Secretion of Tau via an Unconventional Non-vesicular Mechanism

Merezhko, Maria

2018-11-20


http://hdl.handle.net/10138/276993
https://doi.org/10.1016/j.celrep.2018.10.078

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.
Report

Secretion of Tau via an Unconventional Non-vesicular Mechanism

Graphical Abstract

Highlights

- ATP-independent unconventional secretion of Tau directly through the plasma membrane
- Phosphorylated oligomeric Tau clusters in plasma membrane microdomains
- Tau secretion can be manipulated by changing plasma membrane properties
- Heparan sulfate proteoglycans facilitate unconventional secretion of Tau

Authors
Maria Merezhko, Cecilia A. Brunello, Xu Yan, Helena Vihinen, Eija Jokitalo, Riikka-Liisa Uronen, Henri J. Huttunen

Correspondence
henri.huttunen@helsinki.fi

In Brief
Merezhko et al. show that Tau protein is released from cells by an unconventional secretory mechanism that can be manipulated by changing plasma membrane properties. Phosphorylated oligomeric Tau clusters at the plasma membrane, allowing its escape from cells directly through the plasma membrane.
Secretion of Tau via an Unconventional Non-vesicular Mechanism

Maria Merezhko,1,3 Cecilia A. Brunello,1,3 Xu Yan,1 Helena Vihinen,2 Eija Jokitalo,2 Riikka-Liisa Uronen,1 and Henri J. Huttunen1,4,*

1Neuroscience Center, HILIFE, University of Helsinki, 00014 Helsinki, Finland
2Electron Microscopy Unit, Institute of Biotechnology, HILIFE, University of Helsinki, 00014 Helsinki, Finland
3These authors contributed equally
4Lead Contact
*Correspondence: henri.huttunen@helsinki.fi
https://doi.org/10.1016/j.celrep.2018.10.078

SUMMARY

Tauopathies are characterized by cerebral accumulation of Tau protein aggregates that appear to spread throughout the brain via a cell-to-cell transmission process that includes secretion and uptake of pathological Tau, followed by templated misfolding of normal Tau in recipient cells. Here, we show that phosphorylated, oligomeric Tau clusters at the plasma membrane in N2A cells and is secreted in vesicle-free form in an unconventional process sensitive to changes in membrane properties, particularly cholesterol and sphingomyelin content. Cell surface heparan sulfate proteoglycans support Tau secretion, possibly by facilitating its release after membrane penetration. Notably, secretion of endogenous Tau from primary cortical neurons is mediated, at least partially, by a similar mechanism. We suggest that Tau is released from cells by an unconventional secretory mechanism that involves its phosphorylation and oligomerization and that membrane interaction may help Tau to acquire properties that allow its escape from cells directly through the plasma membrane.

INTRODUCTION

Accumulation of misfolded Tau into paired helical filaments and further into insoluble aggregates and neurofibrillary tangles is a hallmark of tauopathies, including Alzheimer’s disease (Lee et al., 2001). Tau is a natively unfolded neuronal microtubule-stabilizing protein that, in pathological conditions, is hyperphosphorylated and detaches from microtubules, leading to misfolding and formation of pathological Tau aggregates in the cytosol. Pathological Tau is released from cells, even in the absence of cell death or membrane leakage, and internalized by neighboring cells, where the misfolded Tau templates misfolding of healthy Tau molecules, propagating Tau pathology (Goedert et al., 2017). Although this pathological spreading of Tau has gained much attention, the secretion mechanism remains unknown, although several routes, such as secretion in extracellular vesicles (EVs), have been suggested (Saman et al., 2012; Simón et al., 2012; Dujardin et al., 2014). Recently, some groups have reported that only a minority of Tau is localized in extracellular vesicles, whereas the vast majority of secreted Tau is found as a vesicle-free protein (Yan et al., 2016; Wegmann et al., 2016; Wang et al., 2017).

Here we show that Tau uses an unconventional secretion route that does not require ATP or Ca2+ but can be regulated by manipulating plasma membrane properties or cell surface heparan sulfate proteoglycans (HSPGs). Tau clusters in microdomains at or near the plasma membrane, independent of vesicles, and the presence of cholesterol and sphingomyelin support Tau secretion. This unconventional secretion process shares several characteristics with both overexpressed human Tau in N2A neuroblastoma cells and endogenous rat tau in cultured cortical neurons. Based on these data, we suggest a membrane microdomain- and HSPG-dependent unconventional mechanism for Tau secretion directly through the plasma membrane.

RESULTS AND DISCUSSION

Overexpressed Tau Is Localized to the Plasma Membrane and Secreted Independent of Vesicles and ATP

For studying the secretion mechanisms of pathological forms of Tau, we used a model where human Tau (isoform 0N4R) is overexpressed in the mouse N2A neuroblastoma cell line, leading to increased levels of free hyperphosphorylated Tau in the cytosol. We first isolated EVs from N2A/Tau cell media and found no Tau present in EVs by western blot (Figure S1A). A protein fragment complementation assay (PCA), a highly sensitive detection method for Tau dimers and oligomers (Figures S2A and S2B), showed that more than 99% of Tau oligomers were secreted in a vesicle-free form (Figure S1B).

To characterize the secreted Tau species in more detail, we used a combination of size-based filter centrifugation and dot blot (Figure S2C). The majority of secreted Tau (~80%) was found to be in the form of dimers, trimers, or tetramers, as shown in Figures S2D and S2E, which is in line with previous reports (Yan et al., 2016; Brunello et al., 2016). Next, we tested the behavior of secreted Tau in an in vitro Thioflavin S aggregation assay. The Tau-conditioned medium alone showed higher Thioflavin S fluorescence compared with mock-conditioned
medium, indicative of the presence of β-sheet aggregates in the conditioned medium (Figure S2F). Moreover, the Tau-conditioned medium was capable of seeding aggregation of recombinant human Tau (ON4R). These results show that Tau secreted by N2A cells has several pathological characteristics.

We then tested whether Tau is secreted by classical secretory processes. Depletion of cellular ATP by 2-deoxyglucose for 30 min had no effect on Tau secretion, strongly indicating that no vesicular transport or membrane fusion processes are involved in the secretion process (Figure 1A). No effect was seen in lactate dehydrogenase (LDH) release, demonstrating that this short treatment did not affect the membrane integrity of the cells. Treatment of cells with the canonical inducer of exocytosis, the Ca	extsuperscript{2+} ionophore ionomycin, did not induce Tau secretion but, instead, decreased it (Figure 1B), further excluding classical exocytosis as a mediator of Tau secretion.

Using light microscopy, total Tau staining with Tau-5 antibody showed cytosolic staining with distinct puncta on the cell surface of N2A/Tau cells (Figure 1C). The T22 antibody, which specifically recognizes Tau oligomers (Lasagna-Reeves et al., 2012), and the phosphospecific antibodies AT8 (Ser202/Thr205) and PHF13 (Ser396) predominantly stained small puncta near the cell boundaries (Figures 1D–1F). Thioflavin S did not stain these sub-plasma membrane Tau structures (Figures 1G–1J), suggesting that they represent the phosphorylated oligomeric forms of Tau not yet converted into aggregates with β-sheet structures. Using a super-resolution microscopy method, direct stochastic optical reconstruction microscopy (dSTORM) Tau-5 staining showed numerous small puncta at the edges of the cells together with some cytosolic signal (Figures 1K and 1L). Analysis of particle size showed a mean diameter of 23 nm and that more than 90% of all detected particles were smaller than 40 nm (Figure 1M).

To visualize whether Tau-containing puncta were membrane-bound organelles, large protein aggregates, or something else, we used immunoelectron microscopy to localize wild-type untagged Tau stained with Tau-5 antibody and Tau-GLuc using anti-HA antibody. In both setups, Tau localized to the plasma membrane in microdomain-like structures but not in vesicles, multivesicular bodies, or any other membrane-bound compartments (Figures 1N–1S; Figure S3). Cytosolic Tau was observed in both diffuse and dense aggregated forms. At the plasma membrane, we observed large (~200 nm) membrane patches associated with diffuse cytosolic Tau clusters just below the plasma membrane (~100 nm) (Figures 1P and 1Q) and smaller membrane microdomains (50–80 nm) with very dense staining (Figures 1R and 1S). The only endomembrane that showed prominent Tau localization was the nuclear membrane, where Tau was localized in nuclear pores (Figures 1N and 1O). Some phenotypic variability was seen between cells, as can be expected in transient expression (quantified in Table S1).

These results suggest a non-vesicular secretion mechanism for Tau, likely involving Tau localization in specific microdomains at the plasma membrane. This is in line with previous studies reporting Tau localization to membranes (Brandt et al., 1995; Kawarabayashi et al., 2004; Pooler et al., 2012) and further suggest that the interaction of Tau with the plasma membrane within specific microdomains may be a prerequisite for non-vesicular release of Tau. Structural compartmentation upon interaction of Tau with membranes (Elbaum-Garfinkle et al., 2010; Jones et al., 2012; Künze et al., 2012; Georgieva et al., 2014; Flach et al., 2012) and/or formation of pore-like structures in membranes (Lasagna-Reeves et al., 2014; Patel et al., 2015) could mediate membrane penetration of Tau.

Manipulation of Plasma Membrane Lipids Affects Tau Secretion

If Tau is secreted directly through the plasma membrane, then manipulating plasma membrane properties should influence this process. We first extracted membrane cholesterol from N2A/Tau cells using methyl-β-cyclodextrin (Mahammad and Parnell, 2015). Treatment with 1 mM methyl-β-cyclodextrin decreased Tau secretion by 47.0% ± 7.2% (Figure 2A), suggesting that cholesterol or membrane order in general affects Tau release. Loading more cholesterol to cells using preformed cholesterol/methyl-β-cyclodextrin complexes increased Tau secretion by 75.8% ± 8.1%, further supporting this view (Figure 2B). Second, we depleted the cells of sphingolipids with two different strategies. Plasma membrane sphingomyelin was degraded by adding exogenous sphingomyelinase (SMase) to the medium, or, alternatively, all sphingolipids were depleted with the serine palmitoyl-CoA transferase inhibitor myriocin.
Both treatments reduced Tau secretion maximally by ~36% (Figures 2C and 2D). Third, N2A/Tau cells were treated with docosahexaenoic acid (DHA), a polyunsaturated fatty acid that reduces membrane order and disrupts lipid raft-like membrane microdomains and whose consumption has been associated with reduced risk of Alzheimer’s disease (Yurko-Mauro et al., 2010; Zhang et al., 2016). Upon DHA treatment, Tau secretion was concentration-dependently reduced, with a maximal inhibition of 93.8% ± 0.9% at 50 μM DHA. Tau secretion was already significantly reduced by 1 μM DHA (Figure 2E), close to the DHA...
concentration found in normal human cerebrospinal fluid (Freund Levi et al., 2014). Cultured neuroblastoma cells typically have a significantly lower level of DHA in their membranes compared with neurons in vivo (Reynolds et al., 2001; Akbar et al., 2005), which may make them particularly sensitive to the effects of DHA.

In summary, these data indicate that the rigid liquid-ordered phase of the membrane enriched in specific lipid species, such as cholesterol and sphingomyelin, are centrally involved in Tau release from cells. Somewhat surprisingly, Tau is more efficiently released from ordered membranes, and Tau secretion can be reduced by increasing the fluidity of cellular membranes.
Interestingly, a similar interdependence of membrane composition and rigidity and membrane permeation was recently reported for arginine-rich cell-penetrating peptides (Wallbrecher et al., 2017).

Cell Surface HSPGs and the Aggregation State of Tau Modulate Secretion Efficiency
Because Tau binds glycosaminoglycans (GAGs) (Goedert et al., 1996; Hasegawa et al., 1997) that are abundantly present at the plasma membrane, we hypothesized that these interactions may play a role in Tau secretion. HSPGs play a central role in the unconventional secretion of FGF2 (Steringer and Nickel, 2018).

Moreover, while this manuscript was under review, Katsinelos et al. (2018) published a paper suggesting that Tau secretion may share some similarities with unconventional secretion of FGF2. NaClO₃ is widely used to inhibit synthesis of PAPS (3′-phosphoadenosine 5′-phosphosulphate), the general sulfate donor in GAG biosynthesis (Venkatachalam, 2003). Treatment of N2A/Tau cells with NaClO₃ resulted in a dose-dependent decrease in Tau secretion (maximal decrease, 37.6% ± 5.4% at 50 mM NaClO₃ (Figure 3A). To reduce the amount of cell surface HSPGs more specifically, we treated cells with heparinase I or heparinase III, two enzymes that cleave heparin and heparan sulfate-type sulfated glycans with different specificities. Both treatments led to a decrease in both intracellular and secreted Tau, with heparinase III having a more consistent effect of reducing Tau secretion by 25.0% ± 3.5% (Figure 3B). Treatment of N2A/Tau cells with chondroitinase ABC to reduce cell surface levels of chondroitin and dermatan sulfate GAGs did not decrease Tau secretion (Figure 3C). These data suggest that cell surface proteoglycans, specifically HSPGs, facilitate Tau secretion and are in line with a previous report showing similar results (Katsinelos et al., 2018).

Because oligomerization plays an important role in unconventional secretion of FGF2 (Steringer and Nickel, 2018), and because at least some of the plasma membrane-localized Tau appears to be hyperphosphorylated and oligomerized, we next tested the effect of inhibition of Tau aggregation on secretion. To narrow down the Tau species that penetrate the membrane, we used four different Tau aggregation inhibitors...
(TAIs), emodin (Pickhardt et al., 2005), BSc3094 (Pickhardt et al., 2007), phthalocyanine tetrarsulfonate (PcTS) (Akoury et al., 2013), and epigallocatechin gallate (EGCG) (Ehrnhoefer et al., 2007; Wobst et al., 2015), which inhibit Tau aggregation at different stages (Bulic et al., 2013; Cisek et al., 2014). Because some of the TAIs are colored, they prevent the use of PCA. Instead, we used a dot blot to study Tau secretion. All four compounds reduced Tau secretion, with effects ranging from 35% to 94% reduction (Figures 3D and 3E). This suggests that formation of oligomeric aggregation intermediates, possibly occurring at the membrane interface, is a prerequisite for unconventional secretion of Tau. EGCG caused the strongest reduction in Tau secretion in the dot blot assay, and because it is a non-colored compound, it was also tested in the PCA-based secretion assay. EGCG reduced secreted Tau oligomers, as detected by PCA, by 63.4% ± 3.6% (Figure 3F). Furthermore, we found that Tau secreted in the presence of EGCG is taken up significantly less effectively compared with the control (Figures 3G–3I). Notably, EGCG not only inhibits Tau aggregation but also modulates membrane properties (Sun et al., 2009; Patra et al., 2008), both of these mechanisms could contribute to the robust overall effect of EGCG on Tau secretion in N2A cells. Overall, together with the finding that phosphorylated oligomeric forms of Tau localize in clusters in sub-plasma membrane microdomains (Figure 1), these results suggest that certain forms of oligomeric aggregation intermediates may be the Tau species capable of penetrating the plasma membrane and exiting cells.

**Endogenous Tau from Primary Neurons Is Also Secreted via a Similar Unconventional Mechanism**

To test whether endogenous neuronal Tau is also secreted via the same or a similar mechanism as overexpressed pathological Tau, we analyzed media from cultures of rat primary cortical neurons (21 days in vitro [DIV]) using Tau ELISA. Similar to N2A/Tau cells, Tau secretion was inhibited by decreasing plasma membrane cholesterol with methyl-β-cyclodextrin, with a maximal decrease of 47.8% ± 0.6% at 1 mM methyl-β-cyclodextrin (Figure 4A). Increasing neuronal plasma membrane cholesterol content by cholesterol:methyl-β-cyclodextrin complexes resulted in a 105% ± 15.6% increase in Tau secretion (Figure 4B). Reduction of sulfated GAGs on the cell surface by treatment of cortical neurons with 50 mM NaClO3 decreased Tau secretion by 45.0% ± 5.3% (Figure 4C). EGCG reduced neuronal secretion of Tau by 35.7% ± 7.1% (Figure 4D). LDH release was not significantly altered by any of these treatments, suggesting that plasma membrane integrity was not affected. These results indicate that not only overexpressed pathological forms of human Tau in N2A cells but also endogenous rat Tau in primary cortical neurons are secreted by an unconventional secretion mechanism.

In summary, our current data demonstrate that Tau uses an unconventional protein secretion pathway to exit cells. Importantly, overexpressed pathological Tau and also endogenous Tau from primary neurons appear to be secreted via a similar mechanism. This process is highly dependent on plasma membrane lipid content and biophysical properties, especially in the presence of cholesterol and sphingomyelin. A number of proteins are released from eukaryotic cells under physiological conditions directly through the plasma membrane in an unconventional secretion process (Rabouille, 2017). We propose that Tau acquires properties, likely associated with its phosphorylation and oligomerization, that promote its plasma membrane interaction and permeation, resulting in secretion. Suppression of plasma membrane interaction and penetration of Tau could be an effective therapeutic target for slowing down disease progression in tauopathies.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - N2A cell culture
  - Primary rat cortical neuron culture
- **METHOD DETAILS**
  - Protein-fragment complementation assay (PCA)
  - Media fractionation
  - Cholesterol:mbCD complex preparation
  - ELISA
  - Immunofluorescence microscopy
  - dSTORM
  - Immunoelectron microscopy
  - Western blot and dot blot assay
  - Thioflavin S assay
  - Size-exclusion dot blot assay
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.10.078.

**ACKNOWLEDGMENTS**

We thank Prof. Stephan Michnick (Université de Montréal, Canada) for providing the humanized GLuc plasmid. Dr. Veijo Salo, Amr Abou Elezz, Mikko Liljestrom, and the Biomedicum Imaging Unit, University of Helsinki are thanked for technical support with dSTORM imaging. This study was funded by the Academy of Finland (grant 296409) and the Finnish Cultural Foundation.

**AUTHOR CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

H.J.H. is an employee and shareholder of Herantis Pharma Plc, which is unrelated to this study.
REFERENCES


SUPPORTING CITATIONS

The following references appear in the Supplemental Information: Ercan et al. (2017); Hoffmann et al. (1997); LoPresti et al. (1995); Mercken et al. (1992); Nieman et al. (1983); Wilson et al. (1984).


# STAR★METHODS

## KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tau-5</td>
<td>Invitrogen</td>
<td>AHB0042, RRID: AB_2536235</td>
</tr>
<tr>
<td>AT8</td>
<td>Thermo Scientific</td>
<td>MN1020, RRID: AB_223647</td>
</tr>
<tr>
<td>PHF-13</td>
<td>Cell Signaling</td>
<td>9632, RRID: AB_2266237</td>
</tr>
<tr>
<td>T22</td>
<td>Sigma</td>
<td>ABN454</td>
</tr>
<tr>
<td>HA</td>
<td>Sigma</td>
<td>H3663, RRID: AB_262051</td>
</tr>
<tr>
<td>Chemicals, Peptides, and Recombinant Proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tau 0N2R</td>
<td>rPeptide</td>
<td>T-1005-1</td>
</tr>
<tr>
<td>Heparinase I and III</td>
<td>Idurol</td>
<td>EC 4.2.2.8 <a href="https://enzyme.expasy.org/EC/4.2.2.8">https://enzyme.expasy.org/EC/4.2.2.8</a></td>
</tr>
<tr>
<td>DHA</td>
<td>Cayman Chemicals</td>
<td>6217-54-5</td>
</tr>
<tr>
<td>Chondroitinase ABC</td>
<td>Sigma</td>
<td>C3667</td>
</tr>
<tr>
<td>Methyl-β-cyclodextrin</td>
<td>Sigma</td>
<td>C4555</td>
</tr>
<tr>
<td>2-deoxyglucose</td>
<td>Sigma</td>
<td>D8375</td>
</tr>
<tr>
<td>Heparin</td>
<td>Sigma</td>
<td>H3149</td>
</tr>
<tr>
<td>Thioflavin S</td>
<td>Sigma</td>
<td>T1892</td>
</tr>
<tr>
<td>Neutral sphingomyelinase</td>
<td>Sigma</td>
<td>C8633</td>
</tr>
<tr>
<td>Myricin</td>
<td>Sigma</td>
<td>M1177</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Sigma</td>
<td>C3045</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>Sigma</td>
<td>407953</td>
</tr>
<tr>
<td>Epigallocatechin-3-gallate (EGCG)</td>
<td>Sigma</td>
<td>50299</td>
</tr>
<tr>
<td>Emodin</td>
<td>Sigma</td>
<td>E7881</td>
</tr>
<tr>
<td>BSC3094 monohydrobromide</td>
<td>Sigma</td>
<td>B7937</td>
</tr>
<tr>
<td>Phthalocyanine teta-sulfonate hydrate (PcTS)</td>
<td>Sigma</td>
<td>P4374</td>
</tr>
<tr>
<td>NaClO3</td>
<td>Sigma</td>
<td>7775-09-9</td>
</tr>
<tr>
<td>Critical Commercial Assays</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytotox 96 Assay</td>
<td>Promega</td>
<td>G1780</td>
</tr>
<tr>
<td>Mouse ELISA kit</td>
<td>Thermo Scientific</td>
<td>KMB7011</td>
</tr>
<tr>
<td>CellTiter-Glo® Assay</td>
<td>Promega</td>
<td>G7570</td>
</tr>
<tr>
<td>Experimental Models: Cell Lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Neuroblastoma cells Neuro-2A (N2A) p. 3-19</td>
<td>ATCC</td>
<td>CCL-131 (59538655)</td>
</tr>
<tr>
<td>Rat primary cortical neurons DIV 15-25</td>
<td>Neuroscience Center, University of Helsinki</td>
<td>N/A</td>
</tr>
<tr>
<td>Recombinant DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phGLuc1 and phGLuc2 plasmids</td>
<td>Prof. Stephen Michnick, University of Montreal; Remy and Michnick, 2006</td>
<td>N/A</td>
</tr>
<tr>
<td>phGLuc1-Tau and phGLuc2-Tau plasmids</td>
<td>Yan et al., 2016</td>
<td>N/A</td>
</tr>
<tr>
<td>Software and Algorithms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GraphPad Prism version 5.08</td>
<td>GraphPad Software</td>
<td><a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a></td>
</tr>
</tbody>
</table>

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Henri Huttunen (henri.huttunen@helsinki.fi).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

N2A cell culture
Neuro-2a (N2A) mouse neuroblastoma cells (RRID: CVCL_0470) were maintained in DMEM (Corning) supplemented with 10% (v/v) of fetal bovine serum (Invitrogen) and 1% (v/v) streptomycin, penicillin and L-glutamine (Lonza) at 37°C in 5% CO₂. Transfection of N2A cells was performed 24 hours after plating using JetPEI reagent (Polyplus) according to manufacturer’s instructions.

Primary rat cortical neuron culture
Primary cortical neurons were prepared from rat embryos as previously described (Kuja-Panula et al., 2003), and maintained in Neurobasal media (Invitrogen) supplemented with 2% (v/v) B27 (Invitrogen) and 1% (v/v) streptomycin, penicillin and L-glutamine (Lonza) at 37°C in 5% CO₂. Experiments were performed between DIV14 and DIV25.

METHOD DETAILS

Protein-fragment complementation assay (PCA)
The PCA system based on split humanized Gaussia princeps luciferase (hGLuc) used in this study has been previously described (Remy and Michnick, 2006). The hGLuc plasmids, with or without HA tag, were cloned on the C terminus of human Tau (isoform 0N4R) in a pcDNA3.1/zeo backbone (Figure S2A). PCA studies were conducted as already described (Yan et al., 2016; Brunello et al., 2016). Briefly, N2A cells on 96-well plates were transfected with phGLuc/Tau plasmids. Luminescence-based detection of PCA signal was done 48 hours post transfection, following injection of native coelenterazine, the substrate for the luciferase with Varioskan plate reader (Thermo Scientific). The detection of the PCA signal is directly proportional to the amount of Tau molecules interacting at a given time in the cells.

For Tau secretion assays, Tau-Gluc expressing N2A cell media was changed to PRF-DMEM with and without treatments for indicated times before PCA measurements. For reading of PCA signal of the conditioned media, plates were spun at 400 g for 5 min in swing bucket rotor (Eppendorf) and media was collected to another plate for signal detection. Cells were placed in fresh media for PCA measurement of intracellular dimers. Both media and cell lysate were also used for measurement of lactate dehydrogenase (LDH) release to assess cell membrane integrity and cell viability. Absorbance was detected at 490 nm with Varioskan plate reader (Thermo Scientific).

For Tau uptake assays, Tau-GLuc expressing N2A cells were treated with 10 μM TAIs for 16 h. Tau-conditioned media was centrifuged at 1500 g for 10 min to remove any cellular debris. Naive N2A cells (with no endogenous Tau expression) were incubated with this Tau-conditioned media for 16 hours. Tau-conditioned media was removed, cells were carefully washed twice with PBS to remove extracellular Tau and placed in fresh media for detection of PCA signal as described above and immunofluorescence analysis of internalized Tau-GLuc. For quantification, background values were subtracted from all images. Mean fluorescence intensity was measured from 18 images of EGCG-treated cells and 17 images of control treated cells (on average, 19 cells per image). The values were normalized by the number of cells per image.

Media fractionation
Separation of conditioned media into vesicle-free, ectosomal and exosomal fractions was performed as previously described (Thery et al., 2006; Yan et al., 2016). Briefly, conditioned media was first cleared from debris by centrifugation at 3000 x g for 30 min, and then centrifuged at 20 000 x g for 1 hour (Sorvall WX Floor Ultra centrifuge) to pellet the ectosomal fraction. While the supernatant containing the exosomal fraction was transferred to a clean tube, the pellet was washed once with fresh media and spun for one additional hour at 20 000 x g, and resuspended into 100 μL of media. In a similar way, the exosomal fraction was pelleted following two centrifugation steps at 100 000 x g for 70 min (Beckman Coulter ultracentrifuge, SW41 Ti rotor), with one washing of the pellet in

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>ImageStudioLight version 5.2.5.</td>
<td>LI-COR Biosciences</td>
<td><a href="https://www.licor.com/bio/products/software/image_studio_lite/">https://www.licor.com/bio/products/software/image_studio_lite/</a></td>
</tr>
</tbody>
</table>
between, before resuspension in 100 μL of fresh media. Flotillin-2 (rabbit monoclonal, Cell Signaling Technology) was used as a marker for the microvesicle-containing fractions (Kowal et al., 2016). The supernatant of the exosomal fraction, corresponding to the vesicle-free fraction of the media, was concentrated with Amicon filters (30 kDa cut-off; Millipore) according to manufacturer’s instructions.

**Cholesterol:mbCD complex preparation**

Cholesterol was first dissolved in chloroform and dried under nitrogen gas. Solution of mbCD in water (300 mM) was added to cholesterol (weight ratio of 1 to 10), vortexed and sonicated for 1 hour at RT until complete dissolution. The complex was stored at −20°C in single use aliquots. The final calculated concentration of cholesterol added to cells was 0.1 mM.

**ELISA**

For the measurement of endogenous Tau secretion to the extracellular space, the media of primary cortical neurons was changed to 250 μL of fresh Neurobasal with or without treatments for 1 hour up to 72 hours before the collection and centrifugation at 400 g for 5 min in swing bucket rotor (Eppendorf) to remove cellular debris. 50 μL of each media sample was used for the measurement of secreted Tau by a mouse-specific ELISA kit (Thermo Fisher) according to manufacturer’s instructions. The sequence identity between the 2N4R isoforms of mouse and rat Tau is 96%. The mouse Tau ELISA kit was found to detect both mouse and rat Tau. LDH release assay was performed as described above for PCA experiments.

**Immunofluorescence microscopy**

Immunofluorescence microscopy was performed as previously described (Kysenius et al., 2012). Briefly, cells were grown on glass coverslips coated with poly-L-lysine and laminin (Sigma), fixed with 4% PFA in PBS for 20 minutes, and washed with PBS before incubation for 1 hour in blocking buffer (1% BSA, 0.1% gelatin, 5% goat or donkey serum, 0.1% Triton X-100 and 0.05% Tween-20 in PBS). Coverslips were then incubated overnight at +4°C with the primary antibodies diluted 1:500 in 1% BSA, 0.1% gelatin and 5% serum. The primary antibodies used in this study were Tau-5 (mouse monoclonal, Invitrogen), HA (rabbit or mouse monoclonal, Cell Signaling Technology), AT8 (mouse monoclonal, Thermo Scientific), PHF13 (mouse monoclonal, Invitrogen) and T22 (rabbit polyclonal, Sigma-Aldrich). Additional information on antibody validation can be found in Table S2 and Figure S4. The secondary antibodies Cy5 (Jackson Immunoresearch) and Alexa Fluor-conjugated (488-goat-anti-mouse and 568-donkey-antirabbit; Invitrogen) were diluted 1:2000 in PBS and incubated 1 hour at room temperature. Coverslips were finally incubated in Hoechst 33342 (Invitrogen) for nuclear staining before being mounted on microscope slides with Prolong Gold antifade reagent (Invitrogen).

**dSTORM**

Direct stochastic optical reconstruction microscopy (dSTORM) was performed as previously described (Salo et al., 2016). Briefly, cells were grown on MatTek 35 mm dishes coated with poly-L-lysine and laminin (Sigma), fixed with 4% PFA in PBS for 20 minutes, and washed with PBS before incubation for 1 hour in blocking buffer (1% BSA, 0.1% gelatin, 5% goat or donkey serum, 0.1% Triton X-100 and 0.05% Tween-20 in PBS). Coverslips were then incubated overnight at +4°C with the primary antibodies Tutu-5 and HA (Invitrogen) were diluted 1:200 and incubated overnight at +4°C. The secondary antibody Alexa Fluor 647-anti-mouse was diluted 1:500 and incubated at room temperature for 1 hour. During imaging, cells were incubated in blinking buffer (0.1 M Tris buffer, 10% glucose, 0.07% cysteamine, 0.75 mg/ml glucose oxidase and 0.04 mg/ml catalase). The buffer was refreshed approximately every hour. Imaging was performed with Nikon Eclipse Ti-E N-STORM microscope and 30 000 images per cell were acquired. Analysis of the localization of single fluorophores activation was done with N-STORM software (Nikon), and images were further analyzed with ImageJ version 1.49 and compiled with Adobe Photoshop CS4.

**Immunoelectron microscopy**

Cells growing on poly-L-lysine coated coverslips were fixed with PLP fixative (McLean and Nakane, 1974) for 2 hours, washed with NaPO3 buffer and permeabilized with 0.01% saponin for 8 minutes, and incubated 1 hour in room temperature with the primary antibodies Tau-5 and HA (Invitrogen) were diluted 1:200 and incubated overnight at +4°C. The secondary antibody Alexa Fluor 647-anti-mouse was diluted 1:500 and incubated at room temperature for 1 hour. During imaging, cells were incubated in blinking buffer (0.1 M Tris buffer, 10% glucose, 0.07% cysteamine, 0.75 mg/ml glucose oxidase and 0.04 mg/ml catalase). The buffer was refreshed approximately every hour. Imaging was performed with Nikon Eclipse Ti-E N-STORM microscope and 30 000 images per cell were acquired. Analysis of the localization of single fluorophores activation was done with N-STORM software (Nikon), and images were further analyzed with ImageJ version 1.49.

**Western blot and dot blot assay**

Western blotting of media fractions was performed as previously described (Yan et al., 2016). Briefly, fractions were boiled at 80°C for 10 min in NuPAGE loading buffer (Invitrogen) and 0.25% mercaptoethanol before loading on 4%–12% Bis-Tris gel (Invitrogen). Resolved proteins were transferred to a PVDF methanol-activated membrane (GE Healthcare) by semi-dry blotting (Bio-Rad).

For dot blot assays, 2.3 million cells were seeded on 10-cm plates and transfected with 9 μg of phGluc-Tau/HA plasmid per well 24 hours after seeding. Culture media was changed to serum- and phenol red-free (PRF) DMEM (Invitrogen) 24 hours
post-transfection. Conditioned media was collected 24 hours later and cleared from debris by a centrifugation at 1500 x g. The cells were washed twice with ice-cold PBS followed by extraction on ice for 30 min in a buffer containing 10 mM Tris-HCl, pH 6.8, 1 mM EDTA, 150 mM NaCl, 0.25% Nonidet P-40, 1% Triton X-100, PhosStop phosphatase inhibitor, and protease inhibitor mixture tablets (Thermo Scientific). Cell debris was removed by a centrifugation at 13 000 x g. 50 μL of lysate and 100 μL of media were pipetted to a 96-well Manifold Spot-Blot unit (Whatman / Schleicher and Schuell), and protein were trapped by filtration through a methanol-activated PVDF membrane (GE Healthcare). Chemiluminescence signal was detected following incubation of the membrane with primary antibodies and horseradish peroxidase-conjugated secondary antibodies and ECL Western Blotting reagent (Thermo Scientific). ImageStudioLite software was used for quantitative analysis of filter trap assay blots and the ratio between the extracellular and intracellular protein was determined.

**Thioflavin S assay**

Tau conditioned media and mock conditioned media from N2A cells were concentrated 100x with Amicon filters (30 kDa MWCO, Millipore) according to manufacturer’s instructions. 100 μL of samples were incubated with or without 0.5 μM recombinant 0N4R Tau (rPeptide), 1% protease inhibitor cocktail (Thermo Scientific) and DTT for 5 days at 37°C with shaking at 250 rpm. As a positive control, 50 μM heparin was incubated with recombinant Tau in the same conditions. For fluorescence measurement, 10 μM Thioflavin S was added to each sample, incubated 30 min and fluorescence was measured at 440 excitation, 550 emission with a Varioskan Flash multiplate reader (Thermo Scientific).

**Size-exclusion dot blot assay**

Tau-GLuc conditioned media from N2A cells was concentrated in parallel using different molecular weight cut-off filters [30 kDa, 50 kDa, 100 kDa (Amicon/Millipore) and 300 kDa (Sartorius)] by centrifugation. Concentrated retained fraction (that did not pass through the filter) was diluted with PRF-DMEM to match the volume of the pass-through fraction. Both fractions were dot blotted on a PVDF membrane and processed as described above under “Western blot and dot blot assay”. The presence of Tau in the media fractions was assessed by Tau-5 staining.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All values were expressed as mean ± SEM. All statistical analyses were performed in IBM SPSS Statistics software (version 25) and the graphs were compiled in GraphPad Prism software (version 5.0a). The statistical details of experiments can be found in the figure legends. Statistical analyses were performed with unpaired, two-tailed t test or one-way ANOVA followed by Bonferroni post-test (ThioS data) or Dunnett post-test (all other experiments). The choice of one post-test or the other was determined by the need to compare all of the data point to each other, or with the control only. If not otherwise specified, “n” represents the number of independent replication of the entity–interventions. In PCA, each independent replica was the average of four technical replicas, while in ELISA each independent replica was the average of two technical replicas. A minimum of 3 independent replicas was used for the statistical analysis. All p values below 0.05 were considered significant. *p < 0.05, **p < 0.01, ***p < 0.001.