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Electron microscopic study of paired helical filaments and cerebral amyloid using a novel en bloc silver staining method *

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Summary. A one step en bloc silver staining method which was originally established to study nucleolar organizer regions has been applied for the demonstration of both paired helical filaments (PHF) and extracellular cerebral amyloids in semi-thin sections and at the electron microscopic level. The three forms of PHF can be visualized: (1) neurofibrillary tangles are shown in all stages from first appearance in form of intracellular patches of PHF to severely degenerated shadow-like "ghost" tangles; (2) neuropil threads are distinctly stained in great numbers; and (3) PHF are easily detected as neuritic components in amyloid plaques. All forms of fibrillar extracellular amyloid structures, i.e. "diffuse", "classical" and "burnt out" plaques, are well demonstrated; congophilic angiopathy reveals amyloid preferentially in arteries and arterioles of the leptomeninges and cortex ranging from small circumscribed patches to large circumferential amounts with occasional plaque-like condensations or broad loose accumulations of amyloid; perivascular cuffs and laminar subpial deposits of amyloid are stained as well. At the electron microscopic level all lesions are clearly visible in non uranyl/lead-stained specimens, characterized by varying numbers of silver grains on a pale background. The detailed demonstration of structures in archival material, which had been stored in paraffin and re-embedded for electron microscopy, is due to the demonstration of argyrophilic structures by the protective colloidal developer of gelatin and formic acid and to the proteolytic resistance of insoluble PHF and extracellular amyloids in plaques and congophilic angiopathy.

Key words: Silver staining – Paired helical filaments – Cerebral amyloid – Electron microscopy

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Introduction

Novel silver staining methods are used at the light microscopical level for a variably selective demonstration of the characteristic lesions in senile dementia of Alzheimer type (SDAT), i.e. neurofibrillary tangles (NFT), neuropil threads (NT) and neuritic plaques (Gallyas 1971; Cross 1982; Gallyas and Wolff 1986), and for extracellular amyloid such as diffuse (Yamaguchi et al. 1990b) and other forms of amyloid plaques (Campbell et al. 1987; Probst et al. 1991). Electron microscopy has revealed NFT (Kidd 1963, 1964; Wisniewski et al. 1976; Miyakawa et al. 1989) and NT as paired helical filaments (PHF) (Braak et al. 1986; Yamaguchi et al. 1990a) and the fibrillar structure of amyloid has long been identified (Terry et al. 1964; Schlote 1965; Miyakawa et al. 1986). The molecular basis for amyloid has been shown to be a small protein with a 42,43 amino acid sequence, independently designated as β- (pleated sheet) protein in cerebral vessels (Glenner and Wong 1984) and as amyloid-A4 (4.2 kDa) protein in plaque cores (Masters et al. 1985). Sensitive immunostaining demonstrated the wide distribution of amyloid-βA4-protein in the brains of patients with Alzheimer's disease (Davies et al. 1988; Ogomori et al. 1989).

Recently we described a new procedure for a simple and effective demonstration of NFT and cerebral amyloids in paraffin sections (Reusche 1991). Here we confirm and extend our results by using this silver staining for the demonstration of both PHF and extracellular cerebral amyloids at the electron microscopic level.

Materials and methods

We selected a total of eight brains, six with SDAT and two with congophilic angiopathy, four of which came from our recently described autopsy series (Reusche 1991). Small pieces of paraffin-embedded tissue were taken from regions where light microscopy had revealed severe morphological changes. The samples were deparaffinized using xylene and rehydrated in a graded ethanol series.

The silver staining procedure was performed according to Ploton et al. (1982) with slight modifications: deparaffinized and re-

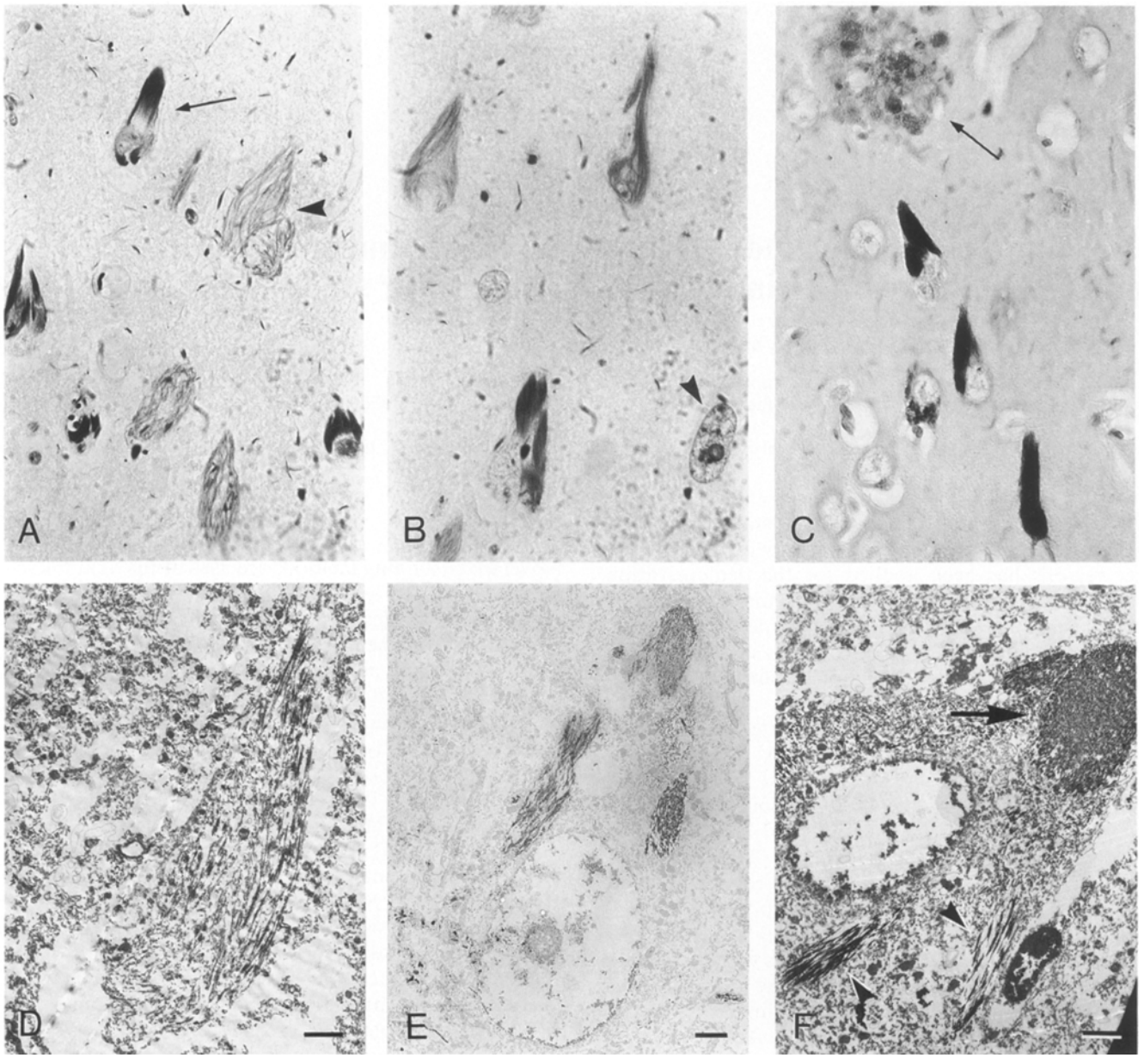

Fig. 1 A–F. Paired helical filaments (PHF) in form of neurofibillary tangles (NFT). **A** NFT (*arrow*) and severely degenerated "ghost" tangles (*arrowhead*); semi-thin section, ×680. **B** Intact neuron (*arrow*) with completely unstained cytoplasm; semi-thin, ×920. **C** Tau-immunoreaction with NFT and neuritic plaque (*arrow*); paraffin, ×480. **D** Ghost tangle; ultra-thin section, ×3000. **E** NFT in non uranyl acetate/lead citrate-stained ultra-thin section, ×2300. **F** Neuron with patches (*arrowhead*) and closely packed (*arrow*) PHF as NFT; ultra-thin, ×3100, *bar* = 2 μm

hydrated specimens were cut in slices of about 0.5 mm, then rinsed in phosphate buffer solution (PBS) overnight at 4° C and immersed in 1.6% phosphate-buffered glutaraldehyde (pH 7.2) for 10 min. Slices were rinsed in PBS, post-fixed in standard Carnoy's solution, rehydrated in a graded ethanol series (each step for 1 h) and silver stained en bloc. The staining solution consisted of one volume of 2% gelatin and 1% formic acid and two volumes of a 50% silver nitrate solution. Staining was performed for 10, 20, 30, 45 and 60 min. The specimens were then rinsed in PBS for 30 min and post-fixed in 2% osmic acid, dehydrated in graded ethanol and propylene oxide, and embedded in Epon 812. Semi-thin sections were examined without counterstaining. Ultra-thin sections were analysed unstained or regularly stained in a uranyl acetate solution with lead citrate post-staining. Ultra-thin sections were studied by use of a Zeiss EM 10A at 60 kV.

Anti-β-protein antiserum against synthetic polypeptide (Kitamoto et al. 1987) and affinity-purified tau-specific antibody (Shin et al. 1989) have been previously characterized and established. After deparaffinization, endogenous peroxidase was blocked. Formic acid pre-treatment was performed for 5 min, to enhance the immunoreactivity (Kitamoto et al. 1987). After washing with tap water and TRIS buffer (50 mM TRIS-HCl, pH 7.6) the tissue sections were incubated overnight with diluted anti-β-protein serum (1:2000) and affinity-purified tau-specific antibodies at 4° C. For detection of primary antibodies the biotin-avidin method (Vectastain, Vector Laboratories, Burlingame, Calif., USA) was used. Diaminobenzidine tetrahydrochloride was used as the colour substrate end-product. Counterstaining was performed with haematoxylin.

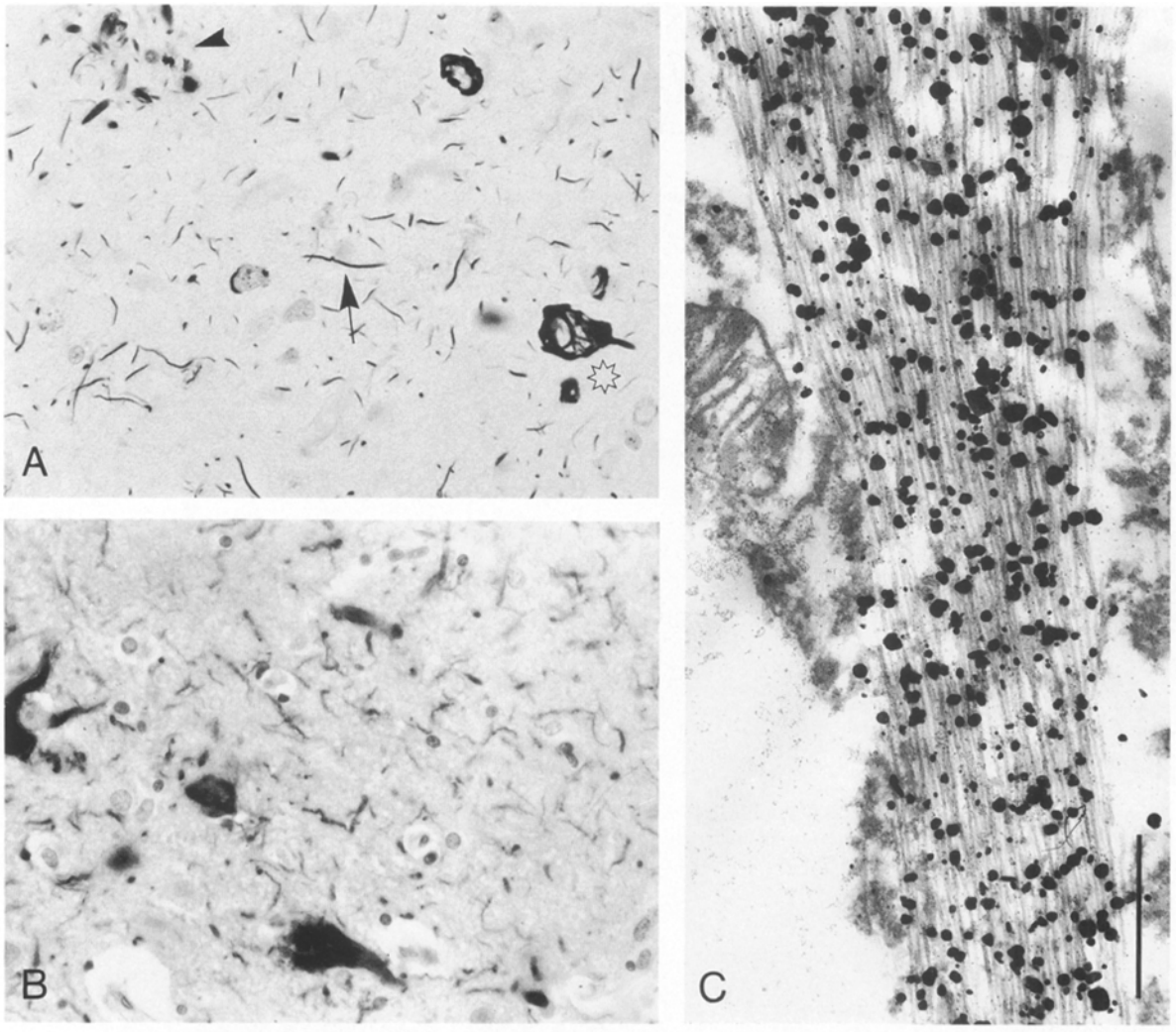

Fig. 2A–C. PHF as neuropil threads. **A** Numerous threads (*arrow*), a neuritic plaque (*arrowhead*), and NFT (*asterisk*); semi-thin, ×460. **B** Tau-immunoreaction with threads and NFT; paraffin, ×520. **C** PHF with mitochondrium; ultra-thin, ×43600, *bar*=0.5 μm

Results

Neuronal lesions with the three forms of PHF were found. NFT were present especially in large neurons of the hippocampus and subiculum. In proximity various stages of degeneration were detected in the same microscopical field (Fig. 1). Intact neurons did not show any staining of cytoplasm (Fig. 1B). PHF in diseased cells were found in a variety of forms ranging from small patches of irregularly arranged PHF to intensely stained and densely packed PHF which were abundant in the cytoplasm. The destruction of the cell resulting in shadow-like contours as so-called ghost tangles represents the final stage of this process (Fig. 1). Severely degenerated PHF had lost most of their argyrophilia, occasionally demonstrable in EM with non-stained specimens.

PHF forming neuropil threads were found in a large amount in the hippocampus, subiculum and temporobasal cortex between NFT, and in accumulations as neuritic plaques (Fig. 2). Intact neuritic processes did not show any significant staining.

PHF were intensely stained as irregularly distributed and thickened neuritic components and thus were detected easily in the core and in the vicinity of plaques (Fig. 3).

All three kinds of PHF showed a distinct positive anti-tau immunoreaction in paraffin sections (Figs. 1, 2).

Diffuse fibrillar amyloid was found in the pre-subicular region, in the hippocampus and in the frontal region, and laminar patterned in the subpial region (Fig. 5). Numerous amyloid plaques as "primitive", "classical", and "burnt out" types were observed in semi-thin sections in the frontal and temporal areas (Fig. 4). Electron microscopy confirmed the fibrillar structure of these silver-stained amyloid deposits (Figs. 5, 6). Congophilic angiopathy occurred primarily in the walls of arterioles and arteries revealing amyloid deposits in various amounts. The morphological changes ranged from thickened, sparsely stained basement membranes and patchy deposits to broad, circumferential accumulations of loose amyloid. The latter lesions were occasionally associated with plaque-like condensations of amyloid and perivas-

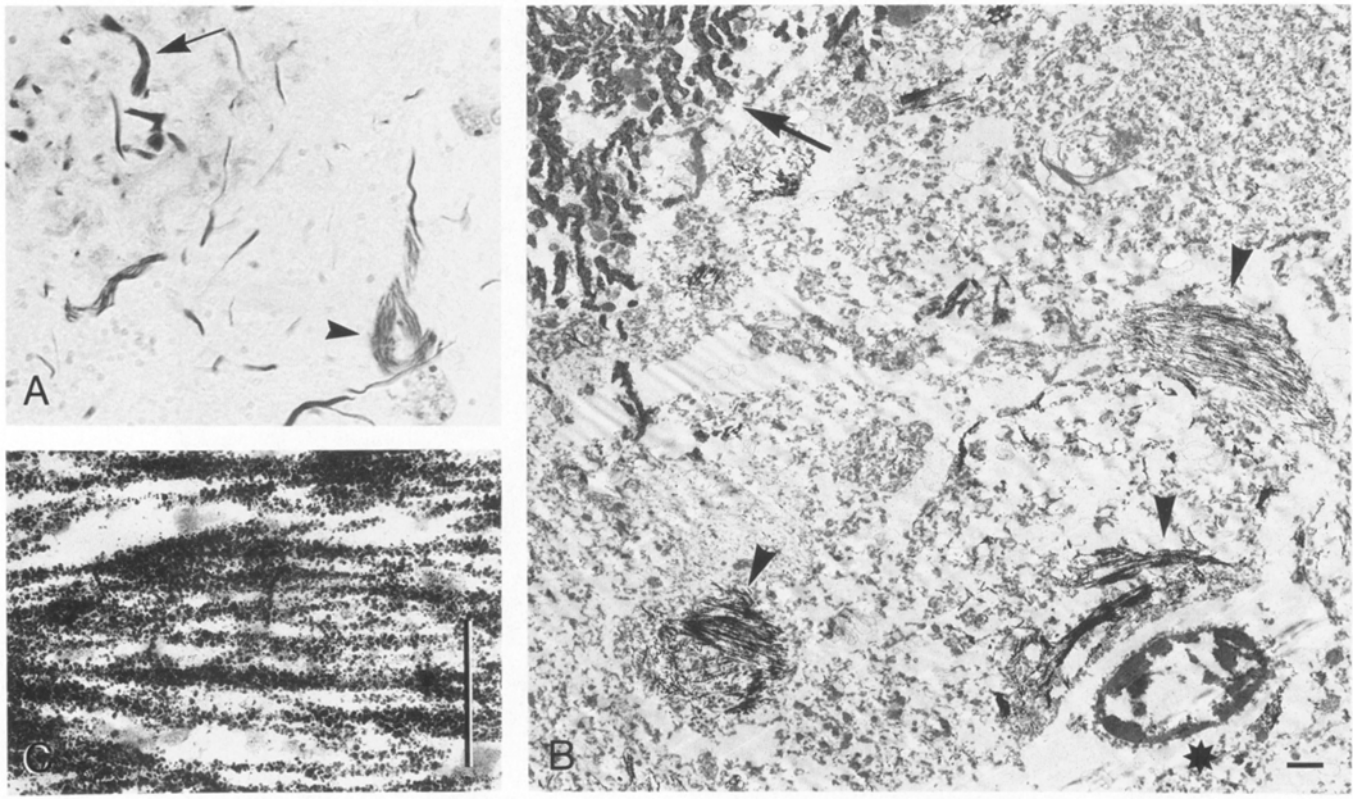

Fig. 3A–C. PHF in neuritic plaques. **A** Intensely stained PHF (*arrow*) within pale amyloid and NFT (*arrowhead*); semi-thin, ×920. **B** Amyloid (*arrow*), neuritic components (*arrowheads*) and microglia (*asterisk*); ultra-thin ×4800, *bar* = 1 μm. **C** Higher magnification of the thickened neuropil thread in **B** bottom left, ×38800; *bar* = 0.5 μm

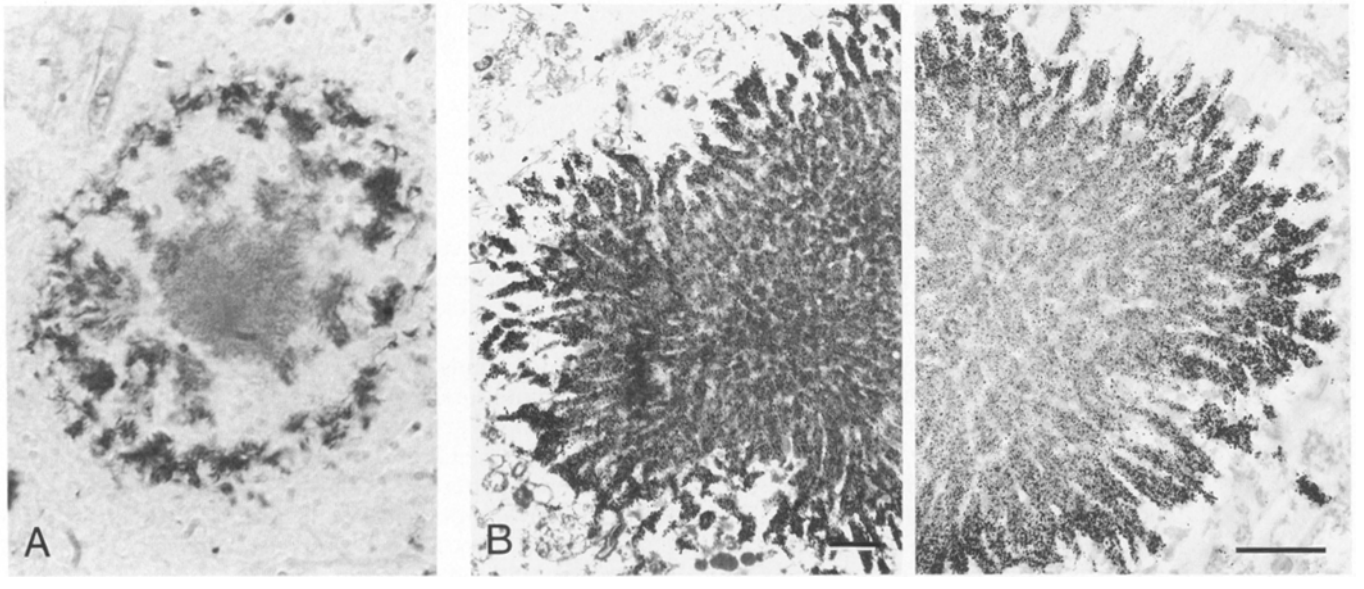

Fig. 4A, B. Amyloid plaque. **A** Classical plaque, semi-thin, ×1000. **B** Burnt out plaque; left ultra-thin, ×6100, right non uranyl acetate/lead citrate-stained, ×11200; *bar* = 1 μm

cular amyloid in the dilated Virchow-Robin space (Figs. 5, 6). In addition several small vessels showed broad accumulations of loose "dyshoric" amyloid (Fig. 5). Occasionally vessels were found in spatial relation to amyloid plaques. All the alterations described were seen in vessels of the subarachnoid space, in pial vessels passing to the cortex, and in cortical and subcortical vessels. All extracellular forms of fibrillar amyloid showed distinct anti-β-immunoreactions.

Distinct silver staining of semi-thin and ultra-thin sec-

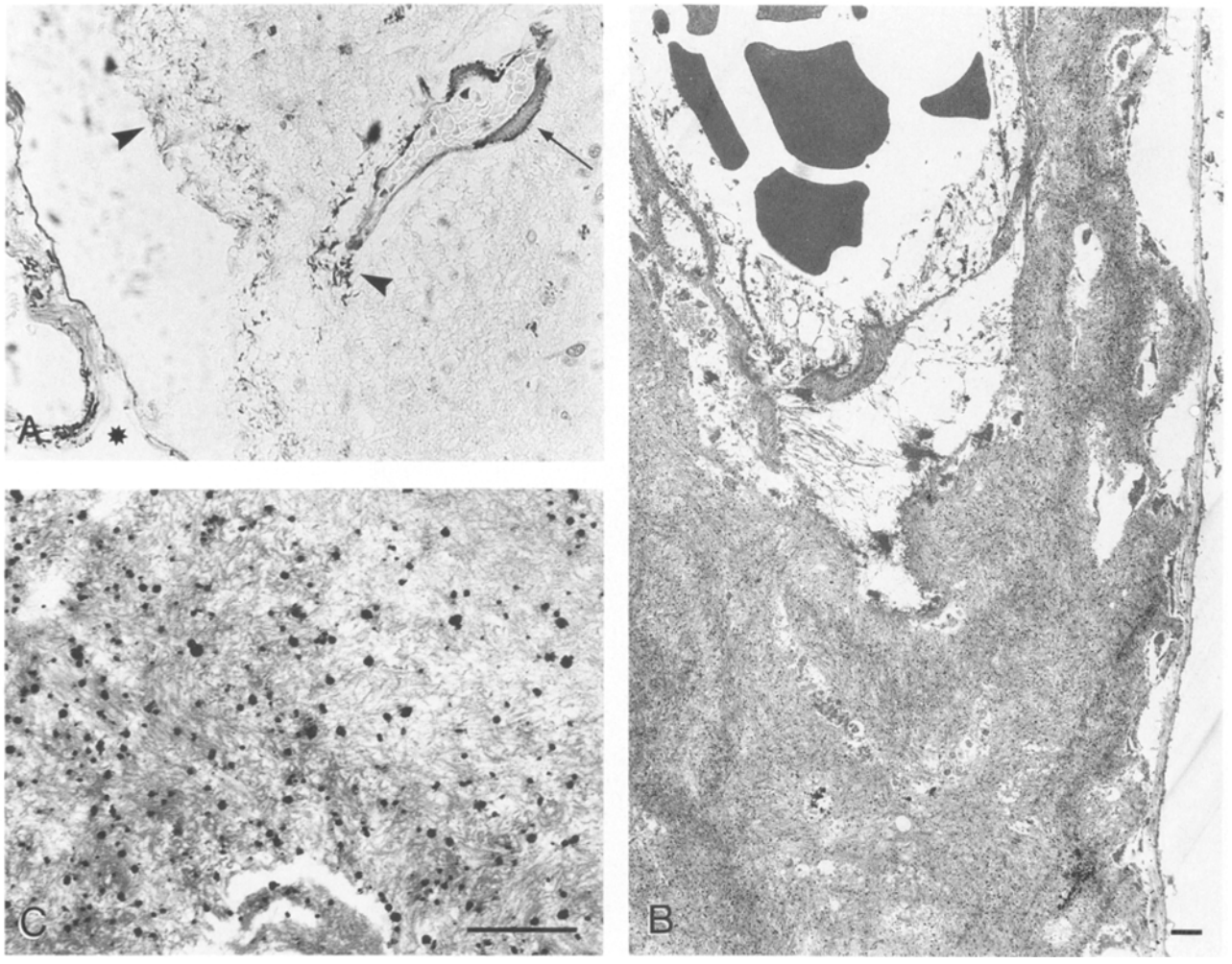

Fig. 5A–C. Congophilic angiopathy. **A** Small vessel with loose "dyshoric" amyloid (*arrow*), perivascular and laminar subpial amyloid (*arrowhead*) and amyloid in a subarachnoid artery (*asterisk*); semi-thin, ×200. **B** "Dyshoric" amyloid, ultra-thin, ×4100; *bar* = 1 μm. **C** Detail from **B** with sparsely silver-stained loosely arranged amyloid fibrils, ×38800; *bar* = 0.5 μm

tions was obtained after treatment with solution for 30–60 min. Beside PHF and all kinds of amyloid, granular intraneuronal lipofuscin and light brownish corpora amylacea were silver-stained.

Discussion

To our knowledge the presented silver staining method has been used to demonstrate both, PHF and extracellular cerebral amyloid in semi-thin and ultra-thin sections for the first time. As in paraffin sections (Reusche 1991) the advantage of the method is the reliable demonstration of PHF in its three forms and of all kinds of extracellular amyloid, including the different types of amyloid plaques and congophilic angiopathy. Other methods demonstrate only either PHF (NT, Braak et al. 1986), or diffuse amyloid (Yamaguchi et al. 1990b). PHF and tau-protein show common epitopes as evidence by double immunogold labelling (Defossez et al. 1988). Originally the technique presented here had been used for staining physiological acidic proteins in nucleolar organizer regions (Ploton et al. 1982). The method is applied in this study for the demonstration of different pathological proteins. Extracellular insoluble amyloid (Masters et al. 1985) is shown in its different forms of plaques and congophilic angiopathy and the three kinds of PHF are shown as intraneuronal NFT, NT and neuritic components in plaques; all these structures show a high resistance against proteolytic degradation, and can even be found in areas of older infarction (Brumback et al. 1991). Apart from PHF and extracellular amyloid, corpora amylacea (consisting of glycoprotein polyglucosan) and lipofuscin are also stained, and are easily distinguished because of difference in colour, size and shape. The advantage of the method is the careful revealing of argyrophilic structures in different kinds of denatured structural proteins and degradation products, by the physical developer of formic acid and gelatin. Because it is easy to perform and is applicable to archival material, this method might even help to provide additional information for the still unsolved problem of the neuronal or haematogenous origin of the cerebral amyloid (Beyreuther and Masters 1991).

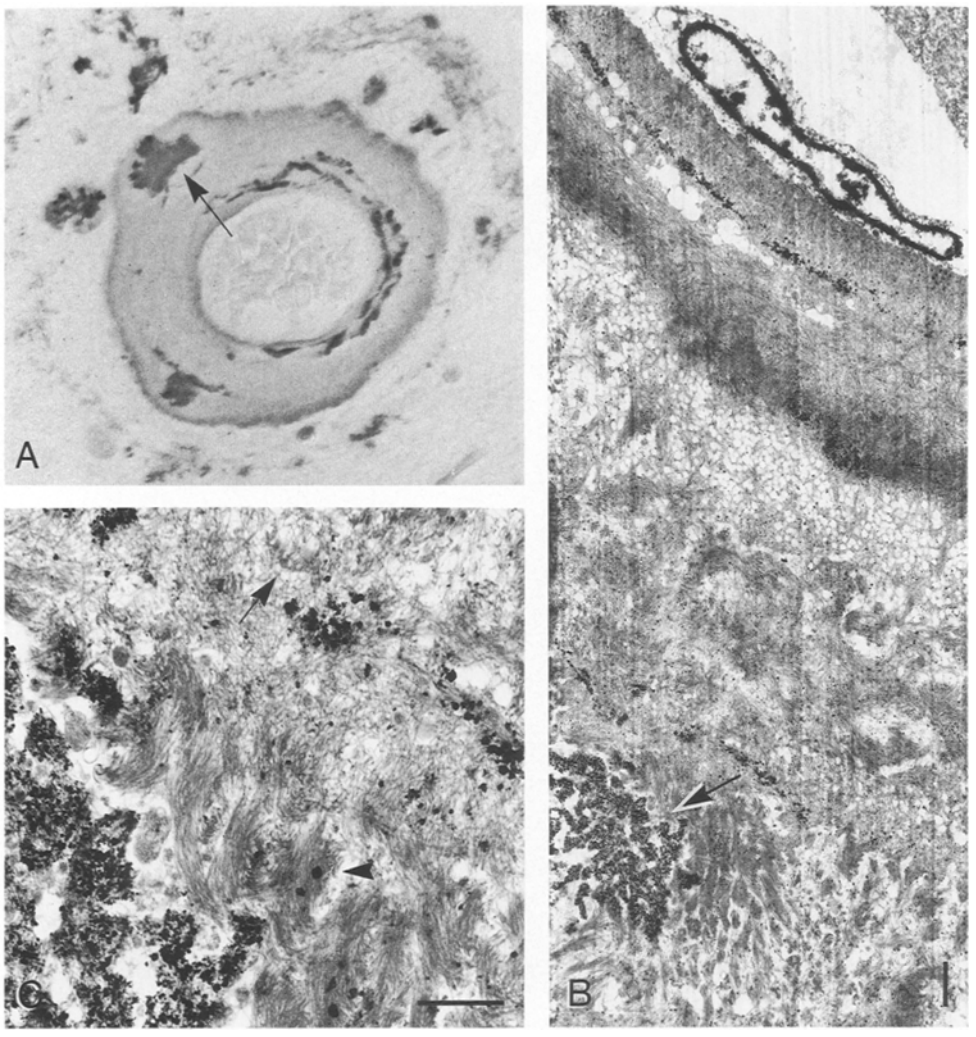

Fig. 6A–C. Congophilic angiopathy. **A** Artery with circumferential loose amyloid and plaque-like condensed amyloid; semi-thin, ×520. **B** Detail of the vessel wall from **A** with plaque-like structure (*arrow*); ultra-thin, ×6600; *bar* = 1 μm. **C** Higher magnification from **B** with loosely (*arrow*) and closely packed sparsely stained amyloidfibrils (*arrowhead*) next to intensely silver-stained amyloid, ×26500; *bar* = 0.5 μm

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