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DnaJ/Hsc70 chaperone complexes control the extracellular release of neurodegenerative-associated proteins

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Abstract

It is now known that proteins associated with neurodegenerative disease can spread throughout the brain in a prionlike manner. However, the mechanisms regulating the trans-synaptic spread propagation, including the neuronal release of these proteins, remain unknown. The interaction of neurodegenerative disease-associated proteins with the molecular chaperone Hsc70 is well known, and we hypothesized that much like disaggregation, refolding, degradation, and even normal function, Hsc70 may dictate the extracellular fate of these proteins. Here, we show that several proteins, including TDP-43, α-synuclein, and the microtubule-associated protein tau, can be driven out of the cell by an Hsc70 co-chaperone, DnaJC5. In fact, DnaJC5 overexpression induced tau release in cells, neurons, and brain tissue, but only when activity of the chaperone Hsc70 was intact and when tau was able to associate with this chaperone. Moreover, release of tau from neurons was reduced in mice lacking the *DnaJC5* gene and when the complement of DnaJs in the cell was altered. These results demonstrate that the dynamics of DnaJ/Hsc70 complexes are critically involved in the release of neurodegenerative disease proteins.

Keywords DnaJ; extracellular; Hsc70; neurodegeneration; tau
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Introduction

The accumulation of pathological proteins associated with neurodegenerative disease, such as the microtubule-associated protein tau, α-synuclein, and huntingtin, follows distinct patterns, suggesting a spreading phenomenon (Braak & Braak, 1991; Spillantini *et al*, 1997; Guo & Lee, 2011; Hansen *et al*, 2011; de Calignon *et al*, 2012; Liu *et al*, 2012; Brettschneider *et al*, 2014; Polydoro *et al*, 2014; Babcock & Ganetzky, 2015; Iba *et al*, 2015). Despite accumulating evidence that neurodegenerative disease-associated proteins are taken up by neurons and spread cell–cell through the brain (Holmes *et al*, 2013; Dujardin *et al*, 2014; Pecho-Vrieseling *et al*, 2014; Calafate *et al*, 2015), there is no clear consensus that these neurodegenerative proteins behave in a canonical prionlike manner (Wegmann *et al*, 2015). Further, the mechanisms regulating the release of these proteins into the extracellular space remain unknown. We speculated that chaperone proteins could play a key part in this process.

The interaction of neurodegenerative disease-associated proteins with the molecular chaperone Hsc70 in particular is well known (Auluck *et al*, 2002; Dou *et al*, 2003; Sarkar *et al*, 2008; Jinwal *et al*, 2010, 2013; Pemberton *et al*, 2011; Yu *et al*, 2014; Monsellier *et al*, 2015). Hsc70 is abundant in metazoans and constitutively expressed in cells (Finka & Goloubinoff, 2013). It is responsible for a myriad of processes to maintain proteostasis in the cell with roles in nascent polypeptide folding (Beckmann *et al*, 1990), protein degradation (Bercovich *et al*, 1997), and clathrin-mediated endocytosis (Chappell *et al*, 1986; Black *et al*, 1991). Many of the important Hsc70 functions are coordinated by a complement of co-chaperones including the DnaJ proteins (Cheetham *et al*, 1992, 1996). DnaJ proteins are structurally diverse, yet all contain a common J-domain which coordinates Hsc70 binding via a HPD motif (Hennessy *et al*, 2000).

Hsc70/DnaJ complexes control refolding, disaggregation (Gao *et al*, 2015; Nillegoda *et al*, 2015), degradation (Jinwal *et al*, 2013; Fontaine *et al*, 2015a), and even normal function (Jinwal *et al*, 2010; Fontaine *et al*, 2015a) of neurodegenerative disease-associated proteins. We hypothesized that an Hsc70/DnaJ interaction may dictate the extracellular fate of these proteins. A logical

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candidate for such a DnaJ is DnaJC5, a protein known to promote exocytosis (Jacobsson & Meister, 1996; Chamberlain & Burgoyne, 1997, 1998). DnaJC5 is a small, neuroprotective protein (Fernández-Chacón *et al*, 2004; Chandra *et al*, 2005; García-Junco-Clemente *et al*, 2010; Tiwari *et al*, 2015) which chaperones SNAP-25 to promote SNARE-mediated exocytosis (Burré *et al*, 2010; Sharma *et al*, 2011) and is important in calcium sensitivity of synapses (Umbach & Gundersen, 1997; Seagar *et al*, 1999; Graham & Burgoyne, 2000; Ruiz *et al*, 2008; Weng *et al*, 2009; Rozas *et al*, 2012; Chiang *et al*, 2014). We hypothesized that a DnaJC5/Hsc70 complex could contribute to the extracellular release of neurodegenerative disease-associated proteins.

Results

We first assessed whether DnaJC5 might regulate extracellular tau release, as the interaction with Hsc70 and tau is well described by our laboratory (Jinwal *et al*, 2010, 2013; Abisambra *et al*, 2013; Fontaine *et al*, 2015a,b). Using a cell model, proteins were overexpressed in HEK293T cells for 42 h, then cells were washed to remove traces of serum proteins prior to incubation in serum-free media for 6 h to assay extracellular release, as detailed in Fig 1A. The conditioned media were free of intracellular contamination, and for mass spectroscopy analyses, vesicular-bound proteins were neither liberated nor digested. Overexpression of DnaJC5 significantly facilitated extracellular tau release, as determined both by dot blot, Western blot, and by quantitative mass spectroscopy using ^{15}N tau as an internal standard (Figs 1A–C and EV1). However, intracellular tau levels were decreased in cells overexpressing DnaJC5 (Fig 1B). To determine whether this was due to DnaJC5-mediated decreases in translation, we performed qRT–PCR on cells overexpressing DnaJC5 and tau. mRNA production of tau was unchanged in DnaJC5-overexpressing cells compared to control (Fig EV2A), suggesting reductions in intracellular tau levels were not due to aberrations in protein translation. Alternatively, intracellular tau reductions due to DnaJC5 overexpression could be attributed to degradation. Cells were treated with epoxomicin for 6 h to block proteasomal degradation, but inhibiting protein degradation did not prevent intracellular tau reductions (Fig EV2B). Finally, overexpression of DnaJC5 was non-toxic by multiple measures (Fig EV2C). Together, these data indicate the reduction in intracellular tau levels following DnaJC5 overexpression was caused by enhanced extracellular release.

Previous reports have shown that endogenous tau release is a normal, physiological process in the brain (Pooler *et al*, 2013; Yamada *et al*, 2014), thereby suggesting endogenous tau should also

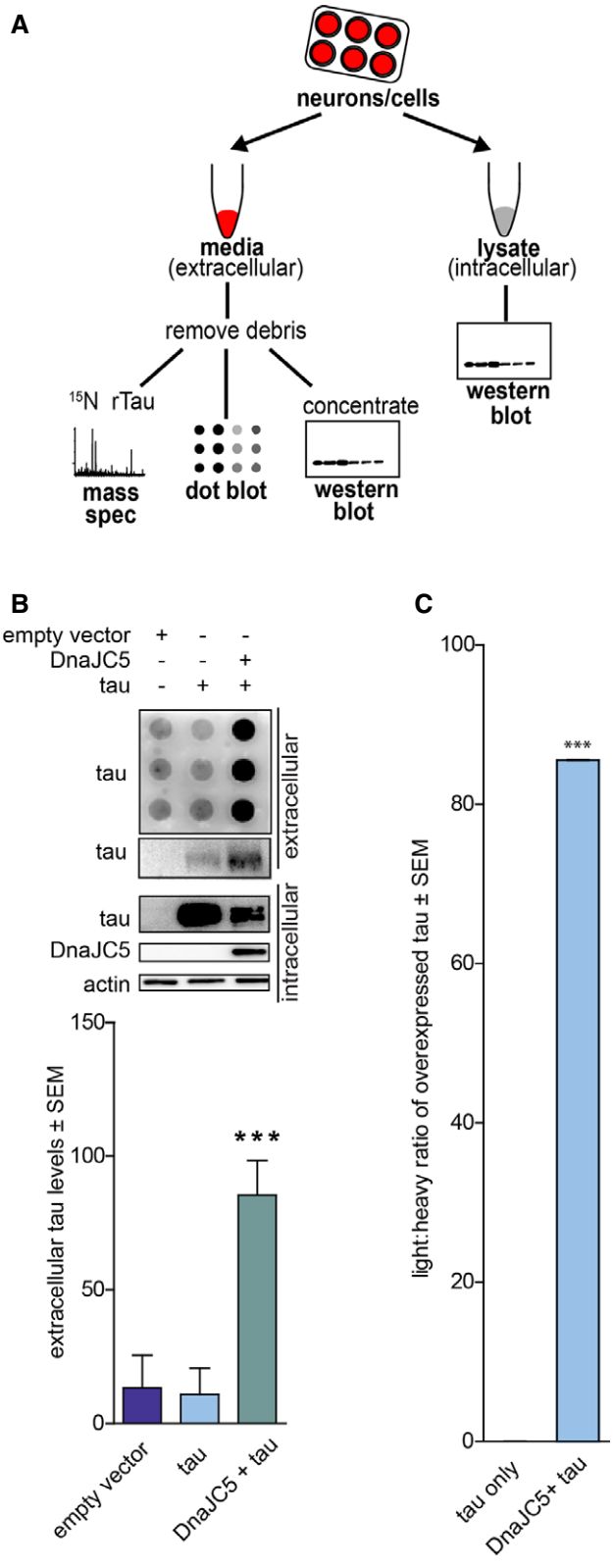

Figure 1. DnaJC5 overexpression facilitates tau release into the extracellular space.

A Schematic illustrating the preparation of samples for media analysis by dot blot/Western blot or mass spectroscopy.

B Dot blot of tau levels in media from HEK293T cells overexpressing tau and FLAG-DnaJC5 compared to empty vector and tau. Intracellular protein levels are shown by Western blot; DnaJC5 was detected by FLAG antibody. Quantification of extracellular tau levels is shown below; data are mean ± SEM, $n = 8$, $^{***}P < 0.001$.

C Light/heavy ratio of tau peptides in label-free mass spectroscopy of media from HEK293T cells overexpressing tau or tau and DnaJC5 compared to 100 ng ^{15}N-labeled recombinant tau. Light peptides represent endogenous tau peptides. Data are mean ± SEM, $n = 3$ biological repeats, $^{***}P < 0.001$.

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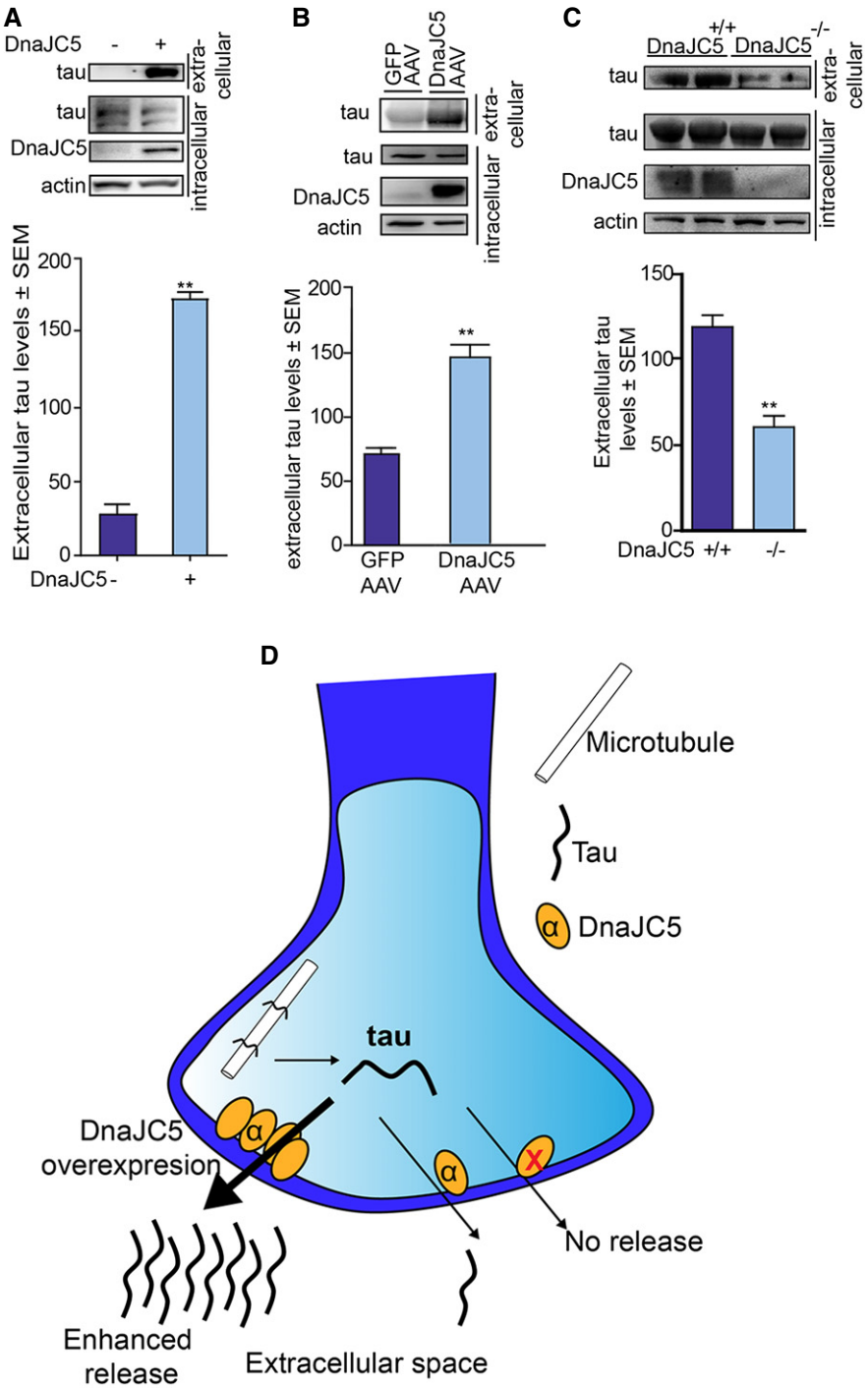

Figure 2. Release of tau is regulated by DnaJC5.

A Western blot of endogenous tau levels from M17 neuroblastoma cells overexpressing FLAG-DnaJC5 or empty vector. Media were concentrated prior to analysis. Intracellular DnaJC5 was detected with FLAG antibody. Quantification of mean extracellular tau levels ± SEM, $n = 3$, $^{**}P < 0.01$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

B Western blot tau levels from concentrated media from *ex vivo* organotypic slice cultures of wild-type mice overexpressing GFP-AAV9 or FLAG-DnaJC5-AAV9. DnaJC5 is detected by FLAG antibody. Quantification of mean extracellular tau levels ± SEM, $n = 3$, $^{**}P < 0.01$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

C Western blot tau levels from concentrated media from *ex vivo* organotypic slice cultures of $Cspa^{+/+}$ or $Cspa^{-/-}$ mice. DnaJC5 was detected by DnaJC5 antibody (Synaptic Systems). Quantification of mean extracellular tau levels ± SEM, $n = 3$, $^{**}P < 0.01$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

D Schematic showing that lack of DnaJC5 (yellow circles) reduces extracellular tau release, whereas overexpression of DnaJC5 results in enhanced release of tau.

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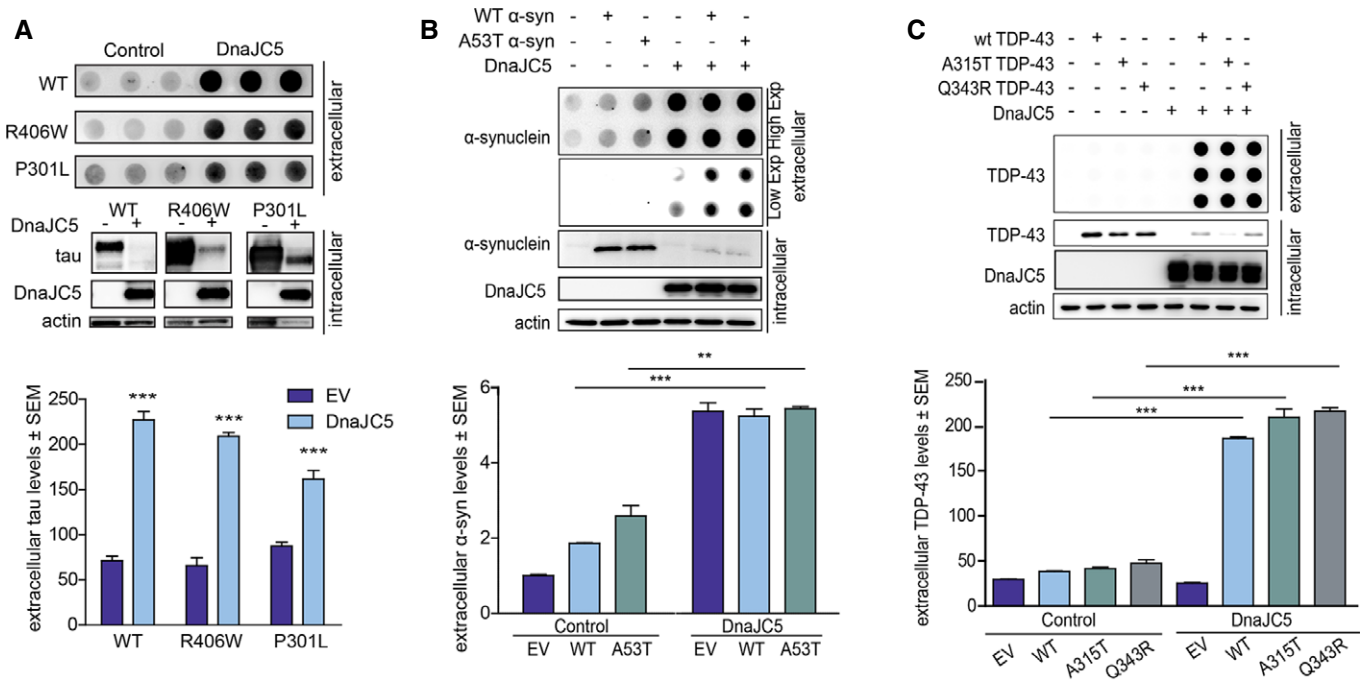

Figure 3. DnaJC5 facilitates the release of neurodegenerative-associated proteins into the extracellular space.

A FLAG-DnaJC5 overexpression facilitates extracellular release of wild-type (WT), P301L, and R406W tau by dot blot in HEK293T cells. Intracellular protein levels are shown by Western blot; DnaJC5 was detected by FLAG antibody. Quantification of extracellular tau levels is shown below; data are mean ± SEM, $n = 3$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

B Overexpression of FLAG-DnaJC5 induces extracellular release of wild-type (WT) and A53T α-synuclein in HEK293T cells. Extracellular protein levels are shown by dot blot (quantification below, mean ± SEM, $n = 3$, $^{***}P < 0.001$, $^{**}P < 0.01$ by one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis), and intracellular proteins levels were analyzed by Western blot; DnaJC5 was detected by FLAG antibody.

C DnaJC5 overexpression induces extracellular release of GFP-tagged wild-type (WT), A315T, and Q343R TDP-43 in HEK293T cells. Intracellular DnaJC5 was detected by FLAG antibody. Extracellular protein levels were analyzed by dot blot (quantification below, mean ± SEM, $n = 4$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis).

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be released by DnaJC5. Indeed, DnaJC5 overexpression facilitated release of endogenous tau from M17 neuronal cell lines and organotypic brain slice cultures from wild-type mice (Fig 2A and B). Endogenous tau was co-localized with the pre-synaptic proteins SNAP25 and synaptophysin in neurons (Fig EV3A, B, D and E) and DnaJC5 overexpression reduced levels of this pre-synaptic tau (Fig EV3C and F), suggesting that DnaJC5 facilitates the release of this "primed" pre-synaptic tau from neurons. To confirm that DnaJC5 is responsible for this enhanced release, extracellular tau levels were analyzed from organotypic slices prepared from mice lacking DnaJC5 (Fernández-Chacón *et al*, 2004; Sharma *et al*, 2012) (*Cspα*$^{-/-}$ mice). DnaJC5/*Cspa*$^{-/-}$ had significantly reduced extracellular tau levels (Fig 2C), thereby confirming that DnaJC5 is a critical mediator of extracellular tau release (Fig 2D).

Not only is extracellular tau found in patients with tauopathies such as Alzheimer's disease, frontotemporal dementia, and other tauopathies (Medina & Avila, 2014), tau pathology is known to spread via synaptically connected brain regions. Therefore, it is likely that extracellular tau plays a critical role in the pathophysiology of these neurodegenerative diseases. DnaJC5-induced release of tau was relevant to disease-associated forms, as DnaJC5 not only facilitated the secretion of wild-type tau, but also mutant tau species associated with tauopathies (Fig 3A). Trans-neuronal spread and release of disease-associated proteins has been described in other diseases as well, including synucleinopathies (Desplats *et al*, 2009; Hansen *et al*, 2011; Masuda-Suzukake *et al*, 2013), polyQ disorders (Pecho-Vrieseling *et al*, 2014; Babcock & Ganetzky, 2015; Pearce *et al*, 2015), and amyotrophic lateral sclerosis (ALS) (Kassubek *et al*, 2014). We speculated that DnaJC5 overexpression could also facilitate the release of some other pathological proteins associated with neurological disease. Indeed, in addition to tau, DnaJC5 facilitated the release of some other disease-associated proteins, such as wild-type and mutant TDP-43 (Fig 3B) and α-synuclein proteins in HEK293T (Fig 3C), but not a polyQ-25 fragment (Fig EV2D), suggesting some level of client selectivity for this process. As with tau, overexpression of DnaJC5 did not reduce mRNA levels of either α-synuclein or TDP-43 (Fig EV2A) and distribution of these proteins in neuronal cells revealed expected subcellular localization without extravagant aggregation (Fig EV4). These results indicate that DnaJC5 could facilitate a common mechanism for the release of neurodegenerative proteins into the extracellular space.

While DnaJC5 and Hsc70 have a known role in synaptic exocytosis through SNAP-25, HEK cells do not express SNAP-25. Despite this, DnaJC5 could facilitate the release of neurodegenerative proteins from these non-neuronal cells. But when DnaJC5 was

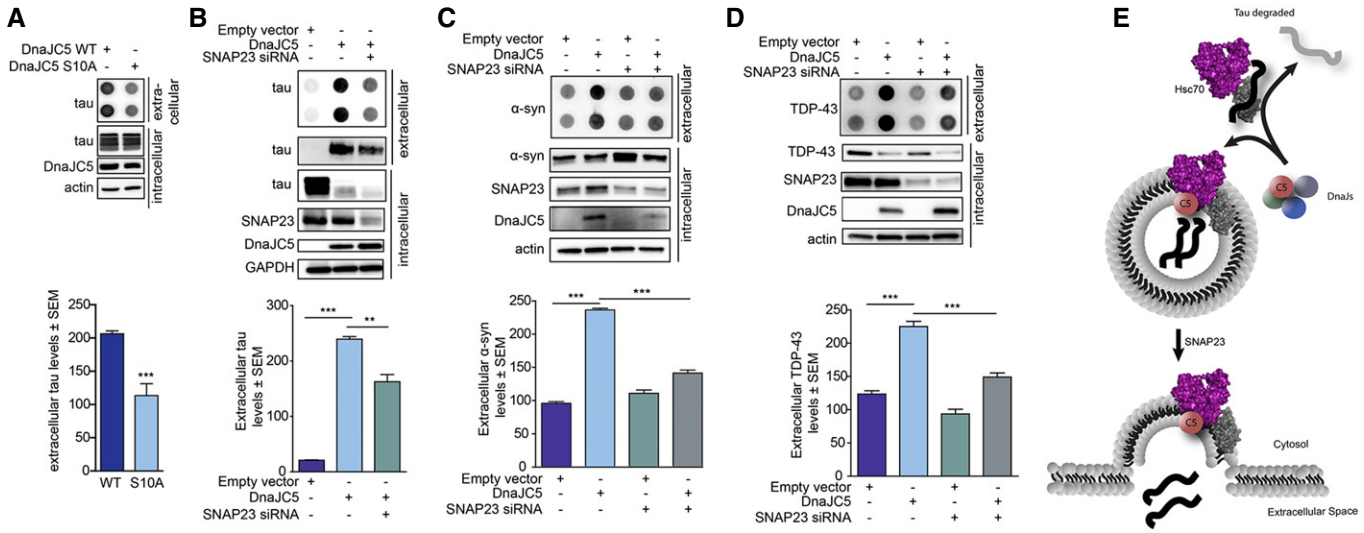

Figure 4. DnaJC5 participates in an alternative exocytosis pathway important for extracellular release.

A Dot blot of tau levels in media of cells overexpressing the phospho-deficient FLAG-DnaJC5 S10A compared to FLAG-DnaJC5 WT. Intracellular levels are shown by Western blot; DnaJC5 was detected by FLAG antibody. Quantification of mean tau levels ± SEM, $n = 6$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

B Knockdown of SNAP23 by siRNA inhibits DnaJC5-mediated tau release in HEK293T cells as shown by dot blot. Quantification of mean extracellular levels ± SEM, $n = 3$, $^{**}P < 0.01$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis. DnaJC5 was detected by FLAG antibody.

C Knockdown of SNAP23 by siRNA inhibits DnaJC5-mediated α-synuclein release in M17 cells as shown by dot blot. Quantification of mean extracellular levels ± SEM, $n = 3$, $^{***}P < 0.001$ by one-way ANOVA with a Tukey's *post hoc* analysis. DnaJC5 was detected by FLAG antibody.

D Knockdown of SNAP23 by siRNA inhibits DnaJC5-mediated TDP-43 release in M17 cells as shown by dot blot. Quantification of extracellular levels is mean ± SEM, $n = 3$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis. DnaJC5 was detected by FLAG antibody.

E Schematic representation of Hsp70 control of DnaJC5-mediated tau release. DnaJ selection on Hsc70 dictates client fate: if a pro-degradation DnaJ binds to Hsc70, clients are sent for degradation. If DnaJC5 binds to Hsc70, client proteins are sent for release by a SNAP23-dependent pathway.

Source data are available online for this figure.

mutated in a way that reduced its exocytotic function (Evans *et al*, 2001), tau release was again abrogated even in HEK cells (Fig 4A). Thus, we hypothesized that a non-canonical exocytosis mechanism was being engaged by DnaJC5. We speculated that the ubiquitously expressed SNARE, SNAP-23, which is known to be involved in alternative exocytosis pathways including lysosomal exocytosis (Rao *et al*, 2004; Boddul *et al*, 2014) and is important for the extracellular release of a variety of molecules including metalloproteases, chemokines, and renin (Kean *et al*, 2009; Frank *et al*, 2011; Mendez & Gaisano, 2013), could be involved in this process. Indeed, siRNA-mediated knockdown of SNAP-23 blocked DnaJC5-mediated extracellular release of tau (Fig 4B). Moreover, α-synuclein (Fig 4C) and TDP-43 (Fig 4D) release were also blocked by SNAP-23 siRNA in M17 neuronal cells. Thus, DnaJC5-stimulated release of neurodegenerative proteins occurs through a non-canonical, SNAP-23-mediated exocytosis pathway, perhaps acting as a release valve for neurodegenerative proteins (Fig 4E).

We next sought to identify the mechanism by which DnaJC5 was enhancing this extracellular triage of these neurodegenerative proteins. In wild-type primary neurons, DnaJC5-AAV overexpression enhanced the association of tau with Hsc70 at the pre-synapse (Fig 5A and B), suggesting that DnaJC5, tau, and Hsc70 were in complex at the synapse. This arrangement is spatially possible as we have previously demonstrated that tau binds to the substrate-binding domain (SBD) of Hsc70 and DnaJC5 binds to a separate region in the nucleotide-binding domain (NDB) (Fontaine *et al*, 2015a). Immunoprecipitation revealed that Hsc70 and DnaJC5 can bind to tau, confirming that this tripartite complex can form (Fig 5C). Therefore, we sought to disrupt this complex and determine whether we could also prevent DnaJC5-mediated tau release. YM-01 is a small molecule inhibitor of Hsc70 which binds to the DnaJ-binding region in the NBD of Hsc70 and inhibits the allosteric inter-domain communication that controls Hsc70 activity (Rousaki *et al*, 2011). This compound does not inhibit the Hsc70–tau interaction, as tau binds to the substrate-binding domain of Hsc70 (Fontaine *et al*, 2015a), but it can compete for DnaJ binding (Abisambra *et al*, 2013). DnaJC5 was immunoprecipitated from cell lysates, then treated with increasing doses of YM-01. Both Hsc70 and tau binding to DnaJC5 decreased with increasing concentrations of YM-01 (Fig 5D). We were also able to determine that the dominant-negative Hsc70 variant E175S, which lacks Hsc70 activity yet can still bind tau (Fontaine *et al*, 2015b), was able to disrupt this complex as well (Fig 5E), thereby demonstrating that both genetic inhibition and chemical inhibition of Hsc70 disrupt this complex.

We then tested the effects of YM-01 in neuronal cells overexpressing DnaJC5. M17 cells were treated with 10 μM YM-01 for 6 h, and the media were analyzed by dot blot. In agreement with previously published studies, YM-01 treatment reduced intracellular tau levels via proteasomal degradation (Abisambra *et al*, 2013) and also reduced DnaJC5-mediated extracellular tau release (Fig 5F). Further, while AAV-mediated DnaJC5 overexpression facilitated tau release in neurons from transgenic mice overexpressing mutant

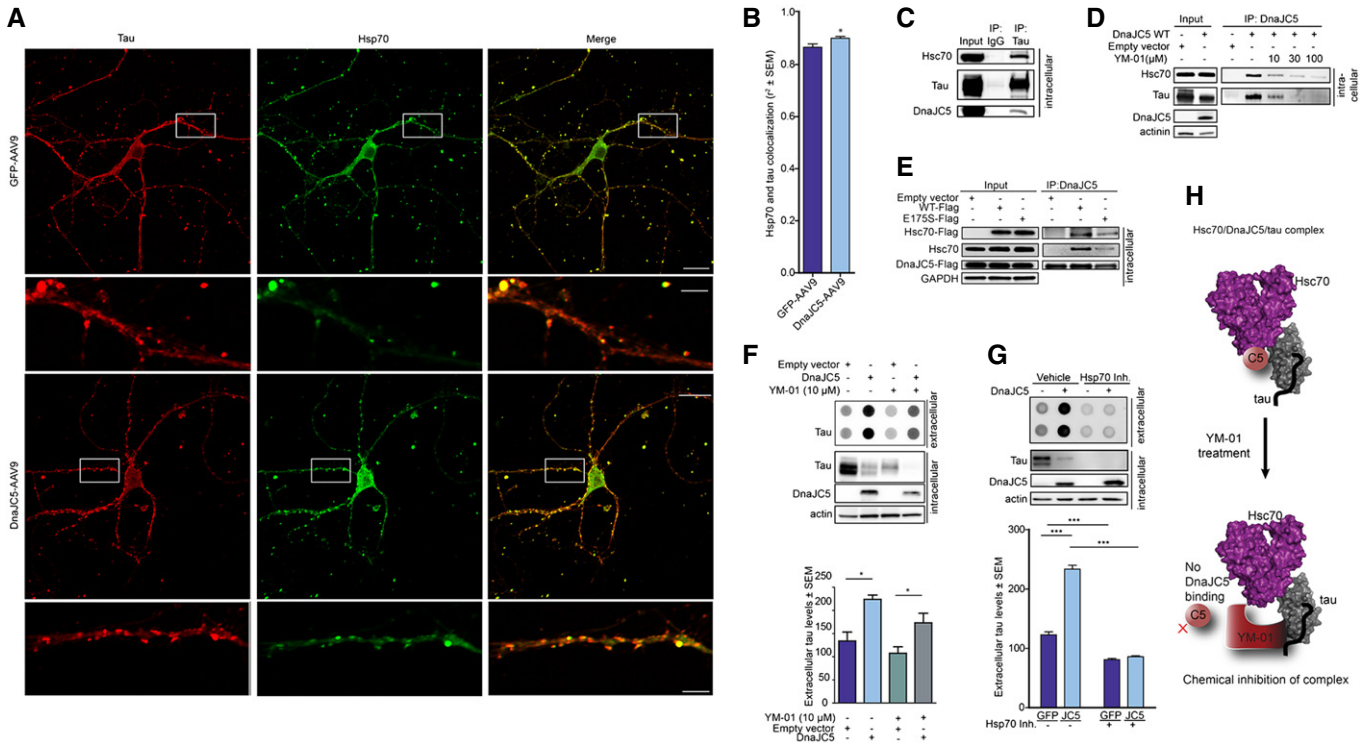

Figure 5. Tau release is controlled by chaperones.

A Confocal micrographs of tau (red pseudocolor) and Hsp70 (green pseudocolor) colocalization (merged image) levels in wild-type primary neurons overexpressing GFP-AAV9 or FLAG-DnaJC5-AAV9. Boxed area are 5× digital zoom on a 60× lens shown below. Scale bars are 20 μm and 5 μm (insets). Only neurons overexpressing virus were imaged.

B Quantification of tau-Hsp70 co-localization (mean Pearson's coefficient ± SEM, $n = 18$, $^*P < 0.05$, one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis).

C Western blot of immunoprecipitated tau from HEK293T reveals tau exists in an intracellular complex with DnaJC5 and Hsc70. DnaJC5 was detected with FLAG antibody.

D Western blot of HEK293T intracellular lysates shows the DnaJC5/Hsc70/tau complex is disrupted by the addition of increasing amounts of YM-01 (0, 10, 30, 100 μM) in a dose-dependent fashion. DnaJC5 was detected by FLAG antibody.

E The DnaJC5/Hsc70 complex is disrupted when Hsc70 activity is inhibited by a dominant-negative (DN) point mutation, E175S. Western blot of immunoprecipitated DnaJC5 from HEK293T cells overexpressing WT or DN E175S Hsc70. DnaJC5 was immunoprecipitated by DnaJC5 antibody (Synaptic Systems). DnaJC5 was detected on Western blot by FLAG antibody.

F Dot blot of tau levels in media of M17 neuroblastoma cells treated with YM-01. Quantification of mean extracellular tau levels ± SEM, $n = 6$, $^*P < 0.05$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis. Intracellular levels shown by Western blot. DnaJC5 was detected on Western blot by FLAG antibody.

G Dot blot of tau levels in media of primary neurons from transgenic overexpressing tau P301L mice treated with YM-01 (10 μM). FLAG-DnaJC5 was transduced into neurons using AAV9. Quantification of mean extracellular tau levels ± SEM, $n = 6$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis. DnaJC5 was detected on Western blot by FLAG antibody.

H Schematic illustrating that the Hsc70/DnaJC5/tau complex can be disrupted by chemical (YM-01) inhibition.

Source data are available online for this figure.

human P301L tau (Fig 5G), treating neurons with YM-01 blocked this DnaJC5-enhanced release (Fig 5G). Thus, disruption of the Hsc70/DnaJC5 complex reduced extracellular tau release in neuronal cells (Fig 5G).

Similarly, DnaJC5-mediated tau release was reduced in a dose-dependent manner in HEK293T cells following YM-01 treatment (Fig 6A), confirming that this mechanism is shared among neuronal and non-neuronal cell types. YM-01 also disrupted the release of α-synuclein caused by DnaJC5 (Fig 6B). While TDP-43 release did not respond well to YM-01 treatment (not shown), a drug that primarily acts in the cytosol (Abisambra *et al*, 2013), it may that the TDP-43 client changes the Hsc70/DnaJC5 complex rendering it insensitive to YM-01. This would be consistent with our previous work showing that client binding can control chaperone complex activity to some extent (Jinwal *et al*, 2013) and suggests the TDP-43-induced structural alterations in Hsc70 were rendered incompatible with YM-01 binding. It could also indicate an entirely distinct process related to trafficking of cytosolic TDP-43 that remains unknown, yet proceeds through a SNAP-23-mediated mechanism.

The high transfection efficiency of HEK293T cells allowed for the overexpression of a dominant-negative form of Hsc70 (E175S) that mimics Hsc70 when it is inhibited by YM-01 (Fontaine *et al*, 2015b). This mutant also abrogated tau release by DnaJC5

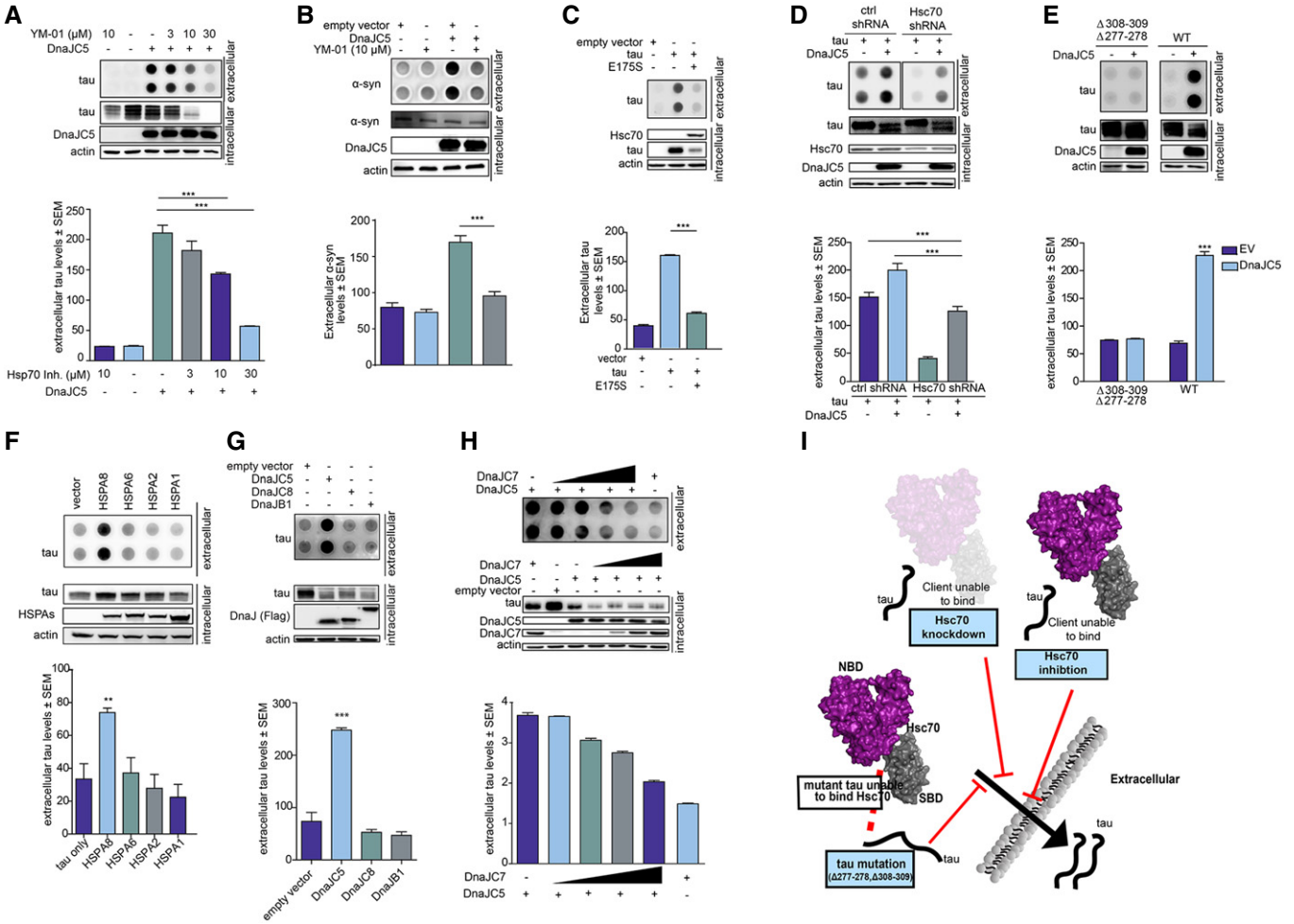

Figure 6. The Hsc70 chaperone is required for DnaJC5-mediated release.

A Chemical inhibition of Hsc70 activity in HEK293T cells overexpressing FLAG-DnaJC5 reduces extracellular tau levels as shown by dot blot. Intracellular levels are shown by Western blot; DnaJC5 was detected by FLAG antibody. Quantification of extracellular tau levels is shown as average ± SEM, $n = 3$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

B Chemical inhibition of Hsc70 activity in HEK293T cells overexpressing FLAG-DnaJC5 reduces extracellular synuclein levels as shown by dot blot. Intracellular levels are shown by Western blot; DnaJC5 was detected by FLAG antibody. Quantification of extracellular synuclein levels is shown as average ± SEM, $n = 3$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

C Dominant-negative Hsc70 E175S blocks tau release when overexpressed in HEK293T cells as shown by dot blot. Intracellular levels are shown by Western blot. Quantification of extracellular tau levels is shown as average ± SEM, $n = 4$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

D Knockdown of Hsc70 reduces extracellular tau release (dot blot) in HEK293T cells overexpressing FLAG-DnaJC5. Intracellular levels are shown by Western blot; DnaJC5 was detected by FLAG antibody. Quantification of extracellular tau levels is shown as average ± SEM, $n = 3$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

E A mutant tau that does not bind Hsc70 is not released when FLAG-DnaJC5 is overexpressed in HEK293T cells. Dot blot and quantification of extracellular tau levels are shown. Intracellular protein levels are shown with representative Western blot; DnaJC5 was detected by FLAG antibody. Data are mean ± SEM, $n = 3$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

F Dot blot of Hsp70 isoform overexpression effect on extracellular tau release in HEK293T cells. Intracellular protein levels are shown with representative Western blot. HSPA variants were detected with V5 antibody. Data are mean ± SEM, $n = 3$, $^{**}P < 0.01$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

G Dot blot of different FLAG-DnaJ overexpression effect on extracellular tau release in HEK293T cells. Intracellular protein levels are shown with representative Western blot. DnaJ levels were detected by FLAG antibody. Data are mean ± SEM, $n = 3$, $^{***}P < 0.001$ one-way analysis of variance with Tukey's multiple comparison *post hoc* analysis.

H Dot blot of increasing FLAG-DnaJC7 levels on FLAG-DnaJC5 overexpression-mediated extracellular tau release in HEK293T cells. Intracellular protein levels are shown with representative Western blot; DnaJ proteins were detected by FLAG antibody. Quantification of extracellular levels are mean ± SEM, $n = 4$.

I Schematic representation of Hsp70 control of DnaJC5-mediated tau release. When clients are unable to bind Hsc70, or if Hsc70 is inactive or knocked down, client proteins are sent for degradation instead of release. Thus, Hsc70 is important for upstream control of the DnaJC5-mediated extracellular release.

Source data are available online for this figure.

(Fig 6C), highlighting that Hsc70 activity can control this DnaJC5-mediated process for tau. To identify whether Hsc70 acted upstream of DnaJC5 in facilitating extracellular protein release, Hsc70 levels were knocked down via shRNA (Fig 6D). Reducing Hsc70 levels indeed blocked DnaJC5-mediated extracellular tau release (Figs 6D and EV5). Finally, when tau was mutated to prevent its interaction with Hsc70 (Sarkar *et al*, 2008), tau release was reduced (Fig 6E), indicating that tau must be able to interact with Hsc70 in order to be released by the complex, and also confirming that DnaJC5 does not indiscriminately triage all overexpressed protein for release.

We next wondered whether other DnaJ/Hsp70 family complexes in addition to Hsc70 and DnaJC5 could also facilitate tau release. However, of the cytosolic Hsp70 variants, only Hsc70/HSPA8 facilitated tau release when overexpressed (Fig 6F). Similarly, while overexpression of other DnaJ proteins reduced intracellular tau levels, none facilitated tau release like DnaJC5 (Fig 6G). In fact, we suspected that shifting the balance of DnaJ proteins could also shift tau triage away from release and toward degradation. Indeed, increasing the expression of the pro-degradation DnaJC7 dose-dependently blocked DnaJC5-mediated release, instead facilitating tau degradation (Fig 6H). Thus, the DnaJ and chaperone landscape within the cell not only controls protein folding and degradation, but also its release to the extracellular space (Fig 6I).

Discussion

Hsc70/DnaJ complexes are critical for mediating many aspects of the triage of neurodegenerative proteins, including their disaggregation (Gao *et al*, 2015; Nillegoda *et al*, 2015), stabilization (Abisambra *et al*, 2012), degradation (Jinwal *et al*, 2013; Fontaine *et al*, 2015b), and now, release. These findings suggest that neurodegenerative disease-associated protein exocytosis from plasma and synaptic membranes is a regulated and physiological process that is still not well understood. The implication that chaperones, particularly the DnaJ/Hsc70 machinery, govern this process is not surprising.

These data illuminate a non-canonical pathway to extracellular release mediated by a SNAP23-dependent mechanism, suggesting a novel route for the release of tau from the neuron. SNARE proteins coordinate vesicle trafficking and exocytosis in all cells (Morelli *et al*, 2014; Rogers *et al*, 2014; Dubuke *et al*, 2015; Kuster *et al*, 2015; Zhu *et al*, 2015), and chaperones play an important role in several of these processes (Joglekar & Hay, 2005; Sharma *et al*, 2011). This has a number of important implications for understanding the propagation of pathological protein aggregates found in several neurodegenerative diseases. SNAP-23 expression is critical for survival (Suh *et al*, 2011; Kaul *et al*, 2015), and found highly expressed in several tissues and both neuronal and non-neuronal cells (Grant *et al*, 1999; Delgado-Martínez *et al*, 2007; Mandolesi *et al*, 2009). While highly analogous to SNAP-25, the SNARE involved in classical neurotransmitter release, SNAP-23 is unique in that it cannot completely rescue SNAP-25 release function in null mice and is not restricted to the synaptic active zone (Delgado-Martínez *et al*, 2007). Thus, it is possible that extracellular protein release via a SNAP-23 may arise in response to overwhelming protein burden of aggregation-prone proteins. Hsc70 is known to direct neurodegenerative clients for degradation by both proteasomal and lysosomal mechanisms. In multicellular organisms, senescent cells in particular sequester misfolded protein aggregates for lysosomal-mediated degradation. This pathway may become problematic if lysosome function becomes compromised (Fornai *et al*, 2008; Poehler *et al*, 2014; Bae *et al*, 2015) or the protein burden is too high, activating a non-conventional secretion process, such as lysosomal exocytosis, or secretory autophagy ("exophagy") (Broadwell & Balin, 1985; Ejlerskov *et al*, 2013). This release may be used as a mechanism of last resort to eject toxic multimers for processing by phagocytic cells. Over time, this degradation pathway itself may become overwhelmed, which then facilitates propagation of these disease proteins through both neurons and glial cells in the brain (Asai *et al*, 2015; Pearce *et al*, 2015).

But how neurodegenerative disease-associated clients enter vesicles for release remains unclear. Since Hsc70 directly interacts with the plasma membrane (Arispe *et al*, 2002) and is known to facilitate the entry of client proteins into vesicular organelles (Bandyopadhyay *et al*, 2008), it is possible that Hsc70 (Fig 6D) is simply delivering neurodegenerative clients directly into the endosomal lumen for eventual extracellular sorting (Sahu *et al*, 2011). Whether this mechanism then directs a constructive or a destructive fate for neurodegenerative-associated proteins remains unknown. It is possible that prionlike proteins are used by neurons to communicate with each other through a non-genetic process (Li & Lindquist, 2000; Shorter & Lindquist, 2006), allowing for rapid communication about a changing environment within a network of multiple coexisting tissues. This process becomes pathogenic when neurodegenerative prionlike proteins misfold, allowing disease-causing conformers to then propagate from neuron to neuron, or neuron–glia–neuron, a process that has been shown in *S. cerevisiae* and other model systems (Tuite *et al*, 2011; Frederick *et al*, 2014). It may be that Hsc70 chaperoning plays an integral role in this communication process, but this remains to be seen.

These data indicate for the first time that there is a unifying, chaperone-dependent mechanism for the release of pathogenic proteins in human neurodegenerative diseases. Further work to delineate subtleties of this mechanism and whether it is for productive or destructive purposes will determine how it contributes to the propagation of these proteins through the brain.

Materials and Methods

Reagents, plasmids, and antibodies

Unless stated otherwise, all chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) including the Hsp70 inhibitor YM-01. The following antibodies were purchased commercially: Hsc70 (Stressgen, Enzo Life Sciences, Farmingdale, NY, USA), tauH150, tau V20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin, α-synuclein, and M2-Flag (Sigma-Aldrich) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TDP-43 (Cell Signaling Technologies, Danvers, MA, USA), SNAP25, synaptophysin, CSPα, SNAP23 (Synaptic Systems). Tau12 and Tau5 were a kind gift from the laboratory of Lester Binder, and PHF1 was a kind gift from Peter Davies.

For mammalian expression experiments, full-length human Hsc70 and rat DnaJC5 (identical amino acid sequence to human

DnaJC5) genes were cloned into pCMV6 (Origene, Rockville, MD, USA) vector with an N-terminal FLAG tag to distinguish between endogenous and overexpressed isoforms. Plasmids for other HSPA isoforms were purchased from Addgene. WT 4R0N tau was cloned as previously described (Jinwal *et al*, 2013). WT, Q434R, and A315T TDP43 plasmids were a kind gift from Benjamin Wolozin. α-Synuclein wt and A53T plasmids were a kind gift from Daniel Lee. Plasmids were cloned into pCMV6 before use for consistency and sequence verified before use. Mutations were introduced via site-directed mutagenesis (Stratagene, La Jolla, CA, USA) and sequences verified before use.

Expression and purification of recombinant ^{15}N-labeled tau

Wild-type 4R0N Tau in pET28a was purified as described previously (Fontaine *et al*, 2015b). Briefly, plasmids were transformed into *E. coli* OneShot BL21 Star (DE3) cells (Life Technologies, Carlsbad, CA, USA) and cultured in M9 minimal media supplemented with 1 g/l ^{15}N-ammonium chloride, 100 μM calcium chloride, 100 μM magnesium sulfate, 0.4% (w/v) glucose, and trace metals and vitamins, induced with 1 mM isopropyl β-D-1-thiogalactopyranoside, and proteins were expressed at 37°C for 3 h. The cells were then harvested, and purification of protein from this resuspension was executed as described previously (Jinwal *et al*, 2013).

Mass spectroscopy

Media were collected from cells, concentrated using a 10,000 MWCO centrifugation filter (Millipore) then processed via filter-aided sample preparation (FASP) as previously described (Wiśniewski *et al*, 2009; Bell-Temin *et al*, 2013). After trypsin digestion using this procedure, samples were analyzed. Proteins were digested with trypsin (Promega) at 1:50 (w:w, enzyme:protein) overnight at 37°C. Peptides were desalted using C18 columns (The Nest Group) and dried in a vacuum centrifuge. Peptide samples were resuspended in 0.1% formic acid in H_2O and analyzed on a Q-Exactive Plus with a 50-cm UPLC column using a 2-h gradient (2–40% acetonitrile) on an EASY-nLC 1000 system (Thermo Fisher). The data-dependent mode of acquisition was utilized in which the top 10 most intense precursor ions were selected for collision-induced dissociation over the course of a 180-min linear HPLC gradient (to 40% acetonitrile). Dynamic exclusion was set for 45 s after one repeat count with a 50 exclusion list size. MS/MS spectra were extracted, and raw files were searched against the most current *Mus musculus* or *Homo sapien*s protein sequence database from UniprotKB using Maxquant (version 1.5.0.30) and Mascot Distiller (version 2.5.1.0) with ^{15}N for quantification of labeled tau. Search parameters included the constant modification of cysteine by carbamidomethylation and variable modification of methionine oxidation. Statistical analysis was performed using Perseus software. Mascot score cutoffs were adjusted to achieve a false discovery rate of 1% for peptide and protein identification.

Cell culture, lysis, and immunoblotting

HEK293T and M17 cells were maintained, transfected, harvested, and immunoblotted as described previously (Dickey *et al*, 2006; Jinwal *et al*, 2013).

Collection of conditioned media from cultured cell, primary neurons, and slices

To collect cultured media, cells or slices were washed three times in PBS to remove serum proteins and incubated in dye-free, serum-free media for 6 h. After clearing debris by centrifugation at 1,467 *g* for 10 min, supernatants were transferred to a fresh tube, protease and phosphatase inhibitors added (EMD Millipore, Sigma-Aldrich) and either processed for mass spectroscopy analysis, concentrated for Western blot analysis, or used directly for dot blot analysis.

Primary neurons

All procedures involving experimentation on animal subjects were done in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of the University of South Florida. *Cspa*$^{+/-}$ mice were a kind gift from Thomas Sudhof (Stanford University) and were maintained as described (Fernández-Chacón *et al*, 2004). Primary neurons were isolated from E16 pups. Briefly, after euthanizing a pregnant dam, pups were extracted, brains removed, meninges removed, and cortices dissected in cold isotonic buffer. Following washing, cortices were minced, digested in trypsin, triturated, then resuspended in DMEM supplemented with 10% FBS, penicillin–streptomycin, and amphotericin B before plating on poly-l-lysine-coated coverslips. The next day, the DMEM was exchanged for Neurobasal medium supplemented with Glutamax and B27 supplement (Life Technologies). For AAV transductions, AAV9 was prepared according to standard methods (Zolotukhin *et al*, 1999). Primary neurons were transduced at DIV4 with 5 μl AAV in PBS at 10^{11}–10^{13} viral particles per μl. At DIV10, neurons were harvested for secreted tau and processed for Western blot as with HEK293T cells or for immunocytochemistry.

Organotypic slice culture and virus transduction

Organotypic cultures were prepared from wild-type C57BL6 mice or *Cspa*$^{+/+}$ and *Cspa*$^{-/-}$ litter mates at 14–21 days of age as previously described (Fontaine *et al*, 2015a,b). At least three litters, with at least three mice per genotype and mice of both sexes were used. DIV2 slices were incubated with 10 μl AAV9 DnaJC5-Flag, or AAV9-GFP at 10^{10} titer for 14 days. After transductions, slices were washed three times in cold PBS, scraped into lysis buffer (MPER; ThermoFisher) containing protease and phosphatase inhibitor cocktails, 50 μl per three sections), homogenized by pipetting then centrifuged for 20 min at 15,493 *g* to remove debris. Supernatants were adjusted for protein concentration and subjected to SDS–PAGE analysis. All Western blots are representative images of $n = 3$ animals, each culture containing 6–10 slices per animal.

siRNA- and shRNA-mediated knockdown

HEK293T and M17 cells were co-transfected with 40 nM siRNA (Dharmacon) or 2 μg shRNA (Sigma) in addition to 2 μg plasmid for overexpression as indicated. After 42 h, cells were then processed for collection of conditioned media and intracellular protein levels assayed by Western blot.

Hsp70 inhibitor treatment

HEK293T cells, M17 cells, and primary neurons were plated and transfected/transduced as indicated. At 42 h, cells were washed in HBSS and treated with 10 μM YM-01 or vehicle control for 6 h prior to collection of conditioned media.

Immunocytochemistry

Primary neurons grown on coverslips were fixed in 4% paraformaldehyde in PBS, permeabilized, blocked, incubated in the appropriate primary antibodies overnight followed by appropriate Alexa-Fluor-conjugated secondary antibodies (Life Technologies). Coverslips were mounted on glass slides using ProLong antifade reagent (Life Technologies).

Microscopy and image analysis

All imaging was performed on an Olympus FV1200 MPE multiphoton laser scanning microscope. Optimal confocal *z*-stack images (0.45 μm slices) were captured using a 60× objective from a minimum of four fields containing at least 10 neurons per condition and two independent primary neuron preps. Image analysis was performed using ImageJ. Briefly, *z*-stacks were background subtracted, despeckled, and thresholds set in each channel to include regions of interest. For transduced primary neurons, masking was applied to include only neurons that expressed virus. To quantify within synapses, as second mask was applied for only the synaptic marker. These parameters were applied identically to all images and then analyzed with the colocalization plugin (Nakamura *et al*, 2007). Pearson's coefficients and mean red and green intensities were calculated.

Analysis of cell toxicity

HEK293T cells were seeded into 96-well plates, transfected for 48 h then analyzed by Alamar Blue (Life Technologies), MTS (Promega), and LDH (ThermoFisher) methods per manufacturer's protocols.

mRNA analysis

Forty-eight hours after transfection with indicated plasmids, the total RNA was extracted from the cells using TRIzol reagent (Life Technologies) according to the instruction and digested with RNase-free DNase I (New England Biolabs). cDNA was prepared through reverse transcription (RT) using the iScript cDNA Synthesis Kit (Bio-Rad), and qPCR was conducted using SYBR Green PCR Master Mix (Applied Biosystems). Triplicate PCRs were conducted, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression was analyzed for each sample in parallel. The primers used for Tau are 5′-caagatcggctccactgaga-3′ (forward) and 5′-caagatcggctccactgaga-3′ (reverse) and for GAPDH are '5-catgttcgtcatgggtgtgaa-3′ (forward) and 5′-cacagtcttctgggtggcagt-3′ (reverse). Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Statistical analyses

Statistical analyses were performed by one-way ANOVA tests with Tukey's *post hoc* analyses, using GraphPad Prism 5.0 software. Any data more than two standard deviations from the mean were excluded.

Expanded View for this article is available online.

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Author contributions

SNF and CAD designed study, analyzed and interpreted results, and wrote the manuscript. SNF and JJS performed microscopy and imaging analysis. SNF, MDM, JJS, DZ, ARS, MK, and JHT performed cell biology experiments. SNF, AL, LJB, LS, JB, and JHT prepared primary and organotypic cultures. AD and DZ prepared AAV. SNF, BAN, DC, and SMS performed and analyzed mass spectroscopy experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

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