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Reduced Adrenomedullin Parallels Microtubule Dismantlement in Frontotemporal Lobar Degeneration

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Abstract

Tau is a microtubule-associated protein highly expressed in neurons with a chief role in microtubule dynamics and axonal maintenance. Adrenomedullin gene (*ADM*) codifies for various peptides that exert broad range of actions in the body. Previous works in our groups have shown that increased *ADM* products are positively correlated to microtubule disruption and tau pathology in Alzheimer's disease brains. In the present study, we explore the involvement of *ADM* in the neuropathology of frontotemporal lobar degeneration that presents with primary tauopathy (FTLD-tau). Proteins from frontal cortices of FTLD-tau patients and age- and sex-matched non-demented controls were analyzed with antibodies against different microtubule components, including adrenomedullin, and synaptic markers. Tau pathology in frontal cortex from FTLD patients was confirmed. Levels of total βIII-tubulin as well as acetylated and detyrosinated tubulins, two markers of stabilized and aged microtubules, were significantly reduced and directly correlated with PSD95 and proBDNF in FTLD-tau patients when compared to non-demented controls. In contrast, no change in actin cytoskeleton was found. Interestingly, changes in microtubule elements, indicators of disturbed axonal preservation, were accompanied by decreased levels of free adrenomedullin, although no association was found. Altogether, reduced levels of adrenomedullin might not be directly linked to the microtubule pathology of FTLD-tau, but based on previous works, it is suggested that downregulation of *ADM* might be an adaptive attempt of neurons to mitigate microtubule disruption.

Keywords Adrenomedullin · Tubulin · Frontotemporal · Tauopathy

Frontotemporal lobar degeneration (FTLD) is a pathological condition that predominantly presents with frontotemporal dementia (FTD) and results from the selective and progressive deterioration of the frontal and temporal lobes of the brain. Depending on the affected regions, patients with FTLD can display progressive changes in behavior, executive dysfunction, and/or language abnormalities, giving rise to distinct clinical symptoms: behavioral variant of frontotemporal dementia (bvFTD), semantic variant primary progressive aphasia (svPPA), and progressive non-fluent aphasia (PNFA). The neuropathology of FTLD is also heterogeneous, and hence, FTLD has been classified into broad categories according to the type of intracellular protein deposits: tau, transactive response DNA-binding protein 43 (TDP-43), or fused in sarcoma (FUS), being each of them either positive or negative for ubiquitin protein [1].

Primary tauopathies are defined by the presence of insoluble and hyperphosphorylated tau proteins in neurons and glial cells. These disorders fall into the clinical spectrum of FTLD (hereinafter referred as pathological subtype FTLD-tau), predominantly presenting with bvFTD, svPPA, and PNFA but also with atypical parkinsonism syndromes such as progressive supranuclear palsy and corticobasal degeneration [2, 3]. The rest of FTLD cases can be assigned to one of the two other

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major pathological subtypes: FTLD-FUS and FTLD-TDP [4]. FTLD is highly linked to family history of neurodegenerative disease (around 40% of cases). Mutations in microtubule-associated protein tau (*MAPT*) gene are frequent in patients with FTLD-tau and cause a subtype of frontotemporal dementia with parkinsonism linked to chromosome 17-tau (FTDP-17T) [5]. Tau is a microtubule-associated protein with a chief role in microtubule stabilization by promoting their polymerization and suppressing their dynamics when assembled. Phosphorylation of tau is important for microtubule dynamics in physiological conditions. However, when tau is abnormally hyperphosphorylated in response to a number of stressors or mutations, tau dissociates from the microtubule cytoskeleton, leading to its instability and causing axonal degeneration [6]. Unbound hyperphosphorylated tau is prone to aggregate and assemble into intracellular fibrillar deposits. In contrast to Alzheimer's disease (AD), where tau deposits are made up of all isoforms, FTLD-tau fibrils are heterogeneous but usually enriched in one of the six tau isoforms, which actually helps defining neuropathological sub-divisions [7, 8]. However, all these pathological phenotypes share a common feature: abnormal hyperphosphorylation of tau is primary and central to the disease by leading to microtubule disruption and axonal degeneration.

Microtubules are composed of globular tubulin proteins. Post-translational modifications (e.g., acetylation, detyrosination) of tubulins and their binding to other cellular proteins orchestrate the dynamics of microtubule polymerization and depolymerization, which are necessary for proper axonal and dendrite organization in neurons. Previous studies identified novel microtubule interactors with prominent role in tubulin dynamics [9]. In particular, two adrenomedullin (*ADM*) gene products, proadrenomedullin N-terminal peptide (PAMP) and adrenomedullin (AM), can bind directly to tubulin and kinesin and other microtubule-associated proteins, and participate in specific functions such as destabilization of microtubule polymerization and increased transport velocity through microtubules [9, 10]. Further, increased *ADM* immunoreactivities were found in apical dendrites and axons in brains from AD patients and mouse models [11, 12], and the fraction of *ADM* peptides bound to tubulin was reported to be increased in aged and AD brains [11, 13]. It is interesting to note that conditional deletion of *ADM* is able to revert aged-related memory impairment and abnormal tau phosphorylation in rodent brains [13]. Taking all these data into account, we decided to explore the status of *ADM* products and its relationship to microtubule dismantlement in a neurodegenerative condition where tauopathy is a primary pathological event, such as FTLD-tau.

To this purpose, brain tissues from FTLD patients were obtained from the Brains for Dementia Research Initiative (BDR, UK). Informed consent was obtained from the patients' next of kin before collection of brains, and the study was approved by the UK National Research Ethics Service. Demented subjects ($n = 10$) fulfilled criteria for the clinical diagnosis of FTLD and pathological classification of FTLD-tau according to recent classification [14]. Controls ($n = 10$) matched for age (62.5 ± 2.2 vs 61.9 ± 3.3, controls and FTLD respectively), sex (5 males/5 females vs 6 males/4 females), and post-mortem delay (34.1 ± 4.9 vs 31.0 ± 4.3 h) did not have dementia or any other neurological diseases and were staged at Braak 0–II. Small frozen and meninge-free pieces of frontal cortices from Brodmann area (BA)10 were used for subsequent analysis, which were performed blind to clinical information. Brain pH measurements were determined for each frontal cortex in deionized water as an index of acidosis associated with terminal coma, and cases were subsequently excluded if the pH was found to be below 6.1. Proteins from BA10 homogenates were isolated in RIPA buffer, resolved by SDS-glycine gel electrophoresis (as described in [11]) and subsequently immunoblotted with different antibodies against cytoskeletal components, including adrenomedullin, and synaptic markers (Table 1).

Table 1 Antibodies

Target	Species	Dilution	Source
Adrenomedullin	Rabbit polyclonal	1:200	In house
Acetylated tubulin	Mouse monoclonal	1:15,000	Sigma T7451
Glu-tubulin	Rabbit polyclonal	1:1000	Millipore AB3201
βIII-tubulin	Rabbit polyclonal	1:1000	Sigma T2200
β-Actin	Mouse monoclonal	1:10,000	Sigma AC74
GAPDH	Mouse monoclonal	1:10,000	Sigma G8795
proBDNF	Rabbit monoclonal	1:500	Abcam EPR1292
PSD95	Rabbit polycolonal	1:1000	Cell Signaling #2507
Tau, pSer202/pThr205 (AT8)	Mouse monoclonal	1:1000	Fisher NM1020
Tau, pSer396/pSer404 (PHF1)	Mouse monoclonal	1:1000	Gift from Peter Davies
Tau, total (Tau12)	Mouse monoclonal	1:1000	Biolegend SIG-39416

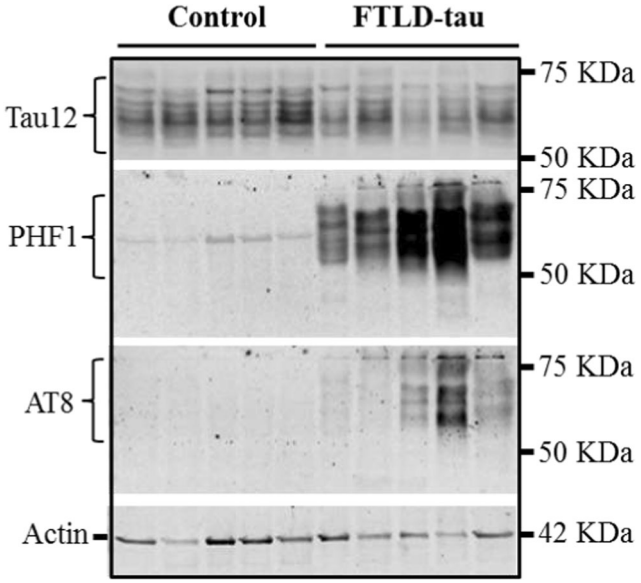

Fig. 1 Representative western blot images showing tau pathology in BA10 frontal cortex of FTLD-tau patients and matched non-demented controls. Antibody Tau12 does not detect significant changes in total levels of tau protein in FTLD-tau frontal cortices. Antibody PHF1 that recognizes Ser-396/Ser-404 shows a more prominent pattern of tau phosphorylation than antibody AT8 that is specific for Ser-202/Thr-205. $N = 10$ (controls), $n = 10$ (FTLD-tau). β-actin is used as internal loading control

As shown in Fig. 1, the antibody Tau12 showed no particular changes in the amount of total tau in BA10 frontal cortex between FTLD-tau patients and control individuals, but the antibody PHF1 that is specific for pSer-396/pSer-404 detected variable but marked pattern of tau phosphorylation in FTLD-tau brains. In contrast, the antibody AT8, which recognizes pSer-202/pThr-205, showed relative increases of tau phosphorylation in most of the cases. These observations confirm the expected tau pathology in the frontal cortex of FTLD-tau brains, although the explanations for the intra-patient variability and the distinct intensities of tau phosphorylation between antibodies are unclear. Perhaps regional differences may account for the distinct intensity signals given by AT8 and PHF1 antibodies, since the former preferentially recognizes the white matter pathology while the latter has preference for gray matter pathology of AD [15].

Neuron-specific βIII-tubulin was significantly reduced in FTLD-tau when compared to controls (Student's t test; $t(18) = 2.12$, $P = 0.024$; Fig. 2). Post-translational modifications of tubulin, in particular, acetylated tubulin and detyrosinated tubulin (glu-tub), both of which associated with microtubule stabilization, were also significantly decreased in FTLD-tau brains ($t(18) = 1.95$, $P = 0.034$; $t(18) = 1.91$, $P = 0.036$, respectively; Fig. 2). However, actin cytoskeleton remained unchanged, as shown by similar levels of β-actin between controls and FTLD-tau groups ($t(18) = -0.23$, $P = 0.41$; Fig. 2). Such decreases in microtubule components undoubtedly represent a disassembly of microtubule cytoskeleton in FTLD-tau pathology and confirm the known effects of tauopathy on microtubule stability and axonal degeneration [6]. Marked decreases of βIII-tubulin and other elements of the neurocytoskeleton, such as neurofilament proteins, but no changes in microfilament β-actin protein have been already

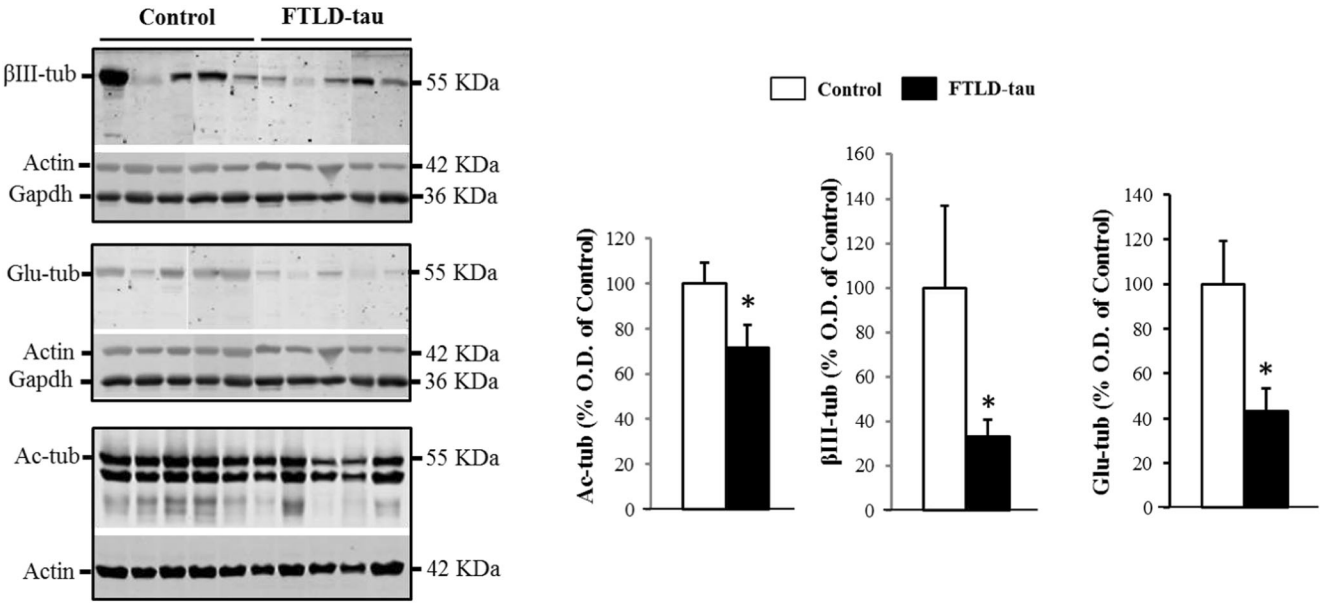

Fig. 2 Changes of various microtubule elements in frontotemporal lobar degeneration with primary tauopathy (FTLD-tau) when compared to non-demented control patients. Left panels show representative pictures of blotting images (full-length blots are shown in Supplementary Data). Right panel shows percentage of optical density (O.D.) values relativized to the control group, as mean ± SEM. βIII-tubulin, detyrosinated tubulin (glu-tub) and acetylated tubulin (Ac-tub), but not β-actin, are found significantly decreased in FTLD-tau BA10 frontal cortex when compared to non-demented controls. $N = 10$ (controls), $n = 10$ (FTLD-tau). $^*P < 0.05$, Student's t test. Gapdh is used as internal loading control

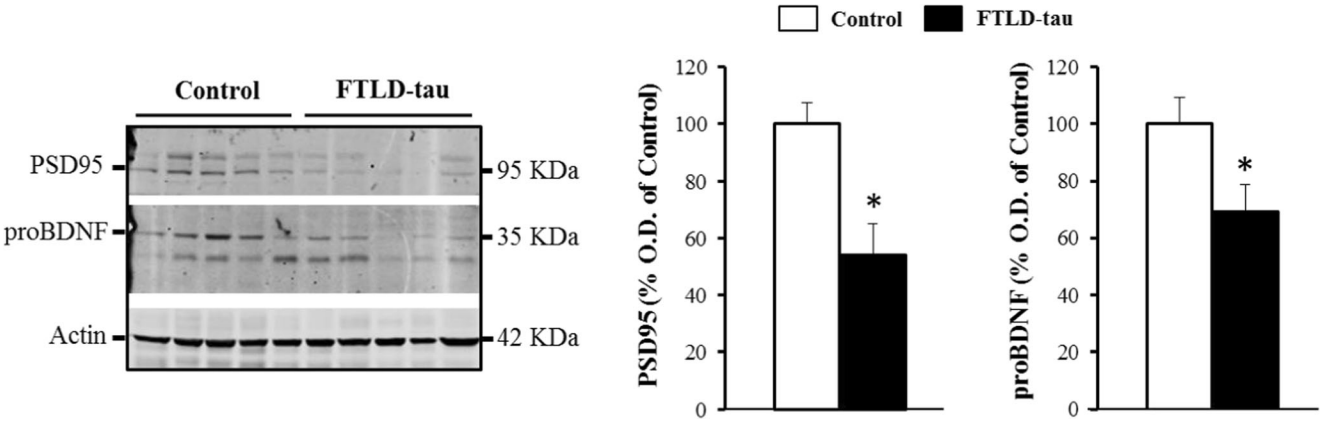

Fig. 3 Decreased levels of PSD95 and proBDNF in BA10 frontal cortex tissue from patients with frontotemporal lobar degeneration with primary tauopathy (FTLD-tau) when compared to non-demented control patients. Left panels show representative pictures of blotting image, and right panel shows percentage of optical density (O.D.) values relativized to the control group, as mean ± SEM. $N = 10$ (controls), $n = 10$ (FTLD-tau). $^*P < 0.01$, Student's t test. β-actin is used as internal loading control

reported in the frontal cortex of Pick disease patients [16], a dementia syndrome that belongs to the subtype FTLD-tau. Notwithstanding, reduced levels of acetylated and detyrosinated tubulins in brains of FTLD-tau patients have not been reported before to our knowledge, but support previous studies reporting decreases of tubulin acetylation and detyrosination in neurofibrillary tangle-bearing neurons of AD brains and in tau-depleted neurons [17–19]. PSD95 and proBDNF proteins, both of which are important for synaptic function and predominantly recruited at post-synaptic sites, were found significantly decreased in FTLD-tau ($t(18) = 3.89$, $P = 0.001$ for PSD95; $t(18) = 2.99$, $P = 0.008$ for proBDNF; Fig. 3), representing the expected disruption of post-synaptic density that takes place in neurodegenerative dementias [20]. Loss of synapses may account for the observed reductions of microtubule immunoreactivities; hence, stronger synaptic disruption should be accompanied by lower levels of tubulins. However, PSD95 was not correlated with either βIII-tubulin (Spearman's rho = 0.301, $P = 0.39$, $n = 10$), acetylated tubulin (Spearman's rho = 0.539, $P = 0.11$, $n = 10$), or detyrosinated tubulin (Spearman's rho = 0.03, $P = 0.93$, $n = 10$) in FTLD-tau brains. This data suggest that loss of microtubule components might not be related to synaptic loss. Instead, this and the lack of changes in other cytoskeletal proteins suggest the specific vulnerability of microtubule cytoskeleton to tau pathology [8, 21].

As previously reported, the anti-AM antibody showed two different bands in the brain at around 14 and 55 kDa (Fig. 4) [11, 13]. The lower band is assigned to proAM protein containing PAMP and AM moieties, while the upper band is purportedly assigned to tubulin-bound ADM peptides [9]. As seen in Fig. 4, significant decreases of proAM (Student's t test; $t(18) = 2.72$, $P = 0.013$) parallel those of microtubule components in FTLD-tau brains, although no changes in tubulin-bound adrenomedullin band were found ($t(18) = 0.46$, $P = 0.33$).

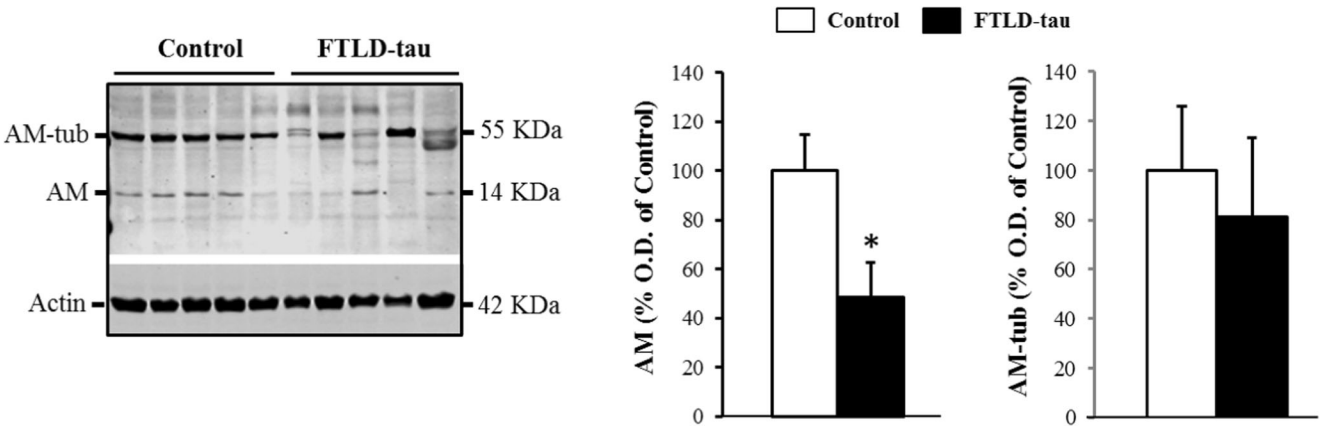

Fig. 4 Changes of ADM gene products in frontotemporal lobar degeneration with primary tauopathy (FTLD-tau) when compared to non-demented control patients. Left panels show representative pictures of blotting images (full-length blots are shown in Supplementary Data). Right panel shows percentage of optical density (O.D.) values relativized to the control group, as mean ± SEM. ProAM peptides (14 kDa) are significantly decreased while tubulin-associated ADM products (AM-tub, 55 kDa) remain unchanged in FTLD-tau BA10 frontal cortex. $N = 10$ (controls), $n = 10$ (FTLD-tau). $^*P < 0.05$, Student's t test. β-actin is used as internal loading control

Since *ADM* peptides have been shown to decorate microtubules [9], the observed reduction of proAM levels might be part of the general disassembly of microtubules in FTLD-tau brains. However, no associations between microtubule components and proAM or tubulin-bound *ADM* have been found in FTLD-tau brains (data not shown). In contrast to previous studies that have reported increased *ADM* in AD brains as a seemingly primary event to microtubule disruption and axonal degeneration [11], the defects of *ADM* that are observed in FTLD-tau brains might not be primary to the neuropathology of these dementia disorders. Instead, as *ADM* downregulation results in microtubule stabilization and increased post-translational modifications of tubulin such as acetylation and detyrosination in vitro [9], as well as improved cognitive performance in aged mice [13], it is more likely that proAM reductions in FTLD-tau brain are the result of an adaptive but barely successful response of neurons to counteract microtubule destabilization. Hence, it will be challenging to explore whether pharmacological manipulation of *ADM* peptides by small molecules (as those reported in [22, 23]) will boost microtubule stabilization under these neurodegenerative conditions. Several microtubule-stabilizing drugs, commonly used in the treatment of cancer, have been tested in animal models of neurodegeneration and they provided particular benefit to tauopathies [24]. In view of the present observations and in line with previous research work, small molecules that specifically target and inhibit *ADM* peptides should be considered candidates for microtubule-stabilizing therapies to treat FTLD-tau in early stages before massive microtubule disassembly takes place.

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Compliance with Ethical Standards

Informed consent was obtained from the patients' next of kin before collection of brains, and the study was approved by the UK National Research Ethics Service.

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