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Differential cerebral deposition of IDE and NEP in sporadic and familial Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is characterized by amyloid β (A β) accumulation in the brain and is classified as familial early-onset (FAD) or sporadic late-onset (SAD). Evidences suggest that deficits in the brain expression of insulin degrading enzyme (IDE) and neprilysin (NEP), both proteases involved in amyloid degradation, may promote A β deposition in SAD. We studied by immunohistochemistry IDE and NEP cortical expression in SAD and FAD samples carrying the E280A presenilin-1 missense mutation. We showed that IDE, a soluble peptidase, is linked with aggregated A β 40 isoform while NEP, a membrane-bound protease, negatively correlates with amyloid angiopathy and its expression in the senile plaques is independent of aggregated amyloid and restricted to SAD cases. NEP, but not IDE, is over-expressed in dystrophic neurites, both proteases are immunoreactive in activated astrocytes but not in microglia and IDE was the only one detected in astrocytes of white matter from FAD cases. Collectively, our results support the notion that gross conformational changes involved in the modification from "natively folded-active" to "aggregated-inactive" IDE and NEP may be a relevant pathogenic mechanism in SAD.

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Conflict of interest

All authors disclose that there are no actual or potential conflicts of interests.

Keywords

Alzheimer's disease; Amyloid β ; Insulin degrading enzyme; Neprilysin; Human brain; Presenilin; Astrocytes

1. Introduction

Alzheimer's disease (AD) is the most prevalent aging-associated brain amyloidoses clinically characterized by dementia and classified as genetic, which is responsible of a rare disease known as familial early-onset AD (FAD), or sporadic late-onset AD (SAD), the most common form of dementia in subjects over 65 years old (Maurer and Hoyer, 2006). FAD is an inherited autosomal dominant disorder with mutations in the genes encoding amyloid precursor protein (APP) or presenilin 1 or 2 (PSEN1, PSEN2) (Haass and De Strooper, 1999). APP is a single-pass transmembrane protein that is sequentially cleaved by the β -secretase (BACE) – a membrane spanning aspartic protease – to generate a membrane-bound C-terminal fragment which is further processed by the γ -secretase complex, in a PSEN-dependent manner, to generate amyloid β (A β) of 40–42 residues (De Strooper et al., 1998; Vassar et al., 1999; Wolfe et al., 1999). In the context of the amyloid hypothesis (Selkoe, 2000), pathogenic mutations in APP induce over-expression of A β 42 or a mutated isoform of A β 40 with increased amyloidogenic properties while mutations in PSEN1/2 proteins alter the normal proteolytic processing of APP resulting in the increase of brain soluble A β 42/A β 40 peptides ratio (Scheuner et al., 1996). Histopathological features of SAD and FAD include the abundance of cortical and hippocampal deposits of A β 42 and A β 40 and of neurofibrillary tangles (NFT) composed of abnormally phosphorylated tau protein. Parenchymal deposition of fibrillar A β develops in a progressive and age-related manner to senile plaques, associated to dystrophic neurites and glial reaction (Dewachter et al., 2000; Poirier, 2005; Shibata et al., 2000; Jellinger, 2002). Furthermore, both types of AD show amyloid angiopathy with the predominance of A β 40 in SAD and A β 42 in the FAD brain microvasculature, respectively (Lemere et al., 1996; Roher et al., 1993). In addition, severe cerebellar and white matter pathology is observed in FAD subjects (Lemere et al., 1996) and it has been suggested that the over-representation of A β 42 in FAD cases produces – as compared to SAD – its earlier deposition and a higher percentage of brain area occupied by amyloid plaques. Mechanisms involved in the development of FAD may be explained by increments on local concentration of A β 42 peptide, while in SAD, causes are still unknown and strongly influenced by genetic and environmental factors such as the apolipoprotein E genotype (Roses, 1997), chronic trauma or cholesterol metabolism (Hartman et al., 2002; Hartmann, 2001; Pappolla et al., 2003). Recent publications support the idea that vascular lesions or defects in blood–brain barrier transport may be pathogenic factors in the formation of the cortical amyloid deposits around or close to capillaries, arterioles and venules (Cullen et al., 2006; Jaynes and Provias, 2006). In addition, it has been suggested that in SAD cases an inefficient A β degradation by brain proteases may contribute to A β accretion and its further accumulation (Perez et al., 2000). Within the brain, the metabolism of A β is mainly regulated by the activity of at least by two major peptidases: neprilysin (NEP) and insulin-degrading enzyme (IDE) as it was demonstrated in knock-out animals for NEP and IDE, respectively (Iwata et al., 2001; Farris et al., 2003). The over-expression of IDE or NEP in the transgenic mice over-expressing the human mutated APP gene (K670N, M671L) responsible for FAD in a Swedish pedigree prevents the amyloid plaque pathology and the early death (Leissring et al., 2003) suggesting that promotion of A β degradation may be a therapeutic target for AD. NEP is a ubiquitous and highly conserved membrane-bound zinc metalloprotease (Turner et al., 2004) expressed in the human brain at relatively low amounts and mainly in the tunica media of cerebrocortical blood vessels and in pyramidal neurons. Endogenous IDE is expressed in the human brain by cortical and subcortical

neurons, it was described within the cytoplasm of the three major components of the vascular wall: endothelial cells, pericytes and smooth muscle cells (Morelli et al., 2004) and it is considered a major soluble protease involved in the degradation of A β in the brain.

Experimental evidences suggest that impairments on A β -degrading proteases activity in the AD brain may be caused by at least 3 physiologic events: (1) decrements of mRNA expression in vulnerable areas of the brain susceptible for A β deposition, such as cortex and hippocampus (Cook et al., 2003; Yakoshima et al., 2001) or decreased protein levels or activity in the brain cortex (Perez et al., 2000; Zhao et al., 2007; Yakoshima et al., 2001), or in brain microvessels (Miners et al., 2006); (2) the presence of post-translational modifications, such as oxidation (Caccamo et al., 2005; Wang et al., 2003) and (3) the deposition of the enzymes, understood as a progressive accumulation of the proteases in the diseased brain with the consequent a loss of its native structure and of functionality. In this context, immunohistochemistry of SAD brains showed clusters of NEP-positive dystrophic neurites partially localized with senile plaques (Akiyama et al., 2001), a limited co-localization of deposits of IDE to amyloid plaques and blood vessels (Bernstein et al., 1999) and a differential deposition of IDE and NEP in vessels from subject with cerebral amyloid angiopathy, while IDE is over-expressed (Morelli et al., 2004) NEP is down-regulated (Miners et al., 2006). Together these results support the concept that both IDE and NEP expression and activity in the human brain participate in a differential manner in the pathogenesis of AD.

Moreover, the overall ageing-related down regulation of A β -degrading proteases was corroborated for NEP (Apelt et al., 2003) but not for IDE (Leal et al., 2006) in transgenic Tg2576 mice brains in which the relevant feature for both proteases was an astroglial up regulation in the vicinity of A β plaques without cortical deposition. In summary, the concept is that in SAD brain “age-dependent” accumulation of A β promotes inhibition of IDE and NEP expression and/or activity while in Tg2576 mice the effect of the “progressive” accumulation of A β on IDE and NEP does not affect the functionality of the endogenous enzymes and noteworthy may promote their expression.

Here we studied by immunohistochemistry the pattern of expression of IDE and NEP in AD brains with different etiologies, namely SAD and FAD carrying the E280A PSEN1 missense mutation and characterized by “accelerated” A β deposition, to test if differences in the rate of A β deposition in the human brain may impact on the expression and conformational state of A β -degrading proteases with potential impact in the progression of the disease.

2. Methods

2.1. Human tissues from AD and control subjects

Brain samples from late-onset SAD ($n = 8$) and control (CTL, $n = 11$) cases were obtained, under the approval of the Institutional Ethical Committees, from the Johns Hopkins University Brain Resource Center (USA) and the Institute for Neurological Research, Fundación Lucha Enfermedades Neurológicas Infantiles (FLENI) (Argentina). Cortical samples of FAD cases carrying the E280A miss-sense mutation in the PSEN1 gene ($n = 8$) were provided by the Neuroscience Group of Antioquia from the University of Antioquia (Colombia). The PSEN1E280A pedigree is the largest in the world affected with early-onset FAD. Patients are residents of the Department of Antioquia (Colombia) and cases included in this study at the age at death were diagnosed as AD according with criteria of the National Institute of Neurological and Communicative Disorders-Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and Diagnostic and Statistical Manual of Mental Disorders (DSM) IV 2 (American Psychiatric Association). Clinical and neuropathological characterization of this pedigree was extensively reported (Lemere et al.,

1996). A brain sample from a subject with hereditary cerebral hemorrhage with amyloidosis Dutch type (HCHWA-D) was used for endogenous amyloid isolation. The clinicopathological and DNA characterization of this patient has been previously reported (Castaño et al., 1996; Levy et al., 1990; van Duinen et al., 1987). SAD and FAD cases were diagnosed clinically with dementia and fulfilled clinical and pathological criteria established by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al., 1991). CTL subjects had no clinical history of dementia and no significant neuropathological changes indicative of AD or other clinically relevant disease. SAD cases were classified as Stage VI using previously published criteria (Braak and Braak, 1991). FAD cases were not staged according to Braak. SAD, FAD and CTL groups were comparable with respect to post-mortem delay and cerebral area analyzed (medial frontal gyrus, MFG). Moreover, SAD and CTL had similar age at death and SAD and FAD groups had a comparable APOE4 allele frequency (0.125). Clinicopathological features of the analyzed samples are summarized in Table 1. At autopsy, brain were fixed in 10% buffered formaldehyde for 2 weeks, then tissue blocks were dissected, processed through alcohols and embedded in paraffin. Consecutive sections of 10 µm thick were mounted on silane-coated slides and stored.

2.2. Immunohistochemistry

Immunostaining was performed on the same cerebral area (MFG) of SAD, FAD and CTL samples to facilitate comparison among cases. MFG is one of the significant and representative neocortical areas to be included in any Alzheimer's disease study both for research and diagnostic purposes (Mirra et al., 1991). The presence and the composition of amyloid deposits were assessed with specific antibodies against the C-terminal 40 or 42 of Aβ species (S40 and S42 polyclonal antibodies, kindly provided by Dr. Mikio Shoji, Okayama University, Okayama, Japan). The pattern of Aβ-degrading proteases expression was determined after antigen retrieval, by microwaving tissues in 0.01 M Tris-HCl, pH 8 for IDE, or by immersing sections 10 min in 100% formic acid followed by 20 min boiling in 10 mM citrate buffer, pH 6 for NEP. Anti-IDE 1C1 or 3A2 (Morelli et al., 2004) and anti-NEP (NCL-CD 10–270, Novocastra, UK) monoclonal antibodies were used. IDE specificity was corroborated in adjacent sections by using 9B12 anti-IDE monoclonal antibody (a generous gift of Richard Roth from Stanford University). Immunoreactivity was revealed with biotinylated anti-mouse IgG followed by incubation with avidin-biotin complex (Vector Laboratories, Burlingame, USA). The reaction product was visualized with 0.05% diaminobenzidine/0.01% hydrogen peroxide. Controls for IDE specificity included the omission of the primary antibody, the incubation of adjacent sections with mouse non-immune IgG, or with 1C1 or 3A2 pre-absorbed with recombinant IDE, as previously described (Morelli et al., 2003). Cellular localization of IDE and NEP by laser scanning confocal microscopy was performed by incubating 1C1 or anti-NEP antibodies alternatively combined with antibodies against specific antigens of astrocytes (anti-glial fibrillar acidic protein (GFAP, DakoCytomation, Glostrup, Denmark), microglial cells (anti-CD45R/B220 rat monoclonal antibody, RA3–6B2, BD Pharmingen, Milan, Italy) or phospho-tau (anti-phospho Tau Ser 422, Calbiochem, San Diego, USA). Brain vessels were stained with anti-human collagen IV IgG (DakoCytomation, Glostrup, Denmark) after antigen retrieval performed with proteinase K (0.025 mg/ml, Sigma, USA) for 20 min at 37 °C. Visualization of the immunoreactivity was performed with Cy3-labelled goat anti-rabbit IgG (GE Biosciences, Miami, USA) and biotinylated anti-mouse IgG followed by Cy5-labelled streptavidin.

2.3. Immuno-electron microscopy

A small formalin-fixed block of MFG from a SAD-BSVI subject was dehydrated in ethanol, embedded in LR Gold (Polysciences, Inc. Warrington, PA USA) and polymerized. After

thin sectioning (750–900 Å) samples were collected on carbon-formvar-coated nickel grids and processed using the immunogold technique. Briefly, grids were blocked with 0.02 M glycine (5 min), pre-treated 50 min with methanol at room temperature for antigen retrieval, blocked with PBS (containing 1% BSA, 5% NGS and 1% Triton-X100) and labeled with anti-IDE monoclonal (1C1) and anti-A β 40 polyclonal (S40) overnight at room temperature. After washing with PBS containing 0.02% Tween and 1% BSA, grids were incubated with a mixture of goat anti-mouse and anti-rabbit IgG (1:5) conjugated to colloidal gold (15 nm and 6 nm, respectively) (Electron Microscopy Sciences, Hatfield, PA, USA) for 2 h at room temperature. Control labeling was performed with an unrelated IgG instead of the primary antibody. After washing with PBS, containing 0.02% Tween and 1% BSA, and with water, sections were post-fixed with 2% PFA/2.5% glutaraldehyde, washed with water, and stained with 2% uranyl acetate. Sections were examined in a Zeiss 10C electron microscope.

2.4. Assessment of the white matter state

White matter perivascular space and myelin density were studied by Eriochromo-Cyanin R histological technique (Kiernan 2007). We determined the perivascular space diameter (PSD) as previously reported (Roher et al., 2003) in brain vessels whose diameter was larger than 1 mm according to this calculation: $PSD = DWM - DV$, where DWM is the external diameter of perivascular space and DV is the diameter of the within vessel.

Myelin sheaths morphology was assessed by myelin basic protein (MBP) immunohistochemistry with anti-MBP antibody (a generous gift from Dr. Anthony Campagnoni, Mental Retardation Research Center, University of California, Los Angeles, USA).

2.5. Image and statistical analysis

Immunostained brain sections were imaged with an Olympus BX50 microscope and immunoreactivity assessed using the Image-Pro Plus software (Media Cybernetics). Laser scanning confocal microscopy imaging was performed with a LSM5 Pascal microscope using a 3-frame filter and Zeiss LSM5 image examiner. To enhance contrast and sharpness, post-processing of images with Adobe Photoshop CS software was conducted using identical values for each image.

For each case from SAD and FAD groups, amyloid plaques positive for Thioflavin-S, A β 42, A β 40 and IDE, respectively, were analyzed in adjacent sections in at least three and up to nine fields (1 field = 0.5 mm²) and the number of immunolabeled deposits per unit area (mm²) was defined. In addition, the number of microvessels positive for collagen IV, Thioflavin-S, A β 40 and IDE respectively, in gray and white matter as well as in leptomeninges was analyzed and the number of immunolabeled deposits per unit area (mm²) was determined. Normality of the variables was assessed by the Shapiro-Wilk test and homogeneity of variances by Levene test. In all cases data were informed as mean $\pm$ standard deviation (S.D.) and median (min; max). IDE-positive plaques were defined as those stained with IDE that co-localized, in adjacent sections, with A β or Thioflavin-S-positive plaques. To compare the average number of IDE positive plaques between SAD and FAD groups the non-parametric Wilcoxon test was performed. To assess for differences in the number of microvessels stained with Thioflavin-S, anti-A β and anti-IDE between groups, the parametric two-tailed Student *t*-test or the non-parametric Mann-Whitney test was applied according with the results of the Shapiro-Wilk and Levene tests. The proportion of Thioflavin-S/collagen IV positive vessels was calculated for each case and the mean value was used to assess differences between SAD and FAD determined by the Mann-Whitney test. To evaluate differences in the perivascular space diameter (PSD) among SAD, FAD-GFAP(+); FAD-GFAP (–) and CTL the procedure was as follows: (1) we determined de

PSD value in vessels >1 mm of diameter in each subject; (2) we categorized the data according to the 4 groups studied (CTL, $n = 53$; SAD, $n = 43$; FAD-GFAP(+), $n = 37$; FAD-GFAP(−), $n = 45$); (3) we applied an un-balanced nested two-factor design (factor1: group; factor 2: number of vessels). Normality of the variables was assessed by the Shapiro-Wilk test and homogeneity of variances by the Barlett's test. A posteriori the Tukey multiple-comparison test was performed by using the R version 2.7.0 (2008-04-22) software (The R Foundation for Statistical Computing. ISBN 3-900051-07-0). Differences in the myelin sheaths thickness were determined by one-way analysis of variances (ANOVA) followed by the Tukey multiple-comparison test using the GraphPad software (GraphPad Software, San Diego, CA).

2.6. Isolation of A β from leptomeningeal vessels

Brain tissue from a subject with hereditary cerebral hemorrhage with HCHWA-D was used to isolate leptomeningeal vascular amyloid, as previously described (Castaño et al., 1996). We used this sample because HCHWA-D is characterized by extensive deposition of A β in the vessel walls of leptomeninges and cerebral cortex. Leptomeninges were dissected from brain coronal sections, cut with scissors into 1–3-mm pieces, and placed in 0.1 M Tris–HCl, pH 8, on ice. The tissue was then washed by resuspension in 0.1 M Tris–HCl, pH 8, containing protease inhibitors (buffer A) and centrifuged at $800 \times g$ for 5 min at 4 °C. The procedure was repeated five times. The material was collected by filtration through a 50- μ m nylon mesh (Spectrum), washed with buffer A without inhibitors, and resuspended in 20 volumes of 2 mM CaCl₂ in 0.1 M Tris–HCl, pH 7.5, containing 0.3 mg/ml collagenase CLS-3 (Worthington Biochemical Corporation, New Jersey, USA) and 10 μ g/ml DNase (Worthington Biochemical Corporation, New Jersey, USA). The mixture was incubated for 18 h at 37 °C. After digestion, the suspension was filtered through a 350 μ m nylon mesh and the filtrate was centrifuged at $10,000 \times g$ for 12 min. The pellet was resuspended in 2% SDS in 0.1 M Tris–HCl, pH 8, and incubated for 2 h at room temperature. The SDS-insoluble material was recovered by centrifugation as above and washed three times with distilled water. The pellet was dissolved in 10 volumes of 99% formic acid (Sigma, Saint Louis, USA) and incubated for 1 h at room temperature. After centrifugation at $10,000 \times g$ for 10 min, the supernatant was analyzed by SDS-PAGE and western blotting.

2.7. SDS-PAGE and western blotting

Amyloid isolated from leptomeninges was run on Tris–Tricine SDS-PAGE. For Western blotting analysis, proteins were transferred onto nitrocellulose membranes (Hybond ECL, GE Biosciences, Miami, USA) and incubated with anti-A β monoclonal antibody 6E10 (Signet Laboratories, Denham, USA) or anti-IDE polyclonal antibody BC2 (Morelli et al., 2004), respectively. Adsorption of BC2 with 20 μ g of recombinant IDE (Morelli et al., 2003) was performed to assess BC2 specificity. Immunoreactivity was detected with anti-mouse or anti-rabbit horseradish peroxidase-labeled IgG and ECL Plus (GE Biosciences, Miami, USA). Immunoblots were scanned with STORM 840 and analyzed with ImageQuant 5.1 software (GE Biosciences, Miami, USA).

3. Results

3.1. Topographic relationship between A β -degrading enzymes and senile plaques

Immunostaining with 1C1 showed that IDE expression was present in the cytoplasm of neurons throughout the cerebral cortex of CTL, FAD and SAD samples (Fig. 1A–C, insets) regardless of the age at death or the presence of amyloid deposits. In agreement with a previous report (Bernstein et al., 1999), we found that in FAD and SAD samples, 1C1 decorated structures in the cortical gray matter (Fig. 1 B–C) with a pattern similar to amyloid plaques and that were stained in adjacent sections with Thioflavin-S (Fig. 1D).

Similar results were obtained when sections were incubated with 3A2 (Fig. 1E) or 9B12 (Fig. 1F) that recognize different IDE epitopes than 1C1, reinforcing that *bona fide* endogenous IDE was being detected. To further assess the specificity of plaque-associated IDE immunoreactivity, adjacent sections were incubated with 1C1 or with normal mouse IgG, respectively. As it is shown in Fig. 1G, mouse non-immune IgG was unable to detect the amyloid plaques revealed with 1C1 (Fig. 1H). Immunoreactivity was not observed when slides were incubated with 1C1 or 3A2 pre-absorbed with recombinant IDE or when anti-IDE antibodies were skipped (not shown). In addition, MFG sections immunostained with anti-NEP showed specific immunoreactivity distributed in the cytoplasm of pyramidal neurons throughout the cerebral cortex in CTL, SAD and FAD cases (Fig. 1I–K, insets) as previously reported (Miners et al., 2006), and positive reaction associated to senile plaques of SAD cases (26 ± 5 positive plaques/mm²) (Fig. 1J) as described (Akiyama et al., 2001). However, we did not observe plaque-associated NEP reactivity in any of the FAD cases analyzed (Fig. 1K). Although there was intra-group subject variability, the number of IDE-positive plaques in the gray matter was significantly higher in SAD as compared to FAD (mean ranks 11.69 plaques/mm² vs. 5.31 plaques/mm², respectively. $W = 42.5$, $p < 0.01$, Wilcoxon test) (Fig. 2).

To further confirm IDE localization in the senile plaque at a ultrastructural level, we performed immuno gold electron microscopy on MFG sections from a SAD case. A representative image of an amyloid cored plaque, delimited in the picture by a dotted-line, is shown in Fig. 3A. A higher magnification of different portions of Fig. 3A (Fig. 3B and C) show clusters of colloidal gold particles of different sizes corresponding to IDE (15 nm; arrows) and A β (6 nm; arrowheads) decorating structures compatible with amyloid aggregates negatively stained with uranyl acetate. Gold particles are not seen outside the limits of the senile plaque, reinforcing specificity to the reaction.

3.2. Composition of A β deposits impact on IDE but not on NEP parenchymal expression

We have observed, in agreement with previous reports, that only a restricted number of senile plaques labeled with anti-A β were positive for IDE or NEP, respectively. This raised the possibility that the expression of these proteases in the senile plaque was related with the A β isoform composition of the deposits. The vascular or parenchymal origin of senile plaques was determined using specific polyclonal antibodies reactive for epitopes VGGVV or GVVIA of the A β 40 and A β 42, respectively. We found in the gray matter of SAD and FAD samples a sub-set of amyloid plaques positive for IDE which partially co-localized with A β 42 and A β 40 deposits (Fig. 4A–F). It is important to note that in all cases analyzed we only detected IDE in senile plaques containing A β 40 regardless of the presence or not of A β 42. After labeling of MFG adjacent sections with anti-NEP, S40 or S42 we found $10.7 \pm 2.5\%$ of the amyloid plaques in SAD cases were decorated with NEP regardless of the A β isoform (Fig. 4G–I). In the white matter we found that the totality of FAD and only three out of eight SAD cases showed positive deposits for A β 42 and A β 40. IDE immunoreactivity was only detected in one case from the SAD group with a mean value of 3 IDE positive plaques/mm². By contrast, we did not observe NEP immunoreactivity in the white matter of any AD cases.

3.3. IDE and NEP are expressed differentially when associated with amyloid angiopathy

To examine whether the extent of amyloid angiopathy in SAD and FAD cases could explain the differential deposition of IDE and NEP in the brain microvasculature we determined the number of vessels positive for Thioflavin-S, IDE or A β 40/mm². We did not detect statistical differences between groups as shown in Table 2. Moreover, no statistical significant differences were detected, by Mann–Whitney test, in the proportion of Thioflavin-S/collagen IV positive vessels from the gray matter (0.57 ± 0.28 vs. 0.45 ± 0.35 ; $U = 25$; $p =$

0.46) and leptomeninges (0.58 ± 0.27 vs. 0.52 ± 0.38 ; $U = 23$; $p = 0.89$). Representative images of cortical microvessels from gray matter of CTL, SAD and FAD cases stained with collagen IV are depicted in Fig. 5A–C. As expected, microvessels from SAD and FAD cases showed co-localization with Thioflavin-S (Fig. 5B and C). We did not find IDE accumulation at the light microscopy level in vessels from CTL subjects (Fig. 5D) while, in accordance with previous reports (Akiyama et al., 2001; Miners et al., 2006), a strong uninterrupted expression of NEP was observed in CTL tissues (Fig. 5E). In contrast to the CTL group, strong positive IDE reaction was detected in vessels of leptomeninges, gray and white matter from SAD (Fig. 5F–H) and FAD subjects (not shown) while we did not detect NEP immunoreactivity in vessels of AD cases. In agreement with the pattern of amyloid plaque-associated IDE, we detected vessels decorated with IDE exclusively when they were positive for A β 40. Furthermore, by using laser scanning confocal microscopy we confirmed the co-localization of IDE with A β 40 (Fig. 5I–K).

To assess for the presence of amyloid-associated IDE biochemically, we performed western blotting of amyloid extracted from a HCHWA-D case, known to have a massive accumulation of A β 40E22Q variant in leptomeningeal and cortical vessels (Castaño et al., 1996). The results revealed the presence of A β 40 (Fig. 6, left panel) and strong immunoreactivity for fragments of IDE (Fig. 6, right panel) ranging from 32 kDa to 68 kDa. The specificity of the reaction was demonstrated by the lack of reactivity when samples were incubated with BC2 pre-adsorbed with recombinant IDE.

3.4. IDE and NEP are over-expressed in astrocytes

For a better characterization of the cellular distribution of IDE and NEP, we analyzed their immunoreactivity together with markers of dystrophic neurites, astrocytes and microglial cells, respectively. Double immunolabelling using anti-NEP or anti-IDE together with anti-phospho Tau or anti-GFAP, respectively, showed co-localization of NEP, but not of IDE, with dystrophic neurites (Fig. 7A and B) and over-expression of both proteases in GFAP-positive astrocytes surrounding parenchymal amyloid deposits in the gray matter (Fig. 7C and D). Interestingly, while NEP reactivity was restricted to the soma of the astrocytes, IDE was distributed throughout the soma and processes. By contrast, we did not detect co-localization of IDE or NEP with microglial cells detected with anti-CD45 antibody (not shown). Next, we analyzed the occurrence of GFAP immunoreactive astrocytes in the white matter in each group. Our results showed that three out of eight cases from the FAD group presented GFAP positive astrocytes and all of them were also reactive for IDE (not shown) but not for NEP. Noteworthy, we did not observe GFAP immunoreactive astrocytes in the white matter of SAD cases. To find a possible relationship between the presence of IDE-immunoreactive astrocytes in the white matter and the myelination state, we explored differences in the perivascular space diameter (PSD) and in the myelin sheaths thickness of fibers localized in the interface between white and gray matter (known as U-fibers). Representative microphotographs of microvessels from the white matter and myelin sheaths, respectively, are depicted in Fig. 8. Since analysis of PSD showed that variances were not homogeneous (Bartlett's K-squared = 8.7219, d.f. = 3, $p < 0.05$) the variable was transformed as suggested (Box and Cox, 1964) (potency = 1/4). Statistics of the un-balanced two-factor design rejected the hypothesis of equality of the means among CTL, SAD, FAD-GFAP(+) and FAD-GFAP(–) ($F = 6.2782$; d.f. = 3, 72; $p < 0.01$) and support the notion that differences were not due to subject variability ($F = 1.7514$; d.f. = 17, 72; $p > 0.05$). A posteriori, the Tukey multiple-comparison test (99% CI) showed that there were significant differences between CTL and SAD ($p < 0.01$) and between CTL and FAD-GFAP(–) ($p < 0.01$), while no statistical significant differences were observed between CTL and FAD-GFAP(+) ($p = 0.25$). Moreover, the myelin sheaths thickness was significantly reduced in SAD (35.37 ± 3.5 ; $n = 43$) ($p < 0.001$); FAD-GFAP(+) (36.5 ± 8.3 ; $n = 37$) ($p < 0.001$) and

FAD-GFAP (-) (45.7 ± 8.5 ; $n = 45$) ($p < 0.001$) as compared to CTL (74.1 ± 18.7 ; $n = 53$). A summary of the statistics are shown in Fig. 8, inset. Our results suggest that there is a direct relationship between the enlargement of the PSD or the reduction of the myelin sheaths thickness and the presence of AD neuropathology. Noteworthy, FAD cases with GFAP (+) and IDE (+) astrocytes in the white matter did not show PSD enlargement as compared to CTL suggesting, as previously reported, a potential protective role of astrocytes to prevent white matter alterations.

4. Discussion

The present study provides a detailed analysis of cellular and amyloid-associated IDE and NEP immunoreactivity in SAD and FAD brains. We found striking differences in the number of plaques positive for IDE or NEP between demented patients with different etiologies of AD. The twofold increase in the number of IDE-containing plaques in SAD as compared to FAD cases and the absence of NEP in the plaques of FAD samples suggest that other factors different from amyloid deposition are involved in the expression and accumulation of these proteases in senile plaques. Although the experiments needed to explain the source of the heterogeneity on IDE or NEP expression between FAD and SAD cases are beyond the scope of this paper, we speculate that these differences are related to etiological and pathogenic differences between the groups under study. The reduced plaque-associated IDE accumulation in FAD may indicate that the time course of the disease is critical for the appearance of protease deposition. It was suggested that soluble A β 42 species are the first to seed the amyloid deposition at an early stage of SAD neuropathology which later may evolve to typical compact fibrillar A β 42 cored or non-cored plaques (Gravina et al., 1995; Güntert et al., 2006; Liu et al., 2006). Finally, in a late stage of plaque maturation, A β 40 isoform is deposited probably arising from inflammation triggered by glial activation or from vascular deposits (Sergeant et al., 2003; Delacourte et al., 2002). Here we describe in FAD and SAD cases vascular localization of IDE, exclusively associated to A β 40 immunoreactive vessels, and lack of NEP vascular reactivity in AD samples. It has been suggested that brain microvessels are actively involved in A β clearance (Roher et al., 2003). In this regard, the evidence that vascular IDE is co-localized with amyloid angiopathy and that NEP is absent in microvessels with amyloid deposits suggest that IDE may be initially over-expressed followed by aggregation and co-deposition with amyloid while NEP expression may be inhibited in the vascular wall. In both cases, these mechanisms concur to a down-regulation of A β -degrading activity. In addition, it was reported that the disruption of vessels may generate amyloid plaques positive for A β 40 (Cullen et al., 2006; Jaynes and Provias, 2006) suggesting a vascular origin of IDE exclusively associated with A β 40-positive plaques in the parenchyma.

We found IDE and NEP localization in cortical neurons from SAD, FAD and CTL groups with similar expression levels regardless of the presence of hyper-phosphorylated tau protein, suggesting that in neurons, A β -degrading proteases expression is not modified by the presence of neurofibrillary tangles. Moreover, our results are consistent with previous reports (Cook et al., 2003) and support the concept that neurons are unable to react against the accumulation of A β by over-producing IDE or NEP. Differences between IDE and NEP expression associated to dystrophic neurites may be partially explained by the different sub-cellular localization of both proteases in neurons: while NEP is mainly an axonal and synaptic membrane protein, IDE is largely located in the cytoplasm and partially secreted (Kuo et al., 1994; Fukami et al., 2002). In contrast, NEP reactivity was restricted to the soma while IDE was distributed in the soma and processes of GFAP immunoreactive astrocytes around A β 40/A β 42 amyloid plaques suggesting that both isoforms of A β are able to stimulate IDE and NEP over-expression by astrocytes as down-stream targets of the inflammatory cascades triggered by fibrillar A β . In agreement with previous observation in

Tg2576 mice (Leal et al., 2006; Apelt et al., 2003), microglial cells were not associated with IDE or NEP reactivity in human brain, suggesting that A β -degrading proteases over-expression by astrocytes may be considered a restricted part of an inflammatory response associated to the dynamic of either a neurodegenerative or neuroprotective process. Although we detected a small number of amyloid deposits in the white matter of SAD and FAD cases, we only found GFAP immunoreactive astrocytes in a sub-set of FAD cases, all of them positive for IDE and not for NEP. Taking into account that the presence of GFAP immunoreactive astrocytes in the white matter is considered a marker of mild and moderate level of SAD pathology (Brun and Englund, 1986) it is reasonable to think that SAD cases analyzed here lacked, due to a late stage of the disease, GFAP positive astrocytes. Noteworthy, it has been reported that the sub-set of FAD cases with GFAP immunoreactive astrocytes showed a lesser degree of pathology in the white matter as compared to the rest of AD cases studied (Sjöbeck et al., 2005). This raises the possibility that IDE over-expression may be linked to neuroprotective pathways involved in the promotion of cell survival and astroglial functional activation (Webster et al., 2006).

To better understand the possible role of IDE and NEP in the neurodegenerative process it is important to compare the neuropathological and biochemical findings in the AD brains with those in transgenic mice carrying human APP with pathogenic mutations. Despite an apparent overall structural resemblance of amyloid plaques from transgenic mice and AD patients, a detailed chemical analysis showed that amyloid from human plaques suffers post-translational modifications such as N-terminal degradation, isomerization, racemization and oxidation, usually attributed to long-lived proteins, and consistent with the time required for human amyloid plaque formation. By contrast, mice plaque amyloid deposits develop in a short period of time, A β peptides lack post-translational modifications and are substantially more soluble than human amyloid (Kuo et al., 2001; Kalback et al., 2002). Moreover, they lack insoluble IDE and NEP (Leal et al., 2006; Apelt et al., 2003). In this context, the accumulation of IDE and NEP associated to human amyloid deposits may reflect the protracted course of the neurodegenerative process in AD.

The presence of IDE in the Tg2576 mice vasculature is rare (Leal et al., 2006) as opposed to our finding in AD cases in the present study. This may also represent a time dependent process or alternatively may result from a differential cerebrovascular response between human and mice to A β accumulation. Finally, we were unable to detect astroglial reactivity in the white matter of young or aged Tg2576 mice (unpublished observation), suggesting that the mechanisms involved in cortical deposition of amyloid in the animal model are not connected with the white matter astroglial activation.

As a concluding remark we propose that regardless of the origin of amyloid-associated IDE, the fact that IDE may be found in the SAD brain as an aggregated protein, and therefore inactive, in a two-excess as compared to FAD cases, may explain the reduced activity of IDE reported in brain soluble fractions from SAD compared to healthy aged subjects (Perez et al., 2000). This result supports a potential mechanism implicated in the reduction of IDE activity in the human brain (Kim et al., 2007; Morelli et al., 2004) in which the conversion of a “soluble active” to an “aggregated inactive” protease unable to degrade A β may have a negative impact on SAD neuropathology.

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Fig. 1.

IDE and NEP distribution in the brain cortex of SAD, FAD and CTL subjects. Panels A–C, representative images of neuronal (inset) and parenchymal (arrow) IDE expression in CTL, FAD and SAD subjects, respectively, revealed with 1C1 antibody. Panel D, Thioflavin-S reactivity of an amyloid plaque (arrow) detected in the adjacent section (Panel C, arrow) with anti-IDE monoclonal antibody. Panels E and F, representative images of IDE reactivity (arrow), compatible with amyloid plaques, in a SAD case detected with 3A2 and 9B12, respectively. Panels G and H, adjacent sections labeled with mouse non-immune IgG and 1C1 antibody, respectively, in order to show 1C1 specificity. The positive plaque (Panel H, arrow) is not observed with mouse non-immune IgG (Panel G). Panels I–K, representative images of neuronal (inset) and parenchymal (arrow) NEP expression in CTL (Panel I), SAD (Panel J) and FAD (Panel K) subjects, respectively. Scale bars = Panels A–K, 70 μ m, Insets, 50 μ m. Arrows indicate the amyloid plaque-like pattern.

Group	N	Mean ± S.D.	Median (Min; Max)	Mean Rank ¹
FAD	8	3.6 ± 3.8	2.79 (0; 9)	5.31
SAD	8	11.5 ± 8.9	8.9 (5.75; 33.33)	11.69*

¹Wilcoxon test. W= 42.5; *, p<0.01

Fig. 2.

Differences in IDE in gray matter of SAD and FAD groups. The graph shows the mean value for the variable “number of IDE positive plaques/mm²” of each case grouped by type of disease. Inset, statistical parameters (mean, standard deviation (S.D.), median and rank (Min, Max) and mean rank) obtained for each group. Statistical significant differences were obtained between FAD and SAD group (*p < 0.05).

Fig. 3.

Ultrastructural localization of IDE and A β in the senile plaque. Panel A, double immunogold electron microscopy of a cored senile plaque (delimited by a dotted-line) with anti-IDE (15 nm particles) and anti-A β (6 nm particles). Portions (framed) of the senile plaque adjacent to the core (asterisk) are enlarged in Panels B and C. Panels B and C, IDE (arrows) and A β (arrowheads) immunoreactivity, respectively, are distributed within the plaque in association with fibrillar or non-fibrillar aggregates. Note that in several clusters, gold particles labeling A β and IDE co-localize. Asterisk, amyloid core. Scale bars = Panel A, 0.5 μ m; Panels B and C, 60 nm.

Fig. 4.

$A\beta$ isoforms impact on IDE but not on NEP plaque-association. Panels A–C, representative images of IDE immunoreactivity in gray matter of SAD (A–C) and FAD (D–F) cases. IDE is absent (Panels A and D, arrowhead) in $A\beta 42$ positive plaques without $A\beta 40$ (Panels B and E, arrowheads) and localizes (Panels A and D, arrow) exclusively at amyloid plaques positive for $A\beta 40$ (Panels C and F, arrows). Panels G–I, representative images of NEP, $A\beta 42$ and $A\beta 40$ immunoreactivity in the gray matter of a SAD case. NEP (Panel G) co-localizes with a sub-set of amyloid plaques positive for $A\beta 42$ (arrows) and its expression is independent of the presence of $A\beta 40$ (arrowhead). Microphotographs of IDE, NEP, $A\beta 42$ and $A\beta 40$ are images of the same region at adjacent sections. Scale bar = Panels A–I, 70 μ m.

Fig. 5.

Vessels from SAD and FAD cases show A β 40 associated-IDE but not NEP immunoreactivity. Panels A–C, representative images of laser scanning confocal microscopy show collagen IV immunoreactive vessels of gray matter from CTL subjects (Panel A) and amyloid angiopathy in FAD and SAD cases by co-localization with Thioflavin-S staining (yellow, Panels B and C respectively). Panels D and E, representative images of CTL cases show negative IDE immunoreactivity (Panel D) and positive NEP immunoreactivity (Panel E) in leptomeningeal (arrow) and gray matter (arrowhead) vessels. Panels F–H, representative images of IDE immunoreactivity at leptomeningeal, gray and white matter vessels, respectively, of a SAD subject. Panels I and J, laser scanning confocal microscopy of a leptomeningeal vessel immunoreactive for IDE (Panel I) and A β 40 (Panel J) showing co-localization (Panel K, purple) of both antigens. Scale bars = Panels A–C, 25 μ m; Panels D–H, 80 μ m; Panels I–K, 25 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 6.

IDE is associated to A β extracted from leptomeningeal vessels of a patient with hereditary cerebral hemorrhage with HCHWA-D. Amyloid preparation was run in 2 Tris/tricine SDS-PAGE gels of 15% and 7% to detect A β and IDE by western blotting, respectively. The membrane incubated with 6E10 (left panel) showed positive reaction compatible with monomers of A β and the membrane incubated with BC2 (right panel, -rIDE) detected IDE fragments which disappear when BC2 was pre-adsorbed with recombinant IDE (right panel, +rIDE), indicating specificity of the reaction. On the left, molecular markers in kilo Daltons (kDa).

Fig. 7. IDE and NEP are over-expressed in astrocytes from the gray matter. Panels A and B, representative images of laser scanning confocal microscopy showing phospho-tau reactive dystrophic neurites (red, arrows) positive for NEP (Panel A, pink, arrowhead), but not for IDE (Panel B, blue). Panels C and D, representative images of laser scanning confocal microscopy showing GFAP immunoreactive astrocytes (red) over-expressing NEP (Panel C, pink) or IDE (Panel D, pink) in the gray matter of a SAD case. Scale bars = Panels A–D, 50 μm. Asterisks, amyloid deposits localization. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 8.

Assessment of white matter state in control, SAD and FAD cases. Panels A, C and E, representative images of white matter stained with Eriochrome Cyanine R showing enlarged perivascular space diameter (PSD) in SAD (Panel C) and FAD (Panel E) as compared to CTL (Panel A). Panels B, D and F, representative images of MBP immunoreactivity of U-fibers from CTL (Panel B), SAD (Panel D) and FAD (Panel F) cases. White calibers show myelin sheath thickness. Scale bars = Panels A, C and E, 100 μm ; Panels B, D and F, 50 μm . Inset, statistical analysis of PSD and myelin sheath thickness of CTL SAD and FAD samples. *Significant increase vs. CTL; $p < 0.05$; ***Significant decrease vs. CTL; $p < 0.001$ analyzed by ANOVA and Tukey's multiple comparison test.

Table 1

Summary of clinico-pathological features of the autopsied cases of demented and control groups.

Group	Number of cases	Sex (M:F)	PMD (h)	AAD (years)	Period from AAO to AAD (years)
SAD	8	3:5	11 ± 7	84 ± 5	10 ± 4
FAD	8	3:5	10 ± 2	58 ± 9**	4.1 ± 2.7*
CTL	11	9:2	10 ± 5	84 ± 10	N.C.

AAD, age at death; AAD, age at onset; F, female; M, male; N.C., no corresponded; PMD, post-mortem delay.

* Significant decrease vs. SAD, $p < 0.0005$.

** Significant decrease vs. SAD, $p < 0.0001$.

Statistic analysis of the number of Thioflavin-S, A β 40 and IDE positive vessels from gray and white matter and leptomeninges of SAD and FAD groups.

Table 2

	Thioflavin-S (+) vessels/mm ² , mean $\pm$ S.D.			A β 40 (+) vessels/mm ² , mean $\pm$ S.D.			IDE (+) vessels/mm ² , mean $\pm$ S.D.		
	GM	WM	LM	GM	WM	LM	GM	WM	LM
SAD	6.0 $\pm$ 4.55	0.125 $\pm$ 0.35	9.55 $\pm$ 4.63	10.49 $\pm$ 6.26	3.92 $\pm$ 2.69	9.09 $\pm$ 3.47	5.14 $\pm$ 2.19	3.68 $\pm$ 2.86	9.0 $\pm$ 3.48
FAD	5.94 $\pm$ 8.47	0.56 $\pm$ 1.116	12.60 $\pm$ 11.41	6.62 $\pm$ 3.34	3.64 $\pm$ 0.88	13.01 $\pm$ 5.72	7.87 $\pm$ 3.67	3.53 $\pm$ 0.96	12.24 $\pm$ 5.39
<i>p</i>	0.49 ϕ	0.441 ϕ	0.55 $\#$	0.163 $\#$	0.628 ϕ	0.148 $\#$	0.105 $\#$	0.401 ϕ	0.207

Tf-S, Thioflavin-S; GM, gray matter; WM, white matter; LM, leptomeninges.

p, value of the significance of the comparison between SAD and FAD in each region analyzed by Student's ($\#$) or Mann-Whitney (ϕ) test.