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

Sarah N Fontaine1,2,†, Dali Zheng1, Jonathan J Sabbagh1,2,†, Mackenzie D Martin1,2,†, Dale Chaput3, April Darling1, Justin H Trotter4, Andrew R Stothert1, Bryce A Nordhues1, April Lussier1, Jeremy Baker1, Lindsey Shelton3, Mahnoor Kahn1, Laura J Blair1, Stanley M Stevens Jr3 & Chad A Dickey1,2,†,**

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, DnaJ5. In fact, DnaJ5 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 DnaJ5 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

Subject Categories Neuroscience

DOI 10.15252/embj.201593489 | Received 16 November 2015 | Revised 25 April 2016 | Accepted 27 April 2016 | Published online 3 June 2016

The EMBO Journal (2016) 35: 1537–1549

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
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.

![Figure 1. DnaJC5 overexpression facilitates tau release into the extracellular space.](source)

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 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.

Source data are available online for this figure.
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 SNAP-25 and synaptophysin in neurons (Fig EV3A, B, D and E) and DnaJC5 overexpression reduced levels of this pre-synaptic tau (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
region in the nucleotide-binding domain (NBD) (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

mutated in a way that reduced its exocytic 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, 2001; Boddula 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

The Authors

Published: June 3, 2016
Sarah N Fontaine et al
DnaJC5 facilitates extracellular tau release

The EMBO Journal
Vol 35 | No 14 | 2016

© 2016 The Authors

1541
human P301L tau (Fig 5G), treating neurons with YM-01 blocked this DnaJCS-enhanced release (Fig 5G). Thus, disruption of the Hsc70/DnaJCS complex reduced extracellular tau release in neuronal cells (Fig 5G).

Similarly, DnaJCS-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 DnaJCS (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/DnaJCS 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 DnaJCS.
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 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., 2013b), 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-Martinez et al., 2007; Mandollesi 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-Martinez 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 (Bandopadhyay 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), ß-actin, 4-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 facilitates extracellular tau release Sarah N Fontaine et al
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, Q343R, 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 15N-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 15N-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 (Wisiórowski 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 H2O and analyzed on a Nexpressive Plus with a 50-μL 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 sapiens protein sequence database from UniprotKB using MaxQuant (version 1.5.0.30) and Mascot Distiller (version 2.5.1.0) with 15N 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 platting 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 1011–1013 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 1010 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 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'-caatgcggctcctgaga-3' (forward) and 5'-caatgcggctcctgaga-3' (reverse) and for GAPDH are 5'-catgctgctagctggtgctgaa-3' (forward) and 5'-caatgcggctcctgaga-3' (reverse). Data were analyzed according to the 2^(-ΔΔCT) 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.

Acknowledgements

The authors wish to thank Thomas Südhof (Stanford University) for the Cspa−/− mouse line and Joe Abisambra (University of Kentucky). This material is the result of work supported with resources and the use of facilities at the James A. Haley Veteran’s Hospital. The contents of this publication do not represent the views of the Department of Veterans Affairs or the United States Government. This work was supported by NS073899 and BX001637 to C.A.D.

Author contributions

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

Conflict of interest

The authors declare that they have no conflict of interest.

References


Boddul SV, Meng J, Dolly JO, Wang J (2014) SNAP-23 and VAMP-3 contribute to the release of IL-6 and TNFα from a human synovial sarcoma cell line. FEBS J 281: 750–765


Fontaine SN, Martin MD, Akouy E, Assimam VA, Borysov S, Nordhues BA, Sabbagh JJ, Cockman M, Ghestewii J, Zweckstetter M, Dickey CA (2015a) The active Hsc70/tau complex can be exploited to enhance tau turnover without damaging microtubule dynamics. Hum Mol Genet 24: 3971 – 3981


