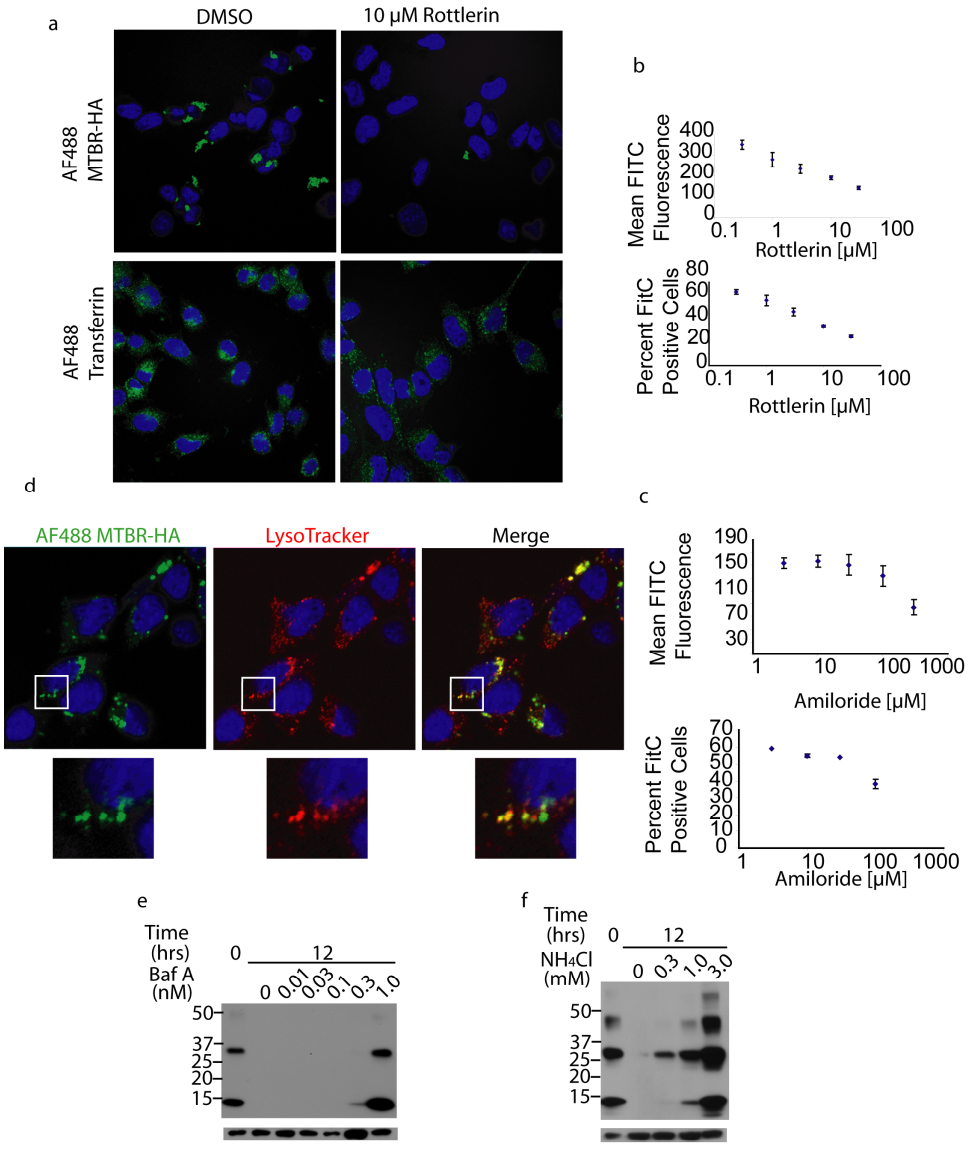

Figure 4. MTBR-HA fibril endocytosis is blocked by macropinocytosis inhibitors rottlerin and amiloride in a dose-dependent manner, and fibrils are internalized into acidified endosomes and degraded by lysosomes.
(**a**) Cells were pretreated with the indicated rottlerin concentration before the addition of 30 nM AF488 MTBR-HA, AF488 buffer, or Tfn488 (5 µg/ml) for 1 hour. Cells were rinsed with PBS, fixed, and mounted for confocal microscopy.
(**b**) Flow cytometry for the effect of rottlerin on MTBR-HA uptake was performed using the same pre-treatment and exposure conditions as in **a**, followed by harvest of the cells with 0.25% trypsin for 5 minutes. The cells were washed,

fixed, resuspended in PBS, and the mean FITC fluorescence and percent FITC positive cells were measured by flow cytometry.
(**c**) Flow cytometry for the effect of amiloride on MTBR-HA uptake was performed using the same pre-treatment and exposure conditions as in **c**, followed by harvest of the cells with 0.25% trypsin for 5 minutes. The cells were washed, fixed, resuspended in PBS, and the mean FITC fluorescence and percent FI-C positive cells were measured by flow cytometry.
(**d**) C17 cells were incubated with 150 nM AF488 MTBR-HA or AF488 alone for 3 hours, then rinsed with PBS and treated with 0.25% trypsin for 1 minute at 37 C. Trypsin was removed and the media replaced, and cells were allowed to recover for 4 hours before the addition of LysoTracker Red DND 99 at 75 nM. After incubation for 1 hour, cells were rinsed, fixed with 4% PFA, DAPI stained and mounted for confocal fluorescence microscopy.
For **e** and **f**, cells were incubated with MTBR-HA fibrils for 3 hours, then the media was replaced with new media containing either (**e**) bafilomycin A or (**f**) NH_4Cl at the indicated concentrations. One well of MTBR-HA-treated cells was harvested at this time as time 0. The remaining cells were incubated for 12 hours, then they were harvested and a western blot using the HA antibody was performed.

We have previously observed that tau fibrils taken into cells can induce normally folded tau within the cytosol to misfold and form fibrils [5]. It is unclear whether this results from failed fibril degradation with possible escape from this pathway. We began by testing whether the fibrils are trafficked to the lysosomal compartment, consistent with other targets for degradation via the endosome/lysosome system. We incubated cells with AF488 MTBR-HA fibrils and LysoTracker DND-99, a marker of acidified endosomes. We then used confocoal microscopy to image the cells and determine the degree of colocalization between tau fibrils and acidified vesicles. 80.2%+/-10.3% of MTBR aggregates co-localized with LysoTracker (n=3) (Fig 4d). We tested for degradation of tau fibrils by incubating cells with fibrils and then treating with chemical inhibitors of the lysosomal degradation pathway. 12-hour inhibition of lysosomal degradation with ammonium chloride or bafilomycin (to block vesicle acidification) dose-dependently blocked the apparent degradation of fibrils taken up by the cells (Fig 4e,f).

Our data suggests that tau fibrils are taken up via an actin-dependent pathway, and are not associated with membrane permeabilization. We confirmed these findings by using electron microscopy to visualize the aggregation internalization process. We prepared tau fibrils with and without AF488 label. To reduce fibril uptake and reduce fibril size, we treated the samples with brief sonication at low energy. We then exposed C17.2 cells to the fibrils for 24 hours prior to processing. We began with thin-section EM to visualize the various samples. This clearly shows the presence of fibrils on the outside of the cell, and

the invagination of the cell membrane in response to their exposure (Fig 5a,b). EM also revealed deep pits which contained the tau fibrils. We excluded the possibility that the fibrils were maintained in these structures by exposing cells to AF488-MTBR fibrils and then quenching with trypan blue. This did not quench the signal apparent in endosomes, which indicated they were not in communication with the extracellular space. Further EM studies indicated that the fibrils were completely enclosed in endosomes (Fig 5c).

Our data are consistent with the idea that tau fibrils gain entry to the cell interior through a macroendocytic mechanism that leads to lysosomal degradation. Although they may be the same process, a strict definition of macropinocytosis only includes the uptake of fluid, and therefore macroendocytosis is a more accurate term for the uptake of tau fibrils. This process is energy and actin-dependent, and is blocked by pharmacologic agents known to interfere with macropinocytosis, such as rottlerin and amiloride. Strikingly, we observed fibrils within membrane-bound structures by EM. Taken together with prior results showing co-localizatin of tau fibrils with the fluid-phase endocytic marker dextran [5], these experiments are most consistent with tau fibril uptake through macroendocytosis.

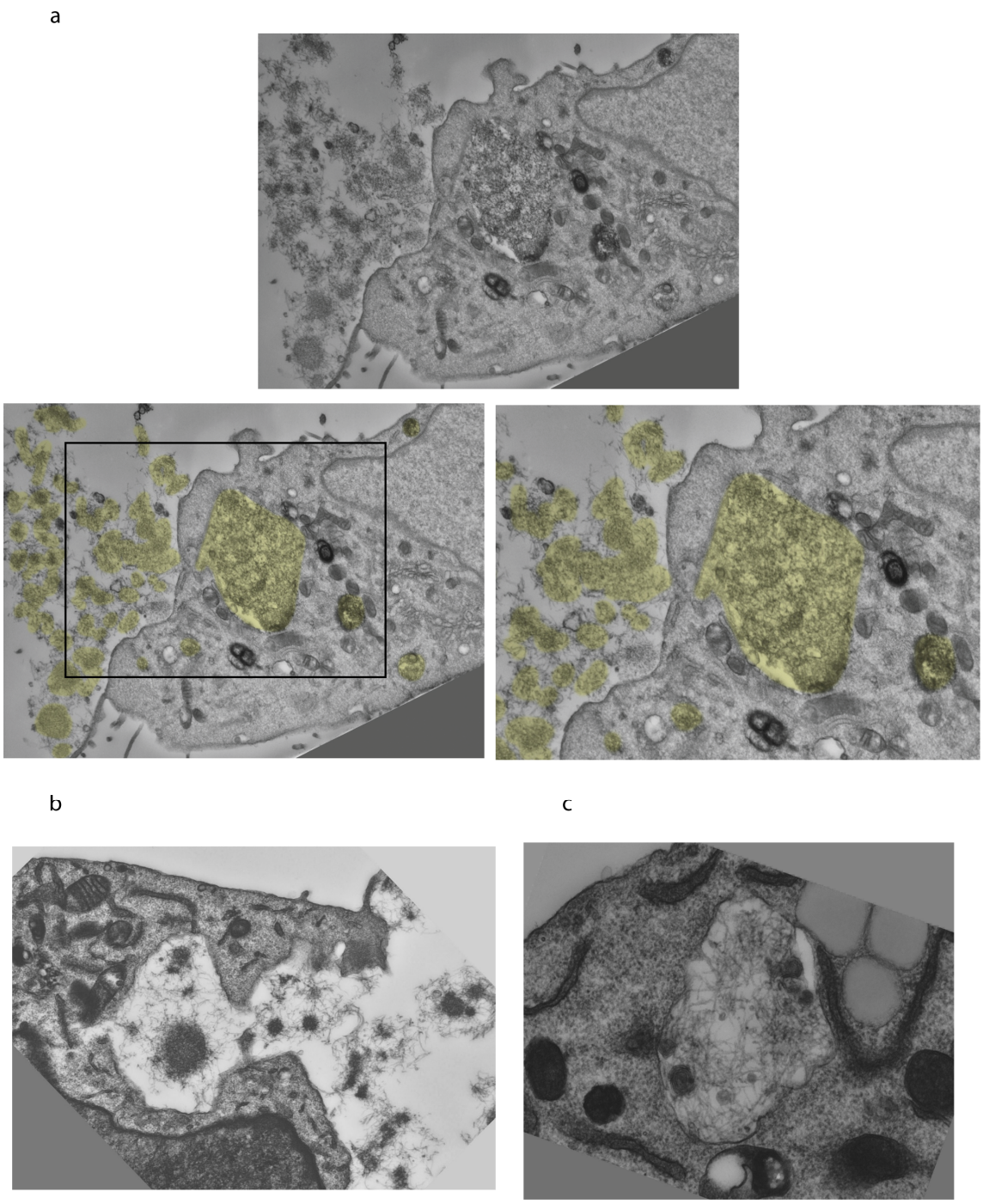

Figure 5. Imaging by thin-section EM shows the membrane engulfment of extracellular MTBR-HA fibrils into membrane-bound vesicles.
(**a**) Membrane engulfment of MTBR-HA fibrils incubated with C17 cells is evident. False-colored images are shown, bottom, with an enlargement of the area of interest on the bottom right.
(**b**) An example of membrane engulfment of large fibrillar MTBR-HA aggregates.
(**c**) A membrane-bound endosome containing MTBR-HA fibrils.

Macroendosomes have few known molecular makers, and are thus predominantly defined by morphology, as we have done here with EM. Beyond the pharmacological inhibitors rottlerin and amiloride and the requirements for energy and actin, macroendocytosis/macropinocytosis is not well-characterized, especially in neurons. Numerous pathogens exploit macropinocytosis for cellular entry and escape into the cytoplasm. For example, *Listeria monocytogenes*, utilizes acidification to escape from the macropinosome into the cytoplasm [55]. Nanoparticle-coupled cargo have also been reported to escape from acidified endolysosomal compartments and interact with cytosolic targets in neurons [56]. Co-localization of fibrils with acidified vesicles is therefore consistent with our EM data, which shows fibrils inside membrane-bound vesicle.

Tau normally functions within the intracellular space, but it is readily detected in the spinal fluid in AD patients, especially as the disease progresses [11]. It is unclear whether this represents release from dying vs. living cells. In AD, neurofibrillary changes spread within the brain in a stereotyped fashion, from the limbic structures toward the neocortical association areas [25-27]. This could represent the involvement of neural networks, as has been previously proposed based on the coincidence of patterns of degeneration with intrinsic connectivity networks. Explanation of the spatio-temporal spread of pathology has mainly focused on the idea of uniquely vulnerable neuronal populations or the existence of diffusible cytotoxic factors. Given the variety of neuronal populations involved in the tauopathies, we favor the latter model. It is possible that tau fibrils are the “toxic factor” that can spread from cell-to-cell, causing progressive degeneration

via trans-cellular uptake. Unlike in the case of prions, where the target protein is a trans-membrane protein, and thus has direct access to the extracellular space, it has been unclear how tau fibrils, or those derived from other intracellular proteins, such as synuclein, might physically gain access to the interior of a cell to propagate pathology. While the mechanisms of propagation remain uncertain, this work helps elucidate the mechanisms of trans-cellular movement of protein aggregation. As molecular mechanisms are elucidated, future exeriments will help test whether the processes described here play a role in pathogenesis.

Methods

Tau Expression, Purification, Fibrillization, and Labeling: Tau MTBR, composed of amino acids 243 to 375 of the wild type 441amino acid tau isoform (P10636-8) (a gift from Dr. Virginia Lee) was prepared as previously described [57]. Briefly, the MTBR was subcloned into the pRK172 bacterial expression vector along with the sequence YPYDVPDYA and an HA tag on the C-terminus. We prepared recombinant WT-HA from Rosetta (DE3)pLacI competent cells (Novagen) as previously described ([57]). Single-use aliquots were stored in 10 mM HEPES and 100 mM NaCl (pH 7.4), and to induce fibrillization the protein was first incubated at 1 hour at room temperature in 12.5 mM DTT, then incubated for 3 hours at room temperature with 10 mM HEPES (pH 7.4), 100 mM NaCl, 150 μM arachidonic acid (Sigma), and 5 mM DTT. For labeling with Alexa Fluor, fibrils or buffer were incubated with 0.05 ng/ul Alexa Fluor 488 carboxylic

acid succinimidyl ester (Molecular Probes) overnight at 4 C with gentle rocking. Labeling reactions were quenched with 100 mM glycine.

Western Blots: C17.2 cells were plated at 50,000 cells/well in a 24-well plate. The following day, the cells were pre-treated for 30 minutes with drug and then incubated with MTBR-HA for the indicated time. Drugs used were: Amiloride hydrochloride hydrate (Sigma), Latrunculin A (Invitrogen)
Cytochalasin D (Invitrogen), Sodium azide (Sigma), Deoxyglucose (Sigma). Cells were harvested by rinsing with PBS, then trypsinizing with 0.25% trypsin for 5 minutes at 37 C. They were diluted in DMEM, spun down for 5 minutes at 1000xg, the media was aspirated and the cells rinsed in PBS, then snap-frozen and stored at –20C. Cell pellets were resuspended in 40 μL of 0.1% Triton/PBS plus an EDTA-free mini protease inhibitor tablet (Roche), incubated 10 minutes on ice, then syringe-lysed. After the addition of loading buffer samples were boiled for 5 minutes and resolved on a 4-15% SDS-PAGE gradient gel (Bio-Rad). After transfer to a PVDF membrane, blots were incubated with the primary antibody. For the detection of MTBR-HA, we used 1:2000 Anti-HA HA11 monoclonal antibody (Covance).
CHC western blots were performed as described previously ([58]). Cells were harvested as above but resuspended in 30 μL of Buffer A (150 mM KCl, 2.0 mM $MgCl_2$, 20 mM HEPES, 10% glycerol, 1.0 mM EDTA, 1.0 mM EGTA, 1.0 mM NaF, 1.0 mM DTT, 0.02% saponin, pH7.2), plus a protease inhibitor tablet at the manufacturer's recommended dilution (Roche). Samples were incubated for 15

minutes at 4º C, then centrifuged for 10 minutes at 14,000xg. Saponin-soluble fractions were boiled for 5 minutes with 4X loading buffer plus DTT and run on a 7.5% gel with a stacking gel on top. Transfer and development was done as above. Clathrin Heavy Chain mouse monoclonal antibody (Covance) was used at 1:1000. Blots were stripped and re-probed with the 1-19-R anti-actin antibody (Santa Cruz) at 1:2000.

siRNA: On the previous day C17.2 cells were plated in 24-well dishes. They were transfected with AllStars Negative control siRNA (Qiagen) or CHC siRNA (Qiagen; target sequence: AAG CAA TGA GCT GTT TGA AGA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 hours cells were trypsinized and plated onto poly-onithine-coated glass coverslips. 24 hours later the cells were either harvested for western blot or assayed for uptake of tau or transferrin. For that assay, cells were incubated with AF488 MTBR-HA for 1 hour or transferrin from human serum conjugated to AF488 (Tfn488) (Invitrogen) at 5 µg/ml was added and cells were incubated for 15 min on ice.

Microscopy and Immunofluorescence: C17.2 cells were plated on poly-ornithine coated glass coverslips. On the following day cells were incubated with 150 nM AF488-containing buffer or AF488 MTBR-HA for the time indicated. Cells were fixed with 4% paraformadehyde for 10 minutes. For CHC immunofluorescence, cells were permeabilized in 0.04% saponin for 15 minutes

at room temp, then blocked in blocking solution (1% fish skin gelatin, 0.1% BSA, 0.02% SDS, 0.1% Nonidet P-40, 0.02% sodium azide) for 1 hour. Coverslips were incubated with DAPI (Sigma) and CHC mouse monoclonal antibody (Covance) in diluted 1:300 in blocking solution for 1 hour at room temp. After rinsing 3 times with washing buffer (10% blocking solution, 0.008% saponin in PBS), coverslips were incubated with 1:500 Alexa Fluor 555 donkey anti-mouse secondary antibody (Invitrogen) for 1 hour at room temp, then washed as before and mounted onto glass slides with ProLong Antifade reagent (Invitrogen).

For staining with LysoTracker DND99 (Invitrogen), C17 cells grown on poly-ornithine-coated coverslips were incubated with 150 nM AF488 MTBR-HA or AF488 alone for 3 hours, then rinsed with PBS and treated with 0.25% trypsin for 1 minute at 37 C. Trypsin was removed and the media replaced, and cells were allowed to recover for 4 hours before the addition of LysoTracker at 75 nM. After incubation for 1 hour, cells were rinsed, fixed with 4% PFA, DAPI stained and mounted for confocal fluorescence microscopy

For propidium iodide staining, cells were co-incubated with AF488 tau fibrils and propidium iodide (1 µg/ml) for 3 hours, then washed twice with PBS and examined by fluorescence microscopy. Cells were incubated for the same time period with 5% ethanol as a positive control.

For phalloidin staining, cells plated on coverslips were incubated with 150 nM AF488 MTBR-HA or AF488-containing buffer for the time indicated, then washed, and fixed as above and stained with 33 nM Rhodamine phalloidin (Invitrogen) for 20 minutes, then rinsed 3X with PBS and mounted as above.

For caveolin-1 staining, cells were plated as above and the following day incubated with 30 nM AF488 MTBR-HA or AF488-containing buffer for the time indicated, then washed, and fixed as above. Fixed cells were incubated with DAPI and a 1:500 dilution of caveolin-1 (4H312) mouse monoclonal antibody (Santa Cruz) in 3% BSA/0.1% Trion/PBS overnight at 4℃. After rinsing 3 times with PBS, cells were stained with goat anti-mouse AF 546 secondary antibody (Invitrogen) at a 1:500 dilution for 1 hr at RT. Cells were rinsed 3 times with PBS, stained with DAPI, then mounted onto glass slides with ProLong Antifade reagent (Invitrogen).

Flow Cytometry: C17.2 cells were plated at 50,000 cells/well in a 24-well plate. The following day, the cells were pre-treated with the applicable drug and then incubated with AF488 MTBR-HA or AF488-containing buffer for the indicated time. Cells were washed with PBS, then harvested with 0.25% trypsin for 5 minutes at 37 C. They were diluted in DMEM, spun down for 5 minutes at 1000xg, the media was aspirated and the cells fixed in 4% PFA for 10 minutes. Cells were spun down again and re-suspended in PBS. Cells were counted in a BD LSR II Flow cytometer and analysis was done using FlowJo software. For percentage-positive cells, FIT-C data was gated so that less than 1% of AF488-buffer-treated cells were positive.

Chapter 3: Investigation of tau fibril co-localization with endocytic markers

Introduction

In order to investigate the cellular location of internalized MTBR-HA fibrils, I examined their co-localization with several markers. Cholera toxin subunit B (CTB) binds to membrane GM1 gangliosides [59] and is used as a marker of lipid rafts, while the Rab proteins are monomeric GTPases that coordinate vesicle formation, transport, fusion and motility. Recombinant Rabs are often used as markers of various stages of endocytic vesicles. Rab5 is a marker of early endosomes, Rab4 is a marker of early and recycling endosomes, Rab7 is a marker of late endosomes, Rab9 is a marker of late endosomes/lysosomes, and Rab11 is a marker of recycling endosomes (reviewed in [49]). Rab34 is a recently discovered member of the Rab family that has been reported to localize to membrane ruffles formed by macropinocytosis [60]. We hypothesized that endocytosed tau fibrils might co-localize with some of these markers of membranes or intracellular vesicles. In order to learn more about the uptake and sorting of endocytosed tau protein, I examined the co-localization of MTBR-HA fibrils with CTB, Rab4, Rab5, Rab7, Rab9, Rab11, and Rab34.

Results

We hypothesized that tau fibrils may co-localize with some Rab protein markers. To determine how tau fibrils progress through the endocytic pathway, I

transfected cells with various recombinant GFP-Rabs and looked for co-localization with AF488 MTBR-HA. Under the conditions used in these experiments, however, I was unable to see co-localization with GFP-Rab4, 5, 7, 9, or 11 (Fig 1a).

To determine whether MTBR-HA fibrils co-localize with CTB, I incubated cells with fibrils and AF555 CTB. AF488 MTBR-HA aggregates appear to occasionally co-localize with vesicles labeled with CTB (Fig 2a), apparently surrounding a core of tau aggregates.

In initial immunofluorescence experiments I detected the robust co-localization of AF488-MTBR-HA fibrils with Rab34 on membrane ruffles (Fig 1b), but further study with recombinant Rab34 YFP constructs failed to bear out this co-localization (data not shown).

a

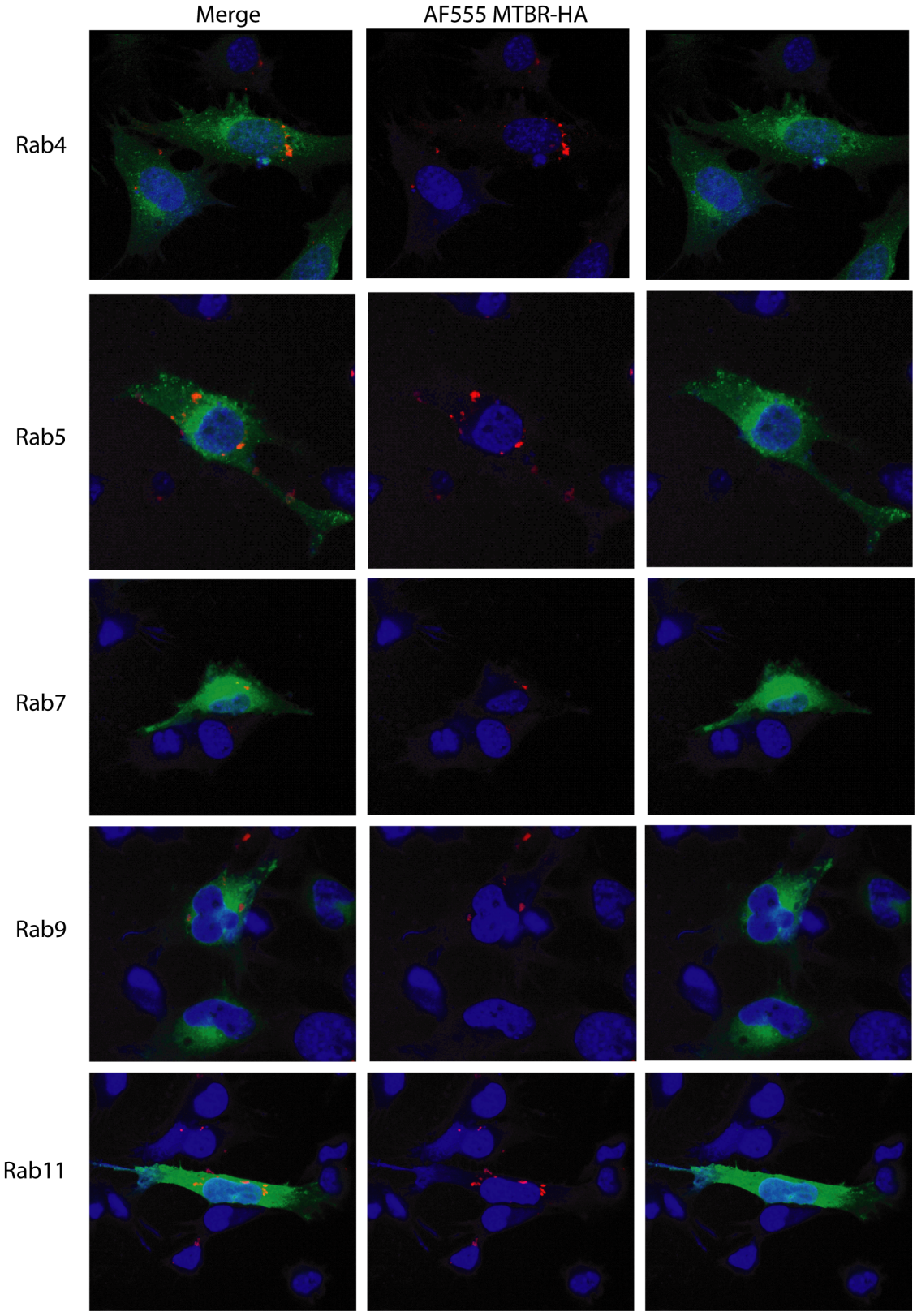

Figure 1. MTBR-HA fibrils do not co-localize with Rab4, Rab5, Rab7, Rab9, or Rab11 GFP constructs.

(**a**) Cells transfected with recombinant GFP Rab constructs were incubated with 150 nM AF555 MTBR-HA fibrils for 3 hours. After the incubation they were trypsinized, allowed to recover for 4 hours, and then fixed and stained for microscopy.

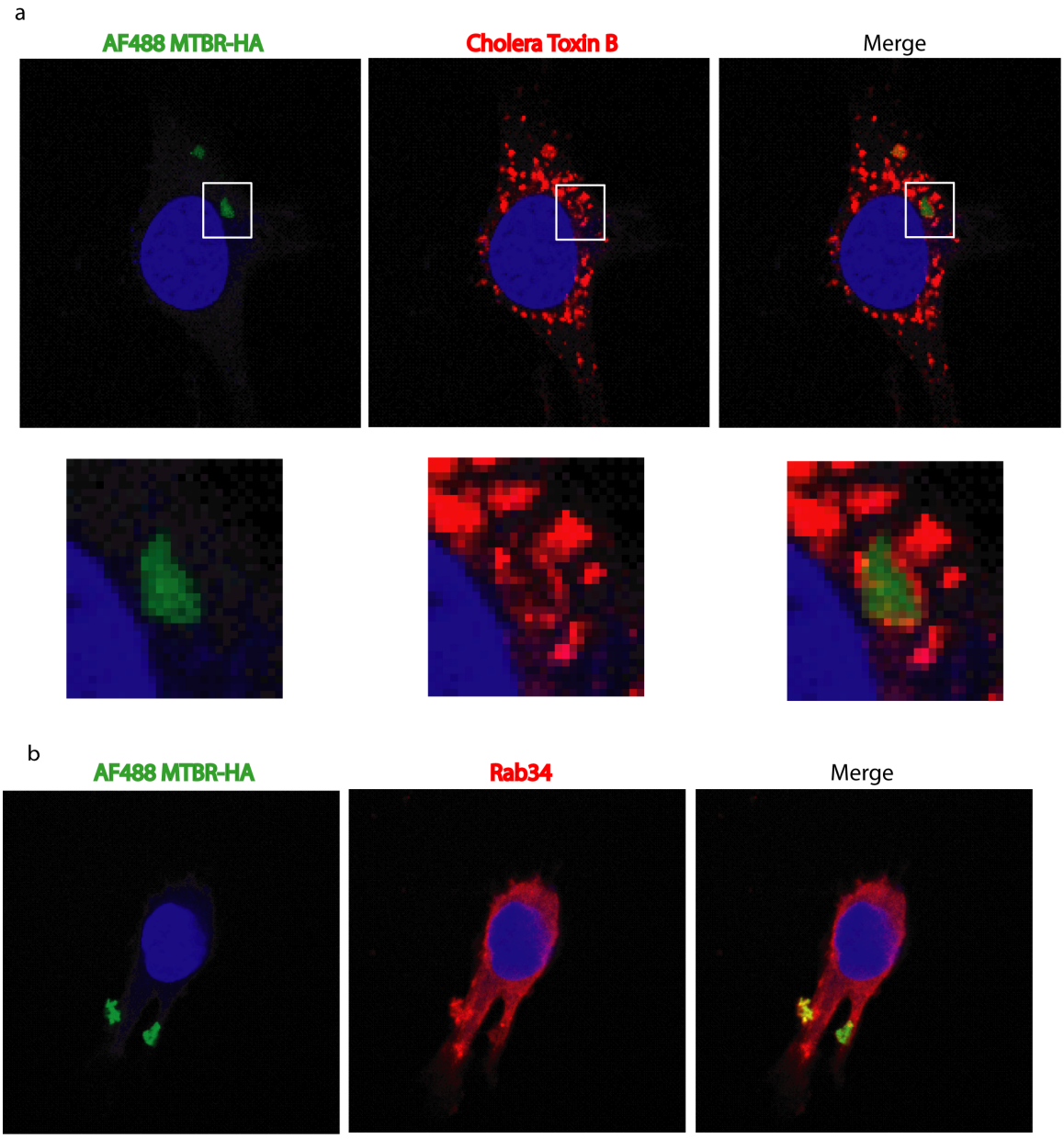

Figure 2. MTBR-HA fibrils are surrounded by CTB and appear to co-localize with Rab34.

(**a**) Cells were incubated with 150 nM AF488 MTBR-HA for 3 hours. For the last 30 minutes of this incubation AF555 CTB (Invitrogen) was added to the media. Following the incubation cells were washed, fixed, stained for DAPI, and mounted for microscopy.
(**b**) Cells were incubated with 30 nM AF488 MTBR-HA for 1 hour, then rinsed with PBS, fixed, and stained. After staining with the Rab 34 (N-16) antibody, DAPI, and donkey anti-goat AF594 secondary anbitody, cells were rinsed and mounted for microscopy.

Methods

***Tau Expression, Purification, Fibrillization, and Labeling*:** Tau MTBR, composed of amino acids 243 to 375 of the wild type 441 amino acid tau isoform (P10636-8) (a gift from Dr. Virginia Lee) was prepared as previously described [57]. Briefly, the MTBR was subcloned into the pRK172 bacterial expression vector along with the sequence YPYDVPDYA and an HA tag on the C-terminus. We prepared recombinant WT-HA from Rosetta (DE3)pLacI competent cells (Novagen) as previously described ([57]). Single-use aliquots were stored in 10 mM HEPES and 100 mM NaCl (pH 7.4), and to induce fibrillization the protein was first incubated at 1 hour at room temperature in 12.5 mM DTT, then incubated for 3 hours at room temperature with 10 mM HEPES (pH 7.4), 100 mM NaCl, 150 μM arachidonic acid (Sigma), and 5 mM DTT. For labeling with Alexa Fluor, fibrils or buffer were incubated with 0.05 ng/ul Alexa Fluor 488 carboxylic acid succinimidyl ester (Molecular Probes) overnight at 4 C with gentle rocking. Labeling reactions were quenched with 100 mM glycine.

***Microscopy and Immunofluorescence*:** The pEGFP-Rab4, pEGFP-Rab5, pEGFP-HA-Rab7, pEGFP-Rab9-HA, and pEGFP-Rab11-HA were a gift from the lab of Dr. Frances Brodsky. Rab34 constructs were a gift from Dr. Takeshi Endo. C17.2 cells were plated on poly-ornithine coated glass coverslips at 10K/ml in 24-well dishes. For Rab transfections, on the following day cells were transfected with the appropriate DNA using Lipofectamine 2000 per the manufacturer's

instructions. On the following day, cells were incubated with 150 nM AF488 MTBR-HA or AF4880-containing buffer for 3 hours, then rinsed with PBS and treated with 0.25% trypsin for 1 minute at 37 C. Trypsin was removed and the media replaced, and cells were allowed to recover in media for 4 hours before fixation with 4% paraformadehyde for 10 minutes.

For Rab34 co-localization, cells were plated on glass coverslips as above. On the following day, cells were incubated with 30 nM AF488 MTBR-HA or AF488-containing buffer for 1 hour, then rinsed with PBS, fixed with 4% PFA for 10 minutes, and stained. Rab 34 (N-16) antibody (Santa Cruz) was used for staining at overnight at 4℃ at 1:100 in 3% BSA/0.1% Triton/ PBS. After rinsing 3 times with PBS, cells were stained with donkey anti-goat AF594 secondary anbitody (Invitrogen) at a 1:500 dilution for 1 hr at RT. Cells were rinsed 3 times with PBS, stained with DAPI, then mounted onto glass slides with ProLong Antifade reagent (Invitrogen).

For CTB staining, cells were incubated with 150 nM AF488 MTBR-HA or AF488-containing buffer for 3 hours. For the last 30 minutes of this incubation AF555 CTB (Invitrogen) was added to the media. Following the incubation cells were washed, fixed, stained for DAPI, and mounted for microscopy.

Discussion

The co-localization of MTBR-aggregates with CTB is consistent with the internalization of tau into membrane-bound vesicles, as observed by EM. CTB binds to GM1 gangliosides in membranes. Not all internalized tau is surrounded

by CTB-labeled membranes, however, which may represent different mechanisms of entry or different sorting pathways. Whether the GM1 gangliosides that CTB binds to are present solely in lipid rafts or are evenly distributed throughout the membrane is controversial, and may depend on the cell type. As further information about the binding and trafficking of CTB is discovered, it may provide us with more context with which to interpret these co-localization results.

There are several possibilities to explain the lack of tau fibril co-localization with Rab4, 5, 7, 9, or 11, one being that under different conditions, at earlier timepoints, MTBR-HA fibrils do co-localize with these proteins. Another possibility is that the tau fibrils are trafficked through a different pathway entirely, one which does not utilize the canonical endocytic vesicle markers. Or, it could be that in the C17.2 cell line, these GFP constructs simply do not behave as they have been reported to in other cell lines. More work will be required to determine which of these possibilities is true.

The co-localization with immunofluorescently-stained Rab34 could signify a true interaction between membrane ruffles and tau fibrils, or it could be a staining artifact. Likewise, the fact that tau fibrils do not co-localize with GFP Rab34 constructs might be an artifact of over-expression of this protein, leading to its inappropriate trafficking, or it might represent a true lack of co-localization of these two proteins. Further study will be required to determine whether tau fibrils actually co-localize with Rab34.

Chapter 4: Conclusions and Future Directions

We hypothesized that tau fibrils can be taken into cells by a specific endocytic process. In this study we have shown that exogenous tau fibrils are internalized by a non-clathrin-mediated, energy- and actin-dependent pathway likely to be classified as macroendocytosis. We clearly visualized this uptake by EM. Once taken into the cell, these fibrils are trafficked to lysosomes, where they are degraded in a process that can be blocked by lysosomal inhibitors. Internalized tau fibrils failed to co-localize with Rab4, Rab5, Rab7, Rab9, or Rab11 under our experimental conditions, but did co-localize with CTB and Rab34, although further work is needed to confirm and expand these results.

This study was undertaken to test the larger hypothesis that extracellular tau can corrupt cellular tau by entering the cell and seeding the misfolding of the endogenous tau protein. In order for this to happen, however, the extracellular tau must enter the cell and come into physical contact with endogenous tau. In this work we sought to discern the specific mechanism by which tau fibrils are able to enter cells. It is possible that tau is internalized by more than one mechanism, perhaps with different processing and outcomes. For the cells that have taken it up, the question of what happens to internalized tau protein aggregates has potentially life-or-death consequences. Degradation of the misfolded protein, release into the cytoplasm, and/or extracellular release may all occur, with differing outcomes for the individual cell and its neighbors. The

cellular localization of internalized tau may provide us with valuable clues to the neurodegenerative process.

Trans-cellular uptake of fibrillar tau proteins may have implications for cell survival regardless of whether seeded misfolding is an important part of neurodegeneration. Neurons and other cells could internalize misfolded extracellular tau, amyloid-ß, or other amyloid protein, and attempt to degrade it. For cells already containing aggregates, or otherwise compromised, the internalization of misfolded extracellular amyloid could be the final push that overwhelms the cell's ability to clear dysfunctional protein. This mechanism of disease could extend to other neurodegenerative diseases, as well, with neurons internalizing the extracellular debris from dead cells, only to be overwhelmed with misfolded protein that they are unable to degrade. The internalization and trafficking of fibrillar tau may therefore represent a general defense mechanism that goes awry in protein misfolding diseases.

The above experiments and data raise many questions that are beyond the scope of this study, such as the existence of a receptor for tau. It might also be enlightening to determine the ability of tau to be excreted, perhaps within exosomes as has been shown for prions and β-amyloid [61, 62]. Preliminary data, not shown here, indicated that cells may be able to transfer tau fibrils to one another, but only if they can come into direct contact. This could be a fascinating observation to follow up on. Other interesting possibilities for future study could utilize mouse primary cultured neurons, cultured hippocampal slices, or mouse models with reduced endocytosis or expressing tau tethered to a secretion signal

sequence. These systems could all be used to learn more about the importance of tau internalization *in vivo*.

Regardless of whether tau aggregation can spread through the brain via anatomical connections, this study will have bearing on our understanding of the ability of amyloid proteins to perturb normal cellular function through endocytic pathways. Information about the cellular internalization of tau could be valuable in the fight to halt the inevitable spread of pathology in tauopathies, and potentially other amyloidopathies as well. By characterizing the pathway and specificity of the endocytosis of tau, it may help us to find new molecular targets to prevent the progression of some types of neurodegeneration.

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