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Colocalization of cholinesterases with β amyloid protein in aged and Alzheimer's brains*

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Summary. The colocalization of β amyloid protein with the enzymes acetyl- and butyrylcholinesterase was assessed using immunocytochemistry for β amyloid protein and a sensitive histochemical technique for cholinesterases. In non-demented aged and Alzheimer's disease brains, double-stained sections for cholinesterases and thioflavin-S showed that all thioflavin-S-positive plaques were also positive for cholinesterases, indicating the presence of these enzymes in all plaques with β-pleated amyloid protein. When amyloid angiopathy was present, cholinesterases were also observed in amyloid-laden vessels walls. Comparison of series of adjacent sections alternatively stained for acetylcholinesterase, β amyloid protein and butyrylcholinesterase, as well as by double histo-immunocytochemical staining, showed either cholinesterase in a proportion of the preamyloid diffuse plaques. These data indicate that cholinesterases are associated with the amyloid protein from very early stages, when the β-pleated structure is being formed. Novel functions attributed to acetyl- and butyrylcholinesterase, such us their proteolytic activity either by themselves or in association with heparan sulfate proteoglycans, may play a role in the aggregation or the consolidation processes taking place at the early stages of diffuse plaque formation.

Key words: β Amyloid – Acetylcholinesterase – Butyrylcholinesterase – Diffuse plaques – Preamyloid deposits

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Despite recent advances in our understanding of the β amyloid protein (AβP) at the molecular and functional levels (for reviews see [33, 53]), the role that this protein plays in Alzheimer's disease (AD) and aging remains unclear. In senile plaques (SP) the pathologically accumulated AβP colocalizes with the serine protease inhibitor alfa-1 antichymotrypsin, in normal aged human and monkey brains and in disorders associating solely these AβP deposits, such as AD [1–3]. AβP is immunologically labelled by heparan sulfate proteoglycans, which are structurally integrated within the characteristic lesions of AD and Down's syndrome [58–60], as well as by dermatan sulfate proteoglycans [61]. In addition, AβP is associated with serum amyloid P component [17, 19], chromogranin A [45], complement factors C1q, C3c and C3d [20, 21, 25, 41, 51], and protein kinase C [38].

Other proteins found within SP include the enzymes acetylcholinesterase (EC 3.1.1.7; AChE) and butyrylcholinesterase (EC 3.1.1.8; BChE) [10, 12, 22, 23, 32, 40, 42–44, 49, 50, 62, 65, 66]. Moreover, the global levels of AChE and BChE, as well as the distribution of their molecular forms, are severely disturbed in AD as compared with normal aged human brains [6, 7, 46, 47, 54, 71]. However, the extensive spread of cholinesterase-positive SP, beyond areas receiving massive cholinergic projections, strongly suggests that the role of AChE (an of the less-well-studied BChE) in SP formation is wider than just acting upon acetylcholine, once thought to be its sole natural substrate [16]. In fact, AChE and BChE might have important functions in addition to their classical involvement as cholinergic enzymes [8, 15, 18, 24, 34–37, 55, 56]. In relationship with the accumulation of AβP in SP, the proposed protease activity of cholinesterases is specially interesting, since it might contribute to the release of the membrane-bound form of the amlyoid precursor protein in aging and AD [57].

In this study the colocalization of both AChE and BChE with AβP is addressed, using immunocytochemical techniques for labelling AβP-positive SP and histochemical techniques for cholinesterases. Special attention will be directed towards the early formed diffuse plaques (DP), reffered to as preamyloid diffuse plaques

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(PDP) [63, 70], where the consolidation of the pathological events related to the evolution of SP may be taking place. Some possible interpretations of the observed results will be discussed.

Materials and methods

Tissue preparation

Tissue from the substantia innominata, cerebellum, hippocampal and parahippocampal regions, as well as temporo-polar, insular, motor and parietal cortex, from nine cases of AD (mean age 81 ± 8.5 years) and four neurologically and clinically intact cases (mean age 83 ± 2.3 years) were obtained at autopsy (postmortem delay 2–9 h). Blocks of tissue (1–1.5 cm) were fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 26–52 h at 4 °C, cryoprotected in graded concentrations of sucrose (10–40 %) in 0.1 M phosphate buffer, and cut on a freezing microtome at 40 μm. The neuropathological criteria used to define AD adhered to the NIH/ADRDA age-adjusted criteria [29] based on sections processed for thioflavin-S (Thio-S) and Bielschowsky silver stains.

Cholinesterase histochemistry

Esterase histochemistry was performed following a modified Karnovsky-Roots technique [28] as previously described [42, 43]. Briefly, cholinesterase staining was obtained by incubating free-floating sections at room temperature for 4 h in a solution (pH 6.8) containing either acetylthiocholine or butyrylthiocholine iodide (Sigma) as a substrate. The reaction product was visualized with diaminobenzidine [64], and intensified with cobalt chloride [4]. As a control, adjacent sections were reacted under the same conditions, eliminating either substrate. Sequential sections to those reacted for either AChE or BChE histochemistry were stained for Thio-S to compare the pattern of labelling. In addition, following the histochemistry for AChE or BChE, selected sections were counterstained with Thio-S.

β-protein immunocytochemistry

Immunocytochemistry using the monoclonal antibody 4G8 (IgG 2b) raised against a synthetic peptide corresponding to amino acids 1–24 of AβP [30] was performed using the biotin-extravidin-phosphatase alkaline method. To enhance the immunoreactivity of amyloid expressing profiles most sections were pretreated with 88 % formic acid (Merck) for 20 min at room temperature [31]. However, selected sections were not pretreated with formic acid to preserve the AChE or BChE histochemical activity in tissue concurrently stained for any of the esterases and the amyloid protein. Endogenous peroxidase was inactivated by incubating sections in a solution of 0.3 % hydrogen peroxidase in methanol for 30 min. The sections were then incubated in 3 % normal rabit serum in phosphate-buffered saline (PBS) with 0.1 % Triton X-100 for 1 h at 4 °C, followed by the primary antiserum (1:1000) overnight at 4 °C. Sections were then incubated in a biotinylated secondary antibody (anti-mouse IgG prepared in rabbit; Biomakor, 1:1000) at room temperature for 1 h, followed by at least 30 min at room temperature in extravidin alkaline phosphatase (Biomakor 1:1000). Fast red was used as a chromogen for revealing the alkaline phosphatase. Control sections were incubated in the vehicle solution without the primary antibody to evaluate the specificity of the staining produced by 4G8.

To compare the pattern of both esterases with that of the amyloid protein, three adjacent series of sections from five AD and three non-demented aged cases were stained: one for AChE, the middle one for β-protein (with formic acid pretreatment) and the last one for BChE.

Double histo-immunocytochemical procedure

Cholinesterase histochemistry followed by AβP immunocytochemistry. In ten cases (six AD and four non-demented aged brains) selected sections were first histochemically reacted for either AChE or BChE. Following histochemistry, sections were wet mounted onto glass slides, the areas of interest were photographed and their locations recorded using the XY coordinates of the microscope stage. A second glass slide was then placed under the slide containing the wet mounted section to outline its border. Sections were then removed from the slide and pretreated with formic acid solution (see above), prior to anti-AβP free-floating immunocytochemistry. Following the immunostaining, the sections were again wet mounted onto the glass slides using the previously drawn section outline for tissue placement and rephotographed.

Some additional sections were histochemically stained for either AChE or BChE using a tenfold greater concentration of substrate and increasing the incubation time to 22 h, followed by AβP immunocytochemistry with formic acid pretreatment.

AβP immunocytochemistry followed by cholinesterase histochemistry. In two AD and one non-demented aged brains, additional sections were concurrently stained for anti-AβP without formic acid pretreatment and then for either AChE or BChE.

Controls for the specificity of these techniques consisted of double-stained sections which omitted eiher the substrate for the appropriate esterase or the primary antibody or both.

Results

In the present study the term DP refers to all AβP-positive deposits without dystrophic neurites [63] and includes both the preamyloid Thio-S-negative accumulations (PDP) and the amyloid Thio-S-positive deposits [67].

The spectrum of morphological appearance of amyloid deposits found in our material closely corresponds to that already described for the 4G8 antibody [69].

In our results both the halo and the core of classical plaques showing a central core were consistently positive for both cholinesterases (Fig. 1a, b). The surrounding rim of the central core was most intensely stained than its inner region (not shown in the figures). The same was true for the isolated core of the "burned-out" plaques (Fig. 1a). In neuritic plaques, the amyloid component was consistently positive for both cholinesterases, whereas the neuritic component was only positive for AChE and/or BChE in some cases (Fig. 1b). DP, including common diffuse parenchimal plaques (Fig. 1c), cerebellar, presubicular plaques (Fig. 1e), ribbon-like subpial deposits (Fig. 1f), DP in the substantia innominata (Fig. 1g) and diffuse deposits surrounding blood vessels (Fig. 1h) were all positive for cholinesterases, except for Thio-S-negative PDP, which were only occasionally positive for either AChE or BChE, as demonstrated in adjacent sections stained for Thio-S and either cholinesterase.

Similar results were obtained in series of three adjacent sections stained for either: (a) AChE, (b) AβP

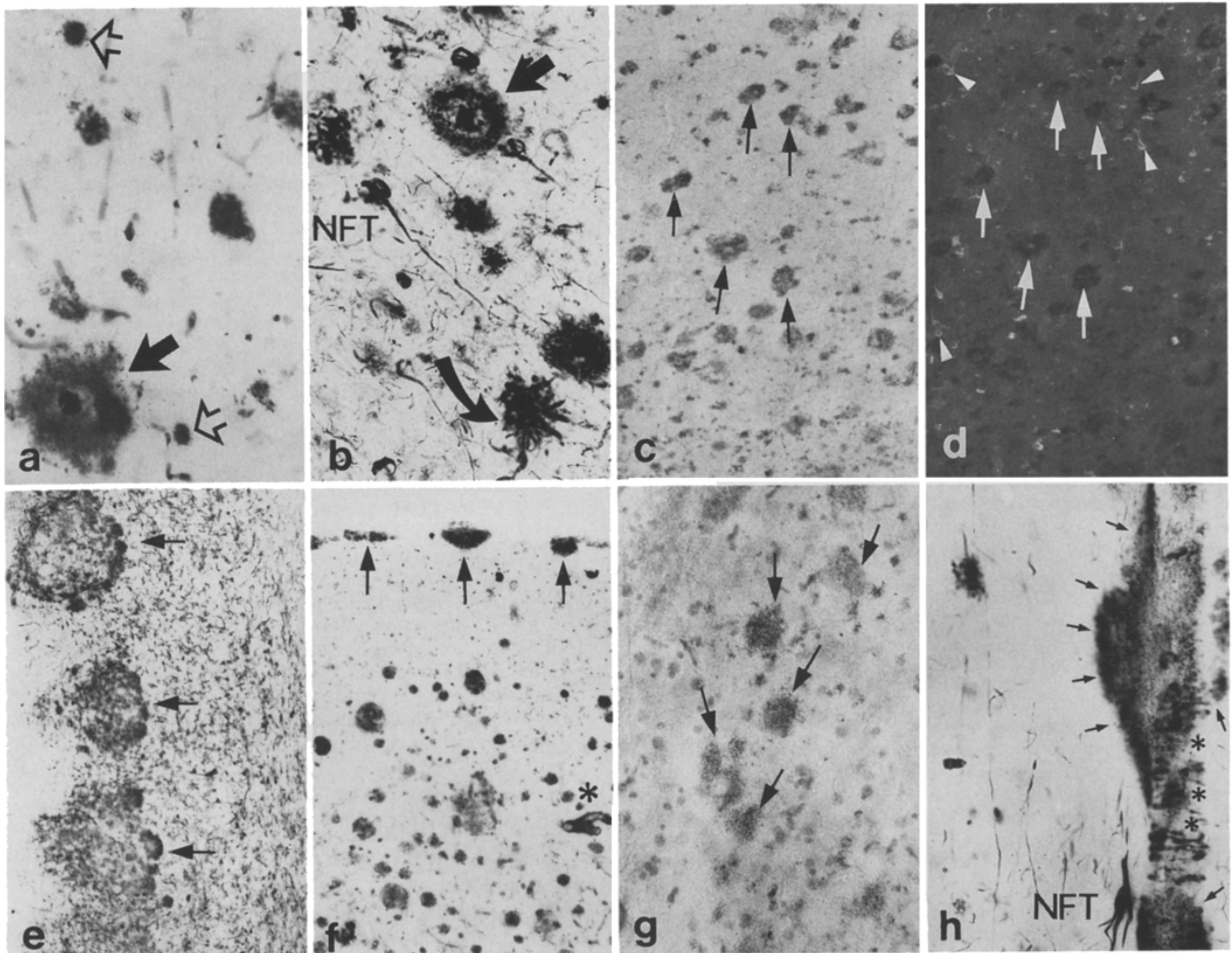
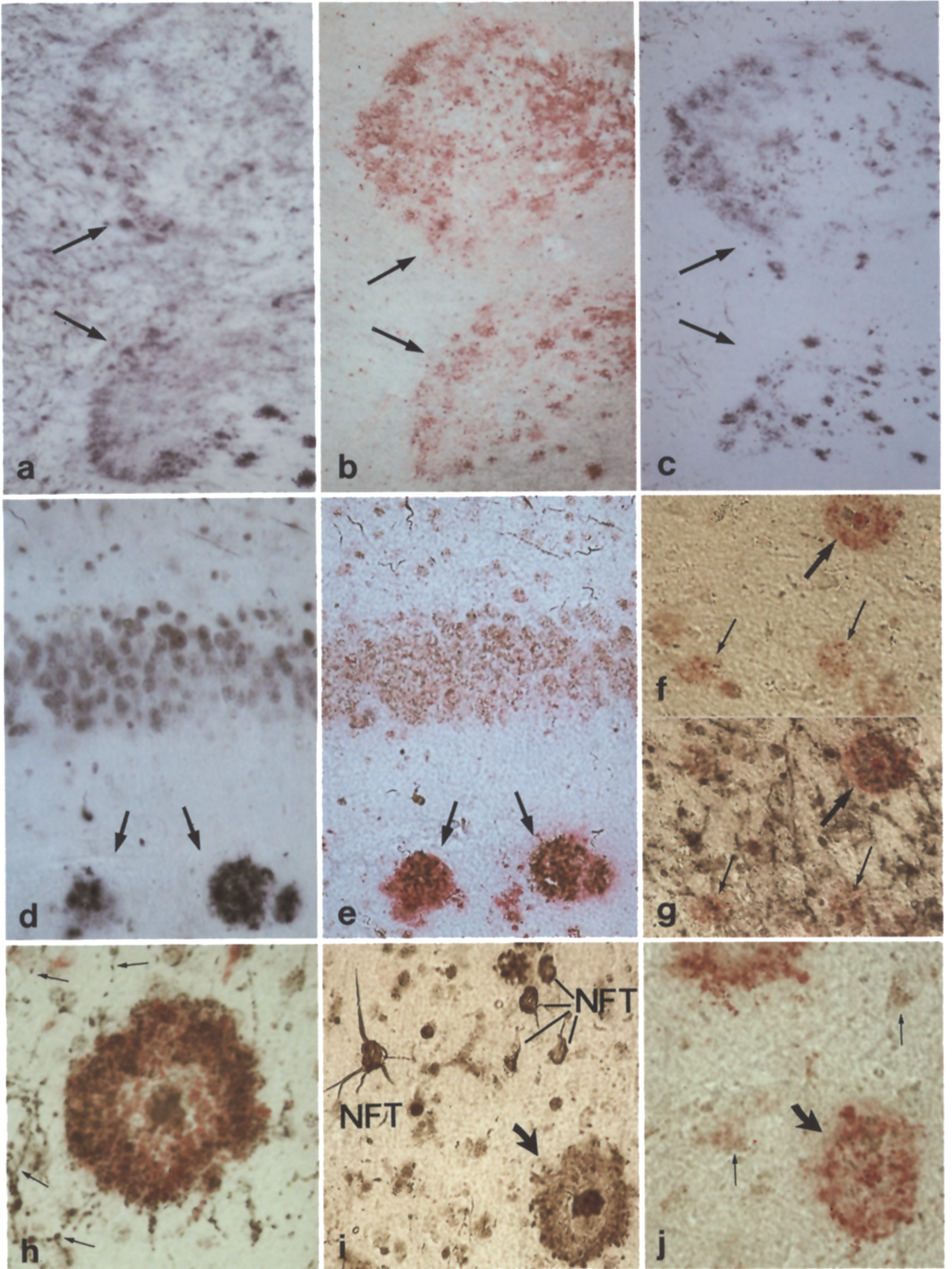


Fig. 1a–h. Cholinesterase histochemistry of senile plaques. **a** Temporal cortex from a non-demented aged brain showing butyrylcholinesterase (BChE) histochemical staining of a classical plaque with a central core (*solid arrow*), and two "burned-out" plaques (*open arrows*); ×100. **b** Temporal cortex from an Alzheimer's disease (AD) brain showing an acetylcholinesterase (AChE)-positive classical plaque with a central core (*straight arrow*) and a neuritic plaque with AChE-positive neuritic component (curved arrow). Neurofibrillary tangles (NFT) and neuropil threads are also AChE positive; ×80. **c** Section from the temporo-polar cortex of an AD brain double-stained for BChE and Thioflavin-S observed with bright field illumination. *Arrows* indicate diffuse plaques with BChE reaction product; ×25. **d** Same section and same field as in **c**, photographed with ultraviolet light. BChE-positive plaques do not show any fluorescence. Many neurofibrillary tangles are only Thioflavin-S positive (*arrowheads*); ×25. **e** AChE-positive accumulations of diffuse plaques in the presubiculum (*arrows*) in the brain of a non-demented aged patient. Some AChE-positive neurons and a dense plexus of AChE-positive fibers are observed in deeper layers; ×10. **f** AChE-positive diffuse subpial deposits (*arrows*) in temporopolar cortex of an AD brain. A blood-vessel (*asterisk*) and abundant AChE-positive senile plaques are also present in deeper layers; ×20. **g** BChE-positive diffuse plaques in the substantia innominata (*arrows*) in the brain of a non-demented aged patient; ×70. **h** BChE-positive diffuse deposits surrounding a longitudinally sectioned blood vessel (*arrows*) in a non-demented aged brain. The vessel wall is also BChE-positive (*asterisks*). NFT and numerous neuropil threads are also BChE-positive; ×60

Fig. 2a–c. Three adjacent sections from the presubiculum of an AD brain showing two accumulations of diffuse plaques (*arrows*). **a** AChE histochemistry, **b** β amyloid protein (AβP) immunocytochemistry, **c** BChE histochemistry. Layer 1 is towards the right. **a–c** ×25. **d, e** Hippocampus from an AD brain. The same section was first reacted for BChE (**d**) followed by AβP immunocytochemistry (**e**). Two senile plaques (*arrows*) are positive for BChE (**d**) and double labeled for BChE and AβP immunocytochemistry (**e**). **d, e** ×60. **f, g** Insular cortex from a non-demented aged brain. The same section was reacted for AβP immunocytochemistry (**f**) followed by AChE histochemistry (**g**). A classical plaque with a central core (*thick arrow*) and two diffuse plaques (*thin arrows*) appear labeled for AβP immunocytochemistry (**f**) and double labeled for AβP and AChE histochemistry (**g**). **g, f** ×70. **h** A classical plaque with a central core from the insular cortex of an AD brain double stained for AβP immunocytochemistry (*red*) followed by AChE histochemistry (*brown*). *Arrows* point to brown varicose, exclusively AChE positive, fibers; ×80. **i** Layer III of temporopolar cortex from an AD brain double stained for AβP immunocytochemistry without primary antibody followed by AChE histochemistry. Only the *brown* reaction product of AChE is observed in NFT and a classical plaque with a strongly positive central core (*arrow*); ×70. **j** Insular cortex from a non-demented aged brain double stained for AβP immunocytochemistry followed by AChE histochemistry without substrate. Only the *red* precipitate of AβP immunocytochemistry is observed in senile plaques. *Thick arrow indicates* classical plaque with a central core. *Thin arrows indicate* lipofuscin granules in neuronal perykaria; ×110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or (c) BChE. Again with this approach the number and distribution of all types of SP were very similar, except for PDP. In those cases in which PDP were abundant, more diffuse deposits were positive for AβP than for either cholinesterase (Fig. 2a–c).

In sections double-stained for cholinesterase and Thio-S, all SP fluorescence was nearly covered by the cholinesterase precipitate (Fig. 1c, d). When neuritic plaques were double stained, some dystrophic neurites appeared fluorescent even after increasing the amount of substrate and the incubation time.

In those cases with amyloid angiopathy some blood vessel walls exhibited cholinesterase-positive ring-like accumulations (Fig. 1h) covering the fluorescent amyloid aggregations revealed by Thio-S.

Double histo-immunocytochemical procedure

Cholinesterase histochemistry followed by AβP immunocytochemistry. Histochemically single-stained sections revealed intense cholinesterase reaction product with its usual dark-blue color (Fig. 2d). However, after concurrent immunocytochemical incubation, the cholinesterase-reaction product appeared light-brown in color (compare Fig. 2d, e). A similar change was observed following the first 5 min of formic acid pretreatment. Thus, it was difficult to distinguish the red color of AβP from the light-brown color of cholinesterase reaction product in the double-stained sections (Fig. 2e). However, by comparing the same plaque photographed before and after the AβP immunocytochemistry (Fig. 2d, e), it was possible to determine that a great proportion of SP were double stained. In those cases with abundant PDP, even after a very long incubation period (22 h) and a tenfold increase in substrate concentration, the cholinesterase histochemistry revealed only a proportion of AβP-positive DP (compare Fig. 3a, b). In addition, careful analysis of individual plaques showed that in many the cholinesterase precipitate occupied a smaller area than the AβP immunostaining (Fig. 2d, e).

AβP immunocytochemistry followed by cholinesterase histochemistry. When pretreatment with formic acid was included in the immunocytochemistry protocol no cholinesterase precipitate was observed in double-stained sections. Since the pretreatment was found to be responsible for this fact, we omitted formic acid pretreatment when AβP immunocytochemistry was followed by cholinesterase histochemistry. Immediately after the double protocol, the cholinesterase reaction product exhibited the usual dark-blue color, but this appeared brown after covering the sections with the water-soluble mounting medium (Apathy; Fig. 2g, h). In these double-stained sections the majority of SP were simultaneously AβP and AChE positive.

The specificity of the double procedures described above was always confirmed in control sections omitting either the antibody (Fig. 2i), the cholinesterase substrate (Fig. 2j), or both.

Discussion

The present results showed a consistent, very clear staining for both AChE and BChE in all types of SP, and in blood vesels with amyloid angiopathy (Fig. 1), extending our previous observations [23, 42, 43]. Other authors have also found that neuritic and classical plaques accumulate cholinesterases within degenerating neurites [10, 22, 32, 40, 49, 50, 62, 65]. To focus our study on the actual relationship between cholinesterases and amyloid, avoiding any confusion due to neuritic staining within the same plaques, the present study was addressed to brain regions, such as presubiculum, substantia innominata and cerebellum, where DP are commonly found in the absence of neuritic plaques [26]. Staining of adjacent sections with either of the cholinesterases or with AβP showed a close correspondence of labelling for adjacent populations of SP (Fig. 2a–c). Following several combinations of sequential immunocytochemical and histochemical protocols on the same sections, all types of parenchimal and perivascular deposits of AβP were shown to colocalize with either AChE or BChE (Fig. 2d–h). Our results are in close agreement with those obtained by Ulrich et al. [66], who also observed the same morphological spectrum of plaques in sections processed for AChE and those immunostained for AβP protein. However, there is some discrepancy between both studies arising from the criteria used for classifying the plaques considered as preamyloid accumulations (preplaques in their terminology). This is a difficult classification that needs to be addressed following not only morphological criteria but also other characteristics of the plaques such as their affinity for Thio-S, which distinguishes preamyloid from amyloid accumulations [63, 67].

Most plaques showed accumulations of cholinesterases. With regard to PDP, some plaques were also stained for AChE or BChE, while others were cholinesterase negative, even after increasing the incubation time and the amount of substrate tenfold (Fig. 3a, b). This suggests that PDP may accumulate cholinesterases while AβP fibrils are forming. In support of this possibility, double-staining techniques for cholinesterases and Thio-S revealed that all fluorescent SP were positive for AChE or BChE (Fig. 1c, d), which indicates that the association between cholinesterases and AβP is already established by the time the β-pleated configuration of the amyloid fibrils is formed. The fact that AβP and cholinesterases do not completely colocalize in PDP may indicate that, in the gradual transformation of the accumulated AβP into Thio-S-positive fibrils, cholinesterases need to accumulate to a certain concentration before being histochemically detected.

Studies on the differences between PDP and other types of SP have shown a gradual involvement of proteases and protease inhibitors during the first stages of SP evolution [1, 13, 52, 53, 57]. Cholinesterases form part of the β amyloid-associated proteins recently identified (and to be found [53]) in SP. As for most of these proteins novel functions have been proposed for cholinesterases [8, 15, 18, 24, 34–37, 55, 56] with special

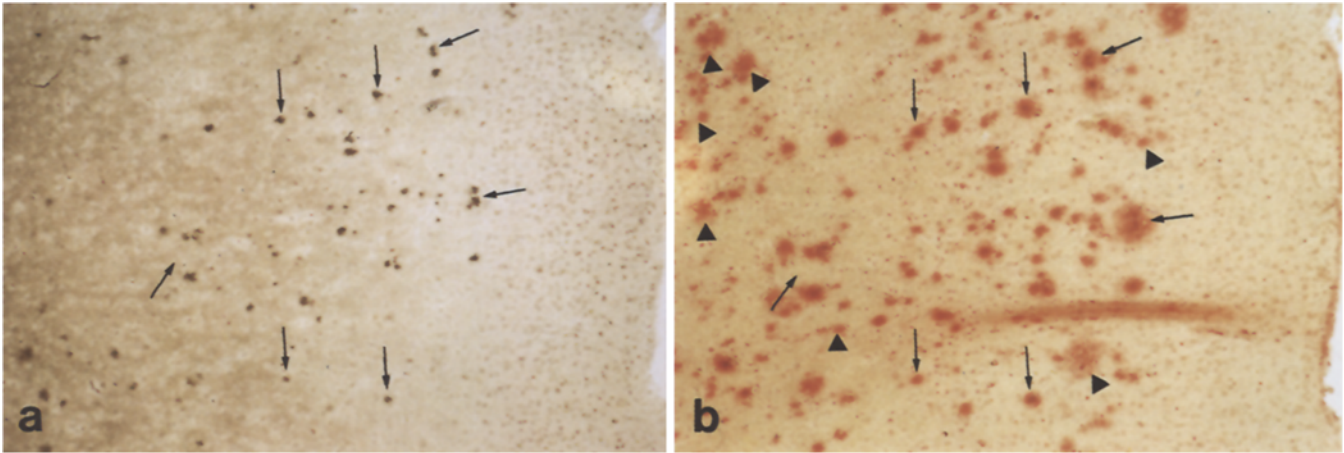

Fig. 3a, b. Temporopolar cortex from a non-demented aged brain. The same section was incubated for 22 h with tenfold amount of BChE substrate (**a**) followed by AβP immunocytochemistry (**b**). In **b,** some plaques are positive for both BChE and AβP immunocytochemistry (*arrows*), whereas other plaques are only AβP positive (*arrowheads*). **a, b** ×10

attention directed to their ability to hydrolyze peptides [14, 16, 18, 37, 55, 56]. Recent studies point out the possibility that this proteolytic ability of cholinesterases may not be intrinsic, but due to a 25-kDa cholinesterase-associated protease [5, 11]. In any case, as suggested by Small et al. [57], either cholinesterases by themselves or the 25-kDa protease associated with them may play a role in the cascade of proteases which results in the release of the membrane-bound form of the amyloid precursor protein. If this is the case, cholinesterases should be present in the very first moments of AβP accumulation. It could be that our histochemical method is unable to detect them until certain levels are reached. However, it seems unlikely that the intrinsic (or associated) cholinesterase protease activity not only contributes to the aggregation of AβP, by specifically acting on the amyloid precursor protein, but also explains the formation of paired helical and straight filaments in which cholinesterases are also localized [23, 42]. An additional possibility for interpreting the observed colocalization of cholinesterases with AβP, is that these enzymes may stabilize the AβP aggregates, once they are formed in PDP. In support of this second possibility, which seems to be more compatible with a parallel accumulation of these enzymes in paired helical and straight filaments, is the proposed interaction of cholinesterases with proteoglycans, as well as with other components of the basement membrane [9, 39, 68].

In AD, the activity of both the A12 and A8 asymmetric forms of AChE is significantly increased (342 % and 406 %, respectively) [71]. In addition, the asymmetric forms seem to predominate in SP [46]. It has been proposed that the A12 form may be anchored to heparan sulfate proteoglycans and other components of the basement membrane through its collagen tail [39]. Indeed, heparan sulfate proteoglycans have been shown to be present in PDP [60]. Both heparan sulfate proteoglycans and cholinesterases are detected at early stages of the plaque development, before the β-pleated amyloid configuration is formed. Moreover, since proteoglycans appear associated with AβP and with paired helical and straight filaments, an active role has been proposed for them in the accumulation and consolidation of all these insoluble structures [59]. Part of this active role might be due to their binding to cholinesterases, in addition to other proteins, such as serum amyloid P component [27] and basic fibroblast growth factor [48].

Although a large amount of new data has been reported dealing with the early stages of the AD pathology, their integration in a global context is far from clear. In this context, if cholinesterases do play a role in these early stages, in addition to their implications as severely altered cholinergic enzymes, a careful and open minded study may be worthwhile. Whether the presence of AChE and BChE in SP and neurofibrillary degeneration could lead to new therapeutical strategies by preventing the consolidation of these cytopathological features remains to be elucidated.

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