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Original Article

Multiple mechanisms of extracellular tau spreading in a non-transgenic tauopathy model

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Abstract: While the interneuronal propagation of neurofibrillary lesions in Alzheimer's disease and other tauopathies now appears to involve the spreading of tau-associated toxicity, little is known about its mechanism. We characterized the movement of human tau through the brain of a non-transgenic lower vertebrate tauopathy model in which full-length wild type and mutant human tau isoforms were expressed in identified neurons, thus permitting the identification and localization of EC tau sources. We describe two distinct patterns of tau spreading that correspond to tau species that lack (MTBR-) and contain (MTBR+) the tau microtubule-binding region. These patterns illustrate the production, migration and uptake of EC tau and resemble some of the extracellular tau deposits typically seen in human brain after repeated traumatic injury in cases of chronic traumatic encephalopathy (CTE). We propose that misprocessed human tau can spread between CNS neurons via a variety of non-synaptic mechanisms as well as synaptically mediated mechanisms.

Keywords: CSF-tau, tau secretion, neuron death, interneuronal lesion spread, chronic traumatic encephalopathy

Introduction

The pathogenesis of Alzheimer's disease (AD) and related neurodegenerative diseases (tauopathies) is associated with hyperphosphorylation, aggregation and proteolytic cleavage of the microtubule-associated protein tau (for reviews, see [1-4]). Aggregated tau lesions (NFTs) spread in a disease-specific pattern through the brain with the development of these diseases, suggesting that interneuronal abnormal transfer of tau protein may be involved [5-7]. Although it is now clear that tau misprocessing is mainly the consequence of abnormal accumulation of beta-amyloid peptide, it has also become evident that tau largely mediates amyloid-beta (Aβ) cytotoxicity in AD. The importance of tau in the pathogenesis of AD is further underscored by the presence of tau in the cerebrospinal fluid (CSF) early in the course of the disease [8-13], where it distinguishes AD both from normal aging [8] and from non-AD tauopathies [14].

Although a good deal is now known about the role of tau misprocessing in AD at the cellular level, we still know very little about the intercellular aspects of AD. However, it now appears likely that some kind of interneuronal transfer of tau protein between neurons plays a major role in the pathogenesis of neurodegenerative tauopathies. Tau secretion from [15-19] and uptake into [20-22] neurons has been observed in a variety of cellular tauopathy models and the spreading of tau lesions between neurons has now been replicated in transgenic mouse models of tauopathy [23-25]. Of particular interest is the observation that secreted tau species closely resembles those typically found in the CSF of AD patients [9,11,12,18,19] and other tauopathies [14] suggesting that CSF tau might be secreted. A "secretory" origin of CSF-tau is consistent with the similarity in size between secreted tau in experimental models to the CSF tau species seen in the early stages of AD [18, 26-27], especially since CSF-tau exhibits secre-

tion-associated features such as exosomal association [9,13]. This possibility is particularly noteworthy since secreted CSF tau could a) be potentially useful as an AD diagnostic [13,19] and b) provides a possible route for tau lesion spreading in addition to the trans-synaptic routes that have been proposed to account for the patterns of progressive lesion spreading in AD [5,6] non AD tauopathies [28-30].

The relative complexity of tau-mediated toxicity has made modeling tauopathy unusually difficult. Tau appears to differ from other disease-associated aggregation-prone proteins (e.g. prion protein (PrP), alpha-synuclein (ASN)) in having an extra toxicity mechanism that is not associated with aggregation, but is centrally involved in the pathogenesis of AD and possibly non-AD tauopathies as well [31-33]. In addition to the aggregation-prone microtubule-binding domain (MTBR) [34-37], the amino terminal of tau appears also to mediate toxicity [38-41]. It is now clear that tau is necessary for most aspects of Aβ cytotoxicity [42-43] and that at least some of these do not require MTBR-mediated aggregation [31,39]. Moreover, unlike Aβ, wild-type tau is not itself toxic in cell culture even when overexpressed unless pharmacologically manipulated [44,45], suggesting that tau toxicity depends on a higher degree of differentiation than that normally present in cell lines. As a consequence, tau toxicity in cell lines has only been generated readily using disease-associated or hyperaggregating mutant tau [46-47]. By contrast, wild type human tau is neurotoxic in situ via a tau-specific mechanism that approximates tau lesions in human disease if sufficiently overexpressed [48-49], especially in subcellular levels of aged individuals [50]. These factors also greatly complicate the identification and characterization of specific mechanisms of tau lesion spreading, since the potential existence of multiple (i.e. MTBR+ and MTBR-) toxic tau species and especially the upstream involvement of Aβ toxicity in AD, but not non-AD tauopathies may account for characteristic differences between lesion spreading patterns in these conditions [33].

In order to analyze the origin, distribution and molecular characteristics of extracellular tau deposits within the brain, we used a non-transgenic model (the lamprey ABC model) in which human tau can be expressed cell-autonomously and in which tau secretion and trans-synaptic tau transfer have been identified unambiguously and characterized at the cellular and subcellular level (**Figure 1**) [18-19,22]. Initial characterization of tau secretion in this system has shown that both MTBR+ and MTBR- tau species are efficiently secreted from ABCs when overexpressed via plasmid microinjection, so long as the N terminus is present [18] and the alternatively spliced exon 2 insert (aa 46-73) is absent [19]. The secretion patterns of MTBR+ and MTBR- tau species differ characteristically from each other with respect to their site of release from neurons [18], and their association with dendritic degeneration, trans-synaptic tau movement and tauopathy mutations [18,22]. However, it has been hitherto unclear whether diffuse tau produced by expression of full length tau isoforms must be MTBR- and whether (or how) the generation, dispersal and transneuronal uptake of EC tau is affected by the presence of tauopathy point mutations.

In this study, we show that both secreted full length (MTBR+) and N terminal (MTBR-) tau species reach the CSF, spread throughout the lamprey hindbrain and are taken up by neurons and glial cells at locations distant from their point of origin. The spreading of both types of EC tau is strongly exacerbated by the presence of the P301L tauopathy mutation. To facilitate the comparison of the biogenesis and migration of secreted tau between models and human disease we analyzed tissue from lamprey and from patients with chronic traumatic encephalopathy (CTE), a condition in with a large amount of extracellular tau are generated episodically [51,52]. We show that EC deposits of exogenous human tau expressed in lamprey neurons resemble EC tau lesions associated with repeated traumatic injury (CTE) in humans. These findings thus suggest that non trans-synaptic mechanisms may coexist with trans-synaptic tau spreading mechanisms in human neurodegenerative conditions associated with tau.

Materials and methods

Animals

Immunostained sections through the hindbrains of 65 ammocoete (larval) sea lampreys that had been induced to express full length (WT and tauopathy mutant (P301L) tau) or a C terminal deletion construct (1-255 tau) in which the MTBR region was absent (Petromyzon marinus) between 8-11 cm in length [18, 53] were the source of the data used for this study. These

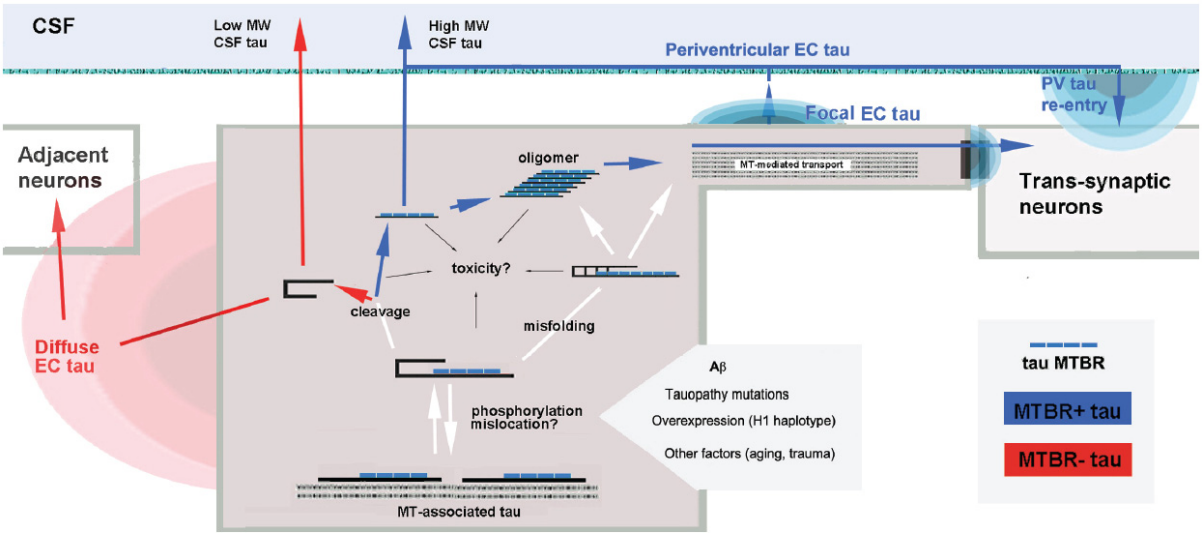

Figure 1. Cellular and extracellular pathways by which human tau may be misprocessed in tauopathy models and in human neurodegenerative disease. In the mature CNS, tau is primarily associated with MTs in neuronal axons, where it is not toxic or associated with neurofibrillary pathology. Conditions predisposing to tau misprocessing include the generation of toxic levels of the Aβ peptide in AD, the presence of exonic point mutations that cause familial tauopathies, elevated baseline tau expression associated with the H1 haplotype, and repetitive brain trauma, which leads to tauopathy (CTE) and is associated with elevated AD risk. All of these can result in increased levels of non-MT associated tau, which may then be mislocated and misprocessed, leading to tau toxicity, stoichiometrically high levels of phosphorylation, altered tertiary structure and susceptibility to proteolytic cleavage by calpains and caspases. Misprocessing and toxicity are associated with the generation and appearance in th CSF of both N terminal tau fragments that lack the microtubule binding region of tau (MTBR- tau, red) or full length of C terminally cleaved tau species (MTBR+ tau, blue) that possess the MTBR. Interneuronal spreading of tau lesions may be due to direct transfer of toxic tau species between neurons followed by either a prionlike templated misfolding of tau in the receiving neuron or by Ca++ dysregulatory effects of oligomeric (MTBR+) tau or toxic N terminal (MTBR-) tau species. While tau lesion spreading is generally thought to occur in association with synapses, we propose that the periventricular and diffuse EC tau migration patterns described here illustrate additional pathways by which lesion spreading might occur in AD and in other tauopathies, including trauma –associated tauopathies such as CTE (arrows).

yielded a total of 104 tau positive cells (48 T24, 6 P301L focals late, 7 1-255, 43 P301L footprints) that were analyzed.

Tau expression in ABCs

Wild type (WT) and mutant 4R0N isoforms of human tau were expressed via microinjected plasmid vectors (pEn1234c, pEn1234c + P301L mutation, and pRcCMVn591), respectively for intervals of 5-40 days and immunohistochemical analysis was performed using a standard horseradish peroxidase-Extravidin technique on dewaxed serial 12 micron paraffin sections as described [15-16,18, 22, 48, 53].

Antibodies

The mAbs Tau1 (1:1000), Tau12 (1:1000), Tau5 (1:1000), PHF1 (1:1000), 9G3 (1:100), AT180 (1:200 and AT8 (1:100) were used to identify the expression of htau and the phosphorylation status of htau in lamprey brain. Tau1, Tau5 and Tau12 were kindly provided by Dr. Lester Binder (Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL). PHF1 was a generous gift from Dr. Peter Davis (Department of Pathology, Albert Einstein of College of Medicine, Bronx, NY). 12E8 (1:200) was a generous gift from Peter Seubert (Neotope Biosciences). AT8 and AT100 were purchased from Innogenetic Corp. (Leuven, Belgium). The polyclonal antiserum against the MTBR K9JA was purchased from DAKO. AT180 was purchased from Pierce pharmaceuticals).

Quantitative analysis of EC tau distribution

We used 2 types of image analysis to describe quantify and compare the distribution and migration of diffuse and periventricular extracellu-

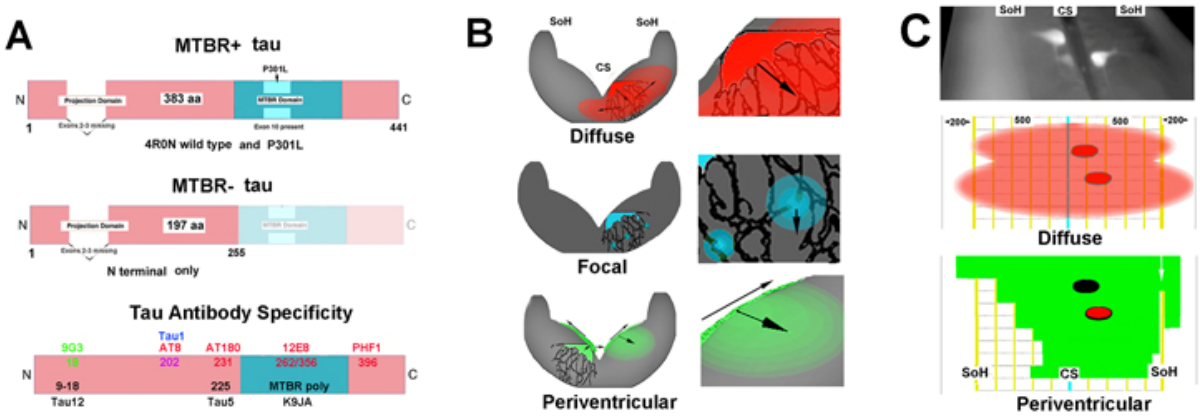

Figure 2. Distribution and quantitative analysis of diffuse and periventricular secreted tau patterns in the lamprey CNS. Panel A: Full length and N terminal half tau constructs were expressed cell autonomously via plasmid injection in lamprey ABCs for 5-40 days. All isoforms used lacked the E2 and E3 inserts. Full length isoforms contained 4 microtubule binding repeat motifs that either lacked (wild type) or contained a tauopathy-inducing point mutation within the second MTBR motif at position 301 (P301L) according to the numbering scheme for the longest of the 6 tau isoforms expressed in the human CNS. The N terminal construct used in this study (bottom) encodes residues 1-44 and 104-255 of human tau. The epitope site and phosphorylation sensitivity of antibodies used in this study. Phospho-specific sites are shown in red (serine/threonine) or green (tyrosine), with a dephosphorylation specific mAb (tau1) shown in blue. Phospho-independent Abs are shown in black. Panel B: Schematics summarizing the characteristic distribution of diffuse (top), focal (center) and periventricular (bottom) tau with respect to the ABC of origin (red, shown in cutaway images in the center of each panel). C: Live image of a lamprey brain (dorsal view) showing the expression of GFP-tagged tau in ABCs. The surface of the hindbrain is dominated by the central sulcus (CS) and the Sulci of His (SoH). Left center: Scoring sheet used in this study illustrating the semiquantitative analysis of the distribution of diffuse (red) and periventricular (PV) tau deposits in serially sectioned lamprey brains. Locations of cell bodies are shown in black. The distribution of PV tau was estimated as occupying a proportion of the ventricular surface for each section using the central sulcus and Sulcus of His as landmarks as shown. Anatomical landmarks such as the CS and SoH were frequently "skipped" (white arrow), suggesting that at least some periventricular tau transport occurs within the ventricle itself.

lar (EC) tau deposits induced by the expression of full length and N terminal half tau as described above.

"Footprint" analysis of EC tau distribution over multiple sections

The parent dataset used in this study consisted of serially sectioned lamprey brains each containing one or more giant identified neurons (ABCs) expressing N terminal (1-255), wild type full length (WT) or tauopathy mutant full length (P301L) 4R0N human tau. In order to determine the pattern and extent of EC tau within the brain as a whole, we created a series of calibrated scale drawings for each brain showing the distribution of diffuse and PV tau as shown by tau12 immunostaining at intervals of 5-7 sections as shown (**Figure 2**). For each drawing, the distributions of tau relative to prominent features (the central sulcus (CS) and sulcus of his (SoH)) were indicated. The extent of diffuse and PV tau with respect with these landmarks was then mapped onto a "footprint" drawing that was then converted to an estimate of surface area covered (PV tau) or neuropil volume occupied (diffuse tau) as described (**Figure 2**). Disambiguation was performed as follows to determine the sources of diffuse and PV tau deposits in cases where multiple tau expressing ABCs were present in a given brain. For diffuse deposits, the cell body nearest to the highest EC tau intensity was assumed to be the source. For PV deposits, ABCs were identified by the presence of high intensity tau emerging from the dorsal soma (somatic PV tau) or axons (axonal PV tau). In addition, the attribution of PV tau to axons was only made in the absence of somatic profiles within 100 microns.

Intensity gradient analysis in individual sections

Distribution gradients of PV tau intensity was taken with the help of the shareware image

Table 1. Definitions and criteria used to identify deposit types and calculate tau distribution gradients

	Top	Bottom	Vector
Diffuse	Next to Soma	Closest point to soma where label = background (brain margin excepted)	Direction defined by shortest possible line
Focal	Next to originating dendrite	Closest point to dendrite surface where label = background	Perpendicular to surface of dendrite profile at the point of greatest extent
PV re-entry	Maximum point in ventricle	Closest point to ventricle surface where label = background	Perpendicular to ventricle surface

PV: periventricular

analysis program ImageJ and Microsoft Excel. The intensity of PV tau immunolabel inside the ventricles that were measured from the maximum point to the point where tau levels out was divided by the length (in microns) between the two points and the intensity gradient slope (steepness) determined using the Measure Particles pulldown in the basic ImageJ program. The direction chosen for all PV gradient measurements were always perpendicular to the ventricle surface. The criteria used for defining EC deposit type and for choosing the top, bottom and gradient direction for the calculation of PV, diffuse and focal intensity gradients are shown in **Table 1**. Confocal images used for making multichannel comparisons in single sections were obtained using an Olympus Fluoview 300 laser scanning confocal microscope, but were not used for either the footprint or gradient analyses described above.

Results

Two different patterns of extracellular tau propagation in the lamprey model

The active secretion of human tau (as opposed to passive tau release after neuronal death) has been characterized in some detail in the lamprey tauopathy model [18,22]. Examination of T12 –immunostained sections from a large number of lampreys with ABCs expressing WT and mutant human tau showed several clearly defined categories of extracellular and interneuronally transported tau, some of which (focal and diffuse tau secretion, anterograde transneuronal tau) have been described in a previous publication [18]. Diffuse tau secretion was defined as occurring ABC somata often in the absence of cytodegenerative changes, whereas focal tau secretion occurred only from dendrites and axons and has been shown to be intimately associated with dendritic degeneration [22]. Focal tau secretion appears to require distal transport of exogenous human tau within dendrites and is accompanied by phosphorylation at multiple sites [18, 22]. Abolition of MTBR function by pseudophosphorylation of sites 262 and 356 in the MTBR (S262D/S356D tau) [55-57] permitted tyrosine 18 (Y18) phosphorylation and dendritic tau transport, but blocked focal tau secretion [18]. The removal of the MTBR (i.e. expression of 1-255 tau) prevented the phosphorylation, distal dendritic transport and accumulation of tau, as well as tau induced toxicity, but potentiated diffuse tau secretion [18]. Expression of 1-255 tau thus replicates the key features of "diffuse" tau deposits generated by full-length constructs (e.g shallow gradient distribution and origination from the soma exclusively). The failure of 1-255 tau to be phosphorylated, to be transported distally and to generate focal dendritic **EC** deposits all suggest that diffuse EC tau is MTBR- and focal tau is MTBR+, but does not establish whether this is true with full length tau expression.

Appearance of diffuse (MTBR-) and focal (MTBR+) EC tau deposits

Diffuse EC tau deposits (as defined above) were characterized in terms of their distribution relative to their sources by gradient analysis and their extent analyzed using multi-section footprint analysis (Methods). We found that diffuse deposits extended for up to several hundred microns from the source ABC. These deposits showed rostrocaudal extents across multiple sections that were comparable to the continuous distributions shown within individual sections, suggesting that all diffuse label was contiguous and thus presumably occurred via propagation through the neuropil not via CSF. Intensity gradients of diffuse deposits invariably consisted of a smooth decrease from the maximum at the source (the ventral soma of the source ABC) to near background levels in neuropil up to 1mm away. Movement of diffuse tau through the neuropil was notably unaffected by the presence of cellular/tissue features such as

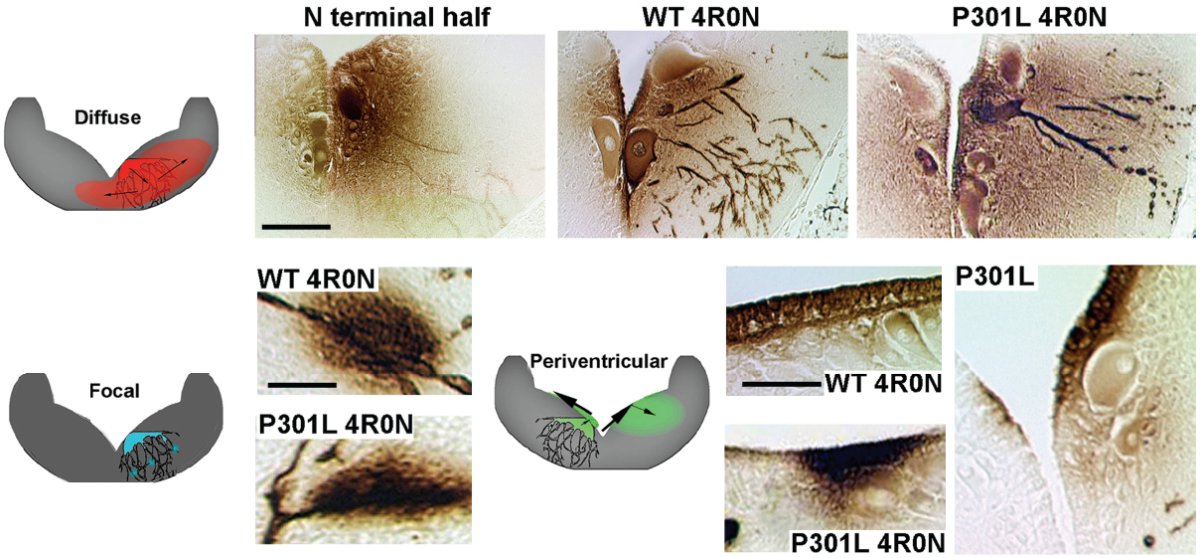

Figure 3. Examples of diffuse, focal and periventricular (PV) extracellular tau deposits in the lamprey brain. The relationship the deposits and their sites of origin from tau-expressing ABCs in the lamprey hindbrain are shown at left as indicated. Photomicrographs of examples of each EC deposit type from wild type (WT) and P301L tauopathy mutant (MTBR+) and a deletion construct (1-255) in which the MTBR region is not encoded (MTBR-) are shown. Scale bars: Diffuse, 100 μm; Focal and PV, 20 μm.

the somata of nearby neurons, periventricular ependymal cell layers and the central sulcus itself (see **Figure**s **3** and **4**), suggesting that diffuse tau distribution occurs via the CSF as well as the CNS neuropil. There was no sign of tau inclusion in endosomes/vesicles at light microscopic level, and the overall distribution displayed a characteristic mildly sloping distribution gradient (**Figure**s **3-4**). All of these characteristics indicate that diffuse EC tau is highly mobile in the lamprey brain and suggest that transcellular movement of diffuse EC tau within the neuropil occurs via a different mechanism than that of other EC tau deposits (described below), which are much more restricted. By contrast, focal EC tau deposits originating from ABC dendrites and/or axons (by definition) [18] were highly localized to their points of origin and exhibited significantly steeper distribution gradients than diffuse EC tau secreted from the ventral somata of tau-expressing ABCs (**Figure 4**). We found focal EC deposits only when a functional MTBR was encoded by the construct being expressed, thereby confirming previous observations [18].

Appearance and distribution of periventricular (PV) tau deposits in the lamprey

EC tau deposits that were centered on the ventricular surface or in the immediate vicinity of ventricular ependymal cells were defined as periventricular (PV) EC tau deposits. These originated primarily from the dorsal surfaces of the somata of ABCs that were expressing tau heavily (e.g. **Figure 5a top**), but also from axons of such neurons in the caudal hindbrain (**Figures 4a** and **b**, right). PV tau accumulates in the sub-ependymal space near the site of secretion (**Figure 4a-b**, **right**, **Figure 6**) and then crosses the ventricular ependymal layer and migrates across the inner surface of the IV ventricle the surface of the brain. In some cases, migration appears to occur via the sub-ependymal space instead of (or as well as) the ventricular ependymal surface (**Figure 7a**). The wide distribution of PV deposits from possible sites of origin (see analysis below) and their concentration at specific sites on the ventricular surface (**Figure 5b**) suggests that PV deposit distribution is via an active distribution mechanism operating along the ventricular surface rather than via diffusion through the CSF, which would favor a more general distribution over the ventricular surface. This interpretation is also supported by the tendency of many PV deposits to "jump" across the central sulcus (CS) and the laterally located sulci of His (SoH) via apposition points (**Figures 3c** and **4a-b**), and for the depth of EC tau on the ependymal surface to exhibit a "thickness gradi-

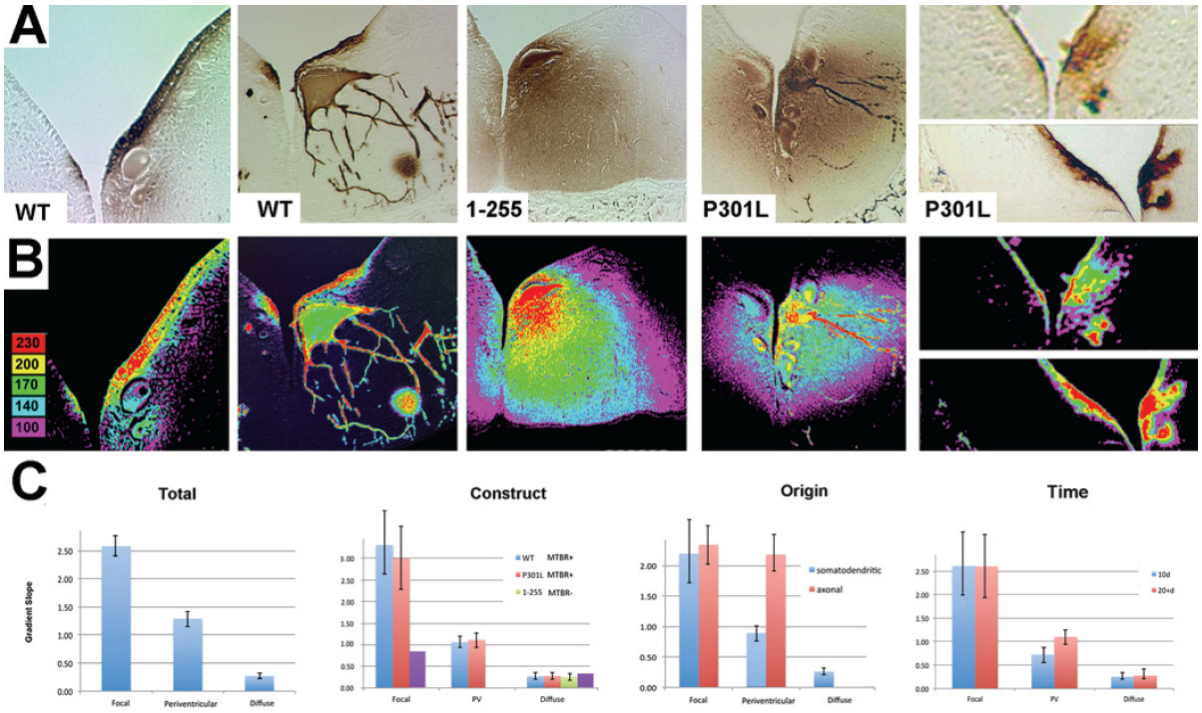

Figure 4. Quantitative analysis of EC tau gradients produced by ABCs expressing MTBR+ and MTBR- tau constructs. Diffuse, focal and periventricular tau deposits differ characteristically from each other in the degree to which they a) vary with respect to the construct expressed and b) with their site of origin in the expressing ABC (i.e. somatic, dendritic axonal). Panel A: Periventricular (PV), focal and diffuse EC tau from each isoform used, immunostained with the mAb Tau12, are shown. Panel B: The typical features of the tau12 immunolabel gradient for each type of EC tau is illustrated using a LUT that corresponds to ranges of DAB immunolabel intensity as shown at left. The images shown are derived from their corresponding image in Panel A. Note that while both diffuse and PV tau species are often intensely immunolabeled at their site of generation (shown as red in the LUT), diffuse deposits are spread over a much larger region of neuropil relative to their intensity that PV and focal deposits, suggesting that the former are significantly more mobile. Panel C: Quantitative comparisons of gradient slopes (i.e. degree of intensity change per unit distance from their site of origin - as defined in Methods) from each EC tau type are shown. Since the data distributions exhibited noticeable skewness with respect to the mean value, both the Student's t-test and the Fisher's Exact test were used to determine significance levels and gave similar results. Bars show the S.E.M. Left: All diffuse deposits exhibited significantly milder slopes than either PV or focal tau deposits, whereas focal deposits had significantly steeper distribution slopes than "re-entering" PV gradients. Both somatic and axonal PV deposits were used in this analysis. Center Left: The absence of the MTBR from the construct expressed abolished focal and PV deposits, whereas its inactivation by pseudophosphorylation (S262D/S356D) made the slopes of fopcal deposits significantly shallower. The presence of the P301L tauopathy mutation did not affect the distribution slope of any of the 3 EC tau deposit types. Center Right: Both focal and PV EC tau derived from ABC axons showed significantly steeper distribution gradients than did tau released from ABC somata. We saw no sign of low slope gradient generation (i.e. axonal diffuse deposits) in this study. Right: While the overall extent of some EC deposits changed between 10 and 20-40 days days of expression (see Figure 5), the slopes of all 3 EC deposit types were not significantly affected. Scale Bars are 100 μm, except for the inset high magnification at right, which is 20 μm.

ent" that is highest at the site of release and decreases with distance (**Figure 5b**). An obvious candidate for mediating such a mechanism is the activity of the ependymal and choroid cilia that are normally responsible for the circulation of CSF [58], but this issue was not investigated further. Similarly, the ability of EC tau to accumulate in and migrate along the sub-ependymal space suggests that elements within this space are capable of binding tau sufficiently strongly to allow a subsequent non-directed dispersal from the site of accumulation, but not so strongly as to immobilize it completely. In order to test the possibility that the tau MTBR is involved in this, we examined PV deposits originating from ABCs that were injected with plas-

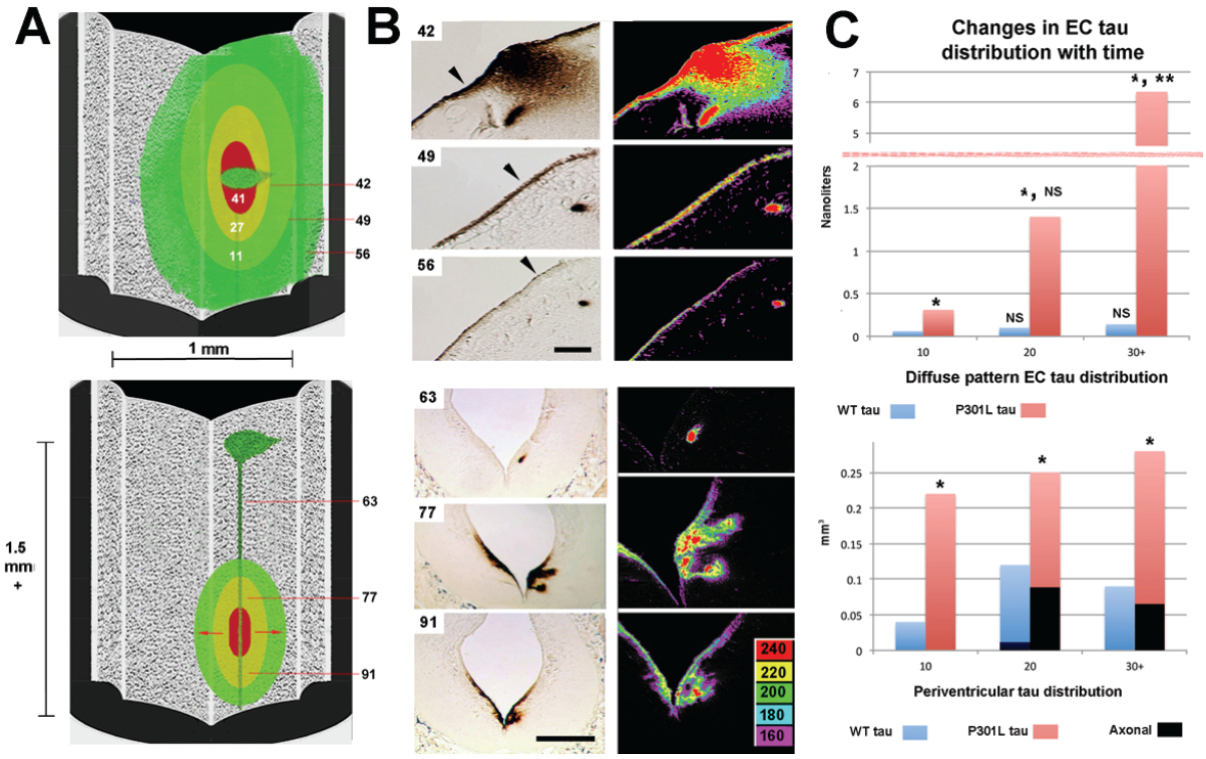

Figure 5. PV and diffuse tau migration in the lamprey brain from the ABC of origin is modulated by the presence of the MTBR and/or the P301L point mutation. Panel A: Schematic representation of PV tau migration in the lamprey hindbrain. PV deposits typically originated from either the dorsal somata (top) and axons (bottom) of tau-expressing ABCs, and spread along the surface of the brain from the point of origin down a concentration gradient (illustrated by red, yellow and green zones, arrows). The numbers at right in both schematics show the slide numbers and the approximate sites of each of the individual sections shown in B. In some cases, maximal extents of PV tau were reached within the first 10 days of expression, with CE tau being present throughout the hindbrain at distances of at least 2 μm from the source somata (not shown). Panel B: Transverse sections taken from the sites indicated in A are shown and a semiquantitative representation of immunolabel intensity is shown for each section (right, see LUT at bottom). Somata (top) and axons (bottom) were identified as sources of EC tau by a) the observation of a focal gradient emerging from the dorsal soma or axon and b) the absence of other candidate sites in that section (or in adjacent sections). Scale bars: top: 50 μm; bottom, 200 μm. Panel C: Quantitative analysis of the "footprint" immunolabel maps for diffuse (top) and PV (bottom) tau. The incidence and extent of diffuse and PV tau within the lamprey brain were modulated by a) the presence of the MTBR, b) the P301L tauopathy mutation and c) the length of time over which tau was expressed in ABCs. We found that the absence of the MTBR resulted in exclusively diffuse secretion. This was present from the earliest time (5 days post plasmid injection) examined and thus confirmed the earlier study of Kim et. al (2010). By contrast, tau secretion was relatively restricted in lampreys expressing WT full length tau constructs, especially with respect to diffuse tau, which was secreted at low levels in a large minority of ABCs examined at all timepoints. There was no significant increase in either the incidence or extent of diffuse EC tau with increasing time post plasmid injection in these animals (top, blue bars). The presence of the P301L point mutation (pink bars) greatly increased the extent of both diffuse and PV deposits. This was especially marked with diffuse deposits, which also became significantly more extensive over time. PV deposit extent also increased with time post injection, but this increase was not significant. Spreading of axonally secreted PV deposits (black bars) was not noticeably different from that of somatic origin, despite the difference in their slops (Figure 4c). Scale Bars: B (top) 50 μm, B (bottom) 200 μm.

mids encoding tau species in which the ability of the MTBR to bind polyanions was manipulated specifically. Reduction of MTBR-mediated binding was either abolished by removal of the MTBR (1-255) or by pseudophosphorylation of residues 262 and 356 (S262D/S356D). Enhancement of MTBR binding was accomplished by N terminal truncation (211-441) [59] to yield a fragment identical in size to the MTBR. We found that removal of the MTBR abolished subependymal tau accumulation, whereas enhancement of MTBR binding caused the immo-

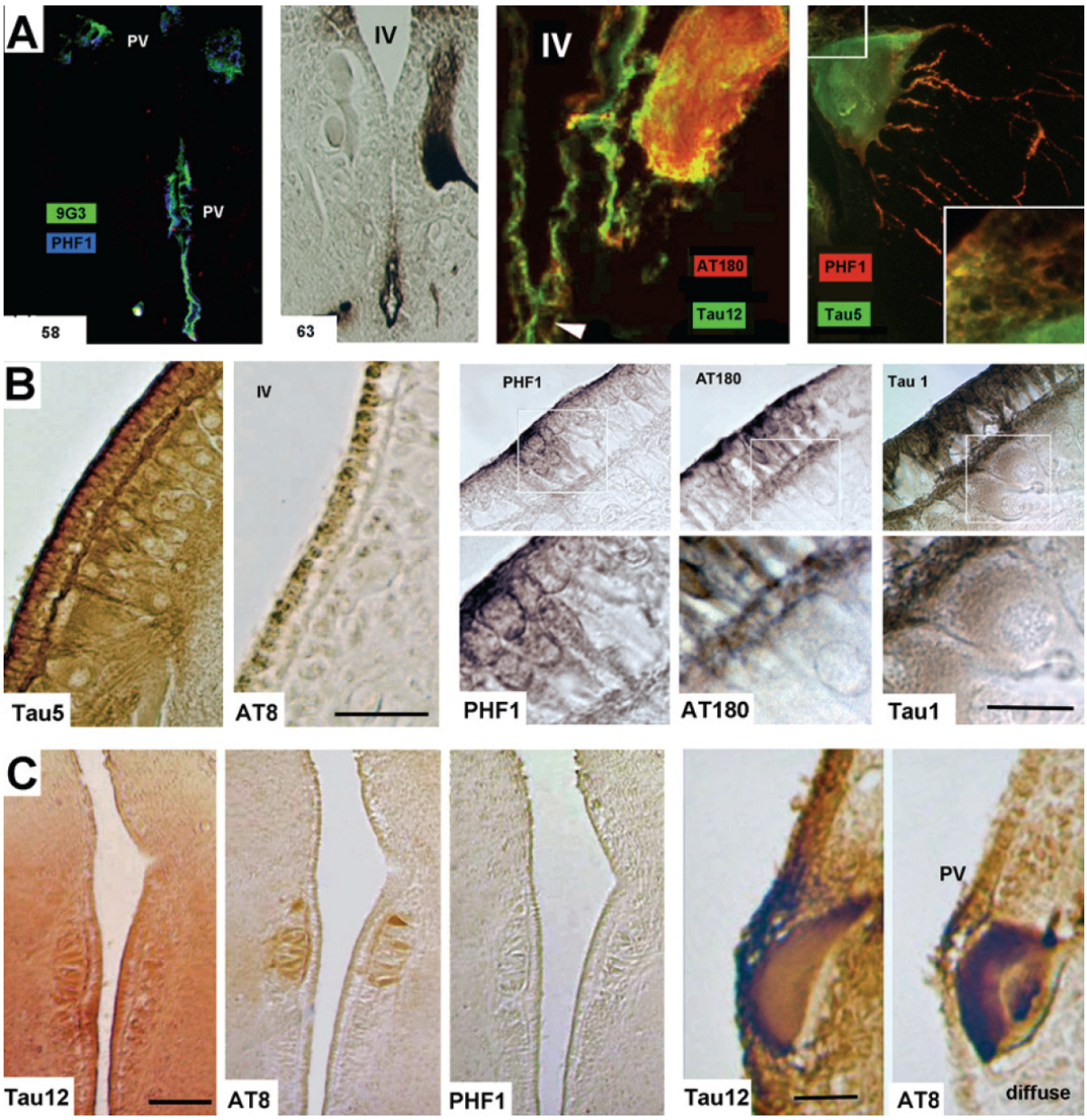

Figure 6 . Phosphorylation state of EC tau species in the lamprey**.** PV tau was typically phosphorylated to a varying extent at one or more of the classic "AD" sites in and around the MTBR, but many of the sections examined showed no significant immunostaining for phosphorylated tau (Ptau), especially at extracellular loci within the brain. Panel A: Confocal imaging of sections adjacent to Tau12/DAB immunolabeled sections shows some MTBR+ character and some phosphorylation of all EC tau species, but especially PV (Panels A, left, Panel B), perimeningeal (Panel A left/ center) and focally secreted tau (C, right). Panel B (left photos) shows the typical finding in this study with respect to PV tau phosphorylation. An AT8 immunostained section adjacent to a section strongly positive for Tau12 showed limited phosphorylation of granular tau deposits. Similar results were seen with other phosphoepitope-specific Abs (B, right). Phosphotau staining was preferentially seen in intracellular loci in both ependymal cells and neurons with each of these mAbs (right, inset area). Most phosphotau (PHF1, AT8, AT180, 9G3) immunopositive examples were from P301L-expressing brains. Panel C: Diffusely secreted tau was generally phosphotau negative, but when present, it was preferentially localized intracellularly as well (left panels). PV tau deposits generally showed more phosphotau immunolabel than did diffuse tau deposits. This can be seen with a direct comparison between PV and diffuse tau (C right panels). Scale Bars: B left: 50 μm, B right (insets) 20 μm, C left 100 μm, C right 50 μm.

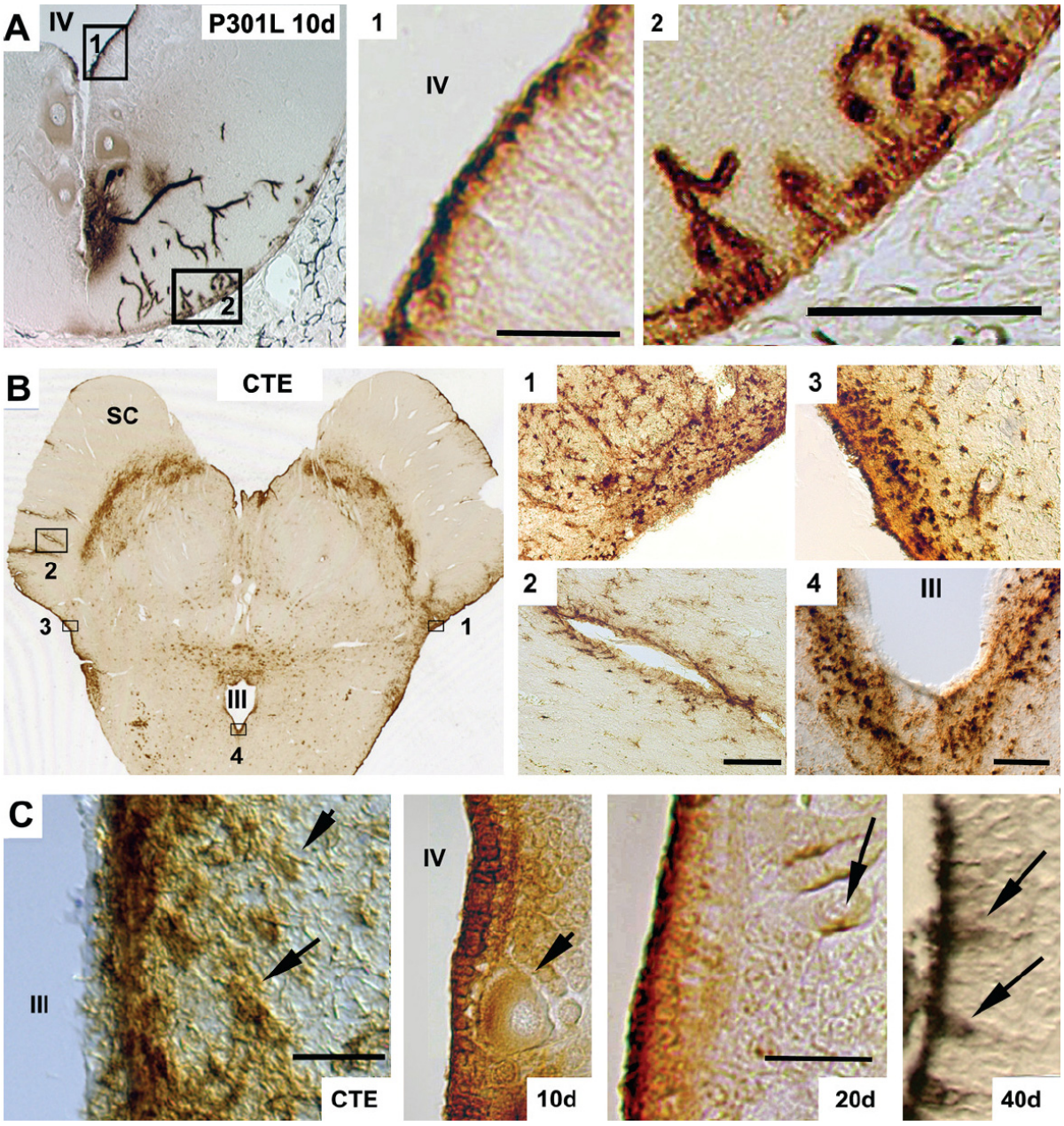

Figure 7 Periventricular and perimeningeal EC deposits of human tau in the lamprey tauopathy model show similarities with tau lesions in chronic traumatic encephalopathy (CTE). Panel A: Low magnification (left) and high magnification views (insets, 1-2) of periventricular and perimeningeal P301L tau deposits at 10 days of expression in the lamprey. This section was immunostained with Tau12. Insets show some tau uptake by and translocation across ependymal and meningeal cell layers, with significant migration along these surfaces. Diffuse and periaxonal focal deposits are also visible in the low magnification image, as are dendritic profiles belonging to the ABC expressing tau. Panel B: Transverse section through the midbrain of a CTE patient showing typical tau lesions at low magnification (left) and high magnification views (insets, 1-4). Tau immunolabel gradients resembling those in the lamprey at low magnification result from the preferential localization of tau+ neurites and NFTs in the vicinity of blood vessels (inset 2) the meningeal surface (insets 1, 3) the ventricular surface (inset 4). Panel C: The predominantly intracellular localization of PV tau in CTE (left) is compared with PV tau deposits in the lamprey, which are initially diffuse in appearance and largely extracellular (10d) but become progressively more condensed and restricted to intracellular sites with time (arrows). Scale Bars: A 50 μm, B inset 2 200 μm, B inset 4 100 μm, C CTE 50 μm, C 20d, 50 μm.

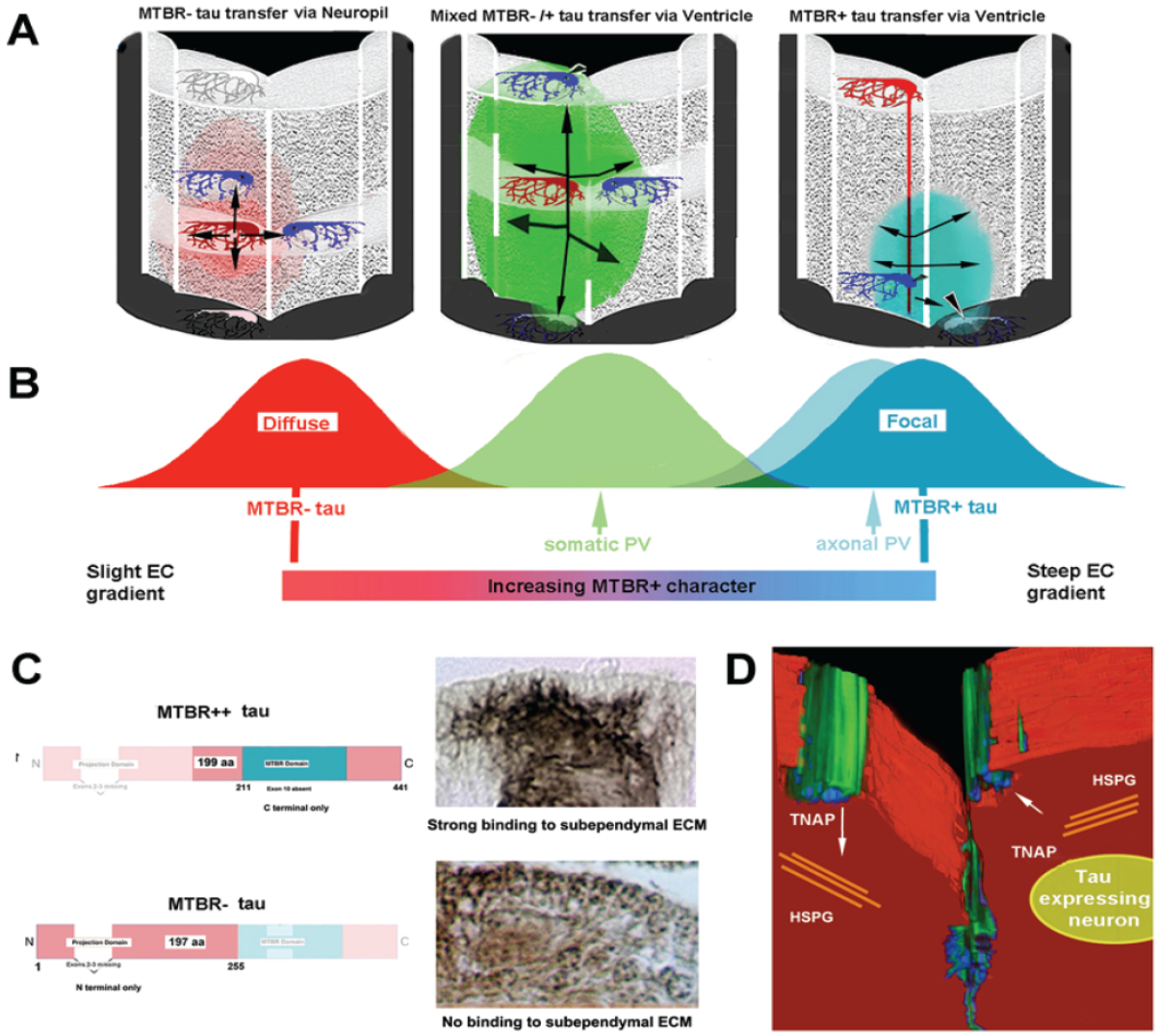

Figure 8. Factors regulating the mobility and phosphorylation state of diffuse and PV EC tau species **.** Panel A: Schematic representations of the migration and uptake patterns of diffuse (left) and periventricular (PV, center and right) in the lamprey model described in this study. Panel B: The distribution mobility gradients and phosphorylation state suggest that diffuse and focal EC tau have relatively well defined characteristics that are not sensitive to the tau species being expressed (N terminal WT, full length WT or full length tauopathy mutant) and which are consistent with their being composed of MTBR- and MTBR+ tau species respectively. By contrast, the mobility of periventricular tau appears to be affected to some extent by its site of origin, with PV tau originating from the axon more closely resembling "focally" secreted tau of dendritic origin. Panel C: The generation of subependymal tau deposits from dorsal somatic regions of heavily expressing ABCs appears to depend on the MTBR. C terminal (211-441) produced exclusively extracellular tau deposits that were tightly localized to the extracellular matrix near the secreting neuron (right top), whereas N terminal (1-255) tau fragments of similar size (left) produced no accumulations in this area. Panel D: We propose that interactions between the MTBR and ubiquitous extracellular ligands in the CNS such as heparin sulfate proteoglycans (HSPGs) may account for the differences in slope between MTBR+ and MTBR- tau species (Figure 4). The preferential dephosphorylation of EC tau (relative to intracellular tau – see figure 6) may be due to the activity of extracellular phosphatases such as TNAP, which has been shown to dephosphorylate extracellular tau in other models and in human brain [72].

bilization of somatically released tau in the subependymal space. This directly implicates MTBR mediated interactions of tau with extracellular matrix elements in PV deposit distribution and also supports our assumption that PV tau is largely MTBR+.

Re-entry of PV tau into the neuropil and uptake by remote neurons and glia

The ability of PV tau deposits to re-enter the neuropil from the ventricle and be taken up by cellular elements at remote locations is key to its relevance to neurofibrillary lesion spread in human disease. This made the unambiguous identification of PV re-entry gradients particularly important for deposits originating from axons, given the known involvement of axonal tau release in trauma-associated tauopathies [60]. We used the following criteria to identify PV re-entry gradients in the lamprey brain: a) the presence of tau deposits at the ventricle with intensity gradients (see below) centered on the ventricular surface, b) the absence of any other cellular or EC tau source in the section being examined (see **Figure**s **3c** and **5a-b** (left) for examples) and c) continuity with perisomatic and periaxonal tau gradients (respectively) at a point remote from any other possible tau source.

Quantitative analysis of the distribution and extent of EC tau deposits in the lamprey

We used densitometric image analysis of individual Tau12 immunostained sections (Methods) at 10, 20 and 30-40 days post plasmid injection to characterize and quantify two key characteristics of EC tau distribution in the lamprey brain, a) the intensity gradient slope (i.e. the degree of localization to a tau source within the neuropil of a given brain section – **Figure 4**) and b) the "footprint" of tau distribution across multiple sections at intervals of either 5 or 7 sections (i.e. the extent of diffuse and periventricular EC tau distribution) relative to tau-expressing ABC(s) shown to be secreting tau (**Figure 5**).

We found that the slopes of PV re-entry intensity gradients were steeper than those of diffuse gradients, but also significantly shallower than either dendritic or axonal focal deposits (**Figure 4c**). However, the PV deposits that were definitively associated with axonal release sites had significantly steeper slopes than PV deposits of somatic origin and were indistinguishable from those of both dendritic and axonal focal deposits (**Figure 4c**, **Figure 8**). The presence of the MTBR domain and the P301L tauopathy mutation both modulated the distribution of PV deposits, but did so in different ways (**Figure 4**). MTBR deletion abolished focal tau secretion and increased diffuse secretion as expected [18], with the "slope" of the 1-255 intensity gradients being identical to those of diffuse deposits from full length WT and P301L tau. While the P301L tauopathy mutation had no effect on the intensity gradients of any EC tau deposit, it greatly increased the incidence of both diffuse and PV tau deposits at all timepoints examined. This effect was most pronounced with respect to the extent of diffuse deposits as seen in "footprint" analyses (**Figure 5**), which increased 10 fold between 10 and 30-40 days of tau expression, both in absolute terms and with respect to control 4R0N WT tau. By contrast, the increases in extent over time seen with WT tau expression were not significant and started from a much lower baseline value. The effect of the P301L mutation on PV deposits was most marked within 10 days of plasmid injection, where it potentiated both the incidence and extent of PV tau deposits (**Figures 4** and **5**).

Tau is localizated to subependymal and perimeningial extracellular matrix

Accumulation of tau immediately under the ventricular ependymal (subependymal) cell layer and along the meningeal margin of the brain appeared to be dependent on the presence of the MTBR. Perimeningeal deposits (**Figure 7c**, **right panel**) were typically accompanied by and similar to focal EC deposits, but emerge from very tips of dendrites extending onto and around meningeal surface. Neither subependymal nor perimeningeal tau deposits were seen with the expression of 1-255 tau, and were typically immunopositive for MTBR-specific antibodies. We found that the removal of the N terminal half of the tau molecule, which abolished almost all tau secretion in cell culture models [19] and during the first 10 days of expression in ABCs [18], did permit the emergence of EC tau in the vicinity of the dorsal soma in a minority of ABCs at longer terms of expression. Interestingly, these deposits were almost exclusively subependymal and were not observed to reach the ventricular surface (**Figure 8c**).

Confocal/Adjacent section analysis of diffuse/PV tau phosphorylation state

In earlier studies, we found that much of the misprocessed tau that is distally transported in

ABC dendrites and then focally secreted appears to be dephosphorylated shortly after secretion, but that at least some focal EC tau retains phosphorylated epitopes [18]. This is unlike most diffusely secreted tau, which is usually negative for phosphotau mAbs even when strong staining with phospho-independent tau mAbs (e.g. Tau12, Tau5) is present in adjacent sections. For this study, we used mAbs specific for phosphorylation sites within (12E8) and at the C terminal end of the MTBR (PHF1) to determine if PV tau did indeed possess MTBR-associated epitopes that would validate it as being MTBR+. We found that PV tau was frequently immunopositive for phosphoepitopes associated with disease, including 12E8 and PHF1, but was somewhat less frequently labeled for the MTBR+ phospho-mAbs. However, the anti-MTBR polyclonal (K9JA) was positive for PV tau when used in this study, suggesting that much PV tau is indeed MTBR+. However, this does not exclude the possible presence of some MTBR- tau in PV tau deposits which may be relevant to the distinct slopes seen with somatic (as opposed to axonal PV).

PV and perimeningeal EC tau deposits in CTE patients

In order to ascertain the relevance of the PV and perimeningeal routes for the spreading of EC tau seen in the lamprey model to human disease, we compared the EC tau deposit types and distribution in the lamprey with immunostained cortical samples taken post-mortem from CTE patients [61] as shown in **Figure 7**. Our rationale for using CTE patients was that the localized EC tau deposits are generated by repeated focal injuries to a variety of loci in CTE [52]. This should be more directly comparable to the effects of localized tau overexpression in the lamprey model than the tau lesions seen in idiopathic dementias such as AD, which are unlikely to show direct evidence of tau lesion migration due to their gradual development [62-63]. Moreover, the coincidence of CTE with elevated AD risk suggests that the tau lesions found with CTE are themselves directly relevant to tau lesion spreading in idiopathic tauopathies. We found a number of features that appear comparable with lamprey model PV and perimeningeal EC tau deposits as described above. Neurofibrillary tau lesions in CTE brainstem (**Figure 7**) and cortex (not shown) showed both periventricular and perimeningeal tau accumulations with overall characteristics similar to those defined in the lamprey model (Methods). A notable difference between the lamprey EC tau pattern and human CTE patterns was that most tau that was distributed in a "gradient" pattern in CTE (**Figure 7b**, **left**) had been internalized (**Figure 7c**, **left)**, presumably due to the extended intervals intervening between the injuries that produced the EC tau in CTE and the death of the subject unlike the situation in the lamprey, where most lesions were examined after 10 days of expression. The lamprey ETC tau deposits became increasingly cellular over time and thus came to resemble those in CTE (**Figure 7c**). The perivascular "halo" tau deposits centered on blood vessels that are widely reported in the EC tau lesions of CTE [60,64] bore a strong superficial resemblance to PV re-entry gradients in the lamprey (**Figure 7B**, **inset 2**).

Discussion

In this study we describe two distinct non-synaptic patterns of long distance spreading of extracellular human tau deposits in the lamprey brain. These "diffuse" and "periventricular" patterns differ characteristically from one another in their sites of origin, degree of localization, migration routes within the brain and their ability to cross-adjacent neurons and ependymal cell layers. We showed that PV tau re-enters the brain from the ventricles after having traveled significant distances within the brain, whereas diffuse tau moves without apparent impediment through the neuropil adjacent to ABC somata and enters adjacent neurons readily. Our results suggest that diffuse and PV tau species correspond to N terminal (MTBR-) and near full-length (MTBR+) tau species respectively. The rates of tau migration is consistent with the possibility that tau can travel via PV and perimengial routes over the course of AD, especially since tau lesions develop at the cellular level over the course of months/years [62-63].

Differences between PV and diffuse tau spreading are correlated with and/or consistent with the presence/absence of the tau MTBR

Tau misprocessing and toxicity is associated with the generation of both MTBR- and MTBR+ tau species, some of which may be both toxic and capable of spreading neurofibrillary tau lesions [31, 39-41, 46, 65-66]. We found that

both MTBR- and MTBR+ extracellular tau species characterized in this and previous studies in the lamprey ABC model could be found at locations remote from their sites of origin by 10 days of tau expression. This suggests that extracellularly localized human tau can move through the brain and can reach the ventricles, meninges and other neurons in neuropil via mechanisms in addition to synaptically mediated transfer (which also has been demonstrated in this model) [18]. This is also consistent with the observations of both MTBR- [9, 51, 67-68] and MTBR+ [13, 68] in the CSF in studies in the various idiopathic and trauma associated tauopathies in humans.

While the mechanisms responsible for EC tau translocation remain largely obscure, our results provide a number of insights that may be of relevance to human tauopathies. Firstly, the absence of intrinsic vasculature from the CNS of the ammocoete sea lamprey [54,69] suggests that tau translocation does not require the circulation to move considerable distances within the brain, although it does not exclude blood-based mechanisms. More importantly, our results strongly suggest that a) the tau MTBR region is at least partially present in PV tau secretion and that it plays some role in its spreading mechanism, and b) the MTBR is either absent from or nonfunctional in diffusely secreted tau fragments. We propose that the tau MTBR domain is a key factor that distinguishes between PV and perimeningeal EC tau deposits based on the following: a) Expression of 1-255 tau lacking the MTBR perfectly reproduced the diffuse tau origin and distribution pattern, but abolished accumulation of tau at distal sites within the dendrites and all focal secretion from the dendrites, confirming earlier findings that the removal or inactivation of the MTBR [55-57] prevents active transport away from the soma [18] in ABCs. b) The much steeper distribution gradients and the lack of free transcytosis (both indicating more restricted mobility) seen with focal vs diffuse deposits is consistent with MTBR-mediated interactions with ubiquitous extracellular matrix elements (e.g. heparan sulfate proteoglycans) known to bind the tau MTBR [70-71] strongly (**Figure 8C-D**). This is also consistent with the greatly reduced slope of peridendritic (i.e. "focal") tau deposits (**Figure 3C**) near ABCs expressing full length tau isoforms with inactivated MTBRs (i.e. S262D/S356D tau), which prevented their identification as such in an earlier study [18]. c) The observed presence of MTBR associated epitopes on PV but not diffuse EC tau (**Figure 6**). d) The origin of PV from periaxonally-secreted tau suggests that this tau was transported to the site of secretion in the axon, and thus is likely to be MTBR+. This is consistent with the identical gradient slopes shown by periaxonal focal deposits and PV reentry gradients associated with axonal tau secretion. The observation that PV tau originating from ABC somata has a significantly shallower distribution gradient than either axonal PV tau or focally secreted tau (**Figure 3**) suggests that a less selective release mechanism may be responsible for tau release from the dorsal soma [18].

The presence of diffuse pattern secretion from ABCs expressing full-length tau isoforms is the result of proteolytic removal of the tau N terminal projection domain from the MTBR during the misprocessing of tau en route to its secretion via both diffuse and focal/PV pathways. Since efficient tau secretion in both the lamprey and in human cellular models requires the tau N terminus [18-19], it seems likely that tau cleavage occurs after the commitment to the "misprocessing pathway" has been made. If so, this would account for the exacerbation of focal and PV as well as diffuse secretion by the P301L mutation.

The phosphorylation state of secreted tau is consistent with the absence of the MTBR from diffusely secreted tau and its presence in PV tau

Both diffuse and PV tau species were found to be phosphorylated at least some of the key disease-associated serine/threonine and tyrosine sites tested (**Figure 2c**). Overall, EC tau species were consistently less phosphorylated than intracellular tau (see examples in **Figure 6b**). This suggests that diffusely secreted tau is inherently less phosphorylated than PV tau, but that both types of tau are dephosphorylated to a large degree at or near the site of production. This is consistent with the recent study by Diaz-Garcia [72], who showed that the activity of the ubiquitous tissue neutral alkaline phosphatase (TNAP) accounts for the relatively dephosphorylated state of EC tau in other cellular models and in human brain (**Figure 6**, schematized in **Figure 8c**). Moreover, the presence of AT8+, AT180 and PHF1+ tau inside ependymal cells

adjacent to PV deposits (**Figure 6a**) and AT8+ in some neurons within the field of diffusely secreted tau (**Figure 6b**) suggest that even diffusely secreted (and presumably MTBR-) tau can be rephosphorylated upon being taken into other cells.

Nature of tau gradients within the brain and the possible role of polyanionic ligands in the extracellular matrix

Key questions raised by the observed differences between diffuse and focal tau include of a) how MTBR- "diffuse" tau might translocate across neuronal membranes and epithelial barriers and b) how the presence of the MTBR might produce the characteristic differences between PV and diffuse deposits in their cell penetration patterns and intensity gradients. MTBR- EC tau exhibited immunolabel gradients within the neuropil that were unaffected by the presence of large cellular elements and even the central sulcus. This is reminiscent of morphogenic protein factors that help establish axial and radial polarity during the earliest phases of embryonic development cases [73]. Like "diffuse" tau, morphogens generate stable concentration gradients within developing tissues irrespective of the presence of cellular elements, show multiple distribution patterns and tend to bind to cell matrix elements, including HSPGs, possibly accounting for the stability of these gradients. Moreover, initial distribution away from their sites of release appears to be largely passive in at least some cases [74]. Interestingly, morphogen oligomerization now appears to be important to their efficient transcytosis in some cases [73]. While the dynamics and regulation of morphogen translocation across membranes are still poorly understood, their distributions within tissues over time can be described in terms of conventional endocytotic/exocytotic mechanisms [75]. Alternatively the translocation might resemble those of "cell penetrating peptides", which tend to be rich in positively charged, nonpolar and in some cases proline residues [76-77]. The amino acid composition of the tau N terminal domain shares some of these characteristics (unpublished analysis), but the actual mechanism of "diffuse" tau secretion remains obscure.

Finally, the striking parallelism between the perimeningial and PV EC deposits generated by tau expression in the lamprey and those seen in the brains of CTE patients (**Figure 7**) suggest that common mechanisms of EC tau production and dispersal exist. If so, these mechanisms likely to be highly relevant to injury associated tauopathy in humans [61] and may also be relevant to the pathogenesis of at least some forms of LOAD [52]. They raise the possibility that non-synaptic mechanisms could play a role in tau lesion spread in human neurodegenerative tauopathies.

Declaration

There are no conflicts of interest for all authors.

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