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ORIGINAL INVESTIGATION

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Further evidence for a synergistic association between *APOE* ε4 and *BCHE*-K in confirmed Alzheimer's disease

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Abstract Recent reports on a potential association between the K-variant of the gene for butyrylcholinesterase (*BCHE*-K) and Alzheimer's disease (AD) are discordant. An initial finding of association through a synergistic enhancement of risk of *APOE* ε4 with late-onset AD has not been confirmed by others. We have conducted a case-control study of histopathologically confirmed AD (n=135) and non-AD (n=70) cases (age of death ≥60 years), in which we have genotyped for *APOE* ε4, *BCHE*-K, and *BCHE*-A1914G, a silent polymorphism 299 bp downstream of the *BCHE*-K mutation. The allelic frequency of *BCHE*-K was 0.13 in the controls and 0.23 in the AD cases, giving a carrier odds ratio (OR_c) of 2.1 (95% C.I. 1.1–4.1) for *BCHE*-K in confirmed AD. The allelic frequency for the *BCHE*-1914G variant was 0.19 and 0.33 in controls and AD cases, respectively (OR_c=2.4; 95% C.I. 1.3–4.5). In an older sub-sample of 27/70 controls and 89/135 AD patients with ages of death ≥75 years, the OR_c was increased to 4.5 (95% C.I. 1.4–15) for *BCHE*-K and 2.7 (95% C.I. 1.0–7.2) for *BCHE*–1914G carriers. The *BCHE*-K association with AD became even stronger in carriers of at least one *APOE* ε4 allele. Only three out of 19 controls compared with 39/81 AD cases carried *BCHE*-K in addition to *APOE* ε4, giving an odds ratio of confirmed AD of 5.0 (95% C.I. 1.3–19) for *BCHE*-K carriers within *APOE* ε4 carriers. Five out of 19 controls and 52/81 AD cases carried *BCHE*-1914G, giving the same odds ratio of confirmed AD of 5.0 (95% C.I. 1.6–16) for *BCHE*-1914G carriers within *APOE* ε4 carriers. In addition, our results suggest strong linkage disequilibrium between *BCHE*-K and *BCHE*-1914G but no major association of the sole *BCHE*-1914G chromosome with AD. We conclude that *BCHE* through its K-variant, rather than a nearby marker, is a susceptibility factor for AD and enhances the AD risk defined by *APOE* ε4 alone in an age-dependent manner.

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Introduction

In humans, butyrylcholinesterase (BChE, EC3.1.1.8) occurs in plasma and in most tissues including certain regions of the brain (for a review, see Darvesh et al. 1998). Despite today's extensive knowledge about allelic *BCHE* variants and their pharmacogenetic impact, its physiological function still remains uncertain (Kalow and Grant 1995). The 574-amino-acid glycoprotein is coded by a single-copy gene on chromosome 3q26.1–26.2, which is part of a linkage group containing the genes for transferrin, BChE, ceruloplasmin and alpha-2HS glycoprotein (Arpagaus et al. 1990; Allderdice et al. 1991; Gaughan et al. 1991). Several dysfunctional *BCHE* mutations have been characterised, both at the phenotypic and genetic level (Primo-Parmo et al. 1996). An increased sensitivity to the muscle-relaxing agent succinylcholine and a tendency for prolonged apnoea seems to be the sole apparent clinical sign of BChE deficiency. Preliminary evidence indicates, however, that BChE is involved in neuritic tissue degeneration and clinical dementia but its role in the normal nervous system remains unclear. Since 1965, several studies have reported the colocalisation of the enzyme with senile plaques and neurofibrillary tangles, the hallmarks of the pathology of Alzheimer's disease (AD; Friede 1965; Carson et al. 1991; Gomez-Ramos et al. 1994; Moran et al. 1994; Gomez-Ramos and Moran 1997) and the severe loss of cholinergic neurons in AD brains has been found to be accompanied by higher than normal levels of BChE (Perry et al. 1978). Recently, it has been suggested that higher BChE levels may play a role in the maturation of senile plaques (Gomez-Ramos and Moran 1997) and in Abeta transformation from a benign to an eventually malignant form (Guillozet et al. 1997). Whether allelic *BCHE* variants exacerbate some of the pathophysiological features of AD remains unknown.

Table 1 Combined *APOE* and *BCHE* genotype distribution (*AD* Alzheimer's disease)

	BCHE-K genotype for ≥60 years (≥75 years)[a]							
	Wild-type		Heterozygous		Homozygous		Total	
APOE	AD	Control	AD	Control	AD	Control	AD	Control
23	3 (2)	3 (1)	0 (0)	3 (0)	1 (0)	0 (0)	4 (2)	6 (1)
24	2 (1)	2 (1)	5 (3)	0 (0)	0 (0)	0 (0)	7 (4)	2 (1)
33	35 (22)	34 (14)	12 (8)	10 (2)	3 (3)	1 (1)	50 (33)	45 (17)
34	32 (20)	14 (7)	25 (19)	3 (1)	4 (3)	0 (0)	61 (42)	17 (8)
44	8 (5)	0 (0)	5 (3)	0 (0)	0 (0)	0 (0)	13 (8)	0 (0)
Total	80 (50)	53 (23)	47 (33)	16 (3)	8 (6)	1 (1)	135 (89)	70 (27)

[a] Ages of death

The K-variant has been identified as by far the most frequent functional mutation of *BCHE* (Bartels et al. 1992). The underlying guanine to adenine transition at nucleotide 1615 causes an amino acid exchange from alanine to threonine at codon 539, which is associated with a 30% activity reduction (Rubinstein et al. 1978). The K-polymorphism is found in various ethnic populations with homozygote frequencies between 1% to 4% (Evans and Wardell 1984; Bartels et al. 1992; Gaffney and Campbell 1994; Whittaker and Britten 1995; Maekawa et al. 1997). Recently, this polymorphism has been reported to occur with higher frequency in late-onset AD patients, especially in carriers of the apolipoprotein E (*APOE*) ε4 allele (Lehmann et al. 1997). Whereas this finding has partly been reproduced (Sandbrink et al. 1998), others have found no association at all (Brindle et al. 1998; Crawford et al. 1998; Roses et al. 1998; Russ et al. 1998; Singleton et al. 1998a) or even a lower frequency within AD cases compared with controls (Hiltunen et al. 1998). One recent study, however, has reported an association of homozygosity for *BCHE*-K with dementia with Lewy bodies (Singelton et al. 1998b). We have re-examined the *BCHE*-K and *APOE* ε4 frequencies in DNA samples of brain tissue of histopathologically confirmed AD cases of the late-onset type. Age-matched brain samples with no neurohistopathologically abnormal signs other than those related to normal ageing served as controls. In addition, a second bi-allelic polymorphism within the BChE gene, *BCHE*-A1914G, which is known to be partially in linkage disequilibrium with the K-variant, has been examined (Bartels et al. 1992).

Materials and methods

Brain material from 135 autopsied AD cases and 70 autopsied control subjects were obtained from the Douglas Hospital Brain Bank in Montreal, Canada. All subjects were Caucasians who originated from French- and English-speaking parts of eastern Canada, participated in this program on a voluntary basis and were enrolled without discrimination. Although no specific sampling effort was undertaken for collecting a population-based sample, the specimens utilised from the Douglas Hospital Brain Bank probably represent the communities of Quebec. The AD-affected subjects were clinically diagnosed and neuropathologically confirmed by using described criteria (Etienne et al. 1986; Aubert et al. 1992). We selected AD patients having ages of death of 60 years and older to bias our AD sample for the late-onset form of the disease. The mean age of death in the AD sample was 78±7.8 years and 74±9.2 years within the controls. The female-to-male ratio was 0.56 and 1.14 for controls and AD cases, respectively. Control cases were identified as having only the typical age-related neuropathological abnormalities post mortem. Brain tissues were stored at –70°C before DNA extraction.

Genomic DNA was isolated from 500 mg brain tissue by using a standard phenol-chloroform extraction method with prior proteinase-K digestion. Genotyping of each of the *BCHE*-K and the *BCHE*-A1914G polymorphisms was performed by using three primers in an allele-specific single-step polymerase chain reaction (PCR) amplification technique (MS-PCR) in a total volume of 50 µl. General PCR conditions were as described elsewhere (Rust et al. 1993). Following DNA electrophoresis in 4% NuSieve agarose gels, fragment detection and genotype determination were performed by viewing ethidium-bromide/UV trans-illumination fluorescence.

For *BCHE*-K-allele detection, MS-PCR amplification was carried out with 500 ng genomic DNA together with 5 pmol of each of the following three primers in a standard 45-cycle PCR with annealing at 60°C: 5'-ctgtactgtgtagttagagaaaatggc-3' (common); 5'-atggaatcctgctttccactcccattccgt-3' (K-allele-specific); 5'-atcatgtaattgttccagcgtaggaatcctgctttccactcccattctcc-3' (wild-type-specific). A single band of 169 bp indicated the wild-type, whereas a single band of 149 bp identified the K-variant at *BCHE* codon 539. Simultaneous visibility of both bands indicated heterozygosity. For the *BCHE*-A1914G polymorphism detection, MS-PCR amplification was performed by using the following three primers at concentrations of 7.5 pmol "A"-specific primer, 2.5 pmol "G"-specific primer and 5 pmol common primer per 50 µl PCR under the above-mentioned conditions and 45°C annealing temperature: 5'-cctttagatcaaggcaaaaatatcaggagc-3' (common); 5'-aactttatattgtgaaatttaattaaagat-3' ("A"-specific); 5'-atgcacataattaactgtagccctttatattgtgaaatttaattaaaccc-3' ("G"-specific). A single band of 186 bp indicated the presence of the 1914A-allele, a single band of 206 bp identified the 1914G-allele, whereas heterozygosity was indicated by the simultaneous visibility of both bands. Set-up and initial validation of both MS-PCR tests were carried out on samples with known genotypes by using DNA-sequencing for the analysis. *APOE* ε2,4,3 genotyping was performed as described previously (McLeod et al. 1998).

A Yates' corrected chi-square test and the Fisher's exact test were used to analyse for any significance, if differences in allelic frequencies were observed between cases and controls. Significance was adjudged to be reached at the 95% confidence level. Confidence intervals (C. I.) for the odds ratios (OR) were calculated and synergy factor analysis was performed by described methods (Fleiss 1979; Lehmann et al. 1998).

Table 2 Odds ratios of confirmed AD for *APOE* ε4 (*n.s.* not significant)

Subjects	No. of cases	No. of controls	Odds ratio (alleles)	95% C. I.	Odds ratio (carriers)	95% C. I.
All ≥60 years[a]	135	70	3.4	2.0–5.8	4.0	2.1–7.6
All ≥75 years[a]	89	27	2.7	1.2–6.1	3.1	1.2–8.1
BCHE-K carriers ≥60 years[a]	55	17	6.9	2.1–23	11.4	3.0–43
BCHE-K carriers ≥75 years[a]	39	4	4.6	n.s.	7.6	n.s.

[a] Ages of death

Table 3 Odds ratios of confirmed AD for *BCHE*-K (*n.s.* not significant)

Subjects	No. of cases	No. of controls	Odds ratio (alleles)	95% C. I.	Odds ratio (carriers)	95% C. I.
All ≥60 years[a]	135	70	2.1	1.1–3.7	2.1	1.1–4.1
All ≥75 years[a]	89	27	3.3	1.2–9.1	4.5	1.4–15
APOE ε4 carriers ≥60 years[a]	81	19	4.2	1.2–15	5.0	1.3–19
APOE ε4 carriers ≥75 years[a]	51	9	6.8	n.s.	8.6	n.s.

[a] Ages of death

Results

A total of 205 Caucasian subjects was genotyped for *APOE* ε2/3/4, *BCHE*-K and *BCHE*-A1914G, an adenine to guanine transition at mRNA nucleotide position 1914 underlying a polymorphism 299 bp downstream of the K-variant position within the 3’-untranslated region of *BCHE* (Bartels et al. 1992). The combined *APOE* and *BCHE*-K genotype distribution for 135 AD cases and 70 controls and an older sub-group with ages of death ≥75 years is shown in Table 1. The genotype distributions of each of the analysed polymorphisms were found to be in good agreement with the expected occurrence assuming Hardy-Weinberg equilibrium. As expected, we found a significantly increased allelic frequency for *APOE* ε4 (0.14 in 70 controls and 0.35 in 135 AD cases; P<0.0001; Fisher’s exact test). The overall OR for AD for *APOE* ε4 carriers was 4.0 (95% C.I. 2.1–7.6) for cases and controls with ages of death ≥60 years and 3.1 (95% C.I. 1.2–8.1) for an older sub-group (Table 2). The *BCHE*-K allelic frequency was 0.13 in 70 controls and 0.23 in the 135 AD cases (P=0.013; Fisher’s exact test) giving a carrier OR of AD of 2.1 (95% C.I. 1.1–4.1) for subjects who died aged 60 years or older. An increased *BCHE*-K allelic frequency was found within older AD subjects (0.25 in 89 AD cases and 0.09 in 27 control cases) giving a carrier OR of AD of 4.5 (95% C.I. 1.4–15) for subjects who died aged 75 years or older (Table 3). These findings suggest a direct association of *BCHE*-K with AD, the association being more pronounced within older patients.

If we took into account each carrier’s *APOE* ε4 genotype, we observed a major exacerbation of the AD association with either *APOE* ε4 or *BCHE*-K compared with carriers of both genetic markers. In subjects who died aged 60 years or older, 4% of control subjects were carriers of both markers, whereas 29% of the AD cases carried *APOE* ε4 plus *BCHE*-K (P<0.0001; Fisher’s exact test). Within older subjects (age of death ≥75 years), 4% of the controls and 31% of the AD cases carried both *APOE* ε4 and *BCHE*-K (P=0.0022; Fisher’s exact test). In all *APOE* ε4 carriers (ages of death ≥60 years), we found that 16% of the controls and 48% of the AD cases were carriers of *BCHE*-K (P=0.011; Fisher’s exact test). These figures were 11% and 52% in controls and in AD cases of ≥75 years, respectively,(Table 4), with the difference still being significant at P=0.031 (Fisher’s exact test). These results gave carrier ORs of 5.0 or 8.6 of confirmed AD for *BCHE*-K in *APOE* ε4 carriers who died aged 60 or older or aged 75 or older, respectively (Table 3). Table 2 shows the ORs of confirmed AD for *APOE* ε4 in *BCHE*-K carriers.

Table 4 Carrier proportions of controls and AD cases for *APOE* ε4 and *BCHE*-K

Subjects	Controls	AD Cases	P-value[b]
All ≥60 years[a]	3/70 (4%)	39/135 (29%)	<0.0001
All ≥75 years[a]	1/27 (4%)	28/89 (31%)	0.0022
APOE ε4 carriers ≥60 years[a]	3/19 (16%)	39/81 (48%)	0.011
APOE ε4 carriers ≥75 years[a]	1/9 (11%)	28/54 (52%)	0.031

[a] Ages of death
[b] Fisher’s exact test

To quantify possible interactions between *APOE* ε4 and *BCHE*-K, we analysed the data with respect to various carrier-status combinations taking subjects who had neither *APOE* ε4 nor *BCHE*-K as a reference (Table 5). For controls and AD cases who died aged 60 or older, the carrier ORs of confirmed AD was 1.1 (not significant) for *BCHE*-K and 2.6 (95% C.I. 1.2–5.8) for *APOE* ε4. Assuming an

Table 5 Odds ratios of confirmed AD for *APOE* ε4 and *BCHE*-K (*n.s.* not significant). The values for *BCHE*-1914G carriers are given in parentheses

APOE ε4 carriers	*BCHE*-K carriers	No. of controls	No. of cases	Odds ratio	95% C. I.
All ≥60 years[a]					
–	–	37 (30)	38 (32)	Reference	–
–	+	14 (19)	16 (23)	1.1 (1.1)	n.s. (n.s.)
+	–	16 (14)	42 (29)	2.6 (2.0)	1.2–5.8 (n.s.)
+	+	3 (5)	39 (52)	12.7 (9.8)	4.1–39 (3.6–26)
All ≥75 years[a]					
–	–	15 (10)	24 (18)	Reference	–
–	+	3 (7)	11 (17)	2.3 (1.4)	n.s. (n.s.)
+	–	8 (7)	26 (20)	2.0 (1.6)	n.s. (n.s.)
+	+	1 (2)	28 (34)	17.5 (9.4)	2.8–108 (1.9–47)

[a] Ages of death

independent mode of action, we expected the OR of carriers of both markers to be 2.9. Instead, we observed a 4.4 times higher OR of 12.7 (95% C.I. 4.1–39). In the older subjects (age of death ≥75 years), we expected an OR of 4.6 under an interaction-free model but found 17.5 (95% C.I. 2.8–108). These results support a concerted mode rather than an independent mode of action of *APOE* ε4 and *BCHE*-K in AD of the late-onset type.

To address further the question of whether the observed *BCHE* association with AD in our cohort was only specific to the K-variant, we genotyped our sample for *BCHE*-A1914G. The “G”-allele frequency was 0.19 in 70 controls and 0.33 within 135 AD cases, giving an overall carrier OR of AD of 2.4 (95% C.I. 1.3–4.5). Previous findings (Bartels et al. 1992) and our data had suggested linkage disequilibrium between the K-variant and the *BCHE*-1914G-chromosome. We found that 16 out of 17 K-allele carriers within the controls and 54 out of 55 within the AD cases were also carriers of the *BCHE*-1914G variant. About two third of the total “G”-allele carriers in both groups (16/24 controls and 54/75 AD cases) were also K-allele carriers. We therefore found, within controls, 2.9 times and, within AD cases, 1.8 times more carriers of both the K-allele and the G-allele than expected, assuming random assortment of both markers within our cohort. Eight out of 70 controls and 19/135 AD cases were identified as carriers of the 1914G-allele but not the K-allele, giving an OR of confirmed AD of 1.3 (not significant) for sole *BCHE*-1914G-allele carrier status. Our results suggest that the observed association of *BCHE*-1914G with AD is more likely through the almost complete inclusion of the K-variant carrier status.

When we analysed the *BCHE*-A1914G data in regard to the carriers’ *APOE* ε4 status and took subjects who had neither *APOE* ε4 nor *BCHE*-1914G as a reference, significant ORs of 9.8 (controls ≥60 years) and 9.4 (AD cases ≥75 years of death) were found for carriers of both *APOE* ε4 and *BCHE*-1914G (Table 5). We suggest that the K-variant alone, rather than in conjunction with the 1914G-variant or the 1914G-allele alone, shows association with AD within our cohort. An individual’s *BCHE*-K status thus modifies the strength of the association of *APOE* ε4 and AD seen in our sample. Since we have observed a 3.5-times higher OR for the *APOE* ε4/*BCHE*-K status and a 4.5-times higher OR for the *APOE* ε4/*BCHE*-1914G status than expected under an independent mode of action, synergism can be hypothesised.

Discussion

Our data are consistent with the well-established finding that *APOE* ε4 is a risk factor for AD (Strittmatter et al. 1993; Poirier et al. 1993). Furthermore, our data confirm the previously reported association of *BCHE*-K with a further increase in the risk of late-onset AD in *APOE* ε4 carriers (Lehmann et al. 1997). Sandbrink and co-workers (1998) have recently reported a significant increase of the *BCHE*-K allele frequency of 0.13 in controls and 0.25 within probable late-onset AD cases; however, synergism with *APOE* ε4 was not apparent in their study. Interestingly, despite the different geographical origin of the populations, we find virtually the same *BCHE*-K increase in our AD cases. In agreement with the findings of Lehmann et al. (1997) and Sandbrink et al. (1998), we have been able to identify an allelic frequency of *BCHE*-K in our confirmed AD sample that is almost twice that in confirmed non-AD controls. In addition, we have found synergism between *BCHE*-K and *APOE* ε4, although to a lower extent compared with Lehmann et al. (1997). Furthermore, the age-dependent increase of *BCHE*-K and the more pronounced than expected abundance of *BCHE*-K/*APOE* ε4 carriers within AD cases is common.

Of the nine published studies so far, six have found little or no evidence of any K-associated AD risk or of an interaction with ε4 (Brindle et al. 1998; Crawford et al. 1998; Hiltunen et al. 1998; Roses et al. 1998; Russ et al. 1998; Singleton et al. 1998a). One group has reported a *BCHE*-K association with AD but no interaction with ε4 (Sandbrink et al. 1998), whereas two have found an age-related *BCHE*-K association with AD being interactive with ε4 (Lehmann et al. 1997; this study). Nonetheless, it has not escaped our

attention that a common finding between these studies is a higher frequency of *BCHE*-K in the AD groups compared with the control groups, despite the fact that all populations come from different geographical regions. Thus, it is possible that the sampling and phenotype assignment of the control population might be a key determinant in the outcome of association studies with *BCHE*-K and AD. It is noteworthy that, in our study and that of Lehmann et al. (1997), histopathological criteria have been used to confirm that the aged-matched controls are free of AD pathology.

We have further investigated whether, in our cases and controls, a genetic *BCHE* marker other than the K-variant might show a stronger association with AD compared with *BCHE*-K. Bartels and colleagues (1992) have previously shown that the K-variant is in linkage disequilibrium with the "G"-variant of the *BCHE*-A1914G polymorphism, which is located within the 3'-untranslated region. In their population, the allele frequency of the K-variant is 0.15 and 0.26 for the 1914G-variant. We have asked whether the 1914G-variant might be a better AD-marker in our study group than the K-variant alone, as the latter only marks a subset of the 1914G-chromosomes. We likewise find that the 1914G variant is more frequent within our cases and controls than *BCHE*-K. Like Bartels and co-workers (1992), our data suggest that *BCHE*-K is, to a certain degree, linked to the chromosome carrying 1914G. The significant carrier OR of AD of 2.4 for *BCHE*-1914G compared with 2.1 for *BCHE*-K is probably a result of the almost complete assimilation of K-variant carriers within *BCHE*-1914G carriers. However, if we analyse carriers of *BCHE*-1914G without *BCHE*-K, the OR of 1.3 (95% C.I. 0.29–5.6) shows that *BCHE*-1914G by itself is not increased in AD cases and that, in our group, *BCHE*-K is the far better marker for the association of BChE with AD. It is noteworthy that *BCHE*-1914G nevertheless gives the highest overall carrier OR compared with *BCHE*-K but lower ORs when stratified for age (2.7 vs 4.5) or age and *APOE* ε4 status (9.5 vs 17.5).

Our analysis of a *BCHE* polymorphism in linkage disequilibrium with *BCHE*-K supports the hypothesis that the chromosome carrying the K-variant is more likely to account for the AD association than other *BCHE* chromosomes. However, a strong linkage of *BCHE*-K with other deleterious mutations, which themselves might show better association with AD than *BCHE*-K, cannot be ruled out. A recent study by Maekawa and colleagues (1997), for example, has demonstrated that, among 53 individuals with reduced BChE activities, the frequency of *BCHE*-K is significantly higher at 0.47 compared with 0.175 in healthy controls. They have concluded that silent BChE genes are linked to the K-polymorphism; they have been able to confirm this for at least 3 of their 12 identified mutations. It is reasonable to speculate that ethnic differences within populations correlate with the extent of linkage disequilibrium of *BCHE*-K with other deleterious *BCHE* mutations. The inter-population variance could possibly account for the conflicting results found so far regarding the association of *BCHE*-K with late-onset AD. The development of a highly dense, genetic map of single nucleotide polymorphisms within the *BCHE* locus of various ethnic populations could help finally to determine which haplotypes or *BCHE*-alleles, defined by specific combinations of sites, might influence AD trait variation (Kruglyak 1997; Chakravarti 1998; Nickerson et al. 1998).

One of the main problems with genetic association studies of AD is the generation of spurious positive results because of the effects of hidden population admixture, sampling biases or other unknown confounding factors. The proper choice of an AD-free control population is critical to the study outcome and there is the added complication of potential phenotype mis-assignments for both the control and disease group. A yet unsolved problem regarding the use of age-matched non-AD controls is the uncertainty as to whether a given individual might develop AD in the future and should be considered as diseased. In order to minimise this risk, we have used post mortem histopathological criteria for phenotype assignment, with the simplified assumption that AD-free pathology at death indicates the complete absence of AD development. We have addressed the problem of population admixture by taking only subjects from the Quebec region of eastern Canada. Because the male-to-female ratios within our cases and controls are not identical, a gender influence as a co-factor on the *BCHE*-K/AD association cannot be fully ruled out. However, a gender influence is not apparent in the work of Lehmann and colleagues (1997). In conclusion, by following the original design with neuropathologically evaluated subjects, the earlier findings of Lehmann et al. (1997) have been fully replicated.

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