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Progressive supranuclear palsy: extensive neuropil threads in addition to neurofibrillary tangles

Very similar antigenicity of subcortical neuronal pathology in progressive supranuclear palsy and Alzheimer's disease

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Summary. Light microscopic immunohistochemical investigations were performed on neurofibrillary tangles (NFT) in four histologically confirmed cases of Alzheimer's disease (AD) and in five patients with a progressive supranuclear palsy (PSP). The antibody panel included antisera to the neuronal microtubule-associated protein, tau, and to isolated paired helical filaments (PHF), as well as mouse monoclonal antibodies (MAbs) to phosphorylated epitopes on high and medium molecular weight neurofilament subunits (RT97 and BF10, respectively). Paraffin sections were also impregnated with the Gallyas silver method, which specifically stains tangles and cortical neuropil threads in AD, but does not stain normal neurofilaments. All tangles in PSP and AD showed consistent immunostaining with antibodies to tau protein and isolated PHF, regardless of their localization. MAbs RT97 and BF10, however, did not stain or only weakly stained, subcortical tangles in PSP and AD, whereas most cortical NFT in AD were intensely immunostained. All tangles in PSP were as heavily impregnated with Gallyas as they were in AD. Furthermore there were extensive networks of Gallyas-positive, tau- and PHF-immunoreactive neurites in subcortical gray areas containing NFT, and bundles of positive axons in white matter tracts interconnecting subcortical nuclei of PSP. Our studies indicate a much more extensive disruption of fibrillar proteins in PSP subcortical neurons than previously reported. They furthermore indicate a very similar antigenic profile of NFT in PSP and AD, as far as subcortical neurons are concerned.

Key words: Progressive supranuclear palsy – Alzheimer's disease – Neurofibrillary tangles – Abnormal neurites – Tau protein

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Neurofibrillary tangles (NFT), as in Alzheimer's disease (AD), are one of the major histological features of progressive supranuclear palsy (PSP). Although differing from AD in localization [12, 19] and ultrastructure [12, 14, 16, 22], PSP tangles have recently been shown to present striking antigenic similarities with tangles in AD [2]. In particular, microtubule-associated protein (MAP) tau antigenicity is prominent in both types of tangles. Furthermore, at least a fraction of PSP tangles were found to react with antibodies to paired helical filaments (PHF), more so with polyclonal than with monoclonal antibodies.

The aim of the present investigation was to compare the antigenic properties of NFT in AD and PSP using a panel of antibodies to tau proteins, PHF antigen and epitopes of phosphorylated neurofilaments. This immunohistochemical study was supplemented by the application of the silver impregnation method of Gallyas [8] which is known to selectively impregnate PHF in AD without staining normal neurofilaments. Similar antigenic properties of NFT were found in AD and PSP when comparing NFT located in subcortical neurons. Extensive involvement by material identical to that in NFT was found in neuronal processes of the subcortical neuropil and in fiber tracts interconnecting subcortical nuclei in PSP, indicating much more diffusely distributed pathology of the cytoskeleton than previously supposed.

Materials and methods

Brain tissue was investigated from five patients (age 63 to 82 years) in whom the clinical diagnosis of PSP was confirmed at histological examination of the CNS. The brains of four elderly, demented patients with morphologically established Alzheimer's disease as well as brain tissues from four elderly, non-demented patients (age 66 to 86) were included in this study. The brains were fixed in 10% buffered formaldehyde for 7 to 10 days.

Table 1. Summary of antibodies used and their specificity

Silver stain and antibodies	Dilutions	*Predominant specificity*
Gallyas silver iodide	–	NFT in situ [2, 8]. PHF and tau polypeptides on SDS polyacrylamide gel [11]
Polyclonal antibodies		
Rabbit anti-PHF	1:1000	NFT in situ (neuronal perikarya, senile plaque neurites) and SDS-isolated PHF from AD brain [5, 6]
Rabbit anti-MAP-tau	1:1000	MAP-tau [5] NFT "in situ" [5] Isolated NFT [5]
Mouse monoclonal antibodies		
Mouse monoclonal RT97	1:1000	Normal axons and NFT in AD [1] Phosphorylated epitopes on high-molecular weight neurofilament subunit [10]
Mouse monoclonal BF10	1:5000	Normal axons and NFT in AD [1] Phosphorylated epitopes on medium size neurofilament subunit [10]

NFT: Neurofibrillary tangles; PHF: paired helical filaments; SDS: sodium dodecylsulfate; AD: Alzheimer's disease; MAP: microtubule-associated protein

Tissue blocks containing neocortical areas, hippocampal region, thalamus and subthalamic structures including the subthalamic nucleus, nucleus lentiformis, nucleus basalis of Meynert, midbrain, pons and cerebellum were embedded in paraffin. Conventional staining methods (H&E, Holmes silver impregnation combined with Luxol fast blue for staining of axons and myelin sheaths) as well as immunocytochemical staining were performed on 4-µm-thick sections.

Silver staining

Silver staining was performed according to the procedure described by Gallyas [8]. Briefly, sections were deparaffinized in xylene/alcohol, rinsed in distilled water and treated for 30 min with 5% periodic acid. After a brief rinse, sections were impregnated in an alkaline silver iodide complex solution for 10 min. Sections were then left in physical developer for 30 min and transferred into a 0.5% solution of acetic acid to terminate development.

Immunocytochemistry

The monoclonal and polyclonal antibodies employed in this study are listed in Table 1 along with their specificities, sources, selected references and dilutions of primary antibodies.

Sections were deparaffinized in xylene/alcohol, rinsed in phosphate-buffered saline (PBS) and endogenous peroxidase activity was stopped with 0.3% hydrogen peroxide in methanol (30 min). Sections were subsequently treated with normal horse (for MAbs) or normal goat serum (polyclonal antibodies). After appropriate dilutions of the primary antibodies (Table 1) incubations were performed overnight at +4°C followed by thorough washing in PBS, treatment with specific biotinylated anti-mouse or anti-rabbit immunoglobulines (30 min), avidin-biotin-peroxidase complex (Vectstain) (30 min) and another wash in PBS. Negative controls were obtained by replacing first antibodies with normal rabbit or mouse serum. Peroxidase activity was developed with diaminobenzidine/H_2O_2 solution.

Table 2. Distribution of NFT and Gallyas-positive threads in progressive supranuclear palsy (PSP)

	NFT	Threads
Neocortex	–	–
Putamen	++	+/++
Globus pallidus	++	+++
Nucleus basalis	+/++	+
Thalamus	+	+
Subthalamic nucleus	+++	+++
Zona incerta	++	++
Substantia nigra:		
pars compacta	+/++	++
pars reticulata	+	+
Area tegm. ventralis	+	++
Nucleus ruber	–	+
Nucleus cuneiformis	++	++
Tectum mesencephali	+	++
Griseum centr. mesencephali	++	+
Edinger-Westphal nucleus	+	+
Oculomotor nuclei	+	+
Locus coeruleus	+++	(+)
Nucleus tegm. pedunculopont.	++	+
Nucleus centralis superior	++	+
Nucleus retic. tegm. pontis	++	++
Nuclei pontis	+	+
Ansa lenticularis	–	+++
Lenticular fasciculus	–	++
Striatopallidal fibers	–	+++

Table 3. Gallyas silver impregnation and immunostaining of NFT

Gallyas silver stain and antibodies	PSP	AD
Gallyas	+++ Numerous threads in subcortical neuropil and fiber bundles	+++ Numerous threads, mainly in neo- and allocortex
Anti-PHF	+++	+++
Anti-tau	+++	+++
MAb BF10	+/0	+/0 subcortical gray +++ cortex
MAb RT97	+/0	+/0 subcortical gray +++ cortex

MAb: Monoclonal antibody
+++: strong silver positivity or immunostain of NFT; +/0: weak to absent reactivity

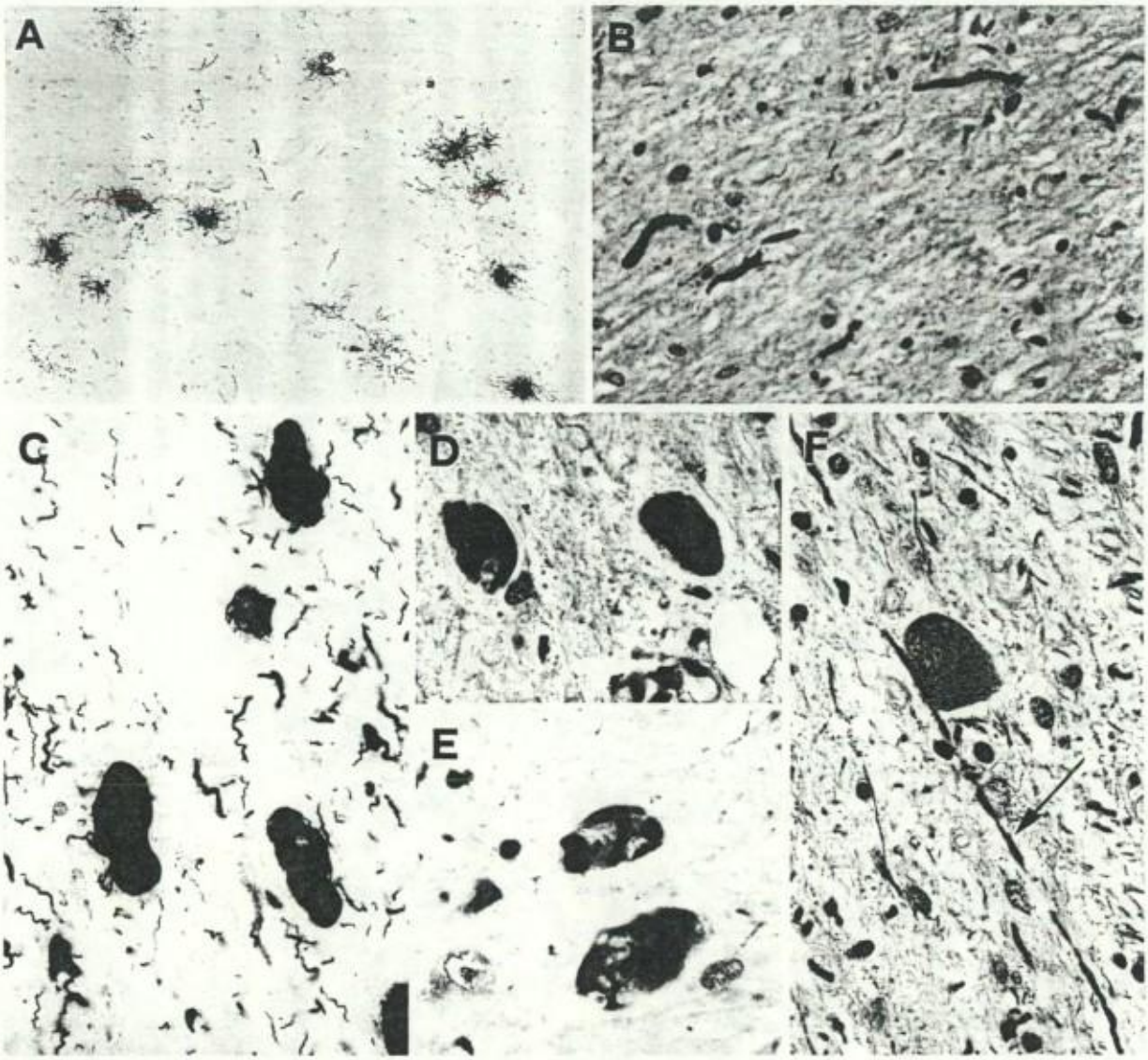

Fig. 1. **A** Small stellate neurons of the putamen showing silver-positive material in the perikaryon and in dendrites. Gallyas stain. **B** Anti-tau labelled, slightly thickened neurites in the lenticular fasciculus. **C–F** Tangles in neurons of the subthalamic nucleus in a progressive supranuclear palsy (PSP) case. **C** Gallyas' silver impregnated tangle and neuropil threads; **D** anti-tau labelling of tangles; **E** anti-paired helical filaments (PHF) labelling. **B, D–F** Counterstained with hematoxylin. **A** ×100, **B–F** ×500

Results

Neurofibrillary tangles in PSP

The distribution and the density of NFT were roughly similar in all PSP cases. The results of semiquantitative estimation of the density of Gallyas-positive tangles in different gray structures are given in Table 2, together with the density of positive neurites in the neuropil and some white matter tracts. The distribution of tangles did not differ from that reported in earlier histopathological studies of PSP [7, 12, 17, 19]. Tangles (Table 3) were consistently impregnated with Gallyas silver iodide method (Figs. 1A, C; 3D) and disclosed intense immunostaining with antisera to tau (Fig. 1E) and to isolated PHF (Figs. 1D; 2A, C). There was no variation in silver impregnation or in the intensity of immunostaining among the different nuclei examined. Only weak to negative reaction was obtained in tangles when using MAbs BF10 and RT97 (Table 3). These antibodies recognize phosphorylated epitopes on the medium- and high-molecular weight neurofilament polypeptides, respectively (Figs. 1F; 2B, D). Most tangles appeared as compact well-delimited round or sausage-like inclusions in neuronal perikarya (Fig. 1C). In the striatum Gallyas-positive, anti-PHF- and anti-tau-reactive material was found in the

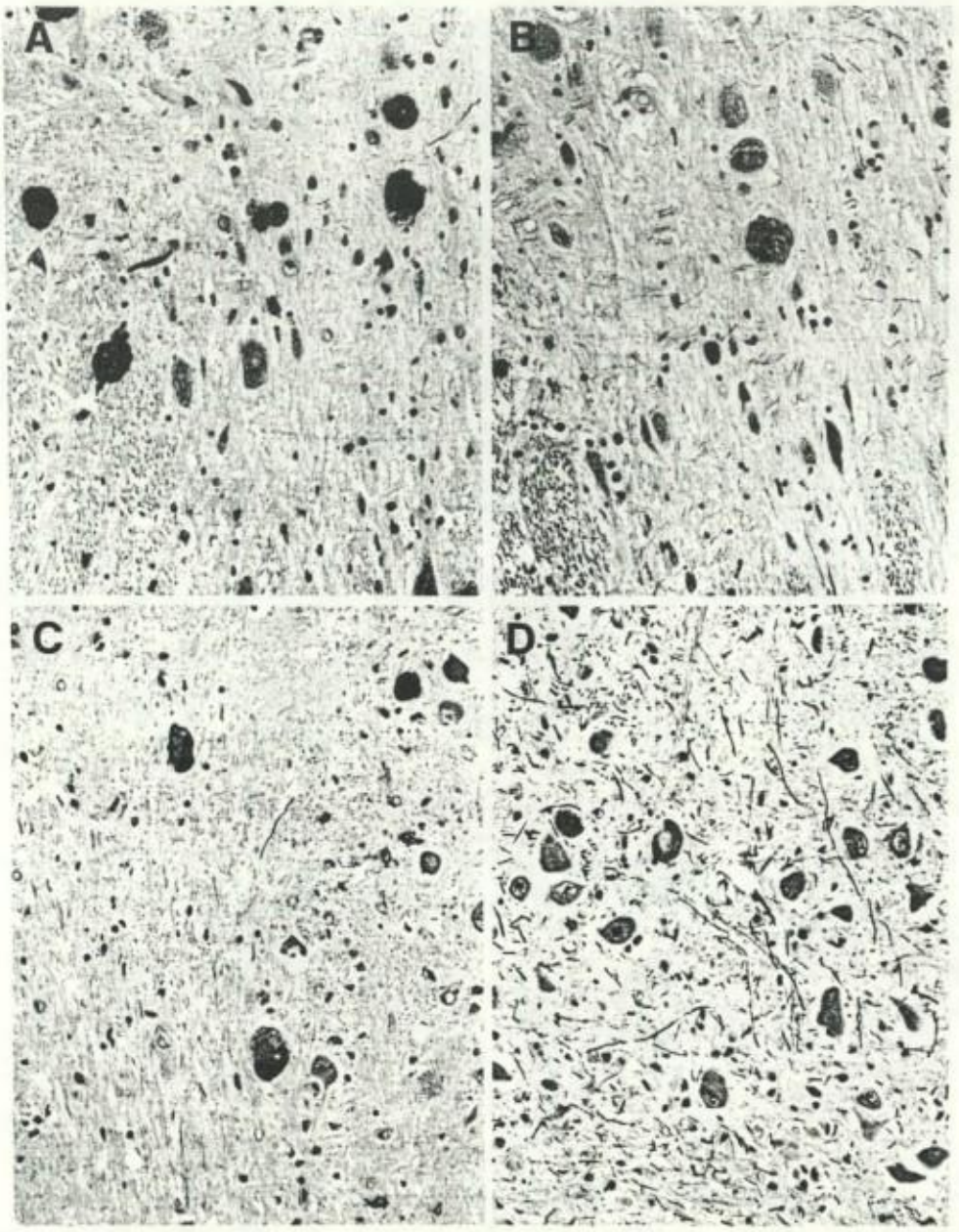

Fig. 2A–D. Tangles in neurons of the nucleus raphé centralis and linearis in the upper pons after labelling with anti-PHF serum (**A, C**) and monoclonal BF10 antibody; (**B, D**) in Alzheimer's disease (**A, B**) and PSP (**C, D**). Immunostaining of tangles with BF10 is very weak or absent in both conditions contrasting with strong anti-PHF reactivity

perikaryon and short dendrites of innumerable small stellate neurons (Fig. 1A). However, a few large ovoid tangles, like those in other nuclei, were also occasionally encountered in this region.

When compared with the Holmes stains of adjacent sections, much higher numbers of intracellular tangles were labelled by the Gallyas silver stain and the antisera to tau and PHF. This was particularly evident in thalamic nuclei and especially in the striatum, where neurofibrillary changes of small stellate cells were generally not stained by the Holmes method.

Neuropil threads in PSP

Numerous thread-like, argyrophilic neurites (threads) were found using Gallyas impregnation. These threads consisted of short, randomly oriented neuritic profiles, dispersed in the neuropil of all subcortical gray structures containing tangles in neuronal perikarya (see Table 2). The highest densities of threads were found in the nucleus subthalamicus (Figs. 1C; 3C, D), the globus pallidus (Fig. 3A) the tectum and tegmentum of the midbrain including substantia nigra (both pars

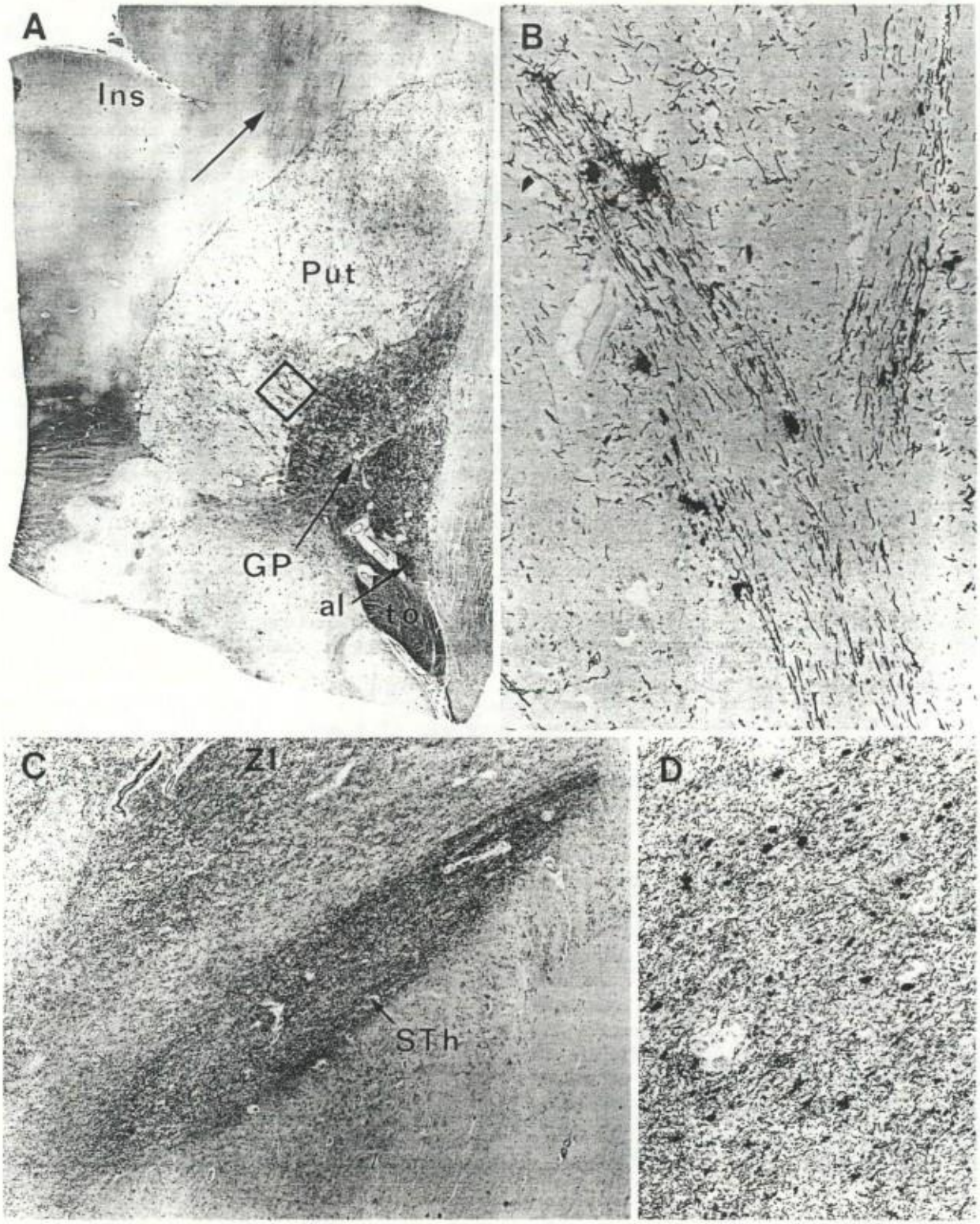

Fig. 3A–D. Gallyas silver-impregnated sections of one PSP case. **A** Low magnification of the lentiform nucleus showing bundles of impregnated axons in the putamen and large numbers of neuritic threads in the globus pallidus (*GP*). Note also nerve fiber impregnation in the ansa lenticularis (*al*). Diffuse staining at *lower left* corner of the picture is non-specific and is not due to impregnation of nerve fibers. *Ins*: Insular cortex. *Arrow* on the top indicates correct dorso-ventral orientation of the figure. **B** Higher magnification of details included in the frame of **A**, showing bundles of impregnated nerve fibers, probably putaminopallidal fibers. **C** Dark appearance of the subthalamic nucleus (*STh*) is due to the great amount of threads and tangles. Note also large numbers of threads in the zona incerta (*ZI*) and in the surroundings of the subthalamic nucleus. **D** Higher magnification of subthalamic nucleus shown in **C**. **A** ×4.5, **B** ×175, **C** ×12, **D** ×70

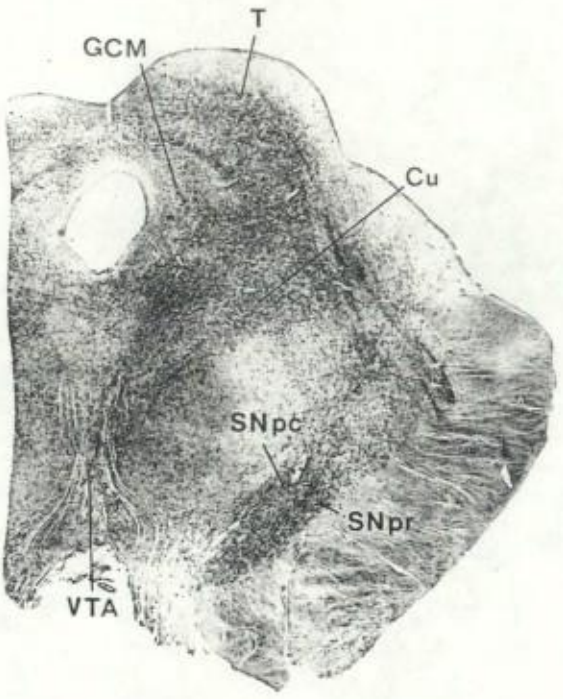

Fig. 4. Midbrain half. Gallyas silver stain. Dark areas correspond to high densities of silver-impregnated nerve fibers (threads). These are mainly distributed through the tectum (*T*), the griseum centrale mesencephali (*GCM*), the cuneiform nucleus (*CU*), the ventral tegmental area (*VTA*) and the substantia nigra pars compacta (*SN pc*) and reticulata (*SN pr*). ×3,5

compacta and reticulata) (Fig. 4) and in the zona incerta (Fig. 3C). A great number of threads were also distributed throughout the midbrain ventral tegmental area (Fig. 4). Apart from haphazardly oriented threads in the neuropil of subcortical gray structures, there were also bundles of parallel Gallyas-positive fibers in many subcortical fiber systems including the medullary laminae of the globus pallidus, the striatofugal fibers to the globus pallidus, the ansa lenticularis (Fig. 3A) and the fasciculus lenticularis. Threads in the neuropil and white matter bundles were also stained with anti-tau (Fig. 1B) and anti-PHF sera (not shown), although the number of immunostained fibers was less than the number of fibers labeled by the Gallyas stain.

Alzheimer's disease

In Gallyas preparations for demonstration of NFT, argyrophilic tangles were found in great numbers in the neo- and allocortex, less often in subcortical nuclei. NFT were intensely labelled when stained with anti-tau and anti-PHF sera, regardless of their cortical or subcortical localization (Table 3). This was in contrast to the variable intensity of labelling when using MAbs RT97 and BF10. Whereas in the neo- and allocortex tangles were consistently and intensely stained by these antibodies, the reaction was generally weak to absent in subcortical gray structures.

A comparison of the pattern of immunolabelling in AD and PSP is illustrated in Fig. 2: neurons bearing tangles in the pontine raphé nuclei show weak to absent BF10 immunostain in both AD and PSP (Fig. 2B, D), whereas tangles are all strongly immunostained by anti-PHF in both conditions (Fig. 2A, C).

In Gallyas-impregnated sections, threads, similar to those of PSP, were found in the cortical neuropil next to impregnated NFT. Some of them were spatially associated with senile plaques, others were not. In general, the density of threads was proportional to that of NFT in the same cortical area. Few Gallyas-positive threads and occasional NFT were found in the lentiform nucleus, the thalamus and subthalamic nuclei. Threads and tangles wer slightly more numerous in the upper brain stem, mainly in midline nuclei and in the periaqueductal gray matter. Very few threads were encountered in myelinated fiber bundles of the striatum and globus pallidus and in the subcortical white matter. A great number of threads, similar in shape to those labeled by Gallyas, were also recognized by our antisera to tau and PHF.

Control cases

In non-demented elderly patients no Gallyas-positive structures or reactivity with antisera to PHF or tau was found in the cortex or in subcortical nuclei and tracts. However, a few Gallyas-positive tangles and threads were observed throughout the neocortex and hippocampus. RT97 and BF10 reactive axons, mainly in white matter tracts, were a common feature of AD, PSP (Fig. 1F) and elderly control cases.

Discussion

Recent immunocytochemical work by Bancher et al. [2] on tangles in PSP and AD has shown that, as previously described for AD, MAP tau is also a major antigenic determinant of tangles in PSP. In the present work we confirm that MAP tau protein immunoreactivity is common to both AD and PSP tangles. Furthermore, all tangles were immunostained using an antiserum to PHF, known by immunoblotting to contain anti-tau antibodies [5]. Both tangles in AD and PSP were intensely impregnated by Gallyas silver iodide, a method that has recently been shown readily and consistently to stain both tau and PHF polypeptides separated on sodium dodecylsulfate polyacrylamide gels [11].

The number of tangles in subcortical nuclei in PSP was much higher when using the Gallyas method or

the anti-tau/-PHF antisera instead of conventional silver impregnation techniques. In the striatum, for instance, a surprisingly high number of small stellate neurons was found to contain pathological material. This is in contradiction to earlier reports describing the striatum to be only slightly affected in PSP and to contain only scarse, if any, NFT [7, 12, 14].

Reduced activity to choline acetyltransferase (CAT) and a decreased number of dopamine receptors have recently been reported in the striatum in PSP and these changes were tentatively explained by a degeneration of cholinergic and dopaminoceptive interneurons in this region [18]. It is possible that the small PHF-containing neurons that we have found in large numbers in the striatum might in part correspond to small cholinergic interneurons, thus explaining biochemical changes observed by Ruberg et al. [18].

Another finding of the present study was that the reactivity of AD tangles, using monoclonal antibodies to neurofilament epitopes, was dependent upon the localization of neuronal cell bodies. This variability of staining reaction contrasted with universal weak reactivity of tangles in PSP to these antibodies. Whereas neurofilament epitopes were consistently labeled in AD tangles located in the cortex, these epitopes were as poorly represented or absent (or at least non-accessible) in subcortical tangles as they were in PSP. These findings confirm recent observation by Tabaton et al. [21] concerning the relationship between the cell location and antigenic properties of tangles in AD and on the absence in pontine tangles of neurofilament epitopes that are present in cortical tangles. According to these authors pontine tangles in AD mostly consist of PHF, like cortical tangles [20], whereas tangles in PSP are made essentially of straight filaments and of an amorphous material [21]. These observations suggest that the antigenic properties of PHF in AD and of straight filaments in PSP are quite similar when observed in the same brain region. They also imply that the localization of the cell bodies is more important in determining the antigenic composition than is the ultrastructure of NFT.

Using the Gallyas silver method, we have found that neurofibrillary changes in PSP are far from being confined to the neuronal cell body, but are widely distributed throughout the neuropil in the form of silver-positive neuritic profiles. These are very numerous in subcortical nuclei containing PHF and in fiber bundles projecting from these nuclei. A number of these neurites were labelled by antisera prepared against isolated NFT and tau protein. However, the number of immunolabelled neurites was definitely less than that of Gallyas-positive neurites, which is in contrast to about equal numbers of tangles demonstrated by the two methods. Similar involvement of the neuropil by a Gallyas-positive, tau- and PHF-reactive material was also evident in our AD cases but in an almost exclusive cortical distribution. Gallyas-positive neuritic profiles ("neuritic threads") have already been described in the cortical neuropil in relation to PHF [4]. These threads have also been reported to contain PHF and to react with an antiserum to PHF [4]. Furthermore, tau immunostaining of threads in AD has recently been reported using polyclonal [13] and MAbs [9].

To our knowledge, no electron microscope study has been performed on subcortical threads of PSP. As we have seen in the case of tangles, identical staining pattern does not necessarily imply identical ultrastructure and it might well be that PSP threads are not made of PHF but of straight filaments like the PSP tangles.

The dispersed network of tau/PHF-reactive neurites in AD and PSP suggests a diffusely distributed abnormality of corresponding proteins extending far beyond neurofibrillary changes in neuronal perikarya. Since tau is known to be enriched in white matter [3, 15], axonal staining might have been expected, for instance in subcortical white matter bundles of PSP cases. However, no tau-reactive structures have been observed in our control cases, which is in keeping with the minimal or absent axonal labelling reported by others in sections of control human brains [13]. Axonal labelling with tau antibodies can be obtained in freshly fixed and processed rat brain and so probably tau associated with normal microtubules in postmortem human brain is antigenically labile, while tau associated with the pathological lesions of AD and PSP has been rendered resistent to tissue fixation and embedding procedures. The finding of tau antigen in perikarya and dendrites further points to an abnormal intraneuronal distribution of tau in AD and PSP, yet the neurons affected are located differently in the two conditions. A further difference consists in a more prominent involvement of axons in PSP than is the case in AD. This leads to intense immunostaining and silver impregnation of many projection systems interconnecting subcortical nuclei.

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