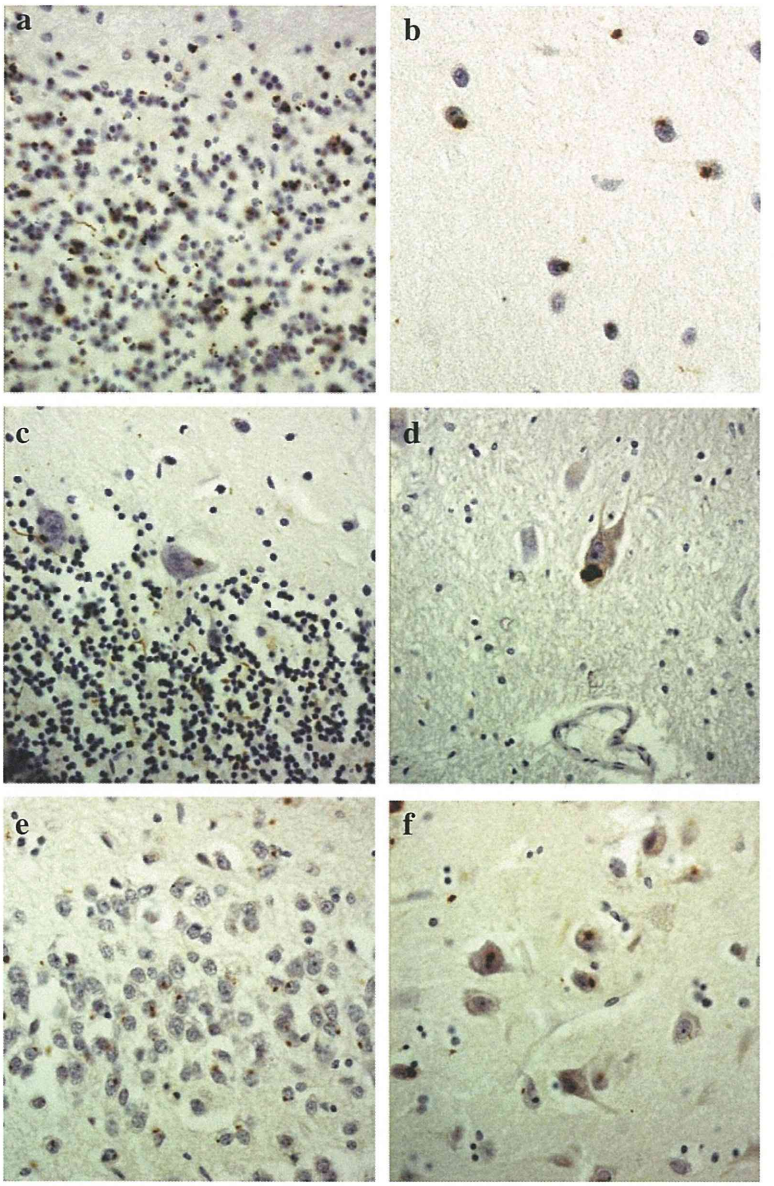

Figure 2 Immunostaining of the cerebellum and hippocampus for p62 proteins shows neuronal cytoplasmic inclusions in granular neurones (a), basket cells (b), Purkinje cells (c) and cells of the dentate nucleus (d) of the cerebellum, and in dentate gyrus granule cells (e) and pyramidal cells of CA4 region (f). Immunoperoxidase–haematoxylin. All × 40 microscope objective magnification.

those prepared by Hasegawa similarly detected NCI in granule cells of the cerebellum, as did Hasegawa's poly-GA antibody (Figure 2a). Again, more granular looking NCI were usually present in basket cells (Figure 2b), occasionally in Purkinje cells (Figure 2c) and neurones in the dentate nucleus (Figure 2d), but none were seen within Golgi neurones, or within Bergmann glia. A punctate, or filamentous, staining was also seen within the molecular layer of the cerebellum, this probably relating to parallel projection fibres (Figure 3a and b). No nuclear inclusions in atrocytes immunostained with p62 appeared to be detected by poly-GA antibody. All FTLD and MND cases also showed abundant, small, rounded NCI within granule cells of the dentate gyrus (Figure 3e), along with spicular or granular inclusions within pyramidal cells of areas CA2/3 and CA4 (Figure 3f), but these were less commonly seen in CA1 and subiculum (Table 2).

Our own custom antibody raised against poly-AP showed rare, and weak, immunostaining of NCI within CA4 neurone in occasional cases were (Table 2), similar in appearance to those seen in such cells on p62 immunostaining, or with poly-GA, poly-GP and poly GR antibodies. The antibody to poly-PR did not stain NCI in any case, but strongly immunostained chromatin granules,

Table 2 Ratings for p62 and DPR antibody immunostaining of neuronal cytoplasmic inclusions for all 15 cases with expansions in *C9ORF72*

Case	CA4						DG						GCC					
	p62	poly-GA	poly-GP	poly-GR	poly-PR	poly-AP	p62	poly-GA	poly-GP	poly-GR	poly-PR	poly-AP	p62	poly-GA	poly-GP	poly-GR	poly-PR	poly-AP
1*	2	2	3	3	0.5	0.5	1	2	0	0.5	0.5	0.5	4	4	3	0.5	0	0
2*	3	3	2	3	0.5	1	2	2	0.5	2	0.5	1	2	3	3	0	0	0
3*	3	3	3	2	0.5	0.5	4	3	3	0	0	0	1	4	2	2	0	0
4*	2	2	3	3	0.5	0	3	2	3	0	0.5	0	3	3	3	0	0	0
5*	2	1	3	3	0.5	0.5	3	3	2	0	0	0	2	3	3	1	0	0
6*	1	2	2	1	0	0	1	2	2	0	0	0	1	1	1	0	0	0
7**	3	3	3	3	0	0	3	3	2	0	0	0	4	4	4	0	0	0
8**	na	na	na	na	na	na	na	na	na	na	na	na	1	1	1	0	0	0
9**	3	2	3	3	0.5	0.5	1	2	1	0.5	0	0	0.5	3	1	0	0	0
10**	3	2	2	2	0.5	0.5	4	3	3	1	0.5	0	3	3	2	0	0	0
11**	3	3	3	3	0	0	4	3	2	2	0	1	0.5	3	2	1	0.5	0
12**	3	3	2	3	0	0	4	3	1	1	0	0	1	3	3	1	1	0
13	0	2	2	2	0	0	0	2	2	0	0	0	1	1	1	0	0	0
14	3	2	3	3	0	1	1	2	2	0	0	0	3	4	2	0	0	0
15	1	1	1	1	0	0	2	2	2	2	0	0	1	1	1	0	0	0
16	2	2	2	0	0	0	2	2	2	2	0	0	3	3	3	0	0	0

Cases #1-6 had FTLD TDP-43 type A histology (*), cases #7-12 had FTLD TDP-43 type B histology (**), case #13 had corticobasal degeneration, and cases #14-16 had Motor Neurone Disease.
CA4 = pyramidal cells of CA4 region of hippocampus; DG = dentate gyrus granule cells; GCC = granule cells of the cerebellum; na = no data.

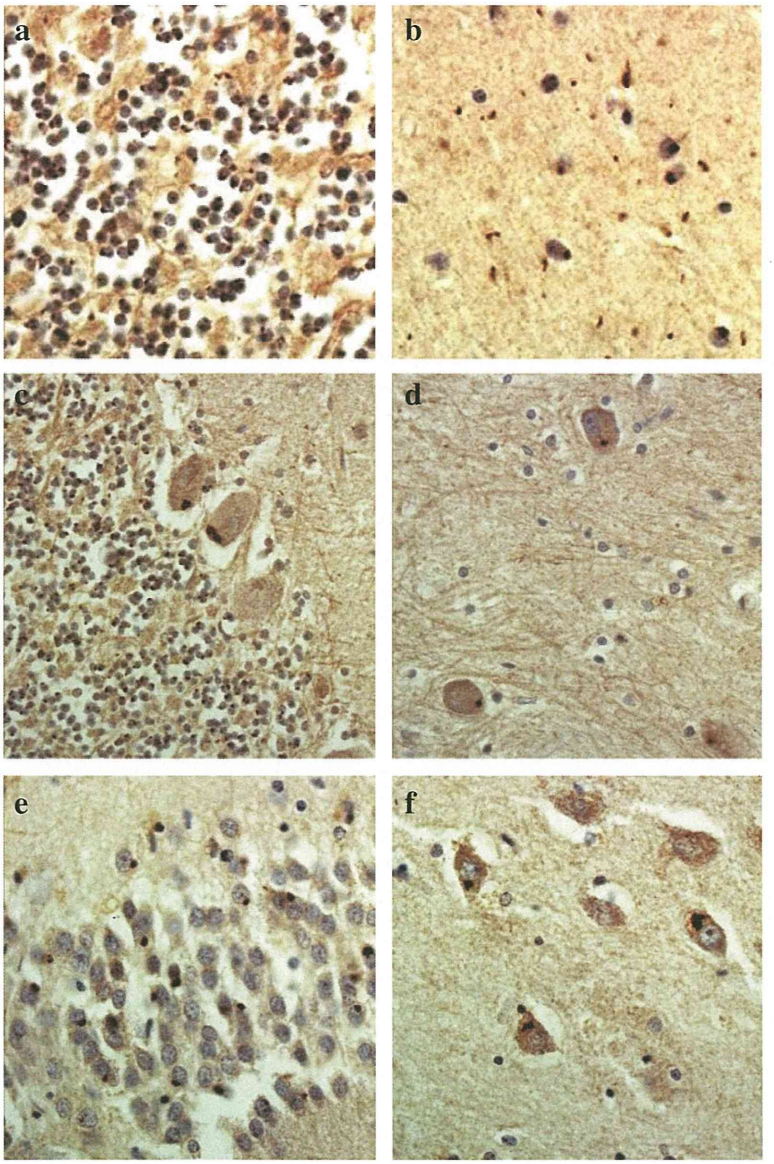

Figure 3 Immunostaining of the cerebellum and hippocampus for poly-GA protein shows neuronal cytoplasmic inclusions in granular neurones (a), basket cells (b), Purkinje cells (c) and cells of the dentate nucleus (d) of the cerebellum, and in dentate gyrus granule cells (e) and pyramidal cells of CA4 region (f), similar to those seen on p62 immunostaining. Immunoperoxidase–haematoxylin. All × 40 microscope objective magnification.

especially in Purkinje cells of cerebellum and CA4 neurones of hippocampus in some cases bearing expansions in *C9ORF72* (Figure 4). However, not all expansion bearers showed any such immunostaining, nor did any of the control cases.

The pattern of immunolabelling of NCI with C9RANT antibody in cerebellum and hippocampus mirrored that reported by others [26], and again appeared to be identical to that seen with both our own poly GP antibody and that prepared by M Hasegawa (not shown).

In all expansion carriers, variable numbers of, but often many, pyramidal neurones within the deeper layers of the adjoining cerebral (temporal) cortex also contained NCI immunostaining with antibodies to DPR in a similar fashion to those in the hippocampus CA regions (not shown).

Cases of FTLD of other histological or genetic subtypes showed no immunostaining of TDP-43 positive NCI, DN or NII, or of tau positive structures (Pick bodies, neurofibrillary tangles). The non-demented control cases showed no relevant DPR immunostaining, and the NII within the HD cases were also unstained by any DPR antibody.

In all instances, immunostaining of NCI with anti-DPR antibodies was robust. There were no apparent effects of variable post mortem delay or prolonged fixation upon

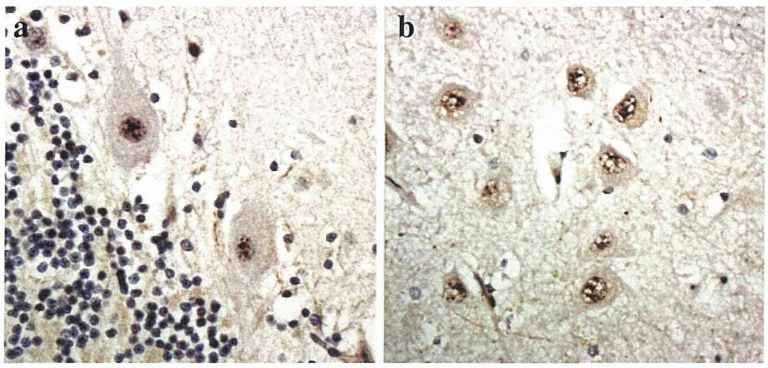

Figure 4 Immunostaining of Pukinje cells of the cerebellum (a) and pyramidal cells of CA4 region of hippocampus (b) for poly-PR protein shows strong immunoreactivity of chromatin. Note lack of staining of NCI in either cell type. Immunoperoxidase–haematoxylin. All × 40 microscope objective magnification.

the quality or quantity of NCI immunostaining, with cases that had been stored in formalin for up to 20 years before new blocks had been taken for processing into wax sections still showing robust immunostaining for DPR, as did cases where post mortem delay periods had exceeded 4 days.

Comparison of p62 and DPR immunostaining between FTLD-TDP subtypes

There were no statistically significant differences between ratings for p62 and DPR immunostaining in either FTLD-TDP type A or type B cases for NCI in granule cells of the cerebellum, or for those of the dentate gyrus, and CA4 neurones, of hippocampus.

Comparisons and correlations between p62 and DPR immunostaining

The numbers of NCI immunostained by each DPR antibody did not always correspond either when compared to p62 immunostaining, or when compared among themselves. Those in CA2, CA3 and CA4 of hippocampus, and those in Purkinje cells and cells of the dentate nucleus appeared numerically similar in p62 and DPR immunostaining (except for poly-AP), though it appeared from microscopic inspection that p62-positive NCI in granule cells of the cerebellum were not all, or always, immunostained by both poly-GR and poly-GP antibodies, and none were stained by poly-AP antibody (Table 2). In the hippocampus only a minority of the p62-positive NCI in the dentate gyrus were immunostained by poly-GR and poly-GP antibodies, and again none were stained by poly AP antibody (Table 2).

Ratings for DPR immunostaining of NCI were correlated with those for each other, and those for p62 immunostaining, in granule cells of cerebellum, and dentate gyrus and CA4 neurones of hippocampus. In general, there were significant correlations between p62, poly-GA, poly GP and poly-GR ratings, but not with those involving poly-AP and poly-PR. Hence, ratings for poly-GA correlated significantly with those for p62 in cerebellum ($p = 0.032$), dentate gyrus ($p = 0.000$) and CA4 ($p = 0.006$), with poly-GR in CA4 region ($p = 0.044$) and with poly-GP in cerebellum ($p = 0.003$). In CA4, ratings for poly-GP correlated with those for poly-GR ($p = 0.007$). In the cerebellum, ratings for poly-GA correlated with those for poly-GP ($p = 0.008$), as did ones for poly-GR with poly-PR ($p = 0.027$). Only in CA4 did poly-AP correlate with poly-PR ratings ($p = 0.014$).

Other observations

Cells containing DPR did not show obvious cell loss, or any other outward signs of neurodegeneration, whether these were granule cells of dentate gyrus or cerebellum, or pyramidal cells of CA regions or cerebral cortex, or Purkinje neurones of the cerebellum. No cases with *C9ORF72* expansion showed excessive tau and Aβ pathology (i.e. commensurate with a pathological diagnosis of AD) for age.

Discussion

In the present study we have observed p62 positive, TDP-43 negative NCI within cerebellar granule cells, and granule cells of the dentate gyrus and pyramidal cells of the hippocampus (see [17-19,24]) in 13 of 84 (15%) cases of FTLD and in 3 of 23 (13%) cases of MND. Interestingly, all cases with expansion showed a FTD, FTD + MND or MND clinical phenotype, and 15/16 bore appropriate TDP-43 protein pathological changes. However, one expansion carrier with clinical FTD (case #13 in Table 1) was of FTLD-tau with CBD pathology. This case was described by us previously [20] and is important as it is only the second case of CBD to be described with expansion in *C9ORF72* (see [32] for details of the other case), though there was no post-mortem confirmation of CBD in this latter instance. The present case demonstrated an expansion in *C9ORF72* both on repeat-primed PCR (not

shown), and on Southern blot (see lane #8 in Figure 1). Although the case showed no TDP-43 pathology, DPR were present in cells of the cerebellum and hippocampus (see Table 2), though curiously those NCI in granule cells of the cerebellum were p62-immunopositive, while those in hippocampus dentate gyrus and CA4 regions were not positive for p62.

None of the other cases with other histological or genetic forms of FTLD showed p62 positive, TDP-43 negative NCI within either cerebellar granule cells or within the hippocampus. The pathological findings described here are therefore broadly consistent with those previously reported by others in unselected series of cases of FTLD and/or MND [17-19,24].

All of the 12 p62 positive cases where frozen brain tissues were available for analysis showed an expansion in *C9ORF72* by Southern blotting, and are therefore consistent with the suggestion [17,18] that this type of p62 pathology is pathognomic for cases of FTLD and/or MND associated with *C9ORF72* mutation. Thus, it can be presumed that an expansion was also present in the other four FTLD cases with relevant p62 pathology where no southern blotting (or repeat primed PCR) was possible due to lack of fresh frozen brain tissue. The lack of association between expansion length and age of disease onset or duration presumably reflects that a minimum size of expansion is required for disease. Present data suggests this might be around 500 repeats, and expansions beyond this are not additionally detrimental.

The exact target protein within the p62 immunoreactive NCI remains uncertain. Here, we performed immunohistochemistry employing antibodies to DPR putatively produced in the brain through non-ATG initiated (RAN) translation of the expansion itself. Although double immunofluorescence labelling was not performed in the present study, microscopic observations on consecutive serial sections stained with each DPR antibody suggested that most, if not all, of the p62-positive NCI within Purkinje and dentate nucleus cells of the cerebellum, and neurones of CA2, CA3 and CA4 of the hippocampus are similarly immunoreactive to poly-GA, poly-GP and poly-GR antibodies (and to a much lesser extent, poly-AP antibody), whereas only a (variable) subset of p62-positive NCI within granule cells of the cerebellum and dentate gyrus appeared immunoreactive to these antibodies. These microscopic impressions were supported by statistical analysis showing good correlations between ratings of inclusion body frequencies as assessed using poly-GA, poly-GP and poly-GR antibodies. Other studies [26,27] where double immunofluorescence labelling was indeed performed substantiate the present observations.

None of the NCI was immunoreactive to poly-PR antibody, though this antibody did immunolabel chromatin granules, especially in Purkinje cells and CA4 neurones, in some *C9ORF72* expansion bearers. Although this kind of immunostaining was not seen in any of the control subjects in such cells, it was not consistently present in all expansion bearers, and so the relevance of this (to pathogenesis) remains uncertain. None of the DPR antibodies were immunoreactive to any other kinds of inclusions (TDP or tau) associated with other histological or genetic forms of FTLD, or the NII containing CAG repeats seen in HD. These findings are consistent with those recently reported by Mori and colleagues [27]. The pattern of DPR immunolabelling in the cerebellum and hippocampus in *C9ORF72* expansion bearers mirrored that reported by Ash et al. [26], and this appeared identical to that shown by our own poly GP antibody, and that prepared by M Hasegawa. C9RANT antibody was raised to a panel of dipeptide repeat immunogens [26], but judging from the similarities between its pattern of immunolabelling and that of our own poly-GP antibody, it might be considered that its specificity, or at least, it avidity is greatest for the poly-GP component of the immunogen mix. The finding of strong and consistent staining with poly-GA, poly-GP and poly-GR antibodies suggests that most of the aberrant translation relates to sense transcripts, though the slight, but variable immunostaining with poly-AP antibody implies some antisense translation might also occur. However, it remains possible that the relative paucity of immunostaining seen here with antibodies to antisense transcripts reflects poor antigen avidity on the part of the antibody rather than indicating an absence of the relevant polypeptide with DPR inclusions *per se*.

Nevertheless, it appears that DPR may not be the only proteins present within these structures since the p62 positive inclusions have also been shown to contain hnRNPA3 [33], and others reported the inclusions in granule cells of the dentate gyrus and cerebellum (at least) to be immunoreactive to ubiquilin-2 [34]. There is little or no DPR immunostaining of TDP-43 in hippocampus or cerebral cortex in either expansion bearers, or patients with other histological forms of FTLD [29], consistent with those observations of NCI based on p62 immunostaining [17-19,24]. Furthermore there was no immunostaining of NII containing expanded poly-Q stretches in patients with HD (see also [27]). Collectively, these findings reinforce the suggestion that p62-, DPR-positive NCI are pathognomic for FTLD (and MND) associated with *C9ORF72* expansions.

It remains uncertain as to how these NCI containing DPR may relate to disease pathogenesis and other fundamental aspects of disease pathology such as TDP-43 positive NCI and neurites. While NCI containing DPR are located in clinically and pathologically relevant regions, such as the hippocampus and adjacent temporal cortex, they are actually present in cells distant from the major TDP-43 changes (layers V and VI in cerebral cortex and

areas CA2/3/4 in hippocampus), although it is acknowledged that cells in the dentate gyrus of the hippocampus may contain one or other, but rarely both, types of inclusion [19,27]. Those cells containing DPR did not show any other outward signs of neurodegeneration, whether these being small granule cells of dentate gyrus or cerebellum, or larger pyramidal cells of hippocampal CA regions, cerebral cortex or Purkinje neurones of the cerebellum. Likewise, there appeared to be no obvious loss (thinning out) of granule cells in either region or depletion of cells from hippocampal pyramidal or cerebellar Purkinje cell layers. Moreover, such changes in the cerebellum appear to be without functional (clinical) consequence, although there have been reports of cerebellar atrophy in expansion bearers [35,36].

Interestingly, there appeared to be no qualitative or quantitative differences between either the number, or the pattern, of DPR stained NCI between FTLD-TDP type A and type B cases, in either hippocampus or cerebellum. This observation begs the question as to the role of *C9ORF72* expansions in determining histological phenotype and their relationship to TDP-43 pathology. It is possible that variability in repeat size may have a role in this, but we were unable to show any obvious differences in repeat size between Type A and type B cases by Southern blot. Alternatively, it is possible that the expansion confers an 'additive' effect, through induction of a process marked by p62 pathology, which is superimposed upon a 'background TDP-43 proteinopathy derived through a similar mechanism to that seen in non-expansion bearers sharing the same TDP-43 histological phenotype. If that were so, then it might be presumed that patients bearing the mutation suffer a 'double whammy', and that the expansion plays no real part in determining the TDP-43 proteinopathy and the basic underlying disorder. However, if this 'chance scenario' were true, then it might be anticipated that *C9ORF72* repeat expansions might commonly occur in association with other disorders, though there is little, and conflicting, evidence for this in, for example, Alzheimer's disease [37-43] or Parkinson's disease and other parkinsonian syndromes such as Corticobasal syndrome [44-47]. Indeed, it is possible that in at least some of these instances where an expansion has been reported in patients with conditions other than FTLD or MND, the underlying condition may still be FTLD, though presenting in an atypical way [39].

Conclusion

Although it is clear that production of DPR from unconventional translation of the expansion is a feature of *C9ORF72* associated diseases, it is far from certain as to whether such changes drive cell damage and loss, and how they might relate to changes in TDP-43 function and contribute clinical disability. It remains an open question whether DPR mediated changes are anything more than a pathological 'curiosity', but nonetheless they appear to provide a specific diagnostic tissue marker for the presence of the genetic expansion.

Additional file

Additional file 1: Figure S1. Relationship between minimum and maximum size of repeat and age at onset/disease duration.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DMAM was responsible for study design, microscopic assessments, data analysis and paper writing. SR, JBC and SPB performed all genetic analyses and Southern blotting, and assisted with preparation of the manuscript. AR provided technical assistance. JCT performed statistical analysis. JSS assisted with case diagnosis and classification. TG and LP provided C9RANT antibody and details of specificity. MMS and MH provided antibodies and details of specificity, and assisted with manuscript preparation. YD performed all immunohistochemical staining. All authors read and approved the final manuscript.

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Extensive deamidation at asparagine residue 279 accounts for weak immunoreactivity of tau with RD4 antibody in Alzheimer's disease brain

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Abstract

Background: Intracytoplasmic inclusions composed of filamentous tau proteins are defining characteristics of neurodegenerative tauopathies, but it remains unclear why different tau isoforms accumulate in different diseases and how they induce abnormal filamentous structures and pathologies. Two tau isoform-specific antibodies, RD3 and RD4, are widely used for immunohistochemical and biochemical studies of tau species in diseased brains.

Results: Here, we show that extensive irreversible post-translational deamidation takes place at asparagine residue 279 (N279) in the RD4 epitope of tau in Alzheimer's disease (AD), but not corticobasal degeneration (CBD) or progressive supranuclear palsy (PSP), and this modification abrogates the immunoreactivity to RD4. An antiserum raised against deamidated RD4 peptide specifically recognized 4R tau isoforms, regardless of deamidation, and strongly stained tau in AD brain. We also found that mutant tau with N279D substitution showed reduced ability to bind to microtubules and to promote microtubule assembly.

Conclusion: The biochemical and structural differences of tau in AD from that in 4R tauopathies found in this study may therefore have implications for prion-like propagation of tau.

Keywords: Alzheimer's disease, Tau, Deamidation, Aging, Microtubule

Background

Intracellular inclusions composed of filamentous tau proteins are defining characteristics of many neurodegenerative diseases, including Alzheimer's disease (AD), Pick's disease, corticobasal degeneration (CBD), and progressive supranuclear palsy (PSP). Tau is a microtubule-associated protein that stabilizes microtubules and promotes their assembly. In adult human brain, 6 tau isoforms are expressed as a result of mRNA splicing. They are divided into two groups, 3-repeat (3R) and 4-repeat (4R) tau isoforms, according to whether or not exon 10 is expressed. Tau pathologies show clear morphological differences among different diseases or disease types, and different tau isoforms are accumulated in the diseased brains, namely, 6 tau isoforms in AD, 3R tau isoforms in Pick's disease, and 4R tau isoforms in PSP and CBD [1,2]. In addition, tau in PSP and tau in CBD are biochemically distinguished by the banding pattern of the C-terminal fragments [3]. However, it remains unclear why different tau isoforms accumulate in different diseases and how they lead to the formation of abnormal filamentous structures and pathologies.

Isoform-specific tau antibodies are useful tools for immunohistochemical and biochemical studies of tau species in diseased brains. In particular, RD3 and RD4 [4], which are specific antibodies to 3R and 4R tau isoforms, respectively, have been widely used to investigate tau pathologies [5-7]. One of the present authors (M.H.) had found that the asparagine residue at position 279 (N279), located in the RD4 epitope, was detected mostly as aspartic acid owing to deamidation of asparagine when PHF-tau in AD brains was subjected to protein sequencing and LC/MS/

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MS analysis after digestion with lysyl endopeptidase [8]. Here, we show that the irreversible post-translational deamidation takes place at N279 (N279D) in the RD4 epitope of tau in AD, but not CBD or PSP, and this modification abrogates the immunoreactivity to RD4. We raised an antiserum against RD4 peptide with N279D in rabbit, and showed that it specifically recognizes 4R tau isoforms regardless of deamidation and strongly stained tau in AD brain. We further show that mutant tau with N279D substitution has a reduced ability to bind to microtubules and to promote their assembly. These results have important implications for immunohistochemical and other studies aimed at understanding the molecular mechanisms of tau accumulation in AD and other tauopathies.

Results

Low immunoreactivities of tau in AD and tau deamidated at N279 to RD4

When Sarkosyl-insoluble fractions of tau from AD, PSP and CBD brains were analyzed by immunoblotting with T46 and RD4, we noticed a lower immunoreactivity of RD4 with abnormal tau in AD compared to that in both PSP and CBD (Figure 1a,b). T46, a monoclonal antibody to the C-terminal region of tau, strongly labeled triplet bands of phosphorylated full-length tau in AD together with smearing substances, and doublet bands together with C-terminal fragments of tau in CBD and PSP (Figure 1a). In contrast, RD4 (1:1000 dilution) stained tau in CBD and PSP relatively strongly, but barely stained the tau bands, and especially the smears, in AD (Figure 1b), though both RD4 and T46 labeled Sarkosyl-soluble tau in these brains (Figure 1c,d). These results suggested that there might be some modification in the RD4 epitope or its vicinity on tau in AD abrogating immunoreactivity. The low affinity of RD4 for tau in AD is consistent with the original report [4], which noted that the RD4 titer appeared to be considerably weaker than those of TP70 and RD3. To confirm that the asparagine residue at position 279 (N279) on tau was deamidated in AD, we performed LC/MS/MS analyses of tryptic peptides of Sarkosyl-insoluble tau prepared from AD brains. As shown in Figure 1e, almost all the VQIINK peptide

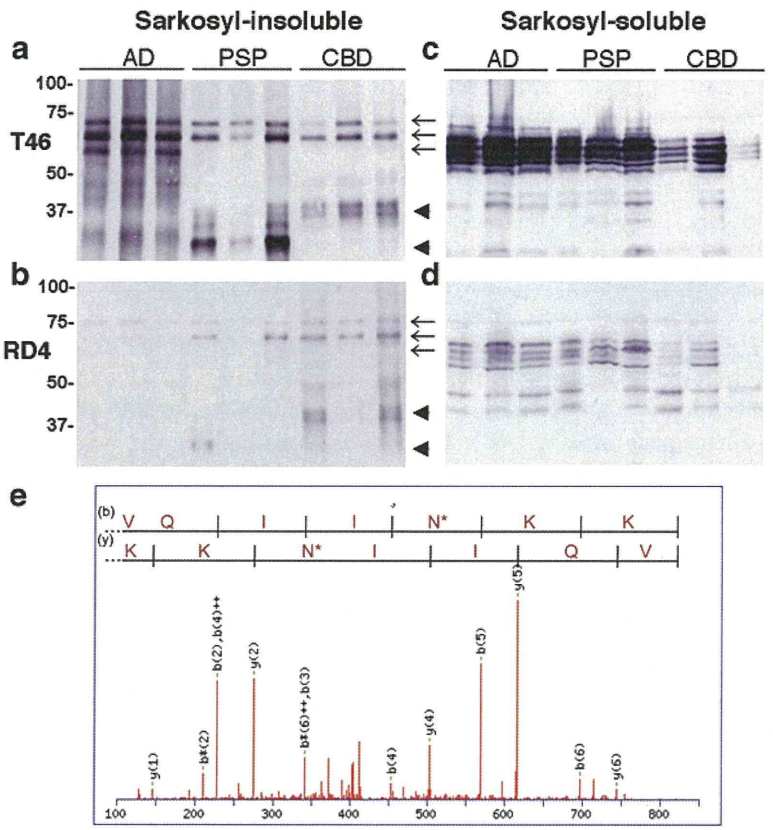

Figure 1 Immunoblot and LC/MS/MS analyses showed a lower immunoreactivity of RD4 with tau in AD and deamidation at N279. Immunoblot analysis of Sarkosyl-insoluble **(a, b)** and soluble **(c, d)** tau from AD, PSP and CBD brains (three cases for each disease) with anti-tau monoclonal antibodies T46 **(a, c)** and RD4 **(b, d)**. Arrows indicated the positions of the 60, 64, 68 kDa triplet tau bands in AD brains, and arrowheads indicate the ~33 and ~37 kDa C-terminal fragments that distinguish PSP and CBD. Identification of deamidated amino acid residue by nano-electrospray tandem mass spectrometry **(e)**. Product ion spectrum of a mass signal of tryptic peptide VQIINK detected in Sarkosyl-insoluble tau from AD brain, showing the b and y ion series. These results identify the site of deamidation as N279, indicated by N*.