

# Dysregulation of human brain microtubule-associated tau mRNA maturation in myotonic dystrophy type 1

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**Intraneuronal aggregates of hyperphosphorylated tau proteins, referred to as pathological tau, are found in brain areas of demented patients affected by numerous different neurodegenerative disorders. We previously described a particular biochemical profile of pathological tau proteins in myotonic dystrophy type 1 (DM1). This multisystemic disorder is characterized by an unstable CTG repeat expansion in the 3'-untranslated region of the DM protein kinase gene. In the human central nervous system, tau proteins consist of six isoforms that differ by the presence or absence of the alternatively spliced exons 2, 3 and 10. Here we show that the pattern of tau isoforms aggregated in DM1 brain lesions is characteristic. It consists mainly of the aggregation of the shortest human tau isoform. A disruption in normal tau isoform expression consisting of a reduced expression of tau isoforms containing the exon 2 was observed at both the mRNA and protein levels. Large expanded CTG repeats were detected and showed marked somatic heterogeneity between DM1 cases and in cortical brains regions analysed. Our data suggest a relationship between the CTG repeat expansion and the alteration of tau expression showing that DM1 is a peculiar tauopathy.**

## INTRODUCTION

Myotonic dystrophy type 1 (DM1) is a dominantly inherited multisystemic disorder caused by unstable expansion of CTG trinucleotide repeats in a gene encoding a serine/threonine protein kinase, named myotonic dystrophy protein kinase (DMPK) (1–7). The normal allele is polymorphic, ranging from 5 to 35 CTG repeats, whereas in DM1 the pathologic allele expands from 50 to multiple thousands of CTG repeats. Expansion of the CTG repeat is correlated with the disease severity and with the anticipation of the age of onset (5,8–11). The clinical expression of DM1 is extremely variable and includes myotonia and progressive muscle weakness, frontal baldness, cataract, cardiac conduction defects, hypogonadism, endocrine deficiency and mental retardation (12).

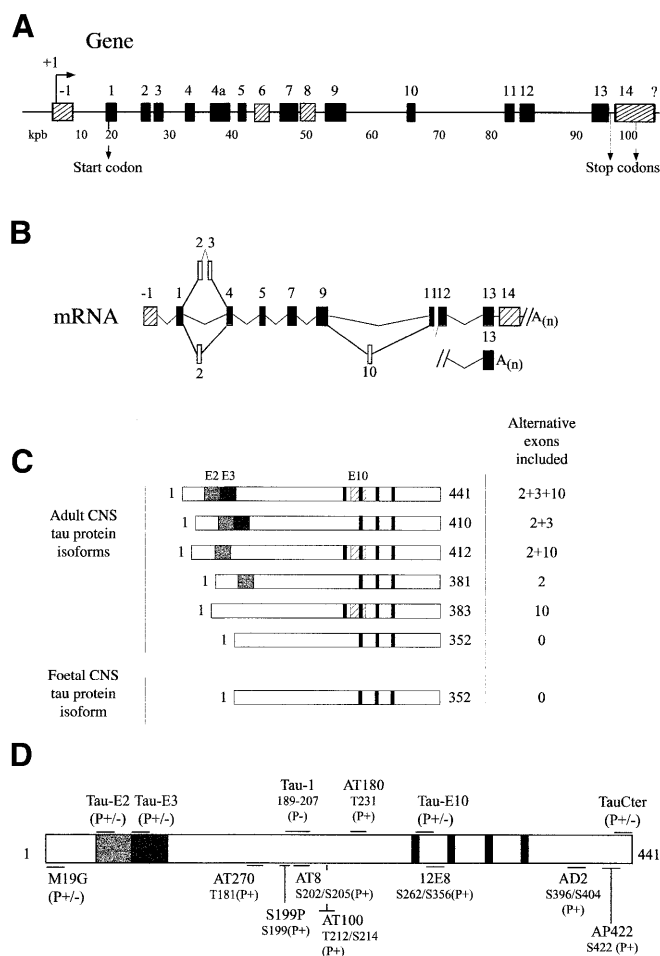
Neurofibrillary tangles (NFT) in the neocortex and in subcortical nuclei of DM1 patients have been described in several studies (13–18). NFT, as described in Alzheimer's disease (AD), consists of intraneuronal accumulation of paired helical filaments (PHF) (19). They result from the aggregation of hyperphosphorylated microtubule-associated tau proteins (20–24), referred to as PHF-tau or pathological tau proteins (25,26).

Tau protein belongs to the family of microtubule-associated proteins (27,28). They are essentially expressed in neurons where their essential function is to regulate the microtubule network. In the adult human brain, alternative splicing of exons 2, 3 and 10 of the single tau gene transcript gives six tau isoforms (29,30) (Fig. 1A). Mutations in the tau gene are responsible for Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (31,32). The mutations are distributed over exons 9–13 and in the introns flanking the exon 10. These mutations lead to alteration of tau protein binding to microtubule or affect the post-transcriptional maturation of tau pre-mRNA, thus changing the tau isoform stoichiometry of expression (31–35).

Yet, tau pathology is observed in 22 neurodegenerative disorders merged as tauopathies (36,37). Four tauopathy classes are distinguishable by the tau protein isoforms that constitute the specific pathological tau electrophoretic profile (37). Thus, in AD the six isoforms determine the pathological tau triplet at 60, 64 and 69 kDa and the minor 74 kDa component (class 1) (38). Tau isoforms containing the exon 10 compose the pathological tau doublet at 64 and 69 kDa (class 2) that is observed in progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) (39). The tau isoforms lacking the exon 10 constitute the typical doublet at 60 and 64 kDa of Pick's disease (PiD) (class 3) (40,41). Depending on the tau gene mutation, FTDP-17 pathological tau patterns belong to classes 1–3 (36,37). In contrast, the pathological tau profile of class 4 is characterized by a major 60 kDa component, a 64 kDa and minor 69 kDa component. It is restricted to DM1 (17). These data suggest that specific sets of tau isoforms are expressed in particular subsets of neurons selectively affected according to the neurodegenerative disorder (42).

Upon multiple molecular mechanism hypotheses, recent data suggest that the expanded CUG repeat in the 3'-untranslated region (3'-UTR) of the DMPK mRNA acts as a gain-of-function mutation by sequestering RNA binding proteins, leading to depletion of transcripts that require this protein for normal

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**Figure 1.** Tau gene structure, pre-mRNA alternative splicing in the human central nervous system (CNS), protein isoforms translated from alternative tau mRNAs and location of the epitopes of tau antibodies. (A) The human tau gene, located on chromosome 17 at position q21, spans >110 kb. It is composed of 16 exons numbered from -1 to 14 (79). The start codon is located in the exon 1 and two stop codons have been described; one in the intron between exons 13 and 14 and the second in exon 14. Note that the 3' end of exon 14 is not completely characterized in human (indicated by a question mark) (80). The initiation of transcription is indicated by +1. (B) Schematic representation of tau mRNAs. In the human CNS, the exons 4a, 6 and 8 are excluded. The exons -1 and 14 are transcribed but not translated. Two cleavage-polyadenylation sites have been described; one in the intron 13/14 and one in the exon 14 (81). The polyadenylated tau mRNA including the exon 14 is less represented in humans. The alternative splicing of the exons 2, 3 and 10 generates six tau protein isoforms (29). Inclusion of exon 3 is associated with inclusion of exon 2. (C) The six human brain tau protein isoforms are presented with the amino acid sequences added by the inclusion or not of exon 2 (E2), exons 2 and 3 (E3) or exon 10 (E10) in the tau mRNAs. When exon 10 is included in the mRNA a fourth microtubule-binding motif (indicated by a black box) is also included in the tau protein isoform (29,79,82). All six isoforms are expressed in the adult human CNS. Note that the tau protein isoform generated from an mRNA lacking the exons 2, 3 and 10 is the sole isoform expressed in the fetal CNS. (D) Location of the epitopes of tau antibodies used in the present study, represented on the longest human brain tau isoform. Phospho-independent tau antibodies are indicated by (P+/-). The phospho-epitopes are indicated by the nature and the number of the phosphorylated amino acid (P+). Tau-1 antibody recognizes a non-phosphorylated (P-) epitope located between amino acids 189 and 207.

gene expression (43,44). The specific pathological tau pattern observed in DM1 suggests that a specific set of tau protein isoforms is implicated in this neurodegenerative disorder (17).

In agreement with the hypothesis of RNA binding protein sequestration, the tau expression could be disrupted in DM1. To evaluate this hypothesis, the brain distribution of tau pathology, the pathological tau phosphorylated sites and the tau protein isoforms implicated in the neurofibrillary process were determined. Expression of normal tau isoforms was investigated at both the protein and mRNA levels. The (CTG)<sub>n</sub> expansion was analysed in multiple brain areas of five cases of DM1. Our results suggest that the (CTG)<sub>n</sub> expansion is altering the processing of tau pre-mRNA splicing, leading to a change in tau protein isoform expression.

## RESULTS

### Biochemical brain mapping of tau pathology

The tau pathology was analysed in more than 20 brain regions as shown for DM1 cases 3 and 5 (Fig. 2A) and the brain distribution of pathological tau in all five DM1 cases is summarized in Table 1. The hippocampus was affected in four out of the five cases (cases 1, 2, 3 and 5). In isocortical brain regions and subcortical nuclei, the tau pathology distribution was inter-individually heterogeneous. As an example, traces of pathological tau were detected in Brodmann's area 7 (superior parietal cortex) of DM1 case 4 (Table 1) whereas other brain regions were spared of any tau pathology. Thus, conversely to other neurological disorders with tau pathology, a non-specific heterogeneous distribution of tau pathology is observed in DM1.

### Biochemical characterization of pathological tau

The number of pathological tau components detected by AD2 antibody was either a single component at 60 kDa for DM1 case 5 or two components at 60 and 64 kDa in DM1 cases 1, 2 and 3 (Fig. 2A). The latter pathological tau profile corresponded to the one previously described (17) and was extended to three additional DM1 cases. Overall, the pathological tau component at 60 kDa was always the more intensively or exclusively labelled component.

Using 2D gel electrophoresis, we previously showed that pathological tau were hyperphosphorylated in DM1 (17). Herein, the phosphorylated sites were investigated using phospho-dependent tau antibodies. The typical triplet of pathological tau proteins and the 74 kDa component was stained by all phospho-dependent tau antibodies except for Tau-1 antibody (AD2 labelling only is shown) (Fig. 2B). All phospho-dependent antibodies strongly detected the pathological tau components at 60 and 64 kDa. AD2, AT180, AT270 and 12E8 also labelled a minor component at 69 kDa. However, the 69 kDa component was observed after an extended exposure. AT100 antibody and AP422 antiserum recognize phospho-epitopes that are directly related to neurofibrillary degeneration found in AD, PSP, CBD and FTDP-17, but completely absent in tau proteins derived from human brain biopsy (39,45,46). Those pathological epitopes were also found in DM1 (Fig. 2B).

Using the isoform-specific tau antisera, the pathological tau 64 kDa component and, to a lesser degree, the 69 kDa component were detected with the Tau-E10 antiserum (Fig. 2B). Pathological tau proteins were detected neither by Tau-E2 nor Tau-E3 antisera (data not shown) suggesting that the shortest tau isoform and the isoform with the exon 10 only compose the pathological tau component at 60 and 64 kDa, respectively. The minor 69 kDa

**Table 1.** Biochemical brain mapping of the tau pathology

DM1		Case 1	Case 2	Case 3	Case 4	Case 5
Hippocampal formation	Area 35	+++	+++	+++	-	+++
	Area 34	+++	+++	-	-	++
	Area 28	+++	+++	-	-	+
Temporal	Area 38	+++	+++	-	-	+
	Area 20	+	++	-	-	ND
	Area 22	-	+	-	-	-
Frontal	Area 4	-	-	+	-	-
	Area 10	-	-	-	-	-
	Area 44	-	-	-	-	++
Parietal	Area 39	-	-	-	-	-
	Area 7	-	-	-	+/-	-
Occipital	Area 17	ND	-	-	-	-
	Area 18	-	-	ND	-	-
	G. Cing	-	++	++	-	-
	Cerebellum	-	-	-	-	-
	Putamen	-	-	ND	-	-
	Amygdala	++	+++	ND	-	+
	NbM	ND	ND	ND	-	++
	Pallidum	-	-	ND	-	-
	N. Caudate	-	ND	ND	-	-
LN	-	ND	++	-	-	

Western blots were performed on brain tissue homogenates from the five DM1 cases. The monoclonal antibody AD2 was used to detect the pathological tau proteins. Pathological tau proteins that were detected are indicated as follows: +++, high amounts; ++, intermediate amounts; +, low amounts; +/-, lightly detected; -, absence of signal. Some brain regions were not available (ND, not determined). The cortical brain areas are numbered according to Brodmann's classification and the brain regions studied are indicated. NbM, nucleus basalis of Meynert; G. Cing, cingulate gyrus; N. Caudate, nucleus caudate; LN, locus niger.

pathological tau component was also detected by Tau-E10 antisera (Fig. 2B) and should correspond to the tau isoform with the exons 2 and 10.

Tau-1 antibody was used to distinguish between pathological and normal tau proteins. Tau-1 antibody selectively labelled the normal tau proteins in AD (Fig. 2B) that are dephosphorylated during the post-mortem delay, whereas aggregates of pathological tau remain hyperphosphorylated (47). Intriguingly, the tau protein profile detected by Tau-1 antibody in DM1 was completely different from the one observed in AD. Tau-1 strongly detected two bands of 47 and 50 kDa. These bands were perfectly aligned with the two lowest normal tau bands in AD, suggesting that normal tau protein expression was altered in DM1.

### Overexpression of tau protein isoforms lacking the exons 2 and 3

Using M19G, tau proteins and N-terminus catabolic products of tau were detected with a similar electrophoretic pattern all over the brain tissue homogenates of DM1 case 3 (Fig. 3A) as well as in the

frontal, temporal, parietal and occipital cortex of the other four DM1 cases (data not shown). When compared with tissue homogenates of a control brain, significant differences were observed. The three uppermost tau bands were less stained, whereas the three bands of lower molecular weight were more strongly stained in DM1 than in control brain homogenate (Fig. 3A).

The change in the intensity of labelling could result from either a selective degradation or a modification in tau protein isoform expression. These two hypotheses were investigated by comparing the electrophoretic profile of tau proteins from non-affected brain regions of the five DM1 cases to that of a control brain (Fig. 3B). M19G and TauCter labelling showed that the six uppermost tau bands aligned with the six recombinant tau protein isoforms (Fig. 3B). According to the distribution of tau protein isoforms, M19G and TauCter antisera faintly detected the tau protein isoform including the exons 2 and 3 in DM1 (Fig. 3B).

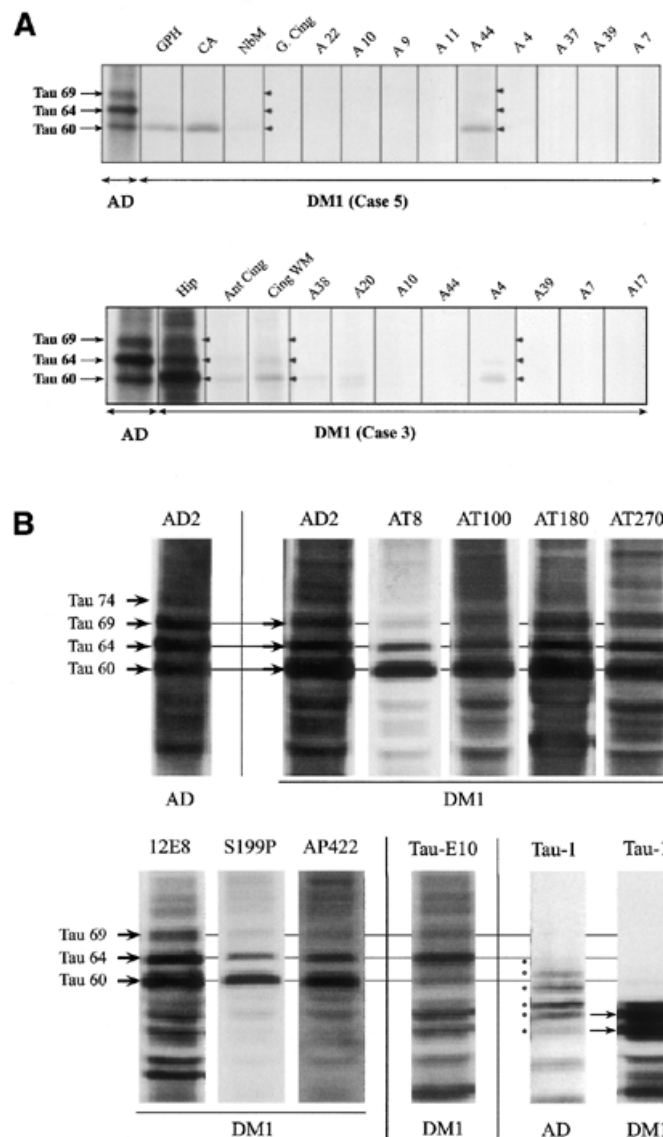
In contrast, the shortest tau isoform and the tau isoform containing the exon 10 only were more strongly stained in DM1 compared with control brain (Fig. 3B). The quantification of the total signal for the six tau protein isoforms was not significantly different between DM1 and control brains, suggesting that the total level of expression was conserved.

### Catabolism is not implicated in the change of tau isoform protein expression

In order to exclude the possibility of a selective degradation of tau protein isoforms in DM1, the exon-specific tau antisera Tau-E2, Tau-E3 and Tau-E10 were used (Fig. 3C). Thus, in the case of a selective catabolism of tau protein isoform, the tau catabolic products containing the exons 2 and 3 should have been in higher amounts in DM1 than in control brain.

The Tau-E2 antiserum labelled the four recombinant tau protein isoforms containing the exon 2, showing the specificity of our antiserum (Fig. 3C). In control brain homogenate, four bands aligned with recombinant tau isoform including the exon 2, as well as catabolic products (Fig. 3C). Because of its lower expression, the longest tau isoform was faintly labelled. A single tau band was labelled by Tau-E2 in DM1. It was detected neither by Tau-E3 nor by Tau-E10 antiserum and it should correspond to the tau isoform containing only the exon 2. The two recombinant tau isoforms containing the exon 3 were stained by Tau-E3 antisera as well as in control brain homogenate. They were also labelled in DM1 but only after a longer time of exposure. The Tau-E10 antiserum labelled the three recombinant tau isoforms with the corresponding sequence, but it also slightly cross-reacted with the tau isoforms lacking the exon 10. It detected five bands in the control brain homogenates, three of which were corresponding to the tau protein isoforms with the exon 10. The two additional bands might correspond to incompletely dephosphorylated exon 10-containing normal tau protein isoforms, as described by Janke *et al.* (48). Nevertheless, in DM1, the Tau-E10 antiserum stained two major bands as well as minor bands, including the shortest tau isoform and the tau protein isoforms containing the exon 10.

Moreover, the tau catabolic products were strongly detected by Tau-E10 antisera whereas the Tau-E2 and Tau-E3 antisera only faintly reacted, suggesting that the lower detection of the tau protein isoforms containing the exons 2 and 3 does not result from a selective tau protein catabolism.



**Figure 2.** Western blot analyses of the brain distribution of pathological tau proteins, their phosphorylation status and the isoform content. **(A)** Pathological tau proteins are detected with AD2 antibody in brain tissue homogenates from DM1 cases 3 and 5. The three major pathological components of AD at 60, 64 and 69 kDa are indicated (Tau 60, 64 and 69). A single pathological tau band at 60 kDa is labelled in the parahippocampal gyrus (GPH), the Ammon's horn (CA), the nucleus basalis of Meynert (NbM) and the area of Broca (A44) of DM1 case 5. The pathological tau doublet at 60 and 64 kDa, as described by Vermersch *et al.* (17), is observed in DM1 case 3. The cortical areas are referred to in accordance with Brodmann's classification. The following abbreviations refer to other brain structures: Ant Cing, anterior cingulate gyrus; G. Cing, cingulate gyrus; Hip, hippocampus; WM, white matter. **(B)** Determination of the phosphorylated sites detected on pathological tau in DM1 case 2 (Brodmann area 38, temporal pole). An AD brain tissue homogenate is loaded and AD2 staining is performed to precisely align the pathological tau components at 60, 64, 69 and 74 kDa (Tau 60, 64, 69 and 74). The corresponding pathological tau components detected in DM1 brain tissue homogenate (DM1 underlined tracks) are indicated by arrowheads. The antibodies used are indicated at the top of the lanes and the epitopes are summarized on the longest tau isoform (Fig. 1D). The normal tau proteins detected by Tau-1 antibody on AD brain homogenate are indicated by dots (Tau-1 lane). The arrows indicate the corresponding normal tau proteins detected by Tau-1 antibody in DM1. It is important to be precise that this experiment was performed for four DM1 cases (cases 1, 2, 3 and 5). In DM1 case 5, it is only the 60 kDa pathological tau component that is detected with the phospho-dependent antibodies (data not shown). The Tau-E10 antiserum (Tau-E10 lane) strongly detects the pathological tau 64 kDa component in DM1 case 2, whereas no staining is observed in DM1 case 5 (data not shown). These results were verified by two-dimensional gel electrophoresis following the procedure described previously (39; and data not shown).

### Alteration of the tau mRNA isoform expression

The altered expression of tau isoform was further investigated at the mRNA level. The quantity of each tau isoform mRNA was determined in four brain regions (temporal, frontal, parietal and occipital cortex) of two controls and three DM1 cases (cases 1, 2 and 3) for which the brain tissue was available. The

typical profile of the six human brain mRNA tau isoforms is shown in Figure 4. No significant quantitative differences were observed between the four brain regions from controls and DM1 cases (data not shown). Therefore, the relative amount of each tau isoform was measured and expressed as the mean of the four brain regions analysed (Fig. 4B). When compared

with control, in DM1 cases, the tau isoform mRNAs lacking the exons 2 and 3 were found in higher amounts, whereas the tau isoform mRNAs containing the exons 2 and 3 were found in lower amounts. Identical results were obtained using primers flanking the exons 2 and 3 (Fig. 4C). However, the total amount of tau transcript was similar between control and DM1 cases, suggesting that the overall tau expression level was not affected in DM1. The tau isoform mRNA expression pattern mirrored the tau protein isoform expression profile, suggesting therefore an alteration of the maturation of tau pre-mRNA in DM1.

#### Determination of CTG repeat sizes

By comparing the CTG repeat lengths among four cortical brain regions in five unrelated DM1 cases, we identified clear intraindividual somatic heterogeneity. As shown in Figure 5 for DM1 case 1 and as summarized in Table 2 for the five DM1 cases, a heterogeneous expansion of the (CTG)<sub>n</sub> track yields different discrete bands in long PCR whose values were indicated. In order to analyse the intertissue variation, four separate reactions for each tissue sample were performed for DM1 case 1. All of the brain samples analysed showed different distributions of larger alleles and a common lower boundary of approximately 250 repeats. These distributions are consistent with a progenitor allele of approximately 250 repeats present in all of the four brain areas analysed. The different larger alleles in each sample probably derived from this single input molecule. As shown in Table 2, larger alleles were detected in the temporal regions containing up to 3300 CTG repeats in four out of the five DM1 cases.

## DISCUSSION

Little is known about the impact of the DM1 genetic defect on the brain. This study is focused on the possible relationship between the CTG repeat expansion and tau pathology in DM1. Three major findings are emerging from the present study: (i) neurofibrillary degeneration with pathological tau protein is a common feature in DM1 and hyperphosphorylated tau proteins are phosphorylated at identical pathological sites found in other neurological disorders; (ii) large expanded CTG repeats are detected and they show a marked somatic heterogeneity between DM1 cases and in the cortical brain regions; and (iii) the major finding is the disruption in normal tau isoform expression, both at the mRNA and protein level, in the brain of patients suffering from DM1, suggesting that the CUG repeat expansion alters the maturation of tau pre-mRNA.

#### Heterogeneous brain distribution of tau pathology in DM1

Pathological tau proteins are found during cerebral aging and in many neurodegenerative disorders. In the former, pathological tau proteins are systematically observed in the hippocampus of persons aged >75 years (49). The biochemical study of the brain distribution of tau pathology in neurodegenerative disorders including AD, PiD, PSP and CBD has shown typical distribution (49–51). Such studies suggest a dynamic spreading of neurofibrillary degeneration following different neuronal pathways that result from distinct etiological factors.

**Table 2.** Somatic heterogeneity and major expanded (CTG)<sub>n</sub> alleles in cortical brain regions

Cases	Cortical regions	Number of expanded alleles amplified	(CTG) <sub>n</sub> expansion size range
1	Tp	5	150–2800
	Frt	9	150–2800
	Par	8	150–1900
	Occ	10	150–2300
2	Tp	3	1000–2400
	Frt	6	1000–2400
	Occ	1	1900
3	Tp	5	150–3300
	Frt	4	150–2250
	Occ	5	150–1650
4	Tp	3	150–2650
	Frt	5	100–1650
	Par	7	100–1400
	Occ	9	150–1200
5	Tp	4	250–500
	Frt	4	250–1650
	Par	5	250–500
	Occ	2	250–530

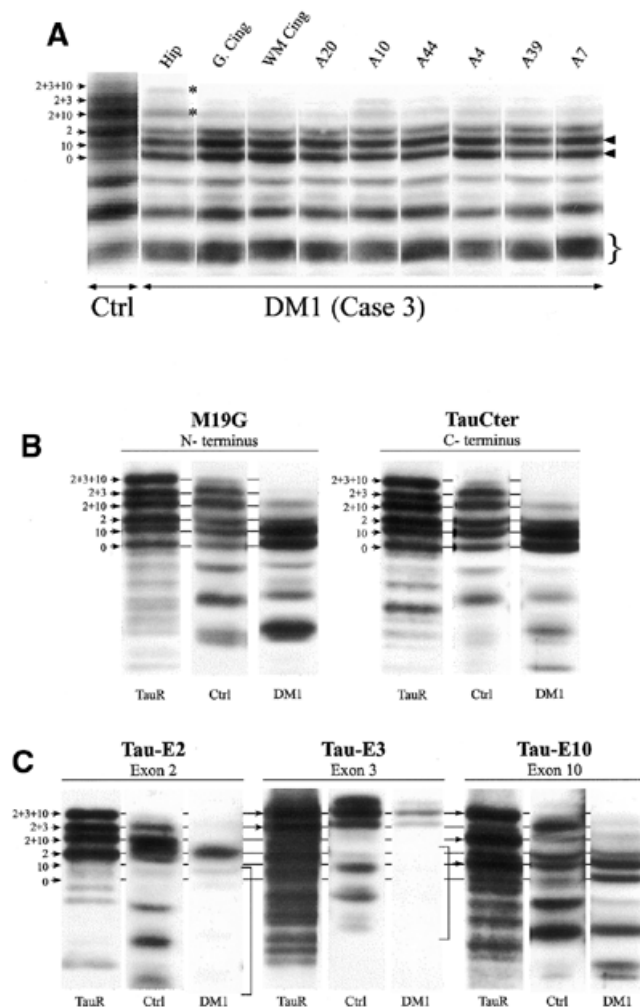
The expanded CTG repeat sizes were determined from genomic DNA that was obtained from brain tissue homogenates. The brain regions analysed include the temporal, frontal, parietal and occipital cortex, except for DM1 case 3 in which the parietal cortex was omitted. The numbers of amplified CTG repeat expansions are indicated in the second column. The size range of the different expanded CTG repeats is indicated in the third column.

In contrast, the brain distribution of tau pathology is inter-individually heterogeneous in DM1. The hippocampal formation was often affected whereas the tau pathology in both isocortical areas and subcortical nuclei is different between the five DM1 cases. DM1 neuropathology has been rarely investigated. At the biochemical level, we show that tau pathology is observed early in the adulthood and that the hippocampal formation is the most vulnerable brain region.

#### Abnormal phosphorylation of tau in DM1

The specific electrophoretic signature of pathological tau has been described in AD, PiD, PSP, CBD and FTDP-17 (37). The particular pathological tau electrophoretic pattern of DM1 should be defined as a single major pathological tau component of 60 kDa, observed in all DM1 cases analysed. The additional minor 64 kDa and traces of the 69 kDa components are only observed in the most severely affected brain regions.

Aggregates of tau proteins in tauopathies are hyperphosphorylated and pathological phosphorylated sites have been well documented in AD, PSP/CBD and PiD, whereas they are absent in biopsy-derived human brain tau proteins (52). AT100 antibody and AP422 antiserum, which are specific for these pathological phosphorylated sites (52), detect pathological tau

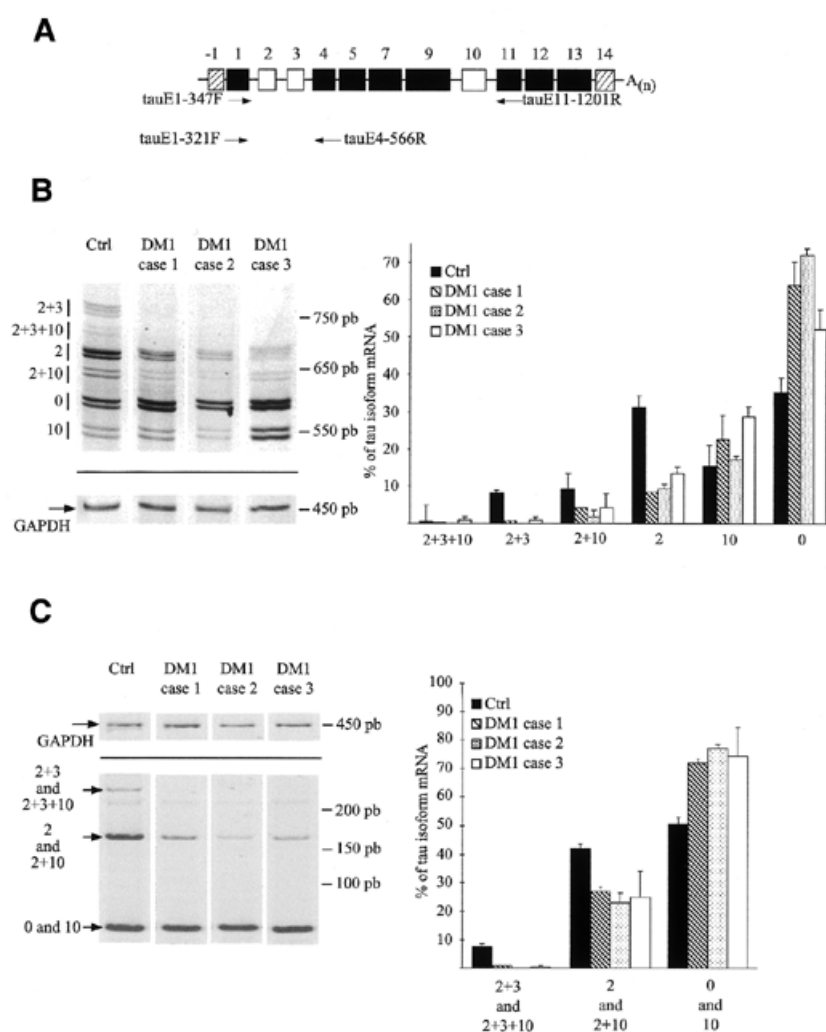


**Figure 3.** Tau protein expression in different brain areas of DM1 case 3. (A) All tau proteins from a control (Ctrl) brain tissue homogenate (Brodmann area 10, frontal pole) are detected by the tau N-terminus antiserum M19G (tau isoforms are indicated by arrows). Different brain areas from DM1 case 3 were analysed. Pathological tau protein bands (asterisks) are detected in the hippocampus homogenate (Hip). The two lowest bands of tau holoproteins (arrowheads) and N-terminus tau catabolic products (bracket) are more strongly detected in DM1 brain tissue homogenates compared with the control brain tissue homogenate. Similar analyses were performed in temporal, frontal, parietal and occipital brain tissue of the five DM1 cases and identical results were obtained (data not shown). The cortical brain regions are indicated according to the nomenclature of Brodmann and the cingulate gyrus (G. Cing) and white matter (WM) were included in the analysis. (B) Western blot analyses were performed using a panel of antisera against the N- (M19G) and C-terminus (TauCter) of tau proteins as well as antisera specific to corresponding sequences of the alternatively spliced exons 2, 3 and 10 [(C) Tau-E2, Tau-E3 and Tau-E10]. In each experiment, the six recombinant tau isoforms (TauR), a control (Ctrl) human brain tissue homogenate and a DM1 brain tissue homogenate (lacking tau pathology; DM1 case 5, temporal pole) were loaded on adjacent lanes. Arrowheads indicate the distribution of tau protein isoforms and the exon content is represented according to the presence or the absence of the alternatively spliced sequences 2, 3 and 10. Tau isoform holoproteins stained in Ctrl and DM1 brain tissue homogenates by M19G, TauCter antisera or the exon-specific tau antisera are aligned according to the six recombinant tau isoform labelling, and the catabolic products are indicated by brackets. Note that the Tau-E2 staining in Ctrl brain homogenate does not fully align with the exon 2 containing recombinant tau protein isoforms. It suggests that normal human brain tau proteins are not completely dephosphorylated, as described previously (48).

proteins from DM1. Phospho-dependent tau antibodies also enable us to distinguish between neurodegenerative disorders. Thus, pathological tau of sporadic PiD are not phosphorylated at the serines 262 and 356 and therefore not detected by the 12E8 antibody (40,41,46,53). Neurofibrillary degeneration in DM1 is distinct from that of PiD since pathological tau proteins are phosphorylated at the serine 262. Together, pathological tau proteins in DM1 possess most of the pathological phosphorylated sites commonly found in other neurological disorders with NFT.

### Normal tau isoform expression is altered in DM1

Pathological tau proteins of DM1 are shown to mainly consist of tau isoforms lacking the exon 2. Hence, the normal tau expression in brain was investigated at both the protein and mRNA level. When compared with the normal tau protein expression of a control case, we showed that total level of expression was conserved in DM1. In contrast, the stoichiometry of the tau protein isoform was dramatically altered in DM1. Using the exon 2- and exon 3-specific tau antisera, lower amounts of tau holoproteins and tau catabolic products were



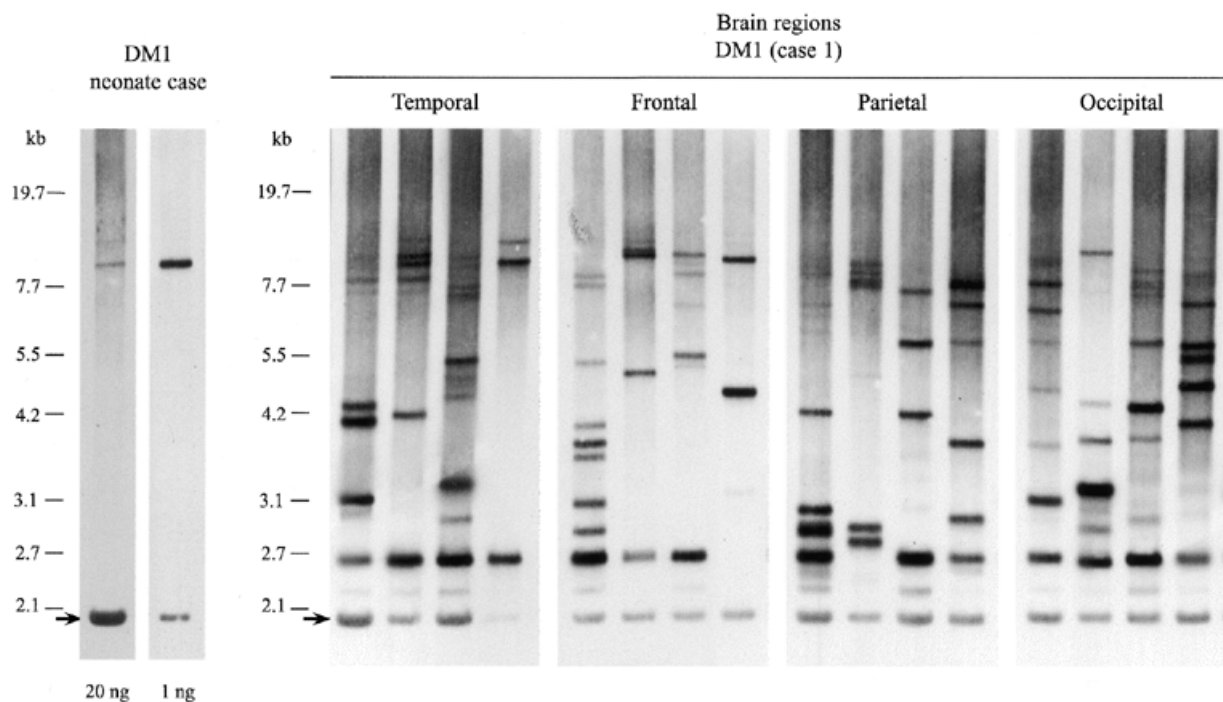
**Figure 4.** Expression profile of whole human brain tau isoform mRNA. RT-PCR products corresponding to the different tau mRNA isoforms were obtained using two sets of primers (A). Primers tauE1-347F and tauE11-1201R allowed the simultaneous amplification of all six isoforms as depicted in (B). Primers tauE1-321F and tauE4-566R were also used in order to differentiate tau mRNA isoforms that contain or lack the exon 2 and/or exon 3, as depicted in (C). The GAPDH was used as internal standard. In (B) the histogram represents the measured percentage of each tau isoform as the mean of two controls and the DM1 cases 1, 2 and 3. In (C) the histogram represents the measured percentage of isoforms containing the exons 2 and 3 (2+3 and 2+3+10), containing the exon 2 (2 and 2+10) and lacking both the exons 2 and 3 (0 and 10). In these two histograms, results are the mean of measurements obtained from four different brain areas (temporal, frontal, parietal and occipital) with standard deviations.

detected compared with that of a control brain homogenate. Moreover, the normal tau protein isoforms lacking the exons 2 and 3 also correspond to that found in pathological tau. The analysis of tau isoform mRNA transcripts also showed a decrease of tau isoforms containing the exon 2 counterbalanced by an increase of tau isoforms lacking the exon 2 and to a lesser extent, the tau isoform with the exon 10 only. Thus, the tau isoform mRNA analysis mirrored the results obtained on tau protein isoforms and demonstrated that the pathological tau profile of DM1 might result from the aggregation of the tau isoforms that are overexpressed.

Change in tau protein isoform expression results from tau gene mutations described in FTDP-17 (34). Indeed, most of the mutations altering the maturation of tau pre-mRNA change the expression of tau protein in favour of tau isoform containing the exon 10 (31,33,35,54,55). Tau isoforms with the exon 10

aggregate in these FTDP-17 and constitute the typical pathological tau doublet at 64 and 69 kDa, and the minor 74 kDa component (32,34). DM1 and FTDP-17 are similar in that the tau isoform expression is altered, characterized by a reduced inclusion of exon 2 or by increased inclusion of exon 10, respectively. DM1 represents the first tauopathy characterized by the alteration of tau exon 2 inclusion. Moreover, to date the function of the exon 2 or exon 3 corresponding sequences remains unknown. Therefore, DM1 is also of particular interest towards the understanding of the function of the latter protein sequence.

Recent data support the idea that DMPK is not responsible for all symptoms of DM1 (43). The CTG repeats could act as *cis*-regulating elements on neighbouring genes, as for SIX5/DMAHP, whose level of expression is reduced in the brain of DM1 patients (56). Transgenic mice with reduced levels of



**Figure 5.** Determination of CTG repeat heterogeneity in DM1 brain tissue. The CTG repeat expansion size was determined from genomic DNA of cortical brain tissue (temporal, frontal, parietal and occipital) regions of DM1 case 1 using a long PCR assay followed by Southern blot and probe-mediated detection of expanded alleles. For each tissue sample, four separate reactions containing 1 ng of input DNA are shown. A control DNA obtained from a neonatal case of DM1 (2000 CTG repeats) with an input DNA of 20 and 1 ng, respectively, is presented in the first two lanes. The arrow indicates the size of the normal allele. DNA molecular weight markers are indicated on the left.

SIX5/DMAHP develop cataracts (57–59). The CUG repeats are located in the 3'-UTR of the DMPK transcripts and RNA mutation dominance hypotheses have been proposed. First, the CUG repeats could act as defective *cis*-regulating elements on the nucleocytoplasmic transport and favour the nuclear retention of the DMPK mRNAs (43,60,61) or modify the splicing of DMPK transcripts (62). Secondly, the CUG repeats cause a gain-of-function by sequestering RNA binding proteins and thus altering gene transcript maturation which requires these proteins for normal gene expression (44,63–65). It is not known whether the change in tau isoform expression observed in DM1 could result from a selective nuclear retention of tau isoform mRNA or alteration in tau transcript maturation.

In DM1, the tau transcripts containing the exon 2 are reduced. However, little is known about the *trans*-regulating factors required for the splicing of the exons 2 and 3 of tau in human brain (66,67). It would be of particular interest to investigate further the regulation of tau splicing and to determine which are the *trans*-acting factors required in this regulation process. Hence, tau is a new molecular target toward understanding the molecular mechanism underlying DM1 in the human central nervous system. Because we demonstrate that tau isoform expression is altered in DM1, this pathology should be considered as a real tauopathy that includes a constellation of non-familial and familial autosomal inherited diseases with different mutations on tau gene, different tau polymorphisms or different etiologies (reviewed in 37).

### Heterogeneity of the (CTG)<sub>n</sub> expansion in brain

Analysis of CTG repeat expansion is generally performed in muscle or lymphoid cells. A tissue-dependent somatic mosaicism in brain was described previously (68). The (CTG)<sub>n</sub> expansions show a high degree of somatic instability. Thornton *et al.* (69) have stated that the expansion size is generally larger in tissues than in lymphoid cells. Five conclusions follow from our results: (i) large expanded alleles (over 3000 CTG repeats) are found in human brain cortex of DM1 patients; (ii) the (CTG)<sub>n</sub> expansion is heterogeneous in the cortical brain regions; (iii) larger expansion is observed in old parental DM1 cases than in the congenital case; and (iv) the presence of expanded alleles is well related to the change in tau expression.

Multiple factors have been related to the variations of the (CTG)<sub>n</sub> expansion size. Increase in size has been associated with ageing, in human and in transgenic mice (70–72). DM1 case 5 (42 years) exhibits the shorter (CTG)<sub>n</sub> expansion size whereas the larger expansion, up to 3000 repeats, was observed in the oldest DM1 case (DM1 case 3, aged 64 years). Intertissue heterogeneity did not reflect subpopulations of cells with the same allele but represented a mutation process derived from a single apparent progenitor allele.

Therefore, our results on human brain tissue are in good agreement with previous studies suggesting that ageing and somatic expansion from a common precursor allele can largely contribute to the instability observed in DM1 (73).

The presence of pathological tau is not related to ageing since it was observed in the DM1 case 5, aged 42 years,

whereas the oldest DM1 case (DM1 case 4) was almost spared. Hence, ageing cannot be considered as the major risk factor for neurodegeneration in DM1. However, there is a direct link between the (CTG)<sub>n</sub> expansion and the change of tau isoform expression. Because of the high degree of somatic mosaicism, it is difficult yet to precisely assign a strict correlation between CTG repeat size and tau isoform dysregulation as well as to determine which neurons have their tau expression altered in DM1. Previous studies support the hypothesis that in the human brain, different vulnerable sub-neuronal populations selectively degenerate in neurological disorders (42). Because of the high heterogeneity of both neurofibrillary degeneration distribution and CTG repeat expansion, distinct neuronal populations might be affected leading to a non-stereotyped spatio-temporal distribution of the tau pathology in DM1. However, the hippocampus might represent the human brain region that is most vulnerable to neurodegeneration, whatever the etiology that is also severely affected in DM1. Thus, any change in balance of tau isoform expression in hippocampal neurons might facilitate the development of neurofibrillary degeneration.

In conclusion, from the study of microtubule-associated tau expression in DM1 brain tissue, we show for the first time an *in vivo* change in the stoichiometry of the six tau isoforms characterized by a lower expression of tau isoforms containing the exon 2. It contrasts with FTDP-17, where tau mutations alter essentially the splicing of exon 10. The dramatic change in tau isoform expression is associated with the presence of large (CTG)<sub>n</sub> expansions. Because of the recent evidence suggesting that expanded CUG repeats could affect RNA metabolism of several genes and act as a gain-of-function mutation, this mechanism is of particular interest for understanding the different pathways leading to neurofibrillary degeneration and tauopathies.

## MATERIALS AND METHODS

### Patients and brain tissue samples

DM1 brains were obtained at autopsy of five patients aged from 42 to 64 years. The brain tissues were obtained from Department of Neurology, Lille, France, in accordance with the local ethic committee protocol (Table 3). Cases 1 and 2 were described previously (17). Cognitive and intellectual impairments were noted in three cases (Table 3; intellectual and cognitive status column). Post-mortem delays were <48 h. The left hemisphere was fixed in formalin (DM1 cases 1–3) and tissue samples were embedded in paraffin for neuropathological examination. The right hemisphere was stored at –80°C for molecular studies.

The brain tissue homogenates were obtained following the same procedure. Briefly, tissue samples from cortical areas of the right hemisphere were dissected according to Brodmann's classification. Areas from the frontal (areas 4, 10, frontal white matter), temporal (areas 20, 21, 22 and 38), entorhinal (area 28), parahippocampal gyrus (area 35), hippocampus (area 34), parietal (areas 7 and 39) and occipital (areas 17 and 18) regions were dissected. The following brain structures were also included: gyrus cingulate (G. Cing), amygdala nucleus, nucleus basalis of Meynert (NbM), putamen, pallidum, nucleus caudate, locus niger (LN) and cerebellum (Table 1).

**Table 3.** Clinical, genetic and neuropathology information of five cases with DM1

Case	Sex	Age of death	Phenotype class <sup>a</sup>	Intellectual and cognitive status	Neuropathology
1	Male	61	Classical adult form	Normal	NFT
2	Male	53	Congenital DM1	Mental retardation, progressive cognitive impairment	NFT
3	Male	64	Adult form	Cognitive impairment	NFT
4	Male	63	Juvenile/Adult form	ND	ND
5	Male	42	Juvenile/Adult form	Mental retardation	NFT

<sup>a</sup>Harper (12).

NFT, neurofibrillary tangles; ND, not determined.

The samples were homogenized at a ratio of 1 g of tissue per 10 ml of 5% SDS Laemmli sample buffer and heat-treated for 10 min at 100°C (74). Brain tissue samples from 10 age-matched controls and 10 AD patients aged from 42 to 80 years were studied in parallel. The latter patients were described previously (49).

### SDS-PAGE and western blot analysis

A sample from a typical AD case was used as an internal standard for western blots to compare the amounts and to align precisely the pathological tau components. For 1D gels, 25 µl of homogenates from DM1 and 10 µl of homogenates from AD were loaded on 7.5–15% SDS-PAGE using the Protean Ixi Cell (Bio-Rad, Marne la Coquette, France). Following SDS-PAGE, proteins were transferred electrophoretically onto nitro-cellulose membranes for 90 min using the Multiphor II Novablot System (Amersham-Pharmacia Biotech, Orsay, France). The proteins were stained reversibly with Ponceau red in order to control the quality of separation and the amount of protein loaded. After blocking with 5% skimmed milk, the membranes were incubated for 2 h or overnight at 4°C with phospho-dependent antibodies or the exon-specific tau antisera (listed below) in 20 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.1% (w/v) of Tween-20 (TBS-T). Following extensive washes, the membranes were incubated with horseradish peroxidase-conjugated secondary mouse or rabbit antibodies (Sigma-Aldrich, St Quentin Fallavier, France). The immunoreactive complexes were revealed, and they were visualized using the ECL™ western blotting system (Amersham-Pharmacia Biotech, Orsay, France).

### Tau antibodies

All of the epitopes recognized by the antibodies used in the present study are represented on the longest human tau isoform (Fig. 1D). AD2 antibody specifically recognizes the phosphorylated serines 396 and 404 (numbering according to the longest human tau isoform) at the C-terminus of tau proteins (75). The AT270, AT8, AT100 and AT180 detect the

phosphorylated serine or threonine residues of tau protein at positions thr181, ser202/ser205, thr212/ser214 and thr213, respectively (a gift from Eugeen VanMechelen, Innogenetics, Belgium) (76). Tau-1 recognizes the unphosphorylated sequence ranging from amino acids 189 to 207 (Boehringer Mannheim GmbH, Germany). 12E8 antibody is raised against the phosphorylated serine 262 and 356 (46) (a gift from Peter Seubert, Elan Pharmaceuticals, USA). The S199P and AP422 antisera selectively detect the phosphorylated residues at positions ser199 and ser422, respectively (39,52). M19G and TauCter antisera are raised against synthetic peptides encoding the first 19 and the last 15 amino acids of tau proteins, respectively. Exon-specific tau antisera Tau-E2 and Tau-E3 are directed against the exons 2 and 3 corresponding sequences (39). Tau-E10 antiserum detects a highly conserved microtubule-binding motif encoded by exon 10 in the half C-terminus of tau proteins (38). The Tau-E10 antibody was obtained after purification of the antisera following affinity chromatography using the longest tau isoform conjugated to an NHS-Fast Flow matrix according to the manufacturer's instructions (Amersham-Pharmacia Biotech, Orsay, France). For analysis of the expression of tau proteins isoforms, a homogenate containing the six recombinant human tau isoforms (77) was provided by Michel Goedert (MRC, Cambridge, UK), as described previously (38,77).

#### Semi-quantitative analysis of tau isoform mRNA

Total RNA was isolated from brain tissue using the RNagents according to the manufacturer's instructions (Promega, France). The RNA concentration was determined by absorption at 260 nm. RT-PCR was performed with 1 µg of total RNA using M-MLV reverse transcriptase (Promega, France) according to standard protocols using random hexamers (5 µmol/l). RT controls were performed and no DNA amplification was observed. PCR amplification of tau transcripts (GenBank Accession no. NM\_005910) was performed with tau primers that recognize the overall tau sequence (exons 1–11). The forward and reverse primer sequences are as follows: tauE1-347 forward, 5'-TGACACGGACGC-TGGCCTGAA-3'; tauE11-1201 reverse, 5'-CACTTGGAG-GTCACCTTGCTC-3'. Following amplification, PCR products were cleaved by *AsnI*, defining six unequivocal distinct fragments at 762 bp (2+3 tau isoform), 722 bp (2+3+10 tau isoform), 676 bp (2+ tau isoform), 636 bp (2+10 tau isoform), 590 bp (0 tau isoform) and 550 bp (10+ tau isoform). Tau mRNA isoform quantification was further investigated using forward and reverse primers: tauE1-321 forward, 5'-TACACCATGCAC-CAAGACCA-3' and tauE4-566 reverse, 5'-GTCTCCAATGC-CTGCTTCTT-3', giving three combinations of tau mRNA isoforms at 245 bp (2+3 tau isoforms), 159 bp (2 tau isoforms) and 73 bp (0 tau isoforms). Amplification of GAPDH mRNA was used as internal control using the forward primer, 5'-CCATGGCACCGTCAAGGCTGA-3' and the reverse primer, 5'-GCCAGTAGAGGCAGGGATGAT-3'. PCR amplification was performed in a final volume of 25 µl containing 25 pmol of each primer, 2.5 mM MgCl<sub>2</sub> and 1 U of *Taq* polymerase (Promega, France), with the following conditions: 5 min at 94°C, followed by 28 cycles of 1 min denaturation at 94°C, annealing for 2 min at 62°C, and 3 min extension at 72°C. A water control was included as a control of DNA contamination.

Aliquots of the PCR products were separated by 5% denaturing or 5% non-denaturing polyacrylamide gel electrophoresis and gels were stained with either silver nitrate or ethidium bromide.

#### Determination of the CTG repeat size

Genomic DNA was prepared from brain tissue (500 mg) by phenol–chloroform extraction. PCR amplification of the CTG repeat region was performed using a long-PCR assay with similar conditions to those described previously (78). This method uses the Expand long template PCR system™ for long target amplifications (Roche Diagnostics GmbH, Mannheim, Germany). Each 50 µl reaction consisted of 1× Expand long PCR buffer [50 mM Tris–HCl pH 9.2, 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>], 0.35 mM of each dATP, dCTP, dTTP, 0.23 mM dGTP, 0.12 mM 7-deaza dGTP, 0.4 µM of primers DMK9003 and DMK 11111 (78), 3.5 U of *Taq* DNA polymerase, 1.75 mM magnesium chloride, and either 20 or 1 ng total genomic DNA. Amplifications were performed in a PTC200 thermocycler (MJ Research, ProLabo, France). Following denaturation for 30 s at 95°C, 35 cycles of amplification were performed: denaturation at 95°C for 15 s, annealing at 61°C for 30 s and extension at 68°C for 6 min. PCR products were resolved on 1% agarose gels. To obtain an accurate detection of large mutant alleles, Southern blot transfer and probe-mediated detection was achieved. Briefly, agarose gels were denatured in 0.4 M NaOH, 0.6 M NaCl and rinsed twice in 2× SSC before being transferred to Hybond N<sup>+</sup> nylon membranes. Blots were prehybridized in 5× SSC, 5% liquid block solution, and 0.02% SDS for 30 min at 59°C. Hybridization was performed in a fresh solution containing 40 pmol 3'-FI-dUTP-(CTG)<sub>10</sub> for 2 h at 59°C followed by two washes in 5× SSC, 0.1% SDS at 42°C for 5 min and two washes in 1× SSC, 0.1% SDS. Detection was achieved using the ECL™ reagents and Hyperfilm-ECL according to the manufacturer's instructions (Amersham-Pharmacia Biotech, Orsay, France). Film exposure times ranged from 30 s to 15 min. DNA molecular weight standards were loaded on each agarose gel (DNA marker IV, Boehringer Mannheim GmbH, Germany).

#### Computer-assisted blot analyses

The western blots, Southern blots and silver-stained gels were scanned with a SHARP JX-325 calibrated digital scanner and the electrophoretic bands were analysed using the Image elite 1D™ software (Amersham-Pharmacia Biotech, Orsay, France). The total tau mRNA signal was normalized to that of GAPDH mRNA and the relative amount of each mRNA tau isoform was expressed as the percentage of the total tau mRNA intensity. Image1D™ software was also used to calculate the sizes of CTG repeat expansion after subtracting the normal allele size (2.05 kb).

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## REFERENCES

- Aslanidis, C., Jansen, G., Amemiya, C., Shuttler, G., Mahadevan, M., Tsilifidis, C., Chen, C., Alleman, J., Wormskamp, N.G., Vooijs, M. *et al.* (1992) Cloning of the essential myotonic dystrophy region and mapping of the putative defect. *Nature*, **355**, 548–551.
- Brook, J.D., McCurrach, M.E., Harley, H.G., Buckler, A.J., Church, D., Aburatani, H., Hunter, K., Stanton, V.P., Thirion, J.P., Hudson, T. *et al.* (1992) Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell*, **69**, 385.
- Buxton, J., Shelbourne, P., Davies, J., Jones, C., Perryman, M.B., Ashizawa, T., Butler, R., Brook, D., Shaw, D., de Jong, P. *et al.* (1992) Characterization of a YAC and cosmid contig containing markers tightly linked to the myotonic dystrophy locus on chromosome 19. *Genomics*, **13**, 526–531.
- Fu, Y.H., Pizzuti, A., Fenwick, R.G., Jr, King, J., Rajnarayan, S., Dunne, P.W., Dubel, J., Nasser, G.A., Ashizawa, T., de Jong, P. *et al.* (1992) An unstable triplet repeat in a gene related to myotonic muscular dystrophy. *Science*, **255**, 1256–1258.
- Harley, H.G., Brook, J.D., Rundle, S.A., Crow, S., Reardon, W., Buckler, A.J., Harper, P.S., Housman, D.E. and Shaw, D.J. (1992) Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. *Nature*, **355**, 545–546.
- Mahadevan, M., Tsilifidis, C., Sabourin, L., Shuttler, G., Amemiya, C., Jansen, G., Neville, C., Narang, M., Barcelo, J., O'Hoy, K. *et al.* (1992) Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene. *Science*, **255**, 1253–1255.
- The International Myotonic Dystrophy Consortium (IDMC). (2000) New nomenclature and DNA testing guidelines for myotonic dystrophy type 1 (DM1). *Neurology*, **54**, 1218–1221.
- Ashizawa, T., Dunne, C.J., Dubel, J.R., Perryman, M.B., Epstein, H.F., Boerwinkle, E. and Hejtmancik, J.F. (1992) Anticipation in myotonic dystrophy. I. Statistical verification based on clinical and haplotype findings. *Neurology*, **42**, 1871–1877.
- Gennarelli, M., Novelli, G., Andreasi Bassi, F., Martorelli, L., Cornet, M., Menegazzo, E., Mostacciolo, M.L., Martinez, J.M., Angelini, C., Pizzuti, A. *et al.* (1996) Prediction of myotonic dystrophy clinical severity based on the number of intragenic (CTG)<sub>n</sub> trinucleotide repeats. *Am. J. Med. Genet.*, **65**, 342–347.
- Hunter, A., Tsilifidis, C., Mettler, G., Jacob, P., Mahadevan, M., Surh, L. and Korneluk, R. (1992) The correlation of age of onset with CTG trinucleotide repeat amplification in myotonic dystrophy. *J. Med. Genet.*, **29**, 774–779.
- Turnpenny, P., Clark, C. and Kelly, K. (1994) Intelligence quotient profile in myotonic dystrophy, intergenerational deficit, and correlation with CTG amplification. *J. Med. Genet.*, **31**, 300–305.
- Harper, P.S. (1989) *Myotonic Dystrophy*. W.B. Saunders, Philadelphia, PA.
- Endo, A., Motonaga, K., Arahata, K., Harada, K., Yamada, T. and Takashima, S. (2000) Developmental expression of myotonic dystrophy protein kinase in brain and its relevance to clinical phenotype. *Acta Neuropathol. (Berl.)*, **100**, 513–520.
- Kiuchi, A., Otsuka, N., Namba, Y., Nakano, I. and Tomonaga, M. (1991) Presenile appearance of abundant Alzheimer's neurofibrillary tangles without senile plaques in the brain in myotonic dystrophy. *Acta Neuropathol. (Berl.)*, **82**, 1–5.
- Mitake, S., Inagaki, T., Niimi, T., Shirai, T. and Yamamoto, M. (1989) Development of Alzheimer neurofibrillary changes in two autopsy cases of myotonic dystrophy. *Rinsho Shinkeigaku*, **29**, 488–492.
- Spillantini, M.G., Tolnay, M., Love, S. and Goedert, M. (1999) Microtubule-associated protein tau, heparan sulphate and  $\alpha$ -synuclein in several neurodegenerative diseases with dementia. *Acta Neuropathol. (Berl.)*, **97**, 585–594.
- Vermersch, P., Sergeant, N., Ruchoux, M.M., Hofmann-Radvanyi, H., Watez, A., Petit, H., Dwaillly, P. and Delacourte, A. (1996) Specific tau variants in the brains of patients with myotonic dystrophy. *Neurology*, **47**, 711–717.
- Yoshimura, N., Otake, M., Igarashi, K., Matsunaga, M., Takebe, K. and Kudo, H. (1990) Topography of Alzheimer's neurofibrillary change distribution in myotonic dystrophy. *Clin. Neuropathol.*, **9**, 234–239.
- Kidd, M. (1963) Paired helical filament in electron microscopy of Alzheimer's disease. *Nature*, **197**, 192–193.
- Brion, J.P., Passareiro, E., Nunez, J. and Flament-Durand, J. (1985) Immunological detection of tau protein in neurofibrillary tangles of Alzheimer's disease. *Arch. Biol.*, **95**, 229–235.
- Delacourte, A. and Defossez, A. (1986) Alzheimer's disease: Tau proteins, the promoting factors of microtubule assembly, are major components of paired helical filaments. *J. Neurol. Sci.*, **76**, 173–186.
- Delacourte, A. and Defossez, A. (1986) Biochemical characterization of an immune serum which specifically marks neurons in neurofibrillary degeneration in Alzheimer's disease. *C. R. Acad. Sci. III*, **303**, 439–444.
- Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.C., Zaidi, M.S. and Wisniewski, H.M. (1986) Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *J. Biol. Chem.*, **261**, 6084–6089.
- Kosik, K.S., Joachim, C.L. and Selkoe, D.J. (1986) Microtubule-associated protein tau is a major antigenic component of paired helical filaments in Alzheimer disease. *Proc. Natl Acad. Sci. USA*, **83**, 4044–4048.
- Delacourte, A., Flament, S., Dibe, E.M., Hublau, P., Sablonniere, B., Hemon, B., Sherrer, V. and Defossez, A. (1990) Pathological proteins Tau 64 and 69 are specifically expressed in the somatodendritic domain of the degenerating cortical neurons during Alzheimer's disease. Demonstration with a panel of antibodies against Tau proteins. *Acta Neuropathol. (Berl.)*, **80**, 111–117.
- Lee, V.M., Balin, B.J., Otvos, L., Jr and Trojanowski, J.Q. (1991) A68: a major subunit of paired helical filaments and derivatized forms of normal Tau. *Science*, **251**, 675–678.
- Cleveland, D.W., Hwo, S.Y. and Kirschner, M.W. (1977) Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. *J. Mol. Biol.*, **116**, 207–225.
- Cleveland, D.W., Hwo, S.Y. and Kirschner, M.W. (1977) Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. *J. Mol. Biol.*, **116**, 227–247.
- Goedert, M., Spillantini, M.G., Jakes, R., Rutherford, D. and Crowther, R.A. (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron*, **3**, 519–526.
- Goedert, M., Spillantini, M.G., Potier, M.C., Ulrich, J. and Crowther, R.A. (1989) Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. *EMBO J.*, **8**, 393–399.
- Spillantini, M.G., Murrell, J.R., Goedert, M., Farlow, M.R., Klug, A. and Ghetti, B. (1998) Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. *Proc. Natl Acad. Sci. USA*, **95**, 7737–7741.
- Spillantini, M.G., Crowther, R.A., Kamphorst, W., Heutink, P. and van Swieten, J.C. (1998) Tau pathology in two Dutch families with mutations in the microtubule-binding region of tau. *Am. J. Pathol.*, **153**, 1359–1363.
- Gao, Q.S., Memmott, J., Lafyatis, R., Stamm, S., Sreaton, G. and Andreadis, A. (2000) Complex regulation of tau exon 10, whose missplicing causes frontotemporal dementia. *J. Neurochem.*, **74**, 490–500.
- Hong, M., Zhukareva, V., Vogelsberg-Ragaglia, V., Wszolek, Z., Reed, L., Miller, B.I., Geschwind, D.H., Bird, T.D., McKeel, D., Goate, A. *et al.* (1998) Mutation-specific functional impairments in distinct tau isoforms of hereditary FTDP-17. *Science*, **282**, 1914–1917.
- D'Souza, I., Poorkaj, P., Hong, M., Nochlin, D., Lee, V.M., Bird, T.D. and Schellenberg, G.D. (1999) Missense and silent tau gene mutations cause frontotemporal dementia with parkinsonism-chromosome 17 type, by affecting multiple alternative RNA splicing regulatory elements. *Proc. Natl Acad. Sci. USA*, **96**, 5598–5603.
- Buee, L., Bussiere, T., Buee-Scherrer, V., Delacourte, A. and Hof, P.R. (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res. Brain Res. Rev.*, **33**, 95–130.
- Delacourte, A. and Buee, L. (2000) Tau pathology: a marker of neurodegenerative disorders. *Curr. Opin. Neurol.*, **13**, 371–376.

38. Sergeant, N., David, J.P., Goedert, M., Jakes, R., Vermersch, P., Buee, L., Lefranc, D., Wattez, A. and Delacourte, A. (1997) Two-dimensional characterization of paired helical filament-tau from Alzheimer's disease: demonstration of an additional 74-kDa component and age-related biochemical modifications. *J. Neurochem.*, **69**, 834–844.
39. Sergeant, N., Wattez, A. and Delacourte, A. (1999) Neurofibrillary degeneration in progressive supranuclear palsy and corticobasal degeneration: tau pathologies with exclusively 'exon 10' isoforms. *J. Neurochem.*, **72**, 1243–1249.
40. Delacourte, A., Sergeant, N., Wattez, A., Gauvreau, D. and Robitaille, Y. (1998) Vulnerable neuronal subsets in Alzheimer's and Pick's disease are distinguished by their tau isoform distribution and phosphorylation. *Ann. Neurol.*, **43**, 193–204.
41. Sergeant, N., David, J.P., Lefranc, D., Vermersch, P., Wattez, A. and Delacourte, A. (1997) Different distribution of phosphorylated tau protein isoforms in Alzheimer's and Pick's diseases. *FEBS Lett.*, **412**, 578–582.
42. Delacourte, A. and Buee, L. (1997) Normal and pathological Tau proteins as factors for microtubule assembly. *Int. Rev. Cytol.*, **171**, 167–224.
43. Mankodi, A., Logigian, E., Callahan, L., McClain, C., White, R., Henderson, D., Krym, M. and Thornton, C.A. (2000) Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. *Science*, **289**, 1769–1773.
44. Miller, J.W., Urbinati, C.R., Teng-Umuay, P., Stenberg, M.G., Byrne, B.J., Thornton, C.A. and Swanson, M.S. (2000) Recruitment of human muscleblind proteins to (CUG)(n) expansions associated with myotonic dystrophy. *EMBO J.*, **19**, 4439–4448.
45. Murrell, J.R., Spillantini, M.G., Zolo, P., Guazzelli, M., Smith, M.J., Hasegawa, M., Redi, F., Crowther, R.A., Pietrini, P., Ghetti, B. and Goedert, M. (1999) Tau gene mutation G389R causes a tauopathy with abundant pick body-like inclusions and axonal deposits. *J. Neuropathol. Exp. Neurol.*, **58**, 1207–1226.
46. Seubert, P., Mawal-Dewan, M., Barbour, R., Jakes, R., Goedert, M., Johnson, G.V., Litsersky, J.M., Schenk, D., Lieberburg, I., Trojanowski, J.Q. *et al.* (1995) Detection of phosphorylated Ser262 in fetal tau, adult tau, and paired helical filament tau. *J. Biol. Chem.*, **270**, 18917–18922.
47. Sergeant, N., Bussiere, T., Vermersch, P., Lejeune, J.P. and Delacourte, A. (1995) Isoelectric point differentiates PHF-tau from biopsy-derived human brain tau proteins. *Neuroreport*, **6**, 2217–2220.
48. Janke, C., Beck, M., Holzer, M., Bigl, V. and Arendt, T. (2000) Analysis of the molecular heterogeneity of the microtubule-associated protein tau by two-dimensional electrophoresis and RT-PCR. *Brain Res. Brain Res. Protoc.*, **5**, 231–242.
49. Delacourte, A., David, J.P., Sergeant, N., Buee, L., Wattez, A., Vermersch, P., Ghozali, F., Fallet-Bianco, C., Pasquier, F., Lebert, F. *et al.* (1999) The biochemical pathway of neurofibrillary degeneration in aging and Alzheimer's disease. *Neurology*, **52**, 1158–1165.
50. Delacourte, A., Robitaille, Y., Sergeant, N., Buee, L., Hof, P.R., Wattez, A., Laroche-Cholette, A., Mathieu, J., Chagnon, P. and Gauvreau, D. (1996) Specific pathological Tau protein variants characterize Pick's disease. *J. Neuropathol. Exp. Neurol.*, **55**, 159–168.
51. Vermersch, P., Robitaille, Y., Bernier, L., Wattez, A., Gauvreau, D. and Delacourte, A. (1994) Biochemical mapping of neurofibrillary degeneration in a case of progressive supranuclear palsy: evidence for general cortical involvement. *Acta Neuropathol. (Berl.)*, **87**, 572–577.
52. Bussiere, T., Hof, P.R., Mailliot, C., Brown, C.D., Caillet-Boudin, M.L., Perl, D.P., Buee, L. and Delacourte, A. (1999) Phosphorylated serine422 on tau proteins is a pathological epitope found in several diseases with neurofibrillary degeneration. *Acta Neuropathol. (Berl.)*, **97**, 221–230.
53. Probst, A., Tolnay, M., Langui, D., Goedert, M. and Spillantini, M.G. (1996) Pick's disease: hyperphosphorylated tau protein segregates to the somatoaxonal compartment. *Acta Neuropathol. (Berl.)*, **92**, 588–596.
54. D'Souza, I. and Schellenberg, G.D. (2000) Determinants of 4-repeat tau expression. Coordination between enhancing and inhibitory splicing sequences for exon 10 inclusion. *J. Biol. Chem.*, **275**, 17700–17709.
55. Grover, A., Houlden, H., Baker, M., Adamson, J., Lewis, J., Prihar, G., Pickering-Brown, S., Duff, K. and Hutton, M. (1999) 5' splice site mutations in tau associated with the inherited dementia FTDP-17 affect a stem-loop structure that regulates alternative splicing of exon 10. *J. Biol. Chem.*, **274**, 15134–15143.
56. Thornton, C.A., Wymer, J.P., Simmons, Z., McClain, C. and Moxley, R.T., III (1997) Expansion of the myotonic dystrophy CTG repeat reduces expression of the flanking DMAHP gene. *Nat. Genet.*, **16**, 407–409.
57. Gennarelli, M., Pavoni, M., Amicucci, P., Angelini, C., Menegazzo, E., Zelano, G., Novelli, G. and Dallapiccola, B. (1999) Reduction of the DM-associated homeo domain protein (DMAHP) mRNA in different brain areas of myotonic dystrophy patients. *Neuromusc. Disord.*, **9**, 215–219.
58. Korade-Mirmics, Z., Tarleton, J., Servidei, S., Casey, R.R., Gennarelli, M., Pegoraro, E., Angelini, C. and Hoffman, E.P. (1999) Myotonic dystrophy: tissue-specific effect of somatic CTG expansions on allele-specific DMAHP/SIX5 expression. *Hum. Mol. Genet.*, **8**, 1017–1023.
59. Sarkar, P.S., Appukuttan, B., Han, J., Ito, Y., Ai, C., Tsai, W., Chai, Y., Stout, J.T. and Reddy, S. (2000) Heterozygous loss of Six5 in mice is sufficient to cause ocular cataracts. *Nat. Genet.*, **25**, 110–114.
60. Davis, B.M., McCurrach, M.E., Taneja, K.L., Singer, R.H. and Housman, D.E. (1997) Expansion of a CUG trinucleotide repeat in the 3' untranslated region of myotonic dystrophy protein kinase transcripts results in nuclear retention of transcripts. *Proc. Natl Acad. Sci. USA*, **94**, 7388–7393.
61. Hamshere, M.G., Newman, E.E., Alwazzan, M., Athwal, B.S. and Brook, J.D. (1997) Transcriptional abnormality in myotonic dystrophy affects DMPK but not neighboring genes. *Proc. Natl Acad. Sci. USA*, **94**, 7394–7399.
62. Tiscornia, G. and Mahadevan, M.S. (2000) Myotonic dystrophy: the role of the CUG triplet repeats in splicing of a novel DMPK exon and altered cytoplasmic DMPK mRNA isoform ratios. *Mol. Cell*, **5**, 959–967.
63. Groenen, P.J., Wansink, D.G., Coerwinkel, M., van den Broek, W., Jansen, G. and Wieringa, B. (2000) Constitutive and regulated modes of splicing produce six major myotonic dystrophy protein kinase (DMPK) isoforms with distinct properties. *Hum. Mol. Genet.*, **9**, 605–616.
64. Philips, A.V., Timchenko, L.T. and Cooper, T.A. (1998) Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. *Science*, **280**, 737–741.
65. Timchenko, N.A., Cai, Z.J., Welm, A.L., Reddy, S., Ashizawa, T. and Timchenko, L.T. (2001) RNA CUG repeats sequester CUGBP1 and alter protein levels and activity of CUGBP1. *J. Biol. Chem.*, **276**, 7820–7826.
66. Andreadis, A., Broderick, J.A. and Kosik, K.S. (1995) Relative exon affinities and suboptimal splice site signals lead to non-equivalence of two cassette exons. *Nucleic Acids Res.*, **23**, 3585–3593.
67. Stoss, O., Olbrich, M., Hartmann, A.M., Konig, H., Memmott, J., Andreadis, A. and Stamm, S. (2001) The STAR/GSG family protein rSLM-2 regulates the selection of alternative splice sites. *J. Biol. Chem.*, **276**, 8665–8673.
68. Ishii, S., Nishio, T., Sunohara, N., Yoshihara, T., Takemura, K., Hikiji, K., Tsujino, S. and Sakuragawa, N. (1996) Small increase in triplet repeat length of cerebellum from patients with myotonic dystrophy. *Hum. Genet.*, **98**, 138–140.
69. Thornton, C.A., Johnson, K. and Moxley, R.T., III (1994) Myotonic dystrophy patients have larger CTG expansions in skeletal muscle than in leukocytes. *Ann. Neurol.*, **35**, 104–107.
70. Fortune, M.T., Vassilopoulos, C., Coolbaugh, M.I., Siciliano, M.J. and Monckton, D.G. (2000) Dramatic, expansion-biased, age-dependent, tissue-specific somatic mosaicism in a transgenic mouse model of triplet repeat instability. *Hum. Mol. Genet.*, **9**, 439–445.
71. Seznec, H., Lia-Baldini, A.S., Duros, C., Fouquet, C., Lacroix, C., Hofmann-Radvanyi, H., Junien, C. and Gourdon, G. (2000) Transgenic mice carrying large human genomic sequences with expanded CTG repeat mimic closely the DM CTG repeat intergenerational and somatic instability. *Hum. Mol. Genet.*, **9**, 1185–1194.
72. Wong, L.J., Ashizawa, T., Monckton, D.G., Caskey, C.T. and Richards, C.S. (1995) Somatic heterogeneity of the CTG repeat in myotonic dystrophy is age and size dependent. *Am. J. Hum. Genet.*, **56**, 114–122.
73. Monckton, D.G., Wong, L.J., Ashizawa, T. and Caskey, C.T. (1995) Somatic mosaicism, germline expansions, germline reversions and intergenerational reductions in myotonic dystrophy males: small pool PCR analyses. *Hum. Mol. Genet.*, **4**, 1–8.
74. Laemmli, U.K. and Johnson, R.A. (1973) Maturation of the head of bacteriophage T4. II. Head-related, aberrant tau-particles. *J. Mol. Biol.*, **80**, 601–611.
75. Buee-Scherer, V., Condamines, O., Mourton-Gilles, C., Jakes, R., Goedert, M., Pau, B. and Delacourte, A. (1996) AD2, a phosphorylation-dependent monoclonal antibody directed against tau proteins found in Alzheimer's disease. *Brain Res. Mol. Brain Res.*, **39**, 79–88.
76. Mercken, M., Vandermeeren, M., Lubke, U., Six, J., Boons, J., Van de Voorde, A., Martin, J.J. and Gheuens, J. (1992) Monoclonal antibodies with selective specificity for Alzheimer Tau are directed

- against phosphatase-sensitive epitopes. *Acta Neuropathol. (Berl.)*, **84**, 265–272.
77. Goedert, M. and Jakes, R. (1990) Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. *EMBO J.*, **9**, 4225–4230.
78. Cheng, S., Barcelo, J.M. and Korneluk, R.G. (1996) Characterization of large CTG repeat expansions in myotonic dystrophy alleles using PCR. *Hum. Mutat.*, **7**, 304–310.
79. Andreadis, A., Brown, W.M. and Kosik, K.S. (1992) Structure and novel exons of the human tau gene. *Biochemistry*, **31**, 10626–10633.
80. Sadot, E., Marx, R., Barg, J., Behar, L. and Ginzburg, I. (1994) Complete sequence of 3'-untranslated region of Tau from rat central nervous system. Implications for mRNA heterogeneity. *J. Mol. Biol.*, **241**, 325–331.
81. Sawa, A., Oyama, F., Matsushita, M. and Ihara, Y. (1994) Molecular diversity at the carboxyl terminus of human and rat tau. *Brain Res. Mol. Brain Res.*, **27**, 111–117.
82. Kosik, K.S., Orecchio, L.D., Bakalis, S. and Neve, R.L. (1989) Developmentally regulated expression of specific tau sequences. *Neuron*, **2**, 1389–1397.

