



Secretion of human tau fragments resembling CSF-tau in Alzheimer's disease is modulated by the presence of the exon 2 insert

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ABSTRACT

Abnormal tau cleavage is prominent in the neurofibrillary degeneration characteristic of Alzheimer's disease (AD) and related tauopathies. We recently showed that cleaved human tau is secreted by specific mechanisms when overexpressed. Here we examined the effect of expressing N-terminal and full length tau constructs in transiently and stably transfected neuronal lines. We show that secreted tau exhibits a cleavage pattern similar to CSF-tau from human AD patients and that tau secretion is specifically inhibited by the presence of the exon 2 insert. These results suggest that tau secretion may play a hitherto unsuspected role in AD and related tauopathies.

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1. Introduction

Abnormal aggregation, phosphorylation and cleavage of the microtubule-associated protein tau are central to the pathogenesis of tau-associated neurodegenerative diseases (tauopathies) including Alzheimer's disease (AD), corticobasal degeneration (CBD) and Pick's disease. Since the presence of the alternatively spliced C-terminal insert (exon 10, or e10) affects both tau:microtubule binding and tau:tau aggregation [1], and is either overrepresented or underrepresented in the neurofibrillary aggregates of CBD and Pick's disease [2,3], it seems likely that e10 plays some role in disease pathogenesis [1,4]. However, there has hitherto been little reason to suspect that the N-terminal alternatively spliced sequences encoded by exons 2 and 3 (e2 and e3) are involved in pathological mechanisms leading either to tau aggregation or the generation of cerebrospinal fluid (CSF) tau. Recently, we found that tau protein fragments can be secreted to the extracellular space in an in situ tauopathy model via two distinct mechanisms, both of which require the presence of an unknown element in the tau N-

terminal domain [5]. These findings suggest that the predominance of N-terminal tau fragments in the CSF of AD [6] might be accounted for by the selective secretion of tau cleavage fragments by living neurons rather than being exclusively due to neuronal death and autolysis. However, the possible effect of N-terminal inserts on tau secretion was not tested in that study since we used only tau isoforms (3R0N and 4R0N) lacking the e2 and e3 inserts [5].

In order to clarify the roles played by either antecedent proteolytic cleavage and/or the presence of e2–3 and other N-terminal elements in modulating tau secretion, we assayed tau secretion of N-terminal half constructs of e2/e3– tau (n591) and e2/e3+ tau (Δ tau) from NB2a/d1 cells after transient expression and of inducibly expressed full length e2– (4R0N tau isoform) and e2+ (4R1N and 3R1N tau isoforms, 4R1N/3R1N) tau isoforms from a human derived tau-expressing cell line (M1C).

2. Materials and methods

2.1. Cell culture and selective induction of tau expression

We used M1C cells derived from human neuroblastoma (BE(2)-M17D) cells (kindly provided by Dr. Ko – Mayo Clinic College of Medicine, FL) to inducibly express wild type human tau, with 4R0N (four microtubule binding repeats and zero N-terminal inserts) tau expression being induced by the tetracycline-off

Abbreviations: AD, Alzheimer's disease; CBD, corticobasal degeneration; CSF, cerebrospinal fluid; TetOff, tetracycline-off expression system; MTBR, microtubule binding region; Ecd, ecdysone; MA, Muristerone A; e2, e3, e10, exons 2, 3 and 10

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expression system (TetOff) and 3R1N/4R1N (three and four microtubule binding repeats with e2 N-terminal insert) tau expression being induced by the Ecdysone (Ecd) (Fig. 1A) [7]. M1C cells were seeded in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, G418 (400 µg/ml), hygromycin (100 µg/ml), zeocin (100 µg/ml), tetracycline (2000 ng/ml).

Tau expression was induced by 1 ng/ml of tetracycline or 1 nmol/ml of Muristerone A (MA) for 10 days (Fig. 1A). Every 2 days, media were collected and fresh media were replaced to M1C cells. At 10 days after tau induction, M1C cells were lysed in Tris–NaCl (TN) buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton-X, 10% glycerol, 2 mM EDTA, protease inhibitor cocktail (Sigma)). Cell lysates were cleared by centrifugation at 10 000×g for 20 min. Total protein concentration of the supernatant was calculated by BCA methods. To concentrate tau protein in medium, Amicon Ultra 10K (Millipore, Billerica, MA) was used according to the manufacturer's protocol.

2.2. Transient transfection

The parent plasmids pRcCMV [5] and pcDNA/V5-DEST [8] were used to transiently express e2/e3-negative (n591) and e2/e3+ (Δ tau, kindly provided by Dr. Lars Ittner) versions (respectively) of the tau N-terminal (residues 1–255) in mouse neuroblastoma NB2a/d1 cells (kindly provided by Dr. Tom, Shea University of Massachusetts Lowell, MA) using Lipofectamine™ 2000 (Sigma) as previously described [5]. Complete medium was replaced with serum-free medium 24 h after transfection which was then collected, cleared and concentrated as described above after 24 h of incubation. NB2a/d1 cell lysates were lysed in Tris–NaCl (TN) buffer and cleared. Supernatant protein concentration was measured using BCA.

2.3. Immunoprecipitation

Protein G magnetic beads (New England Biolabs, Ipswich, MA) were used for direct immunoprecipitation. 30 µl of Protein G magnetic beads were incubated with 1.5 µl of Tau12 (1 µg/µl) or 2 µl of DM1A (1 µg/ml) with continuous mixing at room temperature for 20 min. Tau12 antibody–protein G bead complexes were washed (PBS containing 0.1% Tween 20) and then incubated with 150 µl of concentrated medium or 30 µl of cell lysates with agitation at 4 °C overnight. Ab–tau complexes were then eluted with 60 µl of 0.2 M glycine and then neutralized with 10 µl of 1 M Tris–HCl (pH 9.0).

2.4. Western blot and antibodies

Cell lysates, concentrated medium, and immunoprecipitated samples were analyzed by Western blot. Electrophoresed samples were transferred to polyvinylidene difluoride membrane and incubated with the following primary antibodies: Tau12 (1:10 000), Tau46 (1:10 000), (a kind gift from Dr. Binder), K9JA (1:1000) (DAKO), and DM1A (1:1000) (Sigma) (Fig. 1A). Goat anti-mouse (1:10 000) or anti-rabbit (1:10 000) IgG, with Alkaline Phosphatase conjugated second antibodies and BCIP/NBT substrate was used to reveal immunostaining.

3. Results

We used induced tau expression from M1C cells (Fig. 1) and transient transfection of NB2a/d1 cells (Fig. 2) to characterize tau secretion and its dependence on unknown N-terminal elements [5] in detail. Immunoprecipitation of secreted tau with the N-terminal specific mAb Tau12 was used to analyze the generation and secretion N-terminal-containing tau species from both induced M1C cells and from NB2a/d1 cells that had been transiently transfected with N-terminal (1–255) tau constructs. Results from Immunoblot of M1C cell lysate and raw concentrated medium with Tau12 and K9JA were used to quantitate the ratio of e2–/e2+ tau (Fig. 3). The relative amount of tau retained in cell lysates and secreted to media samples was analyzed by immunoblotting with Tau12 (aa 9–18), K9JA (aa 243–441), and Tau46 (aa 428–441) [9].

3.1. Induction of the 4R0N tau isoform by TetOff induction in transfected M1C cells results in proteolytic generation and selective secretion of large and small tau fragments

Expression of 4R0N tau over 10 days was significantly induced (approximately 15 fold) by reducing tetracycline in the culture medium to 1 ng/ml (Fig. 1A). Increased tau expression was accompanied by the generation of Tau12 and K9JA immunopositive cleavage fragments. These were largely negative for Tau46 in M1C cell lysates, which immunolabeled full length tau only (Fig. 1B). Secreted tau (i.e. Tau12-immunoprecipitated tau from medium) consisted of a select subset of these fragments that contained the Tau12 site plus part of the C-terminal region (243–441) recognized by the K9JA polyclonal (Fig. 1C). Tau46 failed to recognize most of the secreted tau fragments. K9JA-positive, secreted tau species occurred in two distinct groups of bands (22–35 kDa

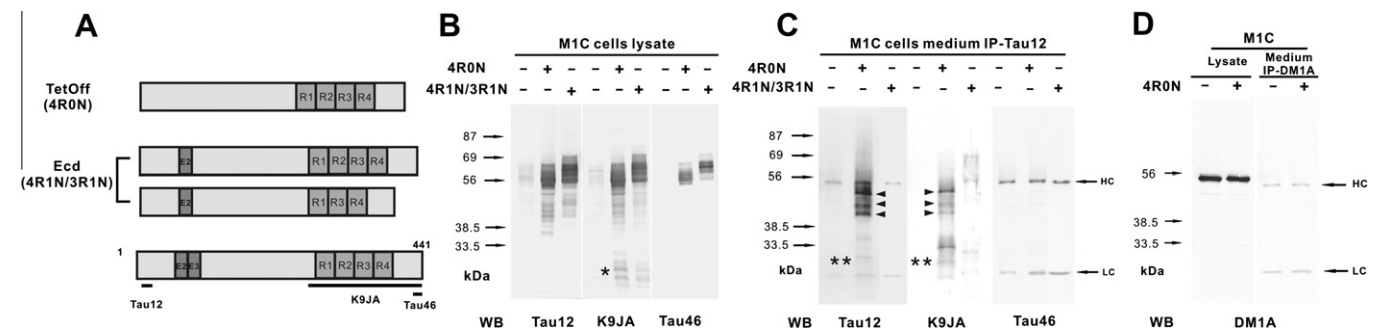


Fig. 1. (A) Either 4R0N tau (top) or both 4R1N/3R1N tau isoforms (center) were induced with reduced tetracycline (1 ng/ml) or via Muristerone A (1 nmol/ml) for 10 days. Bottom: A schematic representation of the longest isoform tau (4R2N–441 residues) and the epitopes recognized with tau antibodies. (B) After tau induction (+) with TetOff (4R0N) and Ecd (4R1N/3R1N) induction for 10 days, the overexpressed tau in M1C cells lysate was characterized using three different tau antibodies. Both 4R0N and 4R1N/3R1N tau expression in M1C cells was strongly induced compared to control (–) cells. Note that most low molecular weight K9JA+ cleavage fragments (asterisk) are not secreted, unlike low molecular weight Tau12 fragments. (C) Tau12 immunoprecipitates from concentrated media were analyzed by Western blot to identify secreted tau fragments containing the N terminus. 4R0N tau was secreted much more efficiently to the culture medium than 4R1N/3R1N tau (compare lanes 2 and 3 of both Tau12 and K9JA blots). Note that several K9JA/Tau12+ high molecular weight fragments (arrowheads) and some low molecular weight fragments (double asterisks) are secreted. (D) To control for possible tau toxicity effects, M1C cells lysates and concentrated culture media from the experiments described above were immunoprecipitated and immunoblotted with DM1A, showing that tubulin was absent from the culture medium.

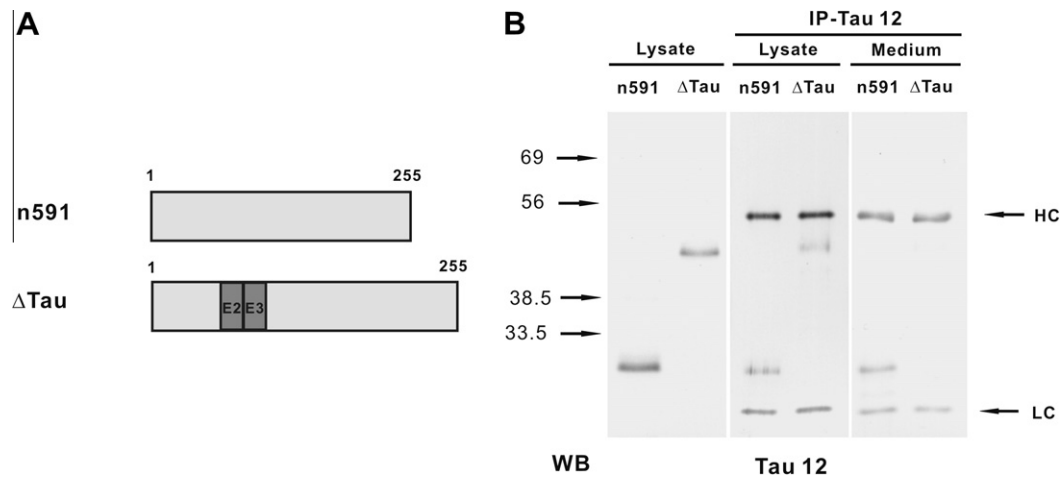


Fig. 2. (A) N-terminal tau constructs (residues 1–255) encoding e2/e3– (n591 – top) or e2/e3+ (Δ tau – bottom) were used to transiently transfect NB2a/d1 cells in tau secretion assays. (B) Cell lysates and Tau12 immunoprecipitates of cell lysate and concentrated media samples from NB2a/d1 cells after transient expression of n591 and Δ tau were immunoblotted with Tau12. To verify that Tau12 antibody recognized and immunoprecipitated N-terminal tau species with and without N-terminal inserts with comparable efficiency, immunoblots of lysates from transfected NB2a/d1 cells were performed with (center lanes) and without (left lanes) Tau12-immunoprecipitation. The presence of N-terminal inserts in the Δ tau significantly decreased the amount of secreted tau detected.

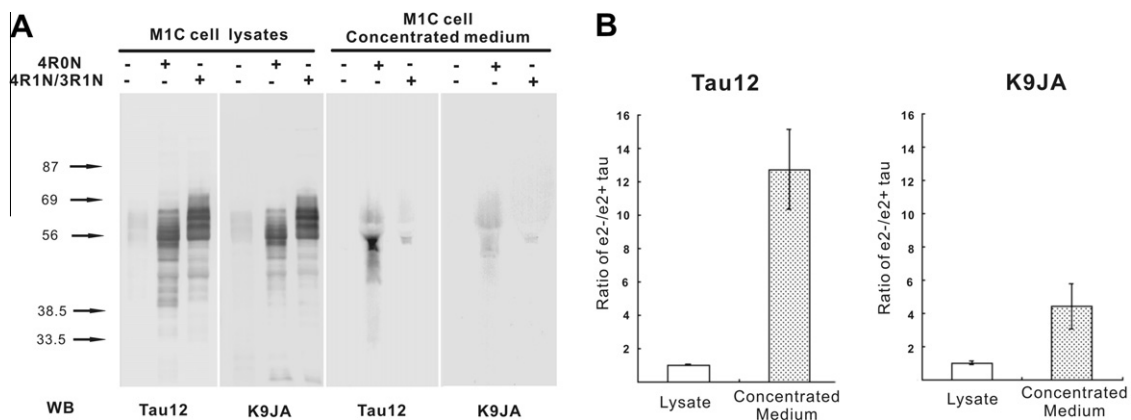


Fig. 3. The relative amount of extracellular (i.e. secreted) tau was significantly increased with the absence of N-terminal insert region (e2) from M1C inducible cells. (A) Tau12 and K9JA were used to determine the total tau in the cell lysate and the concentrated medium. Elevated levels of induced 4R0N and 4R1N/3R1N tau, via TetOff and Ecd system respectively, were present in M1C cells. A higher amount of extracellular tau fragments from 4R0N tau, but not 4R1N/3R1N tau, was found in expressing cells. (B) Quantitative densitometric analysis of the results shown in (A), showing the ratio of secreted versus retained 4R0N to 4R1N/3R1N tau isoforms. Values are represented as mean \pm S.E. from three experiments.

and 40–53 kDa), The individual cleavage fragments were distributed very similarly within in each group (arrow heads, Fig. 1C), suggesting that the lower MW group may have been generated by the removal of an approximately 13 kDa (\sim 160 residue) fragment from the C terminus, which would have to contain most or all of the microtubule binding region (MTBR) (residues 257–367), depending on the isoform. Levels of overexpressed tau sufficient to induce tau secretion at 10 days were not cytotoxic and did not induce passive leakage of tubulin (or tau) to cultured media from M1C cells, since tubulin was not detected when concentrated media were immunoprecipitated and then immunoblotted using the anti-alpha tubulin antibody DM1A (Fig. 1D).

3.2. While a subset of 4R0N tau cleavage fragments are secreted relatively efficiently from TetOff-induced M1C cells, Ecd induced 4R1N/3R1N tau were mostly retained in cell lysates

While the levels of 4R0N and 4R1N/3R1N tau induced (by TetOff and Ecd, respectively) seen in cell lysates was almost identical and generated similar proteolytic cleavage patterns, much less extra-

cellular 4R1N/3R1N than 4R0N tau was found in Tau12-immunoprecipitates from medium (Fig. 1C), indicating that the presence of the e2+ insert (residues 46–73) strongly and specifically inhibited tau secretion either by blocking the secretion event itself or by preventing the generation of secretable cleavage fragments.

3.3. Secretion of N-terminal tau fragments is strongly inhibited by the presence of the e2 insert

In order to determine whether the inhibition of tau fragment secretion from 4R1N/3R1N expressing M1C cells was a direct consequence of the presence of the e2 N-terminal insert, we transfected NB2a/d1 mouse neuroblastoma cells with plasmids expressing N-terminal tau constructs (residues 1–255) which either lacked (n591) or contained (tau) both the e2 and e3 inserts (Fig. 2A). Cell lysates and concentrated cultured media were prepared after 24 h of expression, immunoprecipitated with Tau12, and blotted. While n591 tau was secreted efficiently to the culture medium, Δ tau was not, even though Tau12 appeared to label Δ tau more efficiently in Tau12-immunoprecipitated lysates than did n591 (Fig. 2B). The re-

sult is consistent with the result from induced M1C cells described above (Fig. 1C), and in addition, strongly suggests that the presence of N-terminal insert region acts independently of the C-terminal domain and by directly inhibiting secretion, rather than by preventing the generation of secretable tau fragments.

3.4. Quantitation of e2+ mediated inhibition of tau secretion

For densitometric analysis of tau secretion using Image J, we immunoblotted media samples without Tau12-immunoprecipitation (Fig. 3). Quantitative analysis of M1C lysates and concentrated media showed that while the ratio of e2−/e2+ tau isoforms (4R0N via TetOff of 4R1N/3R1N via Ecd induction) was almost 1:1 (1:0.97 ± 0.02, Mean ± S.E.; n = 3) in lysates, this ratio was increased approximately 13-fold (1:12.71 ± 2.39) in the media samples, a highly significant difference. We found a somewhat smaller but still significant relative decrease (from 1:1 to 1:3.26) in the medium/lysate proportion of e2+ tau fragments when K9JA was used.

4. Discussion

While the C-terminal alternatively spliced insert (e10) of tau, which makes up the second MTBR, has been well studied and has a relatively well defined role in tau biology and pathobiology [10], the functional significance of N-terminal inserts (e2 and e3) remains obscure, despite the fact that e2+ tau makes up over 60% of the tau in the normal adult brain [11], and can be readily incorporated into insoluble neurofibrillary deposits commonly found in a number of neurodegenerative tauopathies [12]. In this study, we found that (a) the secreted tau mainly consists of two groups of tau fragments (molecular weights of 22–35 and 40–53 kDa, respectively) that are immunopositive for mAbs to the N-terminal domain of tau, and resembles in size the tau fragments reported in the CSF [13], and (b) the ability of tau to be secreted from viable neurons [5] is specifically inhibited by the presence of e2.

Increased levels of total tau over age-matched controls have been reported in the CSF of patients with a variety of traumatic and neurodegenerative conditions, including AD, CBD, myotonic dystrophy type 1, Parkinson's disease-related dementia and after ischemic and traumatic brain injury [6,13–17]. In each of these conditions, CSF-tau was found to consist primarily of small (26–33 kDa) and large (52–55 kDa) tau fragments that contain N-terminal tau epitopes similar to those observed here [6,13,17]. Since the calpains and/or caspases activated post trauma and in tauopathies have been shown to cleave tau at sites within the tau N-terminal and on the extreme C-terminal which could yield such fragments [18–21], it seems likely that the tau fragments observed in this study were generated similarly, especially since tau overexpressed above the levels needed to saturate microtubules is likely to be more subject to calpain and caspase activity than normal microtubule-bound tau [10,20,21]. This possibility is consistent with our failure to observe significant levels of secreted tau from stable cell lines expressing tau at low levels (not shown), and raises the possibility that abnormal calpain or caspase activity in tauopathies and/or post trauma could potentiate tau secretion in these conditions.

Our recent finding that tau can be selectively secreted from live neurons, together with observations that tau can be taken up into neurons in culture [22], is toxic when applied extracellularly [23] and can be propagated between neurons in the CNS [24] calls into question the widespread assumption that the generation of CSF-tau is invariably due to the passive tau release consequent to neurodegeneration. The similarity of both secreted and CSF-tau species to calpain and/or caspase cleavage fragments generated post trauma and in association with tau induced neurodegeneration [18–

21] are consistent with the possibility that the proteolytic cleavage on either side of the tau MTBR seen in M1C cells in this study might even potentiate the secretion of specific fragments of overexpressed tau [5], which like the cleavage of “tauopathy” tau [20], is associated with tau toxicity. Our results are therefore consistent with the possibility that the secretion of specific cleavage fragments may play a hitherto unsuspected role in the pathogenesis of neurofibrillary degenerative disease.

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