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Alzheimer-type pathology in melanin-bleached sections of substantia nigra

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Abstract Bleaching of melanin prior to Gallyas staining enabled us to detect an unexpectedly large number of neurofibrillary tangles (NFTs; 62 and 50 NFTs in the upper and the lower substantia nigra, respectively, mostly in the neuronal cytoplasm of the medial zona compacta) in brains of patients who had had Alzheimer's disease. Amyloid-like deposits were quite scarce. In Alzheimer's disease it has been commonly observed that other subcortical nuclei projecting widely to the cerebral cortex also contain a large number of NFTs, although they have few amyloid deposits. In these subcortical nuclei retrograde degeneration may initially affect intraneuronal processes, leading to the preferential development of NFTs in the neuronal cytoplasm.

Key words Bleaching · Gallyas method · Melanin Pigment · Neurofibrillary tangles · Substantia nigra

Introduction

Alzheimer's disease (AD) is morphologically characterized by neuronal depletion and the presence of amyloid deposits and neurofibrillary tangles (NFTs) in the brain. One of the difficulties in identifying nigral NFTs is the presence of melanin, which appears intermingled with neurofibrils (Fig. 1 a). Using a conventional silver staining method alone, it is difficult or almost impossible to identify definitely such structures as NFTs in the substantia nigra (SN). We attempted to visualize nigral NFTs more clearly by bleaching melanin [13] prior to Gallyas staining [2, 4]. The morphological features of AD pathology in the SN are described.

Materials and methods

Autopsied brains from nine patients (five men and four women; mean age at death, SD 65, 9 years, range 48–79 years) with presenile AD were studied. The clinical diagnosis of Alzheimer's disease was made according to the NINCDS-ADRDA criteria [15]. Neuropathological findings of these brains were compatible with AD according to the criteria of Khachaturian [11]. In only three of the patients were extrapyramidal symptoms clinically detected. No more than two Lewy bodies were noted on sections of the SN stained with haematoxylin and eosin, far fewer than observed in Parkinson's disease. Brains from 12 age-matched controls without neuropsychiatric disorders (7 men and 5 women, mean age at death 62.7 years, range 50–74 years) were also studied.

In order to determine which staining method was the most sensitive for visualizing nigral NFTs, five AD brains with a relatively large number of nigral NFTs were selected. Formalin-fixed, paraffin-embedded blocks of the midbrain were obtained. Successive

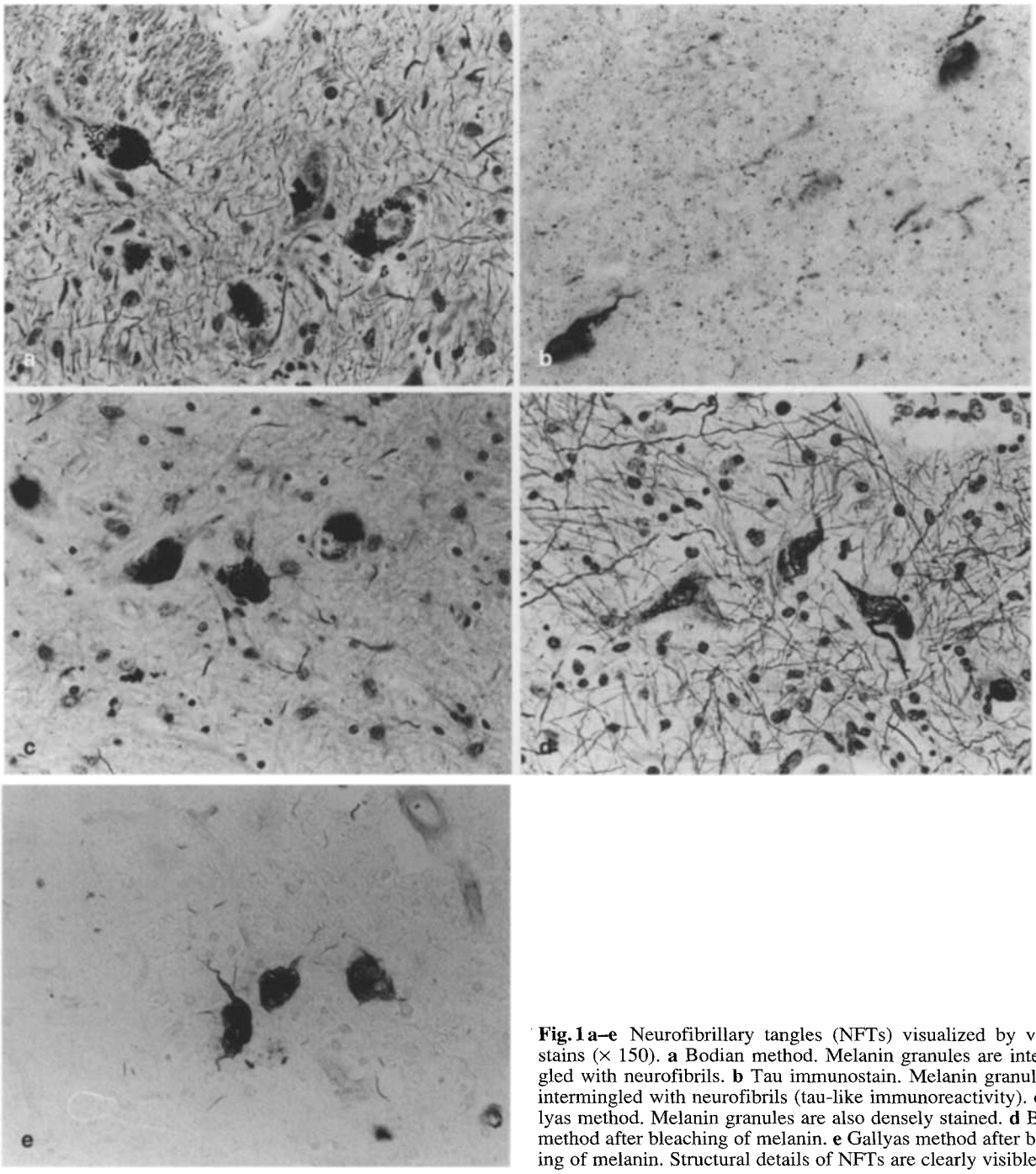

Fig. 1 a–e Neurofibrillary tangles (NFTs) visualized by various stains (× 150). **a** Bodian method. Melanin granules are intermingled with neurofibrils. **b** Tau immunostain. Melanin granules are intermingled with neurofibrils (tau-like immunoreactivity). **c** Gallyas method. Melanin granules are also densely stained. **d** Bodian method after bleaching of melanin. **e** Gallyas method after bleaching of melanin. Structural details of NFTs are clearly visible.

sections (10 μm thick) were stained by the following methods. tau immunohistochemistry (Fig. 1 b), conventional Bodian method (Fig. 1 a), conventional Gallyas method (Fig. 1 c) [2, 4], and bleaching of melanin prior to both the Bodian (Fig. 1 d) and the Gallyas (Fig. 1 e) method. We bleached melanin pigments by immersing the sections in 0.25% potassium manganese peroxide for 60 min followed by immersion in 5% oxalic acid for 5 min [13]. Tau-like immunoreactivity was visualized as deep purple by rabbit polyclonal anti-human tau antibody (1 : 10,000) generously provided by Dr Y Ihara, Brain Research Institute, University of Tokyo, using the avidin-biotin peroxidase method (ABC-Elite, Vector, Burlingame, CA) with 0.01% diaminobenzidine plus nickel ammonium as a chromogen. The numbers of NFTs in the unilateral SN visualized by these five methods were compared.

Sections were obtained from the upper SN (traversing the oculomotor nerve) and from the lower SN (traversing the decussation of the superior cerebellar peduncles) of the nine AD brains and 12 control brains. The Gallyas method after the bleaching pretreatment (the most sensitive method; see Results for details) was utilized to quantify NFTs. NFTs in the unilateral SN of each section

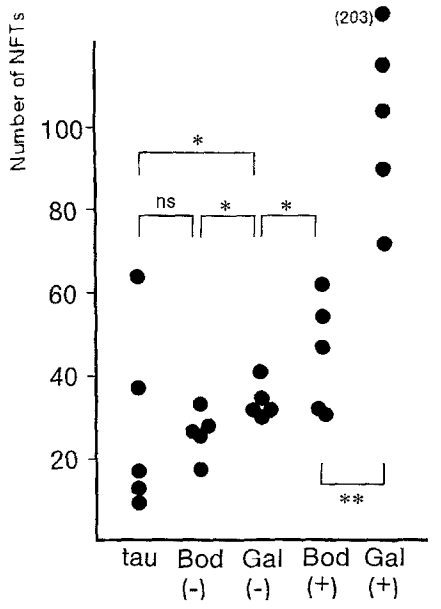

Fig. 2 The numbers of NFTs visualized by various staining methods. *tau* Tau immunostain, *Bod* Bodian method, *Gal* Gallyas method, *(+)* before bleaching of melanin (melanin pigment present), *(−)* after bleaching of melanin (melanin pigment absent); * $P < 0.05$, ** $P < 0.01$, *ns* not significant (Student's *t*-test)

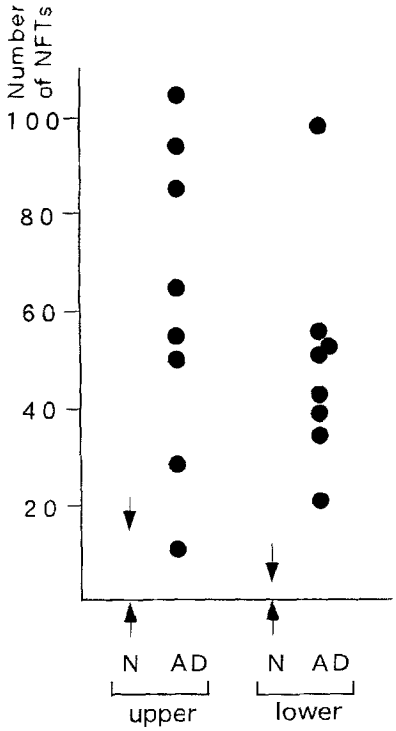

Fig. 3 The numbers of NFTs in the substantia nigra (SN). *N* brains from normal controls, *AD* brains from patients with Alzheimer's disease, *upper* upper SN, *lower* lower SN, *arrows* range of numbers of NFTs in normal controls

were counted three times. The methenamine-Bodian and methenamine-silver methods [12] were utilized to observe argyrophilic deposits such as amyloid.

Results

The numbers of nigral NFTs (the means of three counts) detected by different methods are shown in Fig. 2. Tau immunostaining (Fig. 1 b) visualized 28.4, 20.0 (mean, SD) NFTs. The Bodian method (Fig. 1 a) visualized 25.3, 5.0 NFTs. The Gallyas method (Fig. 1 c) was more sensitive in detecting NFTs (33.5, 3.9) than was tau immunostaining ($P < 0.05$, Student's *t*-test). However, the Gallyas method not only stained NFTs but also stained melanin densely, which inhibited clear visualization of NFTs. It was impossible by the conventional Gallyas method alone to stain NFTs selectively without staining melanin or the background. After bleaching of melanin, the Bodian method (Fig. 1 d) visualized a larger number of NFTs (45.6, 12.0, $P < 0.05$) than the conventional Gallyas method. However, the most sensitive method of all for visualizing NFTs was the Gallyas method after the bleaching of melanin (Fig. 1 e; 116.9, 45.6, $P < 0.01$ vs the Bodian method after the bleaching of melanin). In addition, nonspecific staining of the background was completely avoided with this bleaching pretreatment (Fig. 1 c, e).

Figure 3 shows the number of NFTs in the SN of brains from patients with AD and of control brains visualized by the Gallyas method after this bleaching pretreatment. The upper and the lower SN contained 62, 30 and 50, 21 NFTs, respectively. Most of these NFTs were in the neuronal cytoplasm in the zona compacta. The medial half of the SN contained a larger number of NFTs than the lateral half, as previously reported [21]. Among the 12 brains from the age-matched control patients, no NFTs were detected in 6 cases. Up to three NFTs were found in 5 cases. Fourteen NFTs were identified in the unilateral SN of one exceptional control brain.

Discussion

Numerous NFTs are present in the SN of the brains of people with progressive supranuclear palsy [18], postencephalitic parkinsonism [7, 10] and parkinsonism-dementia complex of Guam [8]. Based on conventional silver-staining methods, nigral NFTs in the AD brain have been considered to be not very numerous [6, 9, 19, 24]. However, we found that the number of detectable NFTs depends on the staining method. The coexistence of melanin results in underestimation of the number of detectable NFTs. We overcame this difficulty by bleaching melanin and found that an unexpectedly large number of nigral NFTs were present in the AD brain. In addition, this bleaching pretreatment decreased nonspecific background staining, regardless of whether melanin was present. After

this pretreatment, one can fully develop the colour of argyrophilic structures. This leads to enhanced sensitivity of the Gallyas method not only in the SN, but also in other areas, such as the cerebral cortex [22]. Most of the nigral NFTs were present mainly in the medial half of the zona compacta [21]. In AD brain, it has been claimed that nigral NFTs are clustered in the medial group of nigral neurons [5, 14, 20], which are believed to project mainly to the frontal cortex [17], where the primary process of degeneration typical of AD takes place. Retrograde degeneration of nigral neurons and subsequent formation of NFTs are reported to account for the clustering of NFTs in the medial portion of the SN [5].

In addition, we used sensitive methods to search for senile plaques and amyloid deposits in the SN [12]. However, such deposits were only rarely identifiable. These neuropathological features, namely a fairly large number of NFTs and substantial depletion of neurons with minimal or no amyloid deposition, have been reported [21]. It is well known that similar pathological features have also been noted in other subcortical nuclei such as the nucleus basalis of Meynert, the locus coeruleus [1], the pedunculopontine nucleus [16] and the nucleus raphe dorsalis [3, 23], all of which project extensively to the cerebral cortex. Melanin-containing neurons in the zona compacta are also known to project extensively to the cerebral cortex or the putamen. It was exclusively the zona compacta that contained NFTs in our study. We assume that these subcortical nuclei are commonly involved in the similar AD pathology with many tangles, essentially without amyloid. The scarcity of amyloid deposition is unlike the primary degenerative process typical of AD. It should be emphasized that the formation of NFTs is generally an intracellular event, while the deposition of amyloid is essentially an extracellular one. We suppose that retrograde degeneration involving these subcortical nuclei may initially affect intraneuronal processes, leading to the development of NFTs in the neuronal cytoplasm rather than extracellular deposition of amyloid.

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