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Role of Tau Protein in Neuronal Damage in Alzheimer's Disease and Down Syndrome

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Neurodegenerative disorders constitute a growing concern worldwide. Their incidence has increased steadily, in particular among the elderly, a high-risk population that is becoming an important segment of society. Neurodegenerative mechanisms underlie many ailments such as Parkinson's disease, Huntington's disease, Alzheimer's disease (AD) and Down syndrome (DS, trisomy 21). Interestingly, there is increasing evidence suggesting that many such diseases share pathogenic mechanisms at the cellular and subcellular levels. These include altered protein misfolding, impaired autophagy, mitochondrial dysfunction, membrane damage, and altered axonal transport. Regarding AD and DS, the first common link comes from observations that DS patients undergo AD-like pathology early in adulthood. Also, the gene encoding for the amyloid precursor protein is present in human autosome 21 and in murine chromosome 16, an animal model of DS. Important functions related to preservation of normal neuronal architecture are impaired in both conditions. In particular, the stable assembly of microtubules, which is critical for the cytoskeleton, is impaired in AD and DS. In this process, tau protein plays a pivotal role in controlling microtubule stability. Abnormal tau expression and hyperphosphorylation are common features in both conditions, yet the mechanisms leading to these phenomena remain obscure. In the present report we review possible common mechanisms that may alter tau expression and function, in particular in relation to the effect of certain overexpressed DS-related genes, using cellular models of human DS. The latter contributes to the identification of possible therapeutic targets that could aid in the treatment of both AD and DS. © 2012 IMSS. Published by Elsevier Inc.

Key Words: Down syndrome, Alzheimer's disease, Tau protein, Amyloid precursor protein, Dyrk1A, Rcan.

Introduction

In neurons, the cytoskeleton constitutes a complex, dynamic and pivotal structure that not only shapes the neuronal architecture, but also plays an essential role in different functions and properties of neurons. These include vesicle transport, transmitter release, neurite elongation, synapse formation, and cone growth as well as regulation of plasticity. Thus, malfunction of this important cytoskeleton network results in common pathophysiological mechanisms that underlie severe diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and Down syndrome (DS). A critical process in such functions depends on the correct, stable assembly of microtubules where tau protein, a microtubule-associated protein (MAP) abundantly expressed in axons, plays a critical role. In the present paper we present evidence of abnormal tau expression and function as a possible mechanism underlying these ailments.

Tau Structure and Its Role in Microtubule Dynamics

Tau is a cytosolic protein encoded by a gene located on the long arm of human chromosome 17 in band 17q21. The

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gene has >100 kb and 16 exons (1). As shown in Figure 1, the protein comprises four regions: (i) an acidic region at the N-terminus that is encoded by exons 1–5; (ii) a proline-rich region that is encoded by exon 7 and the first half of exon 9; (iii) a region responsible for binding to microtubules called microtubule-binding domains (MBDs) that is encoded by exons 9–12; this region contains four imperfect 18-amino acid repeats called R1, R2, R3 and R4, and (iv) a C-terminal region that is encoded by exon 13 (1).

In the adult human brain, exons 4A, 6 and 8 are not expressed (2), whereas exons 2, 3 and 10 undergo alternative splicing (1), generating six isoforms (Figure 1). Thus, depending on the exons that are spliced, the isoforms generated are 0N3R, 0N4R, 1N3R, 1N4R, 2N3R and 2N4R where 0N corresponds to an isoform where exons 2 and 3 are spliced together, 1N where exon 3 is spliced, 2N where both exons 2 and 3 are encoded, 3R where exon 10 is spliced and 4R where exon 10 is encoded. In the adult human brain, the six isoforms are expressed, the 3R and 4R isoforms being expressed in almost equal amounts (3,4). Conversely, in fetal brain, only the shortest isoform (0N3R) is expressed (1).

Both 3R and 4R isoforms of tau promote tubulin polymerization (5). However, the 4R isoform has a greater affinity for microtubules (6,7) and stabilizes microtubules significantly more robustly than the 3R isoform (5). In this regard, changes observed in the expression of these isoforms during the development of the nervous systems seem to have physiological implications because it would allow microtubule behavior to adapt from a more dynamic and plastic network in fetal neurons to a more stable structure in adult neurons. On the other hand, an imbalance in the ratio of the 3R and 4R isoforms seems to contribute decidedly to the pathogenic mechanism of neurological diseases such as DS (8), different types of frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (9,10), Pick's disease (11), progressive supranuclear palsy (12–14) or corticobasal degeneration (15).

Early studies demonstrated that tau phosphorylation also affects its ability to bind microtubules and promote their assembly (16–18), findings that were later confirmed by several groups (19–21). At least 45 phosphorylation sites have been identified in the tau protein, most being serine and threonine residues (22). Among the different kinases that phosphorylate tau physiologically in the brain are glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinase 5 (Cdk5), mitogen-activated protein kinases (MAPK) and SRC family tyrosine kinases (23–25).

Whereas tau phosphorylation plays a physiological role in microtubule dynamics, aberrant hyperphosphorylation of this protein impairs its ability to bind microtubules (26), thus resulting in their disassembly (27,28), tau self-assembly (29), and formation of tau aggregates such as paired helical filaments (PHF) or straight filaments (SF) (29). As we discuss below, both types of intracellular filaments are observed in different neurodegenerative diseases. Among the kinases that importantly contribute to abnormal tau hyperphosphorylation are GSK-3β (30–32) and Cdk5 (33,34), which seem to act cooperatively to induced tau phosphorylation (35) and aggregation (36). Also, the Abelson nonreceptor tyrosine kinase (c-Abl) and AMP-activated protein kinase (AMPK) have also been shown to phosphorylate tau under pathological conditions such as AD

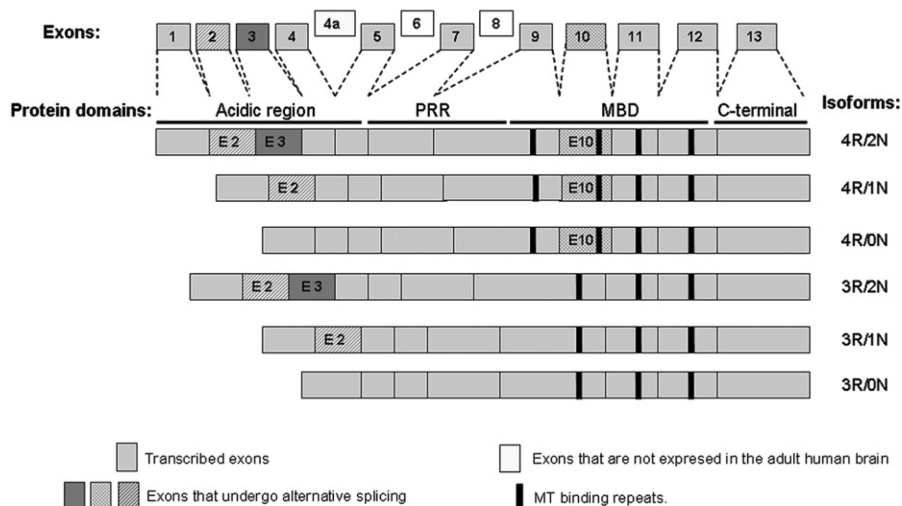

Figure 1. Schematic representation of tau mRNA and protein. The gene has 16 exons, but exons 4A, 6 and 8 are not expressed in the adult human brain. Exons 2, 3 and 10 undergo alternative splicing to generate the isoforms 0N3R, 0N4R, 1N3R, 1N4R, 2N3R and 2N4R. All isoforms contain four common domains: (i) an acidic region at the N-terminus encoded by exons 1–5; (ii) a proline-rich region (PRR) encoded by exon 7 and the first half of exon 9; (iii) a region responsible for binding to microtubules (MT) called microtubule-binding domains (MBD) and that is encoded by exons 9–12; this region contains four imperfect MT binding sites, and (iv) a C-terminal region encoded by exon 13.

(37−40). Another kinase involved in tau hyperphosphorylation and neurofibrillary degeneration is the dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A). In fact, high levels of DYRK1A have been found in cerebral cortex of patients with AD, DS or Pick disease (41). Furthermore, DYRK1A phosphorylates tau at several serine and threonine residues (41), primes tau for phosphorylation with GSK-3β (42,43) and, as we later discuss in more detail, plays an important role in neurofibrillary degeneration in DS (42,44,45).

Tau in Different Neurodegenerative Diseases

The presence of tau protein in PHF of brains from AD patients was described for the first time in 1986 by different laboratories (46−50). In the same year it was also proposed that abnormal tau phosphorylation may be the cause of the neurofibrillary abnormalities observed in diseased brains (47,51,52). One year later, tau was also found in tangles from brain tissue of postmortem patients suffering from DS, Pick's disease, progressive supranuclear palsy, and the parkinsonism-dementia complex of Guam (53). Later, tau-containing tangles were found in brains of patients suffering from other neurodegenerative diseases such as amyotrophic lateral sclerosis (54) or Niemann-Pick Disease Type C (55). Thus, these pioneering findings suggested that tau hyperphosphorylation could constitute a common pathogenic pathway in different neurodegenerative diseases. This idea was later supported by increasing reports of mutations in the gene that codifies tau and that are associated with FTDP-17, an autosomal dominant and progressive neurodegenerative disease clinically manifested by behavioral disorders, motor and cognitive impairment, and characterized as frontotemporal atrophy (56). Hitherto, >30 mutations in the tau gene have been reported in the last 15 years. These include either intronic or coding mutations, most occurring in exons 9−13 (57). These mutations impair both the interaction of tau with microtubules (58,59) and its ability to regulate microtubule dynamics (60−63). They are also associated with aggregation of hyperphosphorylated tau in neurons and/or glial cells forming PHF or SF (64). Furthermore, transgenic mice carrying human tau mutations associated with FTDP-17 develop tau aggregates and motor and cognitive impairments (65−67). Thus, these studies on FTDP-17 mutations have contributed to a better comprehension of tau function and its contribution to neurodegenerative diseases associated with tau aggregates.

Interplay between Amyloid-β Peptide and Tau in Alzheimer's Disease

AD is a neurodegenerative disorder characterized by progressive memory loss and other cognitive impairments leading to severe dementia. At the neuropathological level, this disease is characterized by two types of lesions: extracellular accumulation of insoluble deposits of amyloid-β peptide (Aβ) termed senile plaques (SP) and intracellular neurofibrillary tangles (NFT) composed of aggregates of hyperphosphorylated tau (68). Most AD cases are sporadic with an unknown etiology, but a small proportion of cases are heritable and related to mutations in genes that encode the amyloid precursor protein (APP) or the constituents of the γ-secretase complex, presenilins 1 and 2, which affect Aβ production and deposition (68).

Although SP and NFT are present in both familiar and sporadic forms of AD and are used as postmortem confirmation for AD, only the number and localization of NFTs have been correlated with levels of dementia and such correlation has not been demonstrated for SP (69−71). However, until now, no mutations related to the tau gene have been identified in AD, suggesting that tau pathology in AD would occur downstream to Aβ pathology. In this regard, studies using a transgenic mouse bearing mutations in APP, presenilin 1 and tau revealed that amyloid deposition develops prior to tangle pathology (72), a finding consistent with the hypothesis that Aβ aggregation is the primary event responsible for disease progression (73,74). Aβ is produced by abnormal proteolytic processing of their precursor APP, which is sequentially cleaved by β-secretase and γ-secretase (68). This peptide varies in length, with the 40 amino acids form ($A\beta_{40}$) being the predominant species and the 42 amino acid form ($A\beta_{42}$) the most prone to oligomerization and fibril formation (75−77). More recently, diverse evidence indicates that soluble oligomers of Aβ (Aβos) accumulate in synapses, triggering memory impairments and neural plasticity early before fibrillar amyloid deposition and neurodegeneration takes place (78−82). In this regard, some potential receptors for Aβos have been proposed including the cellular prion protein (PrP^C) (83) and glutamatergic receptors (84−86) through which Aβ oligomers would alter the integrity and function of synapses well before the overt neuronal loss. Nevertheless, because Aβos can exist in several conformations including dimers, trimers, dodecamers, globulomers (amyloid derivative diffusible ligands, ADDLs) and annular protofibrils, a precise mechanism for their toxic activity remains to be elucidated (87).

Several reports have shown an association between Aβ and hyperphosphorylated tau (72,88,89). For instance, soluble Aβ can induce inactivation of phosphatases (90) and activation of tau kinases (91,92), promoting tau phosphorylation (91−94), PHF formation (88,89,94,95) and cognitive impairments (96). Remarkably, it has been described that the direct interaction between tau and Aβ peptide induces tau aggregation and hyperphosphorylation (97). Further, when both proteins decrease, a recovery of cognitive abilities is observed (98,99), suggesting that hyperphosphorylated tau plays a role in the early synaptic

and cognitive damage observed in AD. Moreover, impairments induced by Aβ in forms of synaptic plasticity such as long-term potentiation, which is considered an electrophysiological correlate of learning and memory (100), are mediated by tau phosphorylation, thus providing additional support to tau protein as an element required for the synaptotoxic effects of Aβ oligomers (101). Interestingly, mice overexpressing human tau display impairments in synaptic plasticity and cognition (102). Normally, tau is enriched in axons, whereas in AD it is redistributed to the dendritic compartment (102–104) where it alters axon transport (105) and probably interacts with Aβos (106). In fact, tau phosphorylation induces tau mislocalization and spine remodeling, affecting the synaptic targeting of glutamate receptors in the postsynaptic membrane (104,106–108). Thus, tau reduction may represent an effective strategy to prevent the early synaptic damage induced by Aβ before frank neurodegeneration takes place.

As described above, the dynamic balance between assembly-disassembly of microtubules is crucial to maintain the stability of the cytoskeleton and neuronal morphology and integrity. Disruption of this equilibrium may lead to alterations in the precise formation of neuronal processes (i.e., axons and dendrites) as well as in their functionality as observed in diverse neurodegenerative diseases (109–111). In this sense, MAPs play a pivotal role in this balance by contributing to adequate supply of proteins and organelles essential to neuronal function and viability. In particular, the equilibrium between phosphorylation/dephosphorylation of tau can modulate the stability of microtubules and thus contribute to axonal transport (26,112,113). In AD, as in other tauopathies, abnormal hyperphosphorylated tau loses its capability to stabilize microtubules (114) and acquires a toxic function whereby it sequesters normal tau and other MAPs, aggravating microtubule disruption (27,115,116). Furthermore, Aβos can cause missorting of microtubules (108), disruption of axonal transport (116), and neurotoxicity (117) in a tau-dependent manner (118,119). Interestingly, these effects can be prevented when tau levels are reduced (120). Thus, numerous evidences strongly suggest that neurodegeneration initiated by Aβ can be modulated by tau protein. Tau phosphorylation is regulated by the sequential and concerted action of diverse kinases and phosphatases and the levels of these kinases/phosphatases change in AD (32–43), suggesting that tau hyperphosphorylation is likely to be caused by an imbalance of the complex protein phosphorylation/dephosphorylation systems (121).

Up to now, AD research has been traditionally focused on Aβ and consequently therapies have been directed to prevent amyloid oligomerization and deposition (122). However, a more integrative view of AD would be necessary, which should include downstream targets like tau protein. A growing body of recent evidence suggests that tau is an important and necessary player in synaptic and neural damage observed in AD. In this context, tau participates in two temporally different scenarios: first, alongside with soluble Aβ oligomerization, abnormal phosphorylation of tau can be considered one of the earliest signs of neuronal dysfunction, preceding tau aggregation or amyloid deposition and responsible for the initial cognitive and synaptic dysfunction and secondly, hyperphosphorylated tau aggregates in NFTs along with SPs can modulate the neuronal toxicity and degeneration.

Overexpression of Three Genes Contributes to Tau Phosphorylation in Down Syndrome

Aneuploidy, a term that defines a condition where an abnormal number of chromosomes underlies a given pathology, is an adverse condition for development and generally results in death in utero (123). In the case of trisomies, the resulting disruption of homeostasis is determined by an increased gene dosage (124). In humans, DS is caused by the trisomy of autosome 21, and it represents the hyperdiploid condition that most frequently survives birth, with a current incidence estimated at 1/700 live births (125). The most striking feature of DS patients is mental retardation (126). Interestingly, the condition is also associated with an early onset of AD-like pathology (125), an issue of growing concern as the life expectancy of these patients increases.

Human autosome 21 was the first human chromosome to be fully sequenced (127). Yet, in spite of the most relevant information provided by that investigation, the relationship between specific overexpressed gene products and cellular impairments remains elusive, particularly those related to neuronal dysfunction and AD-like degenerative phenomena. One attractive target that could link both phenomena are tau proteins as various genes present in human autosome 21 could deregulate tau if they are overexpressed. The same could occur in trisomy 16 mice (Ts16), an animal model of DS (128,129) as most of the genes present in human autosome 21 are mapped to murine chromosome 16. Among these DS-related genes, Dyrk1A, Rcan1 and App could affect tau protein composition and function (Figure 2).

Dyrk1A is a serine–threonine protein kinase and an ortholog of the Drosophila minibrain (Mnb). This kinase is required in neuroblast proliferation during postembryonic neurogenesis (130). It is highly expressed in the brain and heart, and it possesses a vast amount of phosphorylation targets in proteins localized in the cytosol as well as the nuclei. As aforementioned, hyperphosphorylation of tau in AD and tauopathies is dependent on the action of several kinases and phosphatases, and distinct active kinases are expressed in association with phospho-tau deposits in neurons and glial cells in these diseases (30–41). Interestingly, DYRK1A phosphorylates tau at Thr212 *in vitro*,

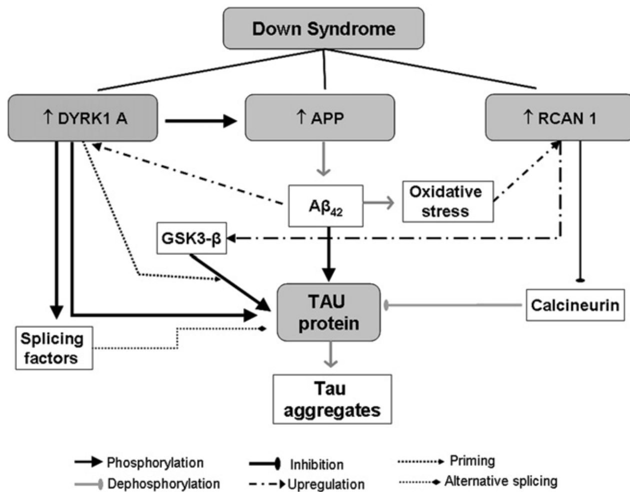

Figure 2. Cooperative contribution of Dyrk1A, APP and RCAN1 to the formation of tau aggregates. DYRK1A, APP and RCAN1 are overexpressed in Down syndrome. Dyrk1A phosphorylates splicing factors such as ASF, SC35 and SRp55, resulting in imbalance in the ratio of isoforms 3R- and 4R-tau, consequently favoring the formation of tau aggregates. Dyrk1A also phosphorylates tau directly in several serine and threonine residues. Particularly, phosphorylation of Thr212 by Dyrk1A primes tau for phosphorylation by GSK-3β, a serine/threonine kinase that hyperphosphorylates tau. Hyperphosphorylation of tau also favors its self-assembly. Furthermore, Dyrk1A phosphorylates APP at Thr668, favoring the amyloidogenic cleavage of APP with the consecutive production of Aβ(42). Aβ(42) also induces tau phosphorylation and increases the expression of both Dyrk1A and RCAN1. RCAN1 is as an endogenous inhibitor of calcineurin, a serine/threonine phosphatase that dephosphorylates tau. RCAN1 also increases the expression of the GSK-3β.

a residue that is phosphorylated in fetal tau and hyperphosphorylated in AD and tauopathies (43). Further, phosphorylation of Thr212 primes tau for phosphorylation by GSK-3 at Ser208 *in vitro*, suggesting a general role for DYRK1A in tau phosphorylation (131). Hence, overexpressed DYRK1A may be a contributory factor of tau hyperphosphorylation and AD pathology in DS (132) (Figure 2). On the other hand, DYRK1A regulates the alternative splicing of tau by phosphorylating different splicing factors such as the alternative splicing factor (ASF) (8), SC35 (133) and serine-arginine rich protein 55 (SRp55) (134), resulting in an imbalance in the ratio of isoforms 3R and 4R (8). Indeed, DYRK1A phosphorylation of ASF results in exclusion of exon 10 in the tau gene, leading to a consequent increase in the 3R isoform. As previously mentioned, the correct balance of 3R/4R is emerging as critical for normal tau function, and its disruption may lead to the typical NFT pathology seen in DS, AD, and various tauopathies.

Rcan1 was initially named dscr1, due to its location in the DS critical region of autosome 21 and later adapt78 for its role in cell adaptation to oxidative stress (135,136). When its role as an endogenous inhibitor of calcineurin was discovered, this protein took its current names of regulator of calcineurin-1 (RCAN1), or calcipressin1. RCAN1 is highly expressed in brain, heart and skeletal muscle (137) and is overexpressed in the brains of DS fetuses (138) and postmortem brain samples from DS patients who suffered AD symptoms (139). Postmortem studies on humans with AD showed a doubling in RCAN1 expression in the cerebral cortex and hippocampus compared to normal controls (140,141). Further, RCAN1 levels in brains with extensive NFTs were three times higher than in controls (141). Calcineurin activity is also decreased in AD (142) and similar to that observed in AD, brains of mice lacking the catalytic subunit of calcineurin exhibit hyperphosphorylation of tau protein and cytoskeletal changes (143). It is then plausible that in a condition such as DS, a gene dosage dependent increase of RCAN1 could determine decreased calcineurin activity, thus contributing to tau hyperphosphorylation by reducing calcineurin phosphatase activity (Figure 2). Interestingly, RCAN1 also increases expression of the GSK-3β (144), which as previously discussed, phosphorylates tau. Abnormal GSK-3β-mediated tau phosphorylation could target the microtubule binding domain of tau, thus affecting its interaction to microtubules (145). The latter could greatly affect microtubule stabilization and dynamics as well as promote tau self-aggregation (32). Hence, in DS, where RCAN1 is overexpressed, it is tempting to speculate that abnormal tau function may be due in part to the upregulation of GSK-3β (Figure 2).

APP was one of the first postulated links between DS and AD pathology. APP is an integral membrane protein that is linked to AD neurodegenerative mechanisms and which is reportedly overexpressed in DS. Furthermore, both Ts16 and Ts65Dn mouse models also exhibit overexpression of APP compared to normal controls (146,147). APP and tau reportedly converge in cellular mechanisms that could greatly compromise neuronal function such as mitochondrial function (148). Also, APP phosphorylation can play an important role in amyloidogenic processing, and protein kinases that phosphorylate APP can also phosphorylate tau (149). Hence, phosphorylation of both tau and APP can be a link in AD pathology and also in DS where APP is overexpressed, with the imbalance further contributing to neurodegeneration. Further, and as previously discussed and depicted in Figure 2, Aβ(42) oligomers contribute to hyperphosphorylated tau (94,150), and both Aβ(42) oligomers and hyperphosporylated tau synergistically contribute to cellular and cognitive impairment in the AD (97−99,101).

The possible concerted actions of the three aforementioned genes are illustrated in Figure 2. When overexpressed, the effects on tau could be i) imbalance of the 3R/4R ratio favoring the expression of 3R isoform, and ii) tau hyperphosphorylation. Interestingly, a positive feedback loop can be deduced from the fact that i) DYRK1A phosphorylates APP at Thr668 (151) favoring the amyloidogenic cleavage of APP (152,153), and ii) Aβ(42) induces an upregulation of both DYRK1A (132) and also RCAN1

(154). The cooperative effects of the three genes could consequently affect tau expression and phosphorylation dramatically and result in abnormal isoform expression and hyperphosphorylation, favoring its aggregation and the destabilization of microtubules.

The Trisomic Cortical Cell Line CTb As a Cellular Model to Study the Contribution of Different Genes to Tau Dysfuntions

Studies at the cellular level are hampered due to inherent difficulties in procuring human tissue samples. Further, murine Ts16 animals do not survive gestation. Our group has overcome the latter limitation by establishing immortalized cell lines from the central nervous system of Ts16 mice as well as from normal, age-matched controls. These Ts16-derived cell lines express neuronal traits and reproduce cell alterations previously described in primary cultures (155,156). Ts16-derived cell lines overexpress APP (157,158) as well as RCAN1 and DYRK1A (159,160). These cell lines could represent valuable models in the elucidation of the proposed above-described mechanisms. Therefore, considering the mechanisms depicted in Figure 2, we analyzed the phosphorylation status of tau protein using an antibody against tau phosphorylated in threonine-181, which is used as a cerebrospinal fluid biomarker for AD (161,162). Furthermore, considering that overexpression of DYRK1A promotes tau exon 10 exclusion thus decreasing the expression of the isoform 4R (Figure 2), we also analyzed the expression of this isoform by RT-PCR using specific primers. As shown in Figure 3, our results evidence higher tau phosphorylation levels in CTb cells derived from the cerebral cortex of a Ts16 animal compared with CNh cells, established from the same territory of an age-matched euploid control. We also observed lower expression levels of the 4R isoform in the trisomic cortical cells CTb as compared with its control CNh cells. These results suggest that the two proposed pathophysiological mechanisms related to tau dysfunction, namely, altered tau expression and phosphorylation, are present in the CTb cell line. Hence, the latter could constitute a model for the study of tau-related dysfunction in AD and DS.

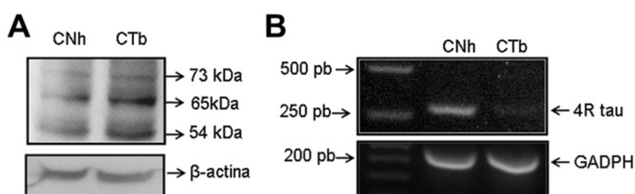

Figure 3. The CTb cell line, derived from the cerebral cortex cells of a trisomy 16 mouse, exhibits increased phosphorylation of tau protein and reduced expression of 4R isoform as compared with control CNh cells. (A) Western blot using an antibody against tau phosphorylated in threonine-181. Note increased expression of phosphorylated tau isoforms. (B) Real-time-PCR performed using specific primers for the 4R isoform. Sequences of forward and reverse primers were 5′-AAGAAGCTGGATCTTAGCAACGTCC-3′ and 5′-TTGGCTTTGGCATTCTCCCT-3′. Amplification of the housekeeping gene, GAPDH, is shown in the lower panel. Forward and reverse primer sequences were 5′-TTTGTGATGGGTGTGAACCACGAG-3′ and 5′-CAACGGATACATTGGGGGTAGGAAC-3′. Note the reduced expression of the 4R isoform in the trisomic condition.

In conclusion, neurodegenerative conditions are one of the last frontiers to be conquered in medicine. The underlying mechanisms for the vast majority of such ailments remain obscure, and current therapies are ineffective both in arresting or even slowing down the course of the diseases. Because many of the illnesses share common mechanisms, potential therapeutic targets can be brought to light once such pathogenic pathways are fully comprehended. The use of adequate animal and cell models will be pivotal in such undertakings and in the exploration of the effects of therapeutic agents. In the mechanisms described herein, cell models such as the CTb cell line could prove quite useful, considering that these cells express abnormal tau expression and phosphorylation and overexpression of relevant genes. A first approach could involve gene knockdown in order to pinpoint specific effects of gene overexpression on tau function, followed by the study of promising lead compounds. In this regard, several molecules have been proposed lately that inhibit some of these gene products (163). CTb cells could then be used as a bioassay for initial screening in order to identify the most effective compounds.

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