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Increased expression of readthrough acetylcholinesterase variants in the brains of Alzheimer's disease patients

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Abstract

Alzheimer's disease (AD) is characterized by a decrease in the enzymatic activity of the enzyme acetylcholinesterase (AChE). AChE is expressed as multiple splice variants, which may serve both cholinergic degradative functions and non-cholinergic functions unrelated with their capacity to hydrolyze acetylcholine. We have recently demonstrated that a prominent pool of enzymatically inactive AChE protein exists in the AD brain. In this study, we analyzed protein and transcript levels of individual AChE variants in human frontal cortex from AD patients by Western blot analysis using specific anti-AChE antibodies and by *quantitative* real-time PCR. We found similar protein and mRNA levels of the major cholinergic “tailed”-variant (AChE-T) and the anchoring subunit, proline-rich membrane anchor (PRiMA-1) in frontal cortex obtained from AD patients and non-demented controls. Interestingly, we found an increase in the protein and transcript levels of the non-cholinergic “readthrough” AChE (AChE-R) variants in AD patients compared to controls. Similar increases were detected by Western blot using an antibody raised against the specific N-terminal domain, exclusive of alternative N-extended variants of AChE (N-AChE). In accordance with a subset of AChE-R monomers that display amphiphilic properties which are upregulated in the AD brain, we demonstrate that the increase of N-AChE species is due, at least in part, to N-AChE-R variants. In conclusion, we demonstrate selective alterations in specific AChE variants in AD cortex, with no correlation in enzymatic activity. Therefore, differential expression of AChE variants in AD may reflect changes in the pathophysiological role of AChE, independent of cholinergic impairment or its role in degrading acetylcholine.

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Keywords

Alzheimer's disease; acetylcholinesterase; human brain; AChE splice variants; readthrough AChE variants

Introduction

Alzheimer's disease (AD) is the most common cause of dementia among elderly people and is characterized by loss of memory and cognitive functions [1]. During the progression of AD many different types of neurons deteriorate, but, in particular, there is a large decrease in cholinergic neurons [2-5] with a reduction in the acetylcholine (ACh) hydrolyzing enzyme acetylcholinesterase (AChE), and choline acetyltransferase (ChAT), the rate-limiting enzyme that synthesizes ACh. As consequence of the cholinergic impairment in AD brain the levels of the neurotransmitter ACh are decreased, and this depletion has been associated with AD clinical symptoms [4-6]. Consequently, a significant portion of the AD therapy field has been focused for many years on developing inhibitors of AChE for prolonging the availability of ACh at the synapse, presumably to ameliorate AD-related symptoms [7, 8]. Although several cholinesterase inhibitors (e.g., donepezil, galantamine, and rivastigmine, among others) have proven to be moderately effective as a palliative therapy, their benefits have a limited duration [9].

A general consensus is that total brain AChE activity levels decrease modestly as dementia progresses (discussed in [10]). However, we recently reported that brain AChE protein levels are unexpectedly similar between AD and non-demented subjects [11]. The discrepancy between AChE activity and protein immunoreactivity levels are attributable, at least in part, to the presence of a large pool of inactive AChE subunits that are relatively spared in the AD brain and cerebrospinal fluid (CSF) [12]. This pool of brain AChE protein has been hypothesized to participate in a variety of non-cholinergic functions [13-16]. Interference of AChE inhibitors with this inactive AChE protein pool is plausible, since cholinesterase inhibitors also interfere in the non-cholinergic functions of AChE [17, 18] and their use in AD therapy may result in unpredictable, off-target effects related with vulnerability in the pathological brain, and loss of efficacy during long-term treatment. Therefore, the characterization of this non-cholinergic functioning AChE pool in the AD brain is of interest.

Importantly, AChE is not only the cholinergic specie. Alternative splicing and alternate promoter usage generates different AChE transcripts with the same catalytic domain, and distinct N- and C-terminal peptides that determine the ability of the molecule to form oligomers [19, 20]. Alternative splicing of AChE is differentially regulated in different cell types, at both the mRNA and post-translational levels. In human brain the most abundant variant of AChE is a “tailed” or AChE-T specie that generates monomeric subunits organized into tetramers which are considered the cholinergic degrading functioning forms [19, 21, 22,]. AChE-T is anchored to neural cell membranes through a proline-rich membrane anchor (PRiMA) subunit, which is an accessory partner for the cellular localization of AChE [23]. In human brain there is also a minor contribution of the rare

“readthrough” or AChE-R variant, which expression is induced by multiple stress stimuli, exposure to cholinesterase inhibitors, and during aging [24, 20]. The AChE-R transcript encodes monomeric, soluble subunits [19]. An alternative upstream promoter produces another version of each of these AChE variants with an extended N terminus, termed N-AChE-T and N-AChE-R [20]. Based on the presence of these multiple alternative splicing transcripts and their encoded proteins, several studies suggest that AChE variants could have alternative functions unrelated to cholinergic neurotransmission [13-16, 25], and possibly unrelated with the cholinergic catalytic activity [26-28]. Thus, the significance of the different species of AChE displaying particular regional, cellular, and subcellular locations may reflect differential physiological roles under normal and diseased states.

In this study we analyzed protein levels of AChE variants in human cerebral cortex obtained postmortem from clinically and pathologically diagnosed AD patients and non-demented controls (NDC) by Western blot analysis using specific anti-AChE antibodies raised against differential domains of AChE variants. Levels of AChE transcripts were also analysed by *q*RT-PCR. Further, we investigated expression levels of the anchor AChE subunit PRiMA-1, a limiting factor for correct localization of cholinergic AChE at the plasma membrane. We found that AChE-R variants were significantly increased in the frontal cortex of AD brain. These AChE-R monomers were further characterized by analysis of their amphiphilic properties.

Materials and Methods

Collection of human brain samples

This study was approved by the ethic committee of the Hospital General Universitario de Elche, and was carried out in accordance with the Declaration of Helsinki. The collection of frozen frontal cortex (Brodmann areas 9/10) samples for AD (11 female and 8 male 80 ± 5 years) and NDC subjects (10 female and 12 male, 69 ± 4 years) was obtained from the following tissue banks: UIPA neurological tissue bank (Unidad de Investigación Proyecto Alzheimer; Madrid, Spain) and CNDR Center for Neurodegenerative Disease Research (University of Pennsylvania, USA). The cases of sporadic AD were selected on the basis of their clinical history of dementia, neuropathological CERAD diagnosis and Reagan Criteria [29, 30] and were categorized as stages V-VI on the Braak scale [31]. Samples from non-diseased individuals corresponded to cases with no clinical dementia and no evidence of brain pathology. The mean postmortem interval of the tissue was between 1.5 and 6 h, with no significant differences between groups.

Preparation of human brain samples for biochemical analysis

Samples (~0.1 g) of human frontal cortex stored at -80°C were thawed slowly at 4°C and homogenized (10% w/v) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (w/v) Nonidet P-40, and 0.5% (w/v) Triton X-100 supplemented with a cocktail of protease inhibitors [32]. The homogenates were centrifuged at $70,000 \times g$ at 4°C for 1 h, and then the supernatants were collected and frozen at -80°C until assayed.

Cell Culture

SH-SY5Y neuroblastoma cells were grown in D-MEM/F12+GlutaMAX™-I (Dulbecco's Modified Eagle medium; GIBCO Invitrogen Corporation) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin solution (P/S; 100 U/mL) (Gibco). Cells were transfected using Lipofectamine™ 2000 (Invitrogen™, Life technologies Paisley, UK) with 4 µg of AChE-T or AChE-R cDNAs under the cytomegalovirus (CMV) promoter-enhancer (a generous gift from Prof Hermona Soreq, Institute of Life Science, Hebrew University, Jerusalem, Israel). The PCI “empty” vector (Promega, Madison, USA) served as negative control. The cells were collected for analysis 48 hours after the transfection.

AChE enzymatic activity and total protein determination

AChE activity was determined by a modified microassay version of the colorimetric Ellman's method [32]. AChE was assayed with 1 mM acetylthiocholine and 50 µM tetraisopropyl pyrophosphoramidate (Iso OMPA), a specific inhibitor of butyrylcholinesterase, a second cholinesterase that co-exists with AChE in brain. One milliunit (mU) of AChE activity was defined as the number of nmoles of acetylthiocholine hydrolyzed per min at 22 °C. Protein concentrations were determined using the bicinchoninic acid method, with bovine serum albumin as standard (Pierce, Rockford, IL).

Sedimentation analysis

Molecular forms of AChE were separated according to their sedimentation coefficients by ultracentrifugation on 5-20% (w/v) sucrose gradients containing 0.5% (w/v) Triton X-100 [32]. Ultracentrifugation was performed at 150,000 ×g in a SW 41Ti Beckman rotor for 18 hr, at 4 °C. Approximately 40 fractions were collected from the bottom of each tube and assayed for AChE activity to identify individual AChE forms (G_4 = tetramers; G_1 = monomers) by comparison with the position of molecular weight markers, catalase (11.4S) and alkaline phosphatase (6.1S). We defined the ratio of AChE forms G_4/G_1 as the proportion of G_4 molecules versus the light form, G_1 . The sucrose fractions containing the light G_1 peaks were separately pooled, dialyzed against Tris buffer, and concentrated by ultrafiltration (Amicon Ultra 10,000 MWCO, Millipore Corporation, Bedford, MA, USA). Monomers of AChE were then characterized by a phenyl-agarose interaction and Western blot assays.

Western blotting assays

AChE subunits and PRiMA-1 levels were detected by immunoblotting. 50 micrograms of protein from brain extracts (equal amount of protein in each lane) were resolved by electrophoresis on 10% SDS-polyacrylamide slab gels. Samples were denatured at 98 °C for 7 min. Following electrophoresis, proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell Bioscience, GmbH), blocked with 5% bovine serum albumin and probed with the following primary antibodies: anti-PRiMA-1 antibody (C16, Santa Cruz Biotech, Santa Cruz, CA, USA), anti AChE-T variants antibody (Ab31276, Abcam, Cambridge, UK), anti AChE-R antibody raised to the unique C-terminus of human AChE-R, an anti N-AChE raised to the extended N-terminus of N-AChE variants (both were a generous gift from Prof Hermona Soreq). A rabbit anti-glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) antibody (Abcam) was used as a loading control. Western blots for different antibodies were performed separately to avoid re-probing of blots. The blots were incubated with the corresponding secondary antibody conjugated to horseradish peroxidase and the immunoreactive signal was detected using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) according to the manufacturer's instructions in a Luminescent Image Analyzer LAS-1000 Plus (FUJIFILM, Japan). For semi-quantitative analysis, protein levels were normalized to GAPDH and the intensity of bands was measured by densitometry with the Science Lab Image Gauge v4.0 software provided by FUJIFILM.

Binding to phenyl-agarose

Amphiphilic AChE monomers were separated from hydrophilic monomers by hydrophobic interaction on phenyl-agarose. Briefly, aliquots of enriched G₁ peaks from sedimentation analysis of NDC and AD samples were mixed with immobilized phenyl-agarose (Sigma, St. Louis, MO, USA) and incubated overnight at 4 °C. Bound amphiphilic AChE light forms were eluted and reserved with the unbound hydrophilic monomers for Western blot analysis.

RNA isolation and analysis of transcripts by qRT-PCR

Total RNA from AD and control frontal cortex was isolated using TRIzol Reagent in the PureLink™ Microto- Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNAs were synthesized by reverse transcription of 1.5 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Life Technologies Paisley, UK), according to the manufacturer's instructions. qRT-PCR amplification was performed using StepOne-Plus™ Real-Time PCR System with Power SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions for analysis of AChE transcripts. GAPDH mRNA was used as the housekeeping marker. The primers used were: AChE-T forward 5'-CTTCCTCCCCAAATTGCTC-3', reverse 5'-TCCTGCTTGCTGTAGTGGTC-3'; AChE-R forward 5'-CTTCCTCCCCAAATTGCTC-3', reverse: 5'-GGGGAGAAGAGAGGGGTTAC-3'; N-AChE forward 5'-GAAAGTCCGAAGTCACCCGTC-3', reverse 5'-CAGGCGGCGTCTGAGAA-3'; GAPDH forward 5'-AGCCACATCGCTCAGACAC-3', reverse 5'-GCCCAATACGACCAAATCC-3'. For the determination of PRiMA-1 transcript levels a TaqMan GenExpression Assay (Hs00930435_m1 for PRiMA-1 and Hs03929097 for GAPDH) with TaqMan PCR Master Mix were employed. Transcript levels were calculated by the comparative 2^{-ct} method with respect to GAPDH cDNA as described previously [33].

Statistical analysis

All data were analyzed using SigmaStat (Version 3.5; Systac Software Inc.) by Student's t-test (two tailed). Results are presented as means ± SEM. *p* values < 0.05 were considered significant.

Results

AChE enzymatic activity levels were determined in frontal cortex extracts from AD and NDC subjects. As expected, AChE activity levels were lower (~40% decrease, $p=0.002$) in AD samples than controls (Fig. 1A). AChE is expressed in the human brain as several molecular forms distinguishable by their molecular weights and hydrodynamic properties [19]. Frontal cortex extracts were fractionated on sucrose density gradients to separate the different AChE molecular forms. As expected in NDC frontal cortex extracts, the cholinergic G_4 species represented the major peak of AChE activity, with a minor contribution of the G_1 forms (Fig. 1B). In agreement with previous reports [34-36] a reduction in the amount of the G_4 form with sparing changes in the lighter forms was observed in AD brain extracts as compared with control samples. Accordingly, a significant decrease (~35%, $p=0.023$, Fig. 1B) in the G_4/G_1 ratio, was observed in AD extracts. The AChE enzymatic activity levels and molecular forms pattern obtained in samples from diverse tissue banks displayed similar differences between NDC and AD groups and no differences between cohorts, indicating consistency of these observations (data not shown).

Sedimentation analysis is not able to differentiate the products of AChE variants when expressed as monomers. The G_4 form is exclusively generated by AChE-T variants, but monomeric forms may be product of both AChE-T and AChE-R variants and also of its N-elongated species N-AChE-T or N-AChE-R. Therefore, we analyzed AChE levels using variant-specific anti-AChE antibodies in frontal cortex. The specificity of these AChE antibodies was tested previously [37] and confirmed here (see Supplemental Fig. 1). An antibody raised against a peptide that maps to C-terminal of the AChE-T subunits detected three major bands of approximately 75, 66, and 55 kDa (Fig 2A), consistent with previous observations [37]. No significant differences in the immunoreactivity of these AChE-T bands were observed between control and AD samples. AChE-R variants were detected with an antibody directed to its unique C-terminus, as revealed by a 55 kDa immunoreactive band (Fig. 2B). Levels of the AChE-R band were significantly higher (~60 % increase, $p<0.001$) in AD brain extracts as compared with controls. Moreover, the levels of N-extended subunits were analyzed using an antibody raised against the extended N-terminal domain of AChE. Because this domain is common to all N-AChE subunits, the antibody cannot distinguish between N-AChE-T and N-AChE-R variants. This antibody resolves a predominant band of approximately 55 kDa and two faint bands of 66 and 75 kDa (Fig. 2C) which could not be quantified reproducibly. Levels of 55 kDa N-AChE immunoreactive band were higher in AD extracts (~50% increase, $p=0.014$), as occurs with the 55 kDa AChE-R species. We also analyzed protein levels of PRiMA-1, the anchorage subunit of the cholinergic G_4 form to the membrane principally expressed in the brain [38, 39]. Similar to a previous report [11], two PRiMA-1 bands of ~22 and 20 kDa were observed in frontal cortex extracts (Fig. 2D), likely corresponding to mature (fully glycosylated) and immature PRiMA-1 [40]. Western blot assays showed no differences in the immunoreactive levels of the mature PRiMA-1 protein band between samples of AD and NDC subjects.

qRT-PCR assays were performed to determine whether changes in AChE protein levels corresponded to alterations in AChE mRNA expression (Fig. 3). Results indicated that there were no significant changes in the levels of the most abundant T-transcript between AD and

control subjects, similar to our protein findings. Consistent with our Western blot analysis, the levels of the minor R-transcript were significantly higher in AD frontal cortex than control samples (Fig. 3). We also designed primers located in exon h1e, common for all N-extended variants [41] to assay levels of N-AChE transcripts. We found no differences between AD and NDC subjects in transcripts that encode N-extended variants. Further, no changes were detected between AD and control subjects in PRiMA-1 mRNA levels (Fig. 3), consistent with protein assessments.

Our results demonstrate in AD frontal cortex an increase in the amount of protein of AChE-R and a subset of N-AChE subunits with similar molecular mass. Technically, we cannot establish by Western blot or *q*RT-PCR whether this subset of N-AChE subunits correspond to N-AChE-R. Therefore, we attempted to further characterize the AChE-R variants based on their expected molecular mass and hydrodynamic properties. Since both AChE-R and N-AChE extended variants are expected to be monomeric species, we analyzed by Western blot G₁ peaks isolated and pooled after ultracentrifugation on sucrose gradients (Fig. 4A). As expected, Western blot analysis confirmed higher levels of the 55 kDa AChE-R band in the monomeric fraction from AD frontal cortex (Fig. 4B), compared with levels from NDC samples. Similarly, resolution of the pooled monomeric peaks with an antibody directed against N-AChE confirmed a significant increase of the 55 kDa N-AChE band in AD samples (Fig. 4B). Since the AChE-R species exist as a soluble form, we expect hydrophilic behavior, whereas N-AChE-R variants, due to their N-elongated fragment that allows them to anchor to plasma membrane, will display amphiphilic properties. Accordingly, the AChE monomeric peaks from AD and control samples were analyzed by interaction with hydrophobic matrix of phenyl-agarose that selectively binds amphiphilic proteins [42]. Western blot assays performed with the anti AChE-R antibody displayed a faint immunoreactive band of 55 kDa in the fraction bound the matrix in NDC (Fig. 4C), indicating that a small percentage of AChE-R subunits displayed amphiphilic properties attributable to N-AChE-R. Interestingly, in AD samples the percentage of the AChE-R species interacting with phenyl-agarose were significantly higher (~190 % increase compared to percentage from NDC; $p=0.013$; Fig. 4D), likely reflecting that the increase of AChE-R variants characterized in AD also involved the N-AChE-R elongated species.

Discussion

The majority of studies of AChE regulation during the progression of AD have been based on the determination of enzymatic activity level. To date, it is well established that the substantial loss of AChE activity is due to the selective depletion of the tetrameric species linked to the plasma membrane [34, 36, 43], the cholinergic enzyme. Despite an overall loss of AChE activity, an increase in the level of a minor monomeric form has been also extensively documented in the AD brain [34, 36, 43]. We hypothesize that a significant increase in a minor AChE form may not cause an imbalance in cholinergic equilibrium but could have functional impact [15, 22] since particular AChE species may exert non-cholinergic roles, including protein-protein interactions, based of their unique N- and C-termini and/or their differential cellular localization.

In the present study we analyzed levels of AChE variants in frontal cortex from AD and NDC subjects. Western blot assays using specific antibodies for AChE variants revealed a complex immunoreactive pattern of 55-75 kDa bands in accordance with previous studies [44, 45]. The differences in molecular mass of the subunits detected by denaturing SDS-PAGE/Western blotting may reflect post-translational processing, such as glycosylation, as the native splice variants are not predicted to have differential molecular mass. Consistent with previous studies, we found that enzymatic levels of tetrameric cholinergic AChE decreased in AD brain. However, no changes were observed in the immunoreactive levels of AChE-T variants between AD and control samples, indicating they are relatively spared in AD. Moreover, expression levels of the 55 kDa AChE-R subunits are increased in frontal cortical samples from AD patients. A similar increase in the immunoreactivity of a 55 kDa subunit is also observed for N-extended variants. Although the 55 kDa immunoreactive band is common to all AChE variants, no changes in AChE-T were detected, confirming that the banding pattern obtained by SDS-PAGE/Western blotting analysis has no direct relationship to specific variants and molecular forms.

Analysis of the lighter AChE forms isolated from a sedimentation gradient served to characterize the amphiphilic behaviour of a subset of R-subunits, which were increased in AD. Amphiphilic properties have been assigned to N-AChE variants, since the hydrophobic signal peptide, which cleavage is prevented by the N-extended terminus, acts like a transmembrane domain [41] similar to neurexin and cyclooxygenase [46]. Thus, the increment of N-AChE in AD frontal cortex likely reflects an increase in N-AChE-R variants.

The increase in AChE-R expression, as well the unchanged AChE-T protein levels were corroborated by the analysis of the transcripts mRNA levels by *q*RT-PCR. Conversely, despite the increment of N-extended proteins in AD samples, we did not find alterations in N-AChE transcripts, likely due to the observation that overexpression only occurs in minor species with low contribution to the entire N-AChE mRNA signal. Similar increment on AChE-R transcript levels were reported by Soreq and co-workers in the dentate gyrus neurons from human AD patients, although they found a decrease on AChE-T mRNA in AD hippocampus [47]. The divergences with our study are not clear, but may be attributed in part to differences in the brain area and cell types analyzed.

Discrepancies that we found in AD brain between the unaltered expression levels of the AChE-T variants and the decrease on the enzymatic activity of cholinergic tetramers may be attributable to a major contribution of inactive subunits to the total immunoreactive levels. The existence of an inactive pool of AChE has been demonstrated in brain, CSF, and plasma [12, 37, 48, 49,]. The inactive AChE species are monomers, thought to be synthesized as a reservoir of precursors of enzymatically active tetramers. However, we and others posit that part of inactive pool includes AChE species with specific roles unrelated with cholinergic neurotransmission (discussed in [11]. Increment on inactive AChE-T forms within AD brain may be due in part to alterations on the oligomerization process of tetrameric forms. For the appropriate processing and cellular localization of this tetrameric AChE, the anchor subunit PRiMA is necessary [23], representing a limiting factor [40]. Indeed, the abolishment of PRiMA expression in a knock-out mouse model leads to a near-total loss of AChE activity with no changes in AChE mRNA levels [23]. We have also analysed the levels of PRiMA-I

and neither protein nor transcript levels are altered in AD brain. We hypothesize that this depletion in tetramers probably results from changes in the post-translational processing of the AChE subunits that compromise the ability to form the active complex.

Interestingly, AChE can interact directly with β -amyloid [50-52]. In this context, AChE-T variants have been suggested as the species able to enhance amyloid-associated neurotoxicity, facilitating the formation of amyloid β -sheets [47]. Indeed transgenic mice model overexpressing AChE-T and the A β PP Swedish mutation (A β PPswe), showed early deposition and more abundant β -amyloid plaques than mice overexpressing mutant A β PPswe alone [53]. In contrast, increases in AChE-R variants in AD may be associated positively with disease progression [54, 55]. The treatment of a cholinergic cell line with low dose of amyloid- β causes a sustained elevation of the AChE-R protein that has been interpreted as part of a neuroprotective effect attenuating the formation of β -amyloid fibrils [56]. Moreover, a transgenic mice model co-expressing AChE-R and A β PPswe, showed reduced cortical amyloid- β plaque burden than A β PPswe mice [47].

We have also determined that amphiphilic N-extended variants of the AChE-R species are increased in AD brains. An increase in N-AChE variants attributed to an increment of the N-AChE-T species has been suggested in cortical neurons of AD patients [57]. These N-AChE-T variants are related with the activation of an apoptotic pathway that includes activation of glycogen synthase kinase 3 β [57]. The significance of the increase in N-AChE-R variants in AD brain remains to be determined.

Taken together, our findings reveal previously unknown expression patterns of AChE variants in AD frontal cortex, likely reflecting specific roles and/or differential regulation for each variant in AD. Understanding the role(s) for AChE variants will be useful for clarifying the mechanisms underlying AChE regulation during the pathogenesis of AD, and whether cholinergic and non-cholinergic functions are dysfunctional. These findings may have strong implications for the re-evaluation of therapies based on AChE inhibitors as therapeutic agents in dementia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Decrease of acetylcholinesterase (AChE) activity levels in the frontal cortex of Alzheimer's disease (AD) patients

(A) AChE specific activity (mU/mg of total protein) was measured in protein extracts from brain cortices of non-demented controls (NDC; n=22) and AD patients (AD; n=19). One milliunit (mU) of AChE activity was defined as the number of nmoles of acetylthiocholine hydrolyzed per min at 22 °C. (B) Equal amount of protein from NDC and AD brain extracts were separated by ultracentrifugation on sucrose gradient and molecular forms of AChE (tetramers: G₄; and light monomers: G₁) were identified in each fraction by comparison with the position of molecular weight markers catalase (C; 11.4S) and alkaline phosphatase (P; 6.1S). Representative profiles for NDC (●) and AD (○) are shown (left panel) in which the G₄/(G₁) ratio were calculated (right panel, n=6 for each group). Represented values are means ± SEM. *Significantly decreased ($p < 0.05$) from NDC as assessed by a Student's *t*-test.

Figure 2. Increased protein levels of AChE splice variants in the frontal cortex of AD patients
Immunodetection of AChE variants and the membrane anchor subunit PRiMA-1 in frontal cortex of non-demented control (NDC, n=12) and AD patients (n=12). Fifty µg of protein from brain extracts (equal amount of protein in each lane) were resolved by electrophoresis and probed with specific primary antibodies raised to: **(A)** the C-terminus of the “tailed” AChE-T variant; **(B)** the C-terminus of “readthrough” AChE-R variant; **(C)** the extended N-terminus of N-AChE variants; and **(D)** the anchor subunit PRiMA-1. Representative blots and densitometric quantification of the immunoreactive bands are shown and expressed as percentage (%) relative to immunoreactive of the 55 kDa AChE band or to the 22 kDa PRiMA band, from the NDC group. For semiquantitative analysis, levels were normalized to the housekeeping protein GAPDH. The results were confirmed in two independent determinations. Values are means ± SEM. * $p < 0.05$ significantly upregulated compared to NDC by a Student's *t*-test.

Figure 3. Transcript levels of the “readthrough” acetylcholinesterase (AChE-R) variant are increased in cerebral cortex of AD subjects

Relative mRNA levels of the transcripts for AChE-T (or “tailed”), AChE-R (or “readthrough”) and N-AChE (or N-extended) splice variants and for proline-rich membrane anchor 1 (PRiMA-1) were analysed by *q*RT-PCR in frontal cortex of NDC (n= 22) and AD subjects (n= 19). For AChE transcript analysis specific primers with Power SYBR® Green PCR Master Mix were employed and the specificity of the PCR products was confirmed by dissociation curve analysis. PRiMA-1 transcripts were measured using a specific TaqMan GenExpression Assay with TaqMan PCR Master Mix. Transcript levels were calculated by the comparative 2^{-C_t} method with respect to GAPDH. The results were confirmed in two independent determinations. Mean value $\pm$ SEM are represented. *Significantly increased ($p < 0.05$) compared to NDC as assessed by a Student's *t* test.

Figure 4. An amphiphilic subset of the “readthrough” acetylcholinesterase (AChE-R) variant is increased in the frontal cortex of Alzheimer's patients

(A) Light AChE monomers (G_1) were fractionated and identified by sucrose gradient centrifugation from frontal extracts of NDC (●; $n=6$) and Alzheimer's subjects (○; $n=6$). Fractions containing AChE monomers were pooled, dialyzed and concentrated by ultrafiltration. Representative sedimentation profiles illustrating the fractions selected for (G_1) peak isolation are shown. Equal amount of protein from enriched AChE light forms fractions were then assayed by immunoblotting using specific antibodies to AChE-R (B) and the N-extended (N-AChE) (C) variants. Representative blot and densitometric quantification of the AChE-immunoreactive bands for each antibody, expressed in arbitrary units (a.u.), are shown. The results were confirmed in two independent determinations with equivalent amounts of protein loaded in each lane. (D) The enriched AChE monomeric fractions were also characterized by hydrophobic interaction on a phenyl-agarose matrix. The original enriched peak (input), the bound (amphiphilic isoforms eluted with Triton X-100), and the unbound (hydrophilic isoforms) were then assayed by Western blotting using the anti-AChE-R antibody, to compare differences in AChE-R amphiphilic behaviour between groups. A representative blot is included (left panel). Immunoreactive bands were quantified and the percentage (%) of the bound fraction respect the input was calculated (right panel). Values are means $\pm$ SEM. * $p < 0.05$ significantly different from NDC group, (Student's t -test).