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The alternative splicing of tau exon 10 and its regulatory proteins CLK2 and TRA2-BETA1 changes in sporadic Alzheimer's disease

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Abstract

Pathological inclusions containing fibrillar aggregates of hyperphosphorylated tau protein are a characteristic feature in tauopathies, which include Alzheimer's disease (AD). Tau is a microtubule-associated protein whose transcript undergoes alternative splicing in the brain. Exon 10 encodes one of four microtubule-binding repeats. Exon 10 inclusion gives rise to tau protein isoforms containing four microtubule-binding repeats (4R) whereas exclusion leads to isoforms containing only three repeats (3R). The ratio between 3R and 4R isoforms is tightly controlled via alternative splicing in the human adult nervous system and distortion of this balance results in neurodegeneration. Previous studies showed that several splicing regulators, among them hTRA2-beta1 and CLK2, regulate exon 10 alternative splicing. Like most splicing factors, htra2-beta and clk2 pre-mRNAs are regulated by alternative splicing. Here, we investigated whether human postmortem brain tissue of AD patients reveal differences in alternative splicing patterns of the tau, htra2-beta, presenilin 2 and clk2 genes when compared with age-matched controls. We found that the splicing patterns of all four genes are altered in affected brain areas of sporadic AD patients. In these affected areas, the amount of mRNAs of tau isoforms including exon 10, the htra2-beta1 isoform and an inactive form of clk2 are significantly increased. These findings suggest that a misregulation of alternative splicing seems to contribute to sporadic AD.

Keywords: alternative splicing, Alzheimer's disease, CDC-like kinase, serine–arginine proteins, tau.

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Tau protein is a cytoskeletal component that is predominantly expressed in axons of mature and growing neurons. The tau gene undergoes extensive alternative splicing, leading to the expression of multiple isoforms in the brain (Andreadis 2005). Exon 10 is one of the alternatively spliced exons and encodes a microtubule binding site. The alternative usage of exon 10 in the adult brain is specific for humans, as rodents

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Abbreviations used: AD, Alzheimer's disease; NFT, neurofibrillary tangle; SR, proteins, serine–arginine rich proteins.

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use this exon alternatively during development, but constitutively at the adult stage. Therefore, in humans, alternative splicing controls the formation of tau proteins containing either four or three microtubule binding domains (4R, 3R). Adult human neurons have a characteristic ratio of these isoforms and perturbation of this ratio leads to the formation of neurofibrillary tangles (NFTs) and cell death. NFTs are a hallmark of several inherited neurodegenerative diseases that are commonly named 'tauopathies'. They include progressive supranuclear palsy, corticobasal degeneration, Pick's disease and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTPD-17). In all of these diseases, the correct ratio between the 3R and 4R isoforms is disturbed by mutation of elements regulating exon 10 alternative splicing (Delacourte and Buee 2000; Spillantini *et al*. 2000).

In recent years, several groups have analyzed the regulation of exon 10 alternative splicing (Gao *et al*. 2000; Hartmann *et al*. 2001; D'Souza and Schellenberg 2002; Jiang *et al*. 2003; Wang *et al*. 2004). Like other alternative exons, exon 10 is regulated by a finely tuned balance of sequences and *trans*-acting factors. Exon 10 contains two enhancers, a GAR (guanidine/adenosine-rich) and an ACE (adenosine/cytosine-enhancer) motif and two weak silencers that are disturbed by mutations occurring in the human population. These elements bind to splicing regulators that either inhibit exon 10 inclusion (SRp30c, SRp55, SRp75, 9G8, U2AF, PTB and hnRNP G) or promote inclusion (hTRA2-beta1, CELF3, CELF4) (Andreadis 2005; Wang *et al*. 2005). From these proteins, TRA2-beta1 appears to be of central importance because it binds directly to the exon 10 GAR-type enhancer (Jiang *et al*. 2003) and can interact with most of the other exon 10 regulatory proteins (SRp30c, SRp55, SRp75, 9G8, U2AF and hnRNP G) (Nayler *et al*. 1998a; Wang *et al*. 2005). Finally, the usage of tau exon 10 is influenced by phosphorylation of splicing factors. The kinases CLK2 and GSK3β have been shown to alter the phosphorylation status of splicing factors, which in turn promote skipping of exon 10 (Hartmann *et al*. 2001; Hernandez *et al*. 2004; Stoilov *et al*. 2004).

In contrast to the inherited tauopathies, there are no obvious mutations in splicing regulatory elements of the tau gene in sporadic tauopathies. However, recent data suggest that the 4R/3R ratio is disturbed in these diseases as well (Umeda *et al*. 2004). We therefore analyzed postmortem brain tissue for the alternative splicing patterns of tau exon 10, htra2-beta1 exon 3 and the alternative exon of clk2. We discovered that in brain tissues affected by sporadic Alzheimer's disease (AD), the fraction of mRNA including exon 10 is increased. Furthermore, we also found that, in addition, the mRNA isoform ratios of proteins regulating exon 10 usage are altered, suggesting that defects in pre-mRNA processing contribute to sporadic AD.

Materials and methods

Cotransfection experiments

Cotransfection procedures were performed as described (Stoilov *et al*. 2004). The human clk2 minigene was constructed by amplifying a fragment containing the human clk2 gene using primers hClk2-BAM-forward (5′-CGCGGATCCAGTGCTCCACCTGCCTTG-3′) and hClk2-NOTI-reverse (5′-TATGCGGCCGCAAGCCCCATATAACCCCAAC-3′). The PCR products were cloned into a TA cloning vector (Invitrogen Life Technologies, Karlsruhe, Germany) and confirmed by sequencing. The insert was then cloned into the ExonTrap vector (Invitrogen Life Technologies). The amplification of the resulting mRNA was performed using primers in the two flanking insulin exons. The size of PCR products from hCLK2 minigene are: 514 and 602 bp.

Brain tissue

Brain samples were obtained from the Kathleen Price Bryan Brain Bank, Durham, USA (temporal and occipital cortex; AD group: Braak III to Braak V, $n = 15$; control group: no tau deposits, Braak I or II, $n = 9$) (Table 1), and from the Wuerzburg-Hirnbank, Wuerzburg, Germany (supplementary motocortex; eight control patients; seven AD patients, Braak III

Table 1 Patient characteristics of the temporal and occipital cortex including case identification number, age, diagnostic category, gender, postmortem interval and neuropathological diagnostic category

Sample number	Diagnostic category	Age	Gender	PM	Neuropathological diagnosis
1	AD	91	f	6:00	AD
2	AD	90	f	2:20	AD, Braak V
3	AD	81	f	3:00	AD
4	AD	64	f	3:36	AD, Braak V
5	AD	100	f	5:50	AD, Braak IV
6	AD	77	f	4:00	AD, Braak III
7	AD	83	m	1:15	AD, Braak IV
8	AD	62	m	1:30	AD, Braak V
9	AD	77	f	4:05	AD, Braak V
10	AD	74	m	1:10	AD, Braak IV
11	AD	79	f	3:45	AD, Braak V
12	AD	72	m	3:30	AD, Braak IV
13	AD	85	f	2:45	AD, Braak V
14	AD	83	m	2:00	AD, Braak V
15	AD	79	m	2:00	AD, Braak IV
17	CON	85	m	2:00	AR
18	CON	73	f	0:30	AR
21	CON	81	m	10:30	AR, Braak I–II
22	CON	82	m	3:15	AR
23	CON	72	f	3:00	AR
24	CON	92	f	2:35	AR
25	CON	80	f	1:10	AR
27	CON	78	f	2:22	AR
29	CON	91	m	7:40	AR, Braak I

f, female; m, male; PM, postmortem interval (h:min); AD, Alzheimer's disease; CON, control subjects; AR, age related.

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Table 2 Patient characteristics of the supplementary motocortex including case identification number, age, diagnostic category, gender, postmortem interval and neuropathological diagnostic category

No.	Diagnostic category	Age	Gender	PM	Neuropathological diagnosis
1	CON	68	f	13:00	AR
2	CON	76	m	18:05	AR
3	CON	64	m	13:55	AR
4	CON	84	m	16:30	AR
5	CON	63	m	10:30	AR
6	CON	88	m	15:00	AR
7	CON	71	f	11:00	AR
8	CON	74	f	< 12:00	AR
9	AD	84	f	< 20:00	AD, Braak III–VI
10	AD	83	f	19:30	AD
11	AD	89	m	02:00	AD, Braak IV–V
12	AD	90	f	08:00	AD
13	AD	81	m	13:40	AD, Braak VI
14	AD	76	f	09:00	AD, Braak V
15	AD	76	f	17:00	AD, Braak IV–V

No.: sample number, internal identification number; f, female; m, male; PM, postmortem interval (h:min); AD, Alzheimer's disease; CON, control subjects; AR, age related.

to VI) (Table 2). Samples from the Kathleen Price Bryan Brain Bank were matched in age ($t = 0421$; df = 22; $p = 0464$), sex ($\chi^2 = 0046$; df = 1; $p = 0831$) and postmortem interval ($t = -1081$; df = 21; $p = 0292$). The brains of the Wuerzburg Hirnbank showed differences concerning age ($t = -2342$; df = 13; $p = 0036$), but not concerning sex ($\chi^2 = 1727$; df = 1, $p = 0189$) and postmortem interval ($t = 0395$; df = 13; $p = 0699$). All tissues were obtained in accordance with the local ethics committee procedures.

Reverse transcription–polymerase chain reaction

Total cellular RNA was isolated using the RNeasy Mini-Kit (Qiagen). cDNA was generated using either oligo-(dT) primers for the tau, htra2-beta and polII reactions or gene specific primer for the clk2 analysis (located in exon 6, 5′-ATGATCTTCAGGGCAACTCG-3′) incubated in the presence of 100 U superscript II (Invitrogen), 20 U RNase inhibitor (Fermentas, St. Leon-Rot, Germany) and 25 pm dNTPs (Amersham, Braunschweig, Germany) for 45 min at 42°C using standard procedures. To determine the linearity of the RT–PCR reaction, various ratios of cDNA containing or lacking the alternative exons were amplified by PCR. A specific number of cycles in the linear range were used for each PCR amplification reaction.

PCR amplification of the tau cDNA was performed under the following conditions: initial denaturation at 94°C for 5 min; 32 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min; 72°C for 7 min final extension (tau primers: forward located in exon 9, 5′-CAAGATCGGCTCCACTGAGAA-3′; reverse located in exon 13, 5′-GGCGAGTCTACCATGTCGAT-3′). The PCR products were visualized with ethidium bromide on 2% agarose gels and the image captured using Eagle Eye (Stratagene, Heidelberg, Germany).

PCR conditions for clk2 and htra2-beta cDNAs were as follows: initial denaturation at 94°C for 5 min; 21 cycles of 94°C for 30 s, 60°C for 30 s (Δ-0.2°/cycle) and 72°C for 1 min, followed by 21 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min; 72°C for 10 min final extension (htra2-β primers: forward located in exon 1, 5′-AAGGAAGGTGCAAGAGGTTG-3′; reverse located in exon 4, 5′-CGGCAATGGGACCATATTTA-3′ and clk2 primers: forward located in exon 3, 5′-AATATCAGCGGGAGAACAGC-3′; reverse located in exon 5, 5′-GTCCCCTCTCCTAAGGTGCT-3′). All PCR products were separated on 2.5% agarose gels.

The PCR reactions for the polII gene was performed under the following conditions: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 1 min; 72°C for 10 min final extension (polII primers: forward, 5′-GGATGAACTGAAGCGAATGT-3′; reverse, 5′-TCAGTCGTCTCTGGGTATTTGA-3′). The PCR products were visualized with ethidium bromide on 2% agarose gels.

PCR conditions for presenilin cDNAs were as follows: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 40 s (presenilin primers: forward located in exon 4, ACCCTGACCGCTATGTCTGT; reverse located in exon 7, ATGATGAGGTAGGCCTGCTG). All PCR products were separated on 2.0% agarose gels.

Results from all PCR analysis are representative of at least two independent experiments.

Statistical evaluation

The density of each band was measured (TotalLab; Phoretix, Newcastle-upon-Tyne, UK) and the relative ratio between the isoforms was determined. Statistics were performed using the SPSS 11.0 Software (Statistical Package for Social Sciences, SPSS Inc, Chicago, IL, USA, 2001). *t*-tests were used to test for differences concerning the relative expression between the two groups in the respective brain regions. Analyses used two-tailed estimation of significance, an α-significance level of $p < 0.05$ was defined to be statistically significant.

Results

Htra2-beta1 and clk2 reciprocally regulate the splicing patterns of their genes and regulate tau exon 10 usage

We previously showed that tau exon 10 usage is increased by hTRA2-beta1 (Jiang *et al*. 2003) and decreased by CLK2 mediated phosphorylation (Hartmann *et al*. 2001). CLK2 binds and phosphorylates hTRA2-beta1 (Stoilov *et al*. 2004). Like almost all pre-mRNAs of splicing regulatory proteins, htra2-beta1 and clk2 pre-mRNAs undergo alternative splicing (Hanes *et al*. 1994; Nayler *et al*. 1998a). Skipping of exons 2 and 3 of htra2-beta pre-mRNA generates an inactive protein, hTRA2-beta3 (Stoilov *et al*. 2004), whereas skipping of exon 4 of clk2 pre-mRNA generates a frameshift resulting in the inactive variant, clk2tr (Duncan *et al*. 1997). clk2tr mRNA is subject to nonsense-mediated decay and therefore is not translated into any protein (Hillman *et al*. 2004). To test whether these proteins can influence each other's splice site selection at the pre-mRNA level, we

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employed a minigene construct consisting of the alternative exon flanked by the constitutive exons. As shown in Fig. 1(a), an increase of hTRA2-beta1 promotes skipping of clk2 exon 4, most likely through binding to an htra2-beta1 motif AAGAGCGA present in the 3′ part of the clk2 exon 4. Similar to the situation in clk1 (Duncan *et al*. 1997), clk2 promotes skipping of its exon 4 generating a frameshift resulting in an inactive form (Fig. 1a). We then performed similar assays with the expression constructs encoding the inactive proteins hTRA2-BETA3 and CLK2-KR. CLK2-KR is an inactive CLK2 mutant because it cannot bind ATP. We tested this mutant to determine whether clk2tr protein might influence pre-mRNA splicing if the mRNA escapes nonsense-mediated decay under special conditions. We found that these inactive forms have no effect on clk2 pre-mRNA splicing (Fig. 1b). Interestingly, our previous research has revealed that CLK2 influences the splicing pattern of tra2-beta by promoting exon skipping and the formation of the inactive protein isoform TRA2-beta3 (Stoilov *et al*. 2004).This suggests that the amount of active TRA2-beta1 and CLK2 is controlled through a feedback of alternative splicing decisions (Fig. 1c).

Together, these results indicate that CLK2 promotes exon skipping in the tra2-beta, clk2 and tau genes resulting in the formation of the inactive htra2-beta3 isoform, the inactive clk2tr variant and the tau isoform lacking exon 10. The formation of CLK2 is influenced by hTRA2-beta1, which promotes the formation of the inactive clk2tr isoform, the mRNA of which is subjected to nonsense-mediated decay and is not transcribed into protein (Hillman *et al*. 2004). Finally, TRA2-beta1 promotes the formation of the 4R tau isoform (Jiang *et al*. 2003; Wang *et al*. 2005). Given the intricate relationship between the three genes (Fig. 1c), we analyzed the distribution of the splicing variants in brain areas affected by AD.

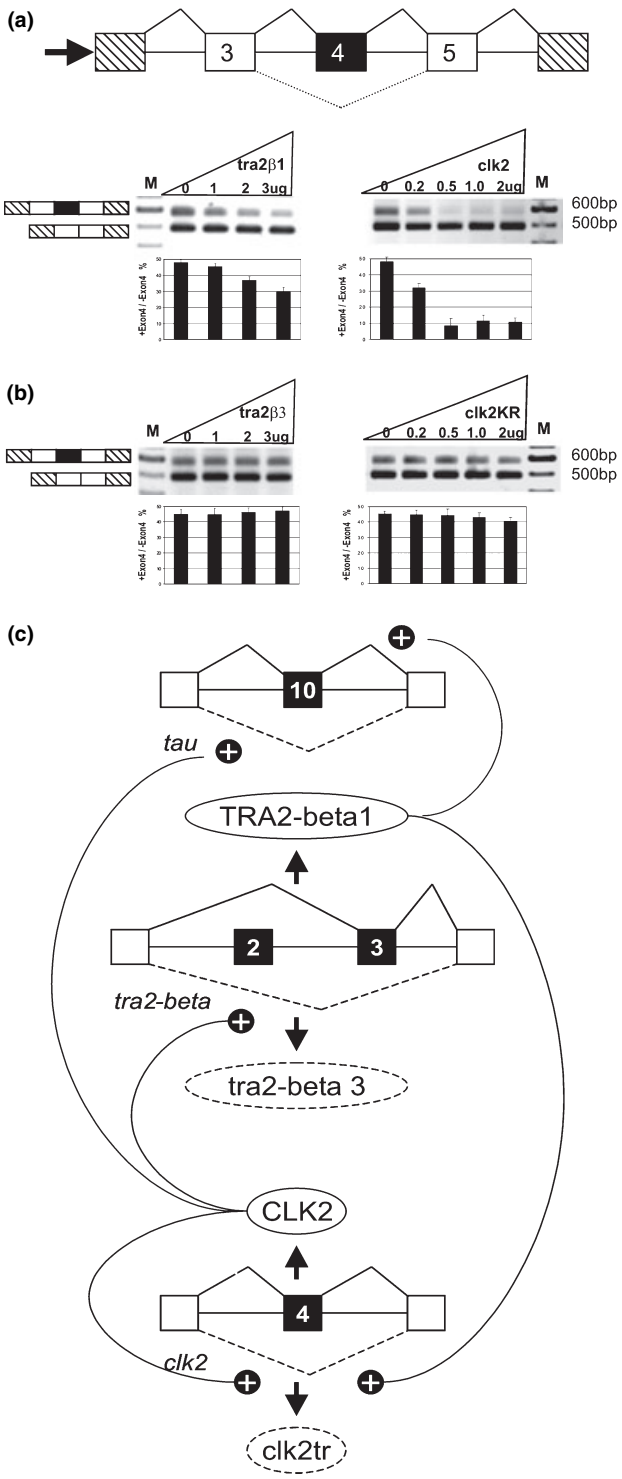

Fig. 1 Interdependency of the splice site selection of tau, tra2-beta and clk2. (a) hTRA2-beta1 and CLK2 promote skipping of clk2 exon 4. A minigene consisting of clk2 exon 3, 4 and 5 was cotransfected with increasing amounts of hTRA2-beta1 and CLK2 expression cDNAs. The mRNA formed was analyzed by RT–PCR. Exons are shown as boxes, introns as lines. The alternative exon is black, insulin exons from the exon trap vector are striped. The structure of the PCR products is schematically indicated on the left. Numbers indicate μg of cDNA construct transfected, M: marker. (b) hTRA2-beta3 and CLK2-KR have no influence on clk2 exon 4. An experiment similar to (a) was performed using constructs expressing htra2-beta3 and CLK2-KR. CLK2-KR is a catalytic inactive mutant of CLK2. In (a) and (b), error bars indicate standard deviations of at least three independent experiments. (c) Summary of the splice site regulation of tau exon 10, tra2-beta1 and clk2. Proteins active in splice site selection are indicated as an oval with a thick line. Inactive variants are indicated with a dashed line. clk2tr mRNA (tr: truncated) is most likely subject to nonsense-mediated decay and therefore is not translated into protein. The regions of the pre-mRNAs subjected to alternative splicing are schematically indicated, introns as horizontal lines, exons as boxes, alternative exons as black boxes. Splicing patterns are indicated by connecting lines.

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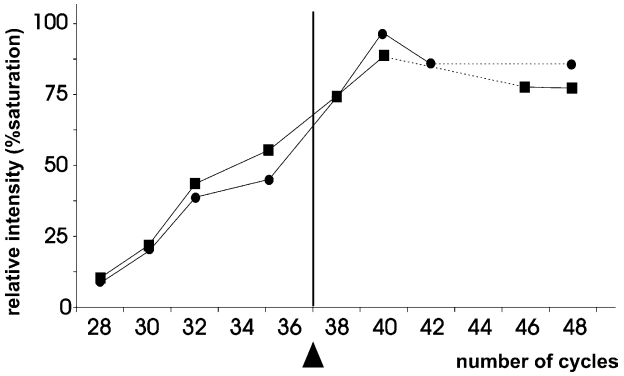

Fig. 2 Amplification profile of tau cDNAs with and without exon 10. The number of PCR cycles is plotted against the percentage of saturation. Circles indicate the product corresponding to tau cDNA lacking exon 10, squares indicate cDNA containing exon 10. cDNA (1 ng) corresponding to both isoforms was amplified. The PCR signal was linear between 28 and 40 cycles, and 37 cycles (black arrow) were employed for other experiments.

Increased usage of tau exon 10 in brain areas affected by Alzheimer's disease

First, we investigated whether the alternative splicing pattern of tau exon 10 is altered in human brain tissues from AD patients by semiquantitative RT–PCR. The semiquantitative nature of the assay was determined by RT–PCR using a defined quantity of tau cDNA. As shown in Fig. 2, the assay was linear between 28 and 40 cycles and we therefore subsequently amplified cDNA using 37 cycles. Using different cDNAs (± exon 10), we found that the ratio between exon inclusion and exon skipping reflected the amount of cDNA in the starting reaction (data not shown). To analyze the ratio of tau exon inclusion and exon skipping in tissue, mRNA was reverse transcribed using oligo-(dT) primers and cDNA was amplified using gene-specific primers located in tau exon 9 and 11. Using this assay, the ratio of exon 10 containing and lacking isoforms was determined in various brain regions. We analyzed the temporal cortex as this region is known to develop abundant neurofibrillary tangles in early stages of AD. As control regions from affected individuals, occipital cortex and supplementary motocortex were employed, which constantly demonstrate less NFT formation and functional impairment in AD (Braak and Braak 1995).

These measurements were compared with tau isoform ratios in aged-matched individuals not suffering from AD. When compared with control patients, the mRNA from AD affected brains showed an increase of exon 10 in temporal cortex ($t = -2964$; df $= 20$; $p = 0008$; Fig. 3). In contrast, there are no significant differences between those groups in control regions (occipital cortex: $t = 0214$; df $= 20$; $p = 0832$; supplementary motocortex: $t = -1016$; df $= 8$; $p = 0339$). We conclude that there is a statistically significant increase of exon 10 containing tau mRNA in brain areas affected by AD.

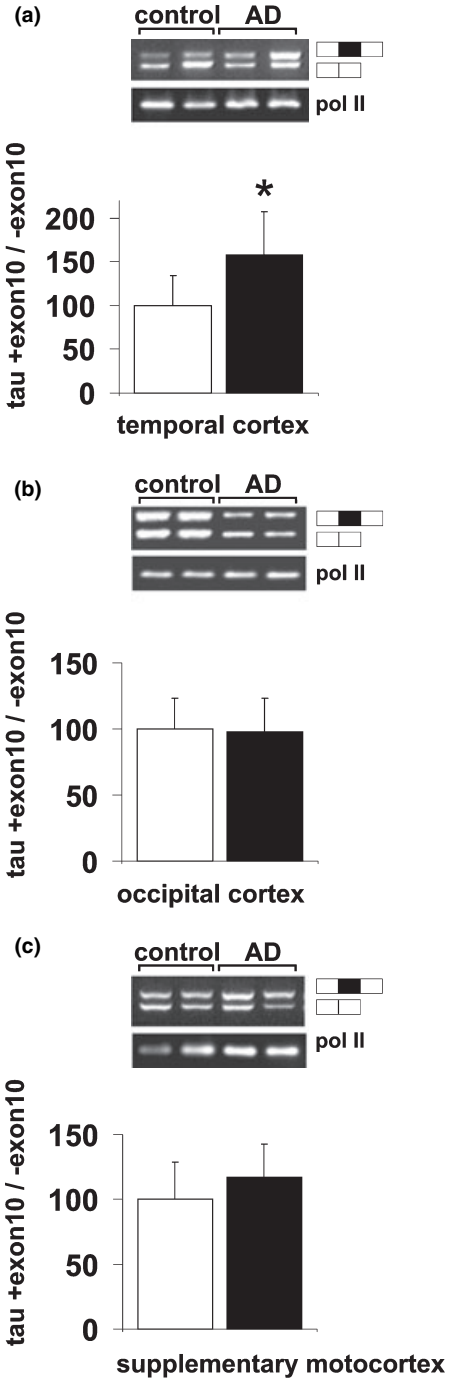

Fig. 3 Exon 10 usage in brain regions affected with Alzheimer's disease (AD). Results from RT–PCR analysis performed in temporal (a), occipital (b) and supplementary motocortex (c) from two representative AD and control patients are shown on top of each panel. The structure of the gene products is schematically indicated. pol II: indicates amplification of polII as a loading control. From each experiment, the ratio between exon 10 inclusion and exon 10 skipping was calculated. The mean of this ratio for control tissue was arbitrarily set to 100%. The mean and standard deviation of each set of experiments are included.

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Expression of clk2 exon 4 is decreased in brain areas of Alzheimer's disease patients

Alternative splice site selection is frequently regulated by phosphorylation (Stamm 2002) and we have previously demonstrated that the SR-protein kinase CLK2 influences tau exon 10 splicing, where it strongly promotes exon 10 skipping (Hartmann *et al*. 2001). Similar to other clk kinases, CLK2 is regulated by alternative splicing. Skipping of exon 4 causes a frameshift resulting in a mRNA that becomes subjected to nonsense-mediated decay and therefore is not translated into any protein (Hillman *et al*. 2004).

In order to determine whether clk2 is altered in brain regions affected by AD we examined clk2 exon 4 inclusion by RT–PCR (Fig. 4). We found a strong decrease of exon 4 in brain areas affected by AD (temporal cortex: $t = 3725$; df $= 19$; $p = 0001$). Unexpectedly, in the control regions (occipital cortex: $t = 12\ 549$; df $= 17$; $p < 0001$ and supplementary motocortex: $t = 2545$; df $= 9$; $p = 0031$) there was also a marked decrease of exon 4 when the controls were compared with age-matched controls. We conclude that there is a decrease of the mRNA encoding active CLK2 protein in the brain of individuals affected by AD, which extends beyond the areas specifically altered in AD.

The ratio between htra2-beta1 and htra2-beta3 is altered in brain areas affected by Alzheimer's disease

The inclusion of tau exon 10 is stimulated by hTRA2-beta1, a member of the SR-related protein family of splicing regulators (Jiang *et al*. 2003). Similar to most other alternative splicing regulators, the htra2-beta gene undergoes alternative splicing and forms two proteins: TRA2-beta1, which regulates exons containing the sequence GHVV-GANR, and TRA2-beta3, which is inactive. On the protein level, TRA2-beta1 and TRA2-beta3 differ in the presence of the first RS domain. On the RNA level both isoforms differ in the presence of exon 3. CLK2 promotes the formation of htra2-beta3 (Stoilov *et al*. 2004). Since both tau and clk2 splicing are altered in brains of AD sufferers, we employed an RT–PCR assay to determine the ratio between tra2-beta1 and tra2-beta3 isoforms. The expression pattern of the isoforms htra2-β1 and htra2-β3 was altered in the temporal cortex of AD patients when compared to controls ($t = -2060$; df $= 15$; $p = 0057$; Fig. 5). Both control regions, comprising the occipital cortex ($t = 1226$; df $= 11$; $p = 0246$) and supplementary motocortex ($t = 0555$; df $= 9$; $p = 0592$), revealed no statistically significant differences between the AD and control group. We therefore conclude that the ratio between hTRA2-beta1 and hTRA2-beta3 is specifically altered in brain areas affected by AD.

Increased skipping of presenilin 2 exon 5 in Alzheimer's disease

Recently, an alternatively spliced form of the presenilin 2 gene lacking exon 5 was found in human brains with

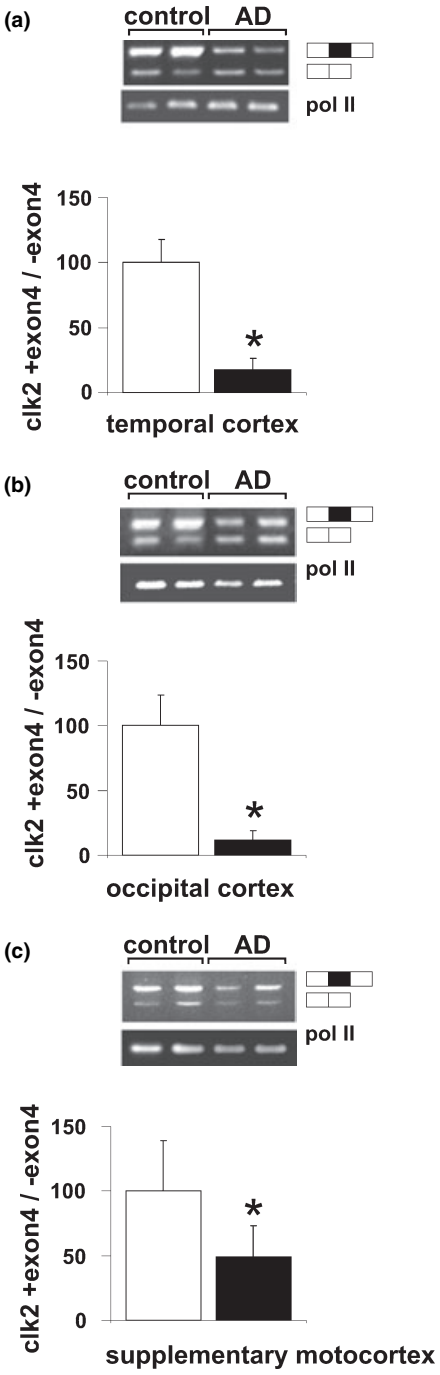

Fig. 4 Ratio of clk2 isoforms in brain regions from Alzheimer's disease (AD) patients and controls. Results from RT–PCR analysis performed in temporal (a), occipital (b) and supplementary motocortex (c) from two representative AD and control patients are shown on top of each panel. The structure of the gene products is schematically indicated. pol II: indicates amplification of polII as a loading control. From each experiment, the ratio between exon 4 inclusion and exon 4 skipping was calculated. The mean of this ratio for control tissue was arbitrarily set to 100%. The mean and standard deviation of each set of experiments are included.

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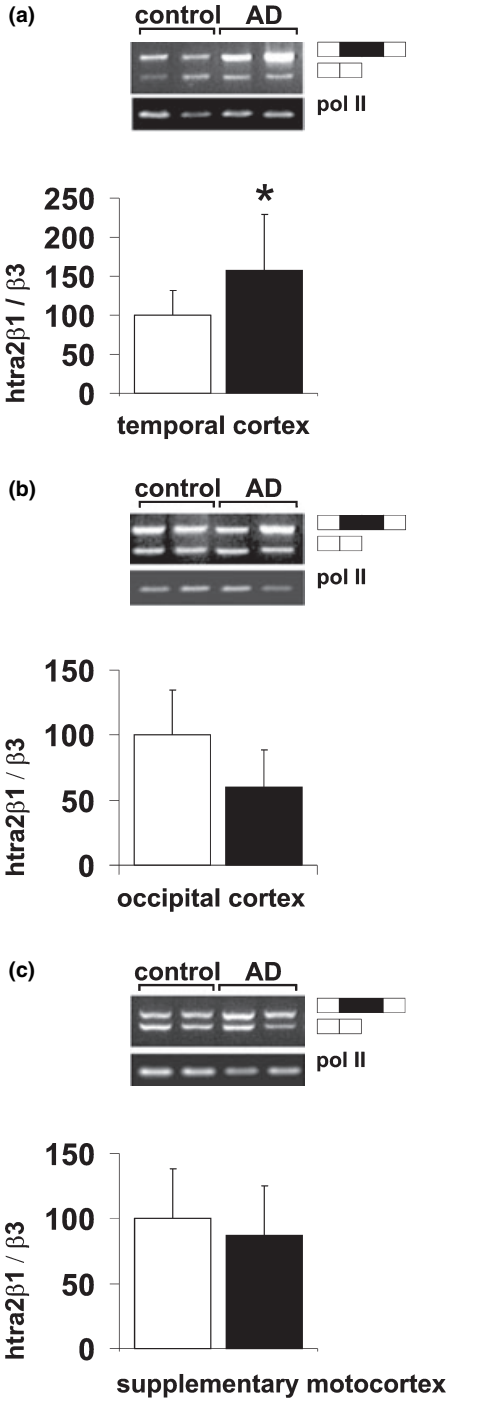

Fig. 5 Ratio of tra2-beta isoforms in brain regions from Alzheimer's disease (AD) patients and controls. Results from RT–PCR analysis performed in temporal (a), occipital (b) and supplementary motocortex (c) from two representative AD and control patients are shown on top of each panel. The structure of the gene products is schematically indicated. pol II: indicates amplification of polII as a loading control. From each experiment, the ratio between exon 3 inclusion and exon 3 skipping was calculated. The mean of this ratio for control tissue was arbitrarily set to 100%. The mean and standard deviation of each set of experiments are included.

sporadic Alzheimer's disease. It was shown that skipping of this exon was induced by hypoxia (Higashide *et al*. 2004). We thus determined presenilin 2 exon 5 usage in our samples. Presenilin 2 exon 5 showed a stronger variation than other exons, since three classes of samples could be discriminated: one class of samples contained both exon 5 inclusion and skipping variants, one class contained only exon 5 inclusion and one class only exon 5 skipping (Figs 6a–c). As can be seen in Fig. 6(d), samples completely

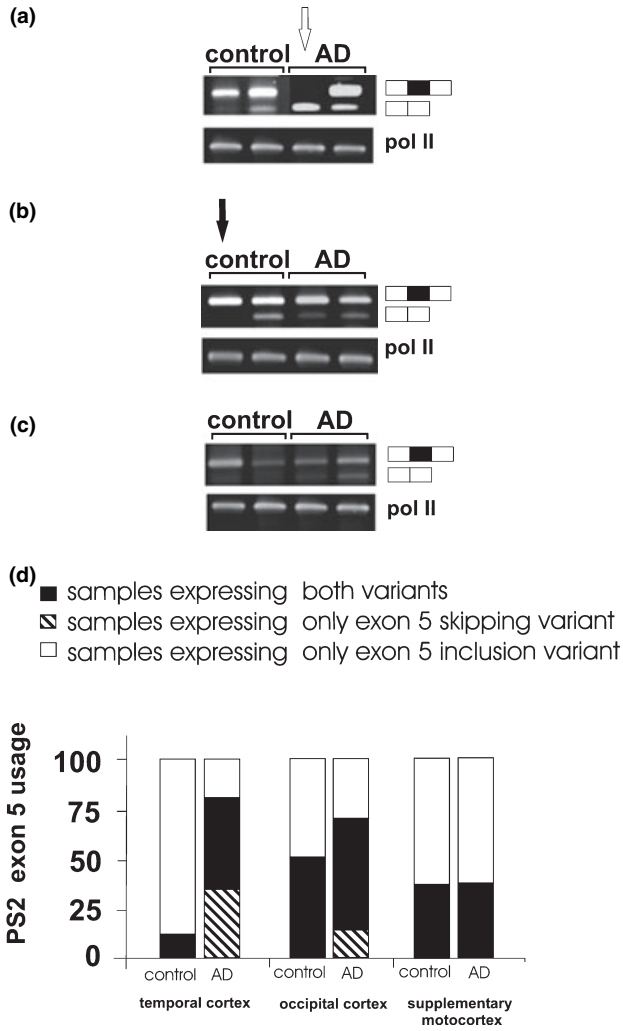

Fig. 6 Presenilin 2 isoforms in brain regions from Alzheimer's disease (AD) patients and controls. Results from RT–PCR analysis performed in temporal (a), occipital (b) and supplementary motocortex (c) from two representative AD and control patients are shown. The structure of the gene products is schematically indicated. pol II: indicates amplification of polII as a loading control. Since a substantial number of samples completely lacked presenilin exon 5 inclusion (open arrow) or skipping (black arrow), no ratios of isoforms could be determined. We therefore classified samples as expressing both variants or only the variant either including or excluding exon 5. This evaluation is shown in (d).

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GCCT	**TT AGAAA** AACACA	Ps2	intron 5	79-96
CAAATTA	**GAGGAAAA**TAGACC	Ps2	intron 5	95-115
GCTA	**C AAGA G** CGAT	Clk2	exon 4	75-88
GCTCC	**AAGGAAG** ATTCCAG	tra2-beta1	exon 3	77-95
GCTCA	**AAGGATA** ATATCA	tau	exon 10	53-70
AATTAA	**TAAGAAG** CTGGATC	tau	exon 10	8-28
TCCAGAA	**GT GGAAG** TGCT	tra2-beta1	exon 3	16-33
	gHVVGANR	consensus		

Fig. 7 Tra2-beta1 motif in exons misregulated in Alzheimer's disease (AD). TRA2-beta1 binds *in vivo* to the sequence GHVVGANR (H = A,T,C; V = G,A,C; R = A,G; N = A, G, C, T). PS: presenilin. Inspection of alternative exons misregulated in AD using the 'RNA workbench' at http://www.ebi.ac.uk/asd/shows that this motif occurs in each exon or adjacent intron. Numbers indicate the position of the sequences that can be found in the ASD database (Thanaraj *et al.* 2004).

lacking exon 5 usage are found only in individuals suffering from Alzheimer's disease, being more frequent in temporal cortex than in occipital cortex. The relative frequency of the presenilin isoform skipping exon 5 was significantly higher in AD patients compared to controls in the temporal cortex ($\chi^2 = 7.44$; df = 2; $p = 0.024$; Fig. 6). Both control regions, comprising the occipital cortex ($\chi^2 = 2.37$; df = 2; $p = 0.306$) and supplementary motocortex ($\chi^2 = 0.00$; df = 2; $p = 1.0$), revealed no statistically significant differences between the AD and control group. Thus, the usage of presenilin exon 5 seems to be specifically altered in brain areas affected by AD.

Discussion

Our data demonstrate that tau mRNAs containing exon 10, which codes for the 4R protein isoform, are relatively increased in the temporal cortex of AD patients. It has been shown that a distortion of the 3R and 4R tau isoform ratio leads to enhanced NFT formation and neurodegeneration (Andreadis 2005). For example, the disruption of the proper balance of the 3R and 4R repeat isoforms through mutations in splicing regulatory regions causes frontotemporal dementia with parkinsonism linked to chromosome 17 (FTPD-17) (Spillantini and Goedert 2000). We found an increase of the 4R tau isoform in the brain regions affected by sporadic AD. This is in agreement with previous data showing that the 4R tau isoform is up-regulated in brain areas from Alzheimer's disease patients with a heavy burden of neurofibrillary tangles (Yasojima *et al*. 1999). The increase of the 4R tau isoforms is quantitative and therefore semiquantitative PCR is necessary for its detection. Furthermore, the tau exon 10 ratio varies between individuals and brain areas. This could explain why there is a variability between studies (Umeda *et al*. 2004) and why some studies did not describe differences in exon 10 usage in Alzheimer's disease (Chambers *et al*. 1999; Boutajangout *et al*. 2004). Since previous studies have reported an absence of mutations in tau pre-mRNA associating with AD (Poorkaj *et al*. 2001), we analyzed the splicing patterns of two pre-mRNAs encoding proteins that regulate tau exon 10 splicing, tra2-beta and clk2 (Hartmann *et al*. 2001; Jiang *et al*. 2003; Kondo *et al*. 2004; Wang *et al*. 2004, 2005). Results from the investigation revealed a statistically significant change in the ratio of clk2 isoforms. In brain tissue of AD patients, the mRNA for the clk2tr isoform, encoding a non-functional mRNA, is increased in comparison to the full-length isoform encoding the active kinase. These findings point to a defect in splicing regulation associated with AD. A decrease of CLK2 activity favors the inclusion of exon 10 and the formation of 4R tau isoforms and could explain the observed changes in tau exon 10 splicing. However, the activity of CLK2 is not only regulated by its abundance, but also by its phosphorylation state (Nayler *et al*. 1998b). At this point, we can only speculate that the CLK2 activity is reduced in brains affected by AD. We therefore analyzed tra2-beta1 as an example of another pre-mRNA that is regulated by CLK2. CLK2 activity promotes the formation of the tra2-beta3 isoform. Our finding that the ratio between tra2-beta3 and tra2-beta1 is increased in temporal cortex affected by AD strongly suggests a decrease of CLK2 activity in this area, since CLK2 promotes tra2-beta3 formation (Fig. 1c). Considering that it has been well established that tra2-beta1 promotes tau exon 10 inclusion (Jiang *et al*. 2003; Kondo *et al*. 2004), it is natural to assume that a relative increase of tra2-beta1 would favor exon 10 inclusion. It is therefore possible that an abnormally low activity of CLK2 could contribute to tau exon 10 missplicing, since a low concentration of CLK2 would fail to correctly promote both tau exon 10 and tra2-beta exon 3 inclusion. Surprisingly, we found a down-regulation of mRNA encoding active CLK2 kinase also in supplementary motocortex, a brain region not affected by Alzheimer's disease. For sporadic Alzheimer's disease, aberrant splicing of presenilin 2 exon 5 has been demonstrated previously (Sato *et al*. 1999). We therefore determined presenilin 2 exon 5 usage in our samples and found pronounced changes similar to tau exon 10 and tra2-beta1 exon 3 in temporal cortex, but no changes in supplementary motocortex. Interestingly, the intron upstream of exon 5 harbors a TRA2-beta1 binding site. Thus, so far, all alternative exons that are changed in AD contain TRA2-beta1-binding sites (Fig. 7). It is therefore possible that changes in TRA2-beta isoforms cause abnormal splicing of other pre-mRNAs containing TRA2-beta1-binding sites. Since alternat-

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ive pre-mRNA splicing is regulated by combinatorial control involving numerous, often antagonistic factors, it is not possible to predict what other mRNAs might be affected. This combinatorial control could also explain why alterations of CLK2 isoforms in supplementary motocortex have no influence on the splicing patterns of tau exon 10 and tra2-beta1, since this brain area might express other factors that compensate the loss of CLK2.

The sequencing of several genomes has underlined the importance of alternative splicing. Indeed, cDNA expression array data indicate that up to 75% of all human genes are subject to alternative splicing (Johnson *et al*. 2003), which serves as a major mechanism for creating functionally different proteins from a surprisingly small number of genes in humans (Stamm *et al*. 2005). It is therefore interesting to note the increasing number of human diseases suspected to be caused by abnormal regulation of splicing (Stoilov *et al*. 2002; Faustino and Cooper 2003). Whereas the majority of the diseases associated with missplicing are caused by mutations in regulatory regions, a number of missplicing events occur without any obvious mutations in the affected genes. The most likely reasons for these changes are alterations in regulatory factors, suggesting that splicing could be a genetic modifier of disease (Nissim-Rafinia and Kerem 2002). Pre-mRNA splicing modulations are frequent during senescence, which could be caused by age-dependent changes in the splicing machinery (Meshorer and Soreq 2002). Our findings suggest that missplicing events originating from the tra2-beta1 system contribute to the pathogenesis of sporadic AD.

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