REGULAR PAPER

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Silver staining profiles distinguish Pick bodies from neurofibrillary tangles of Alzheimer type: comparison between Gallyas and Campbell-Switzer methods

Abstract Silver staining profiles of Pick bodies (PBs) and their relation to tau-like immunoreactivity were examined on hippocampal sections and compared with those of neurofibrillary tangles of Alzheimer type (NFTs). Pairs of mirror sections were double-fluorolabeled with an anti-paired helical filament tau (AT8) antibody and thiazin red (TR), a fluorochrome that identifies fibrillary structures such as NFTs. One of the paired sections was subsequently stained using the Gallyas method (GAL), and the other using the Campbell-Switzer method (CS). By comparison of the same microscopic field on fluorolabeled sections and on both silver-stained paired sections, four different profiles of each structure could be distinguished: AT8 immunoreactivity, affinity to TR, argyrophilia with GAL or CS staining. PBs, containing mainly three-repeat (3R) tau, were positive for CS but not for GAL and its affinity to TR was, at most, weak. This selective affinity of PBs to CS is in sharp contrast with tau-positive structures of corticobasal degeneration/progressive supranuclear palsy, which are positive for GAL but not for CS, as we reported previously. This contrast is explainable if the argyrophilia with CS is related to deposits containing 3R tau, while that with GAL is linked to those containing four-repeat (4R) tau. Indeed, NFTs, containing both 3R and 4R tau, are positive for both CS and GAL, as expected. Taken together, differences in molecular composition of tau protein in these deposits are linked to their argyrophilic properties that are dependent on the staining method. Although explanations for these empirical differences are not yet available, awareness of this clear distinction is potentially of diagnostic and pathological relevance.

Keywords Argyrophilia · Campbell-Switzer · Diagnosis · Gallyas · Repeat tau

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Introduction

Degenerative tauopathies are characterized by tau-positive deposits, and some of them are considered to be diagnostic hallmarks for some clinicopathological entities. Tauopathies are currently classified according to the difference in their molecular species [three-repeat (3R) or four-repeat (4R)] of pathologically phosphorylated tau [26]. Disease-specific definition of tau-positive deposits, however, is still a challenge because differentiation based on the their immunohistochemical profiles are so far not very successful in spite of this biochemical difference [5, 6, 10, 19, 38]. Because of the lack of reliable routine histological staining that discriminates these molecular species of tau, combination of biochemical analyses and tau immunostaining is considered the current standard to delineate tau pathology.

Initial observations on these deposits, now known to contain tau protein, were based on silver staining methods. These methods are still widely used for diagnosis of these tauopathies and Alzheimer's disease (AD) [1, 2, 7, 9, 12, 14, 15, 22, 36]. Further modifications of silver staining methods have been reported [3, 4, 17, 27] that claim an improved sensitivity with lower background and easier standardization of the procedure. Because the Gallyas method (GAL) [12] and its modification [4] are highly successful in visualizing innumer-

able lesions, it may appear that the differences between silver staining methods are based on their difference in sensitivity. Indeed, various silver staining methods have been demonstrated to have a different sensitivity in detecting AD-related deposits [8, 11, 18, 23, 33, 34, 35, 37].

In our previous study [32], we found that tau-positive lesions in corticobasal degeneration (CBD)/progressive supranuclear palsy (PSP) were positive for GAL but negative for the Campbell-Switzer method (CS) [7, 25], another sensitive silver staining method that labels neurofibrillary tangles of AD (NFTs). Because both GAL and CS label equivalent number of NFTs, this discrepancy between CS and GAL observed in CBD/PSP may represent a feature unique to CBD/PSP. This implies that differences in silver staining methods are related not only to their sensitivities but also to the characteristics of each tau deposit. This prompted us to examine argyrophilic features of another type of tau-positive inclusions, Pick bodies (PBs), that contain predominantly 3R tau. Although little is known about how these argyrophilic properties are formed [13, 16], ''argyrophilia'' is now found to be dependent not only on the sensitivity of each staining procedure but also on the disease process, and, therefore, is of potential importance in histological distinction of tauopathies. Moreover, this difference in argyrophilic property may possibly represent a different architecture or molecular composition of the deposits.

Materials and methods

Four cases of PB disease (PBD) and four cases of AD were enrolled in this study. Pathological diagnosis of AD was based on the published criteria [21]. Demographic data on these cases are shown in Table 1. Brains were fixed in formalin and embedded in paraffin. Serial hippocampal sections were stained either with GAL [4, 12] or CS [3, 7] and the corresponding argyrophilic structures were compared. Mirror section pairs (4 μm thick) from the hippocampus were subjected to subsequent studies to identify possible relation between argyrophilia and tau-like immunoreactivity, as reported previously [32]. Pairs of mirror sections were first incubated at 4°C for 2 days with an anti-paired helical filament tau antibody (AT8, 1:10,000, Zwijndrecht, Belgium [20]) and the target epitope was visualized with an anti-mouse IgG conjugated with Alexa 488 (1:500, Molecular Probe, Eugene, OR). Sections were then incubated with thiazin red (TR, 1:30,000, Wako, Tokyo, Japan) for 15 min. After being observed under a confocal microscope (Leica TSC/SP, Heidelberg, Germany), one of the section pair was stained with GAL and the other with CS to compare argyrophilic properties of each AT8- or TR-positive structure. Identification of the same microscopic field on the fluorescence images (AT8 and TR) and on the corresponding silver-stained (GAL and CS) pair-wise images allowed us to compare staining profiles of each structure based on four different properties; AT8 immunoreactivity, affinity to TR, argyrophilia with GAL and that with CS.

Results

On hippocampal sections from AD brains, CS visualized NFTs and neuropil threads (NTs) as well as innumerable senile plaques (SPs) (Fig. 1A). On the neighboring section, GAL also visualized NTFs to an equivalent extent and a larger number of NTs (Fig. 1B), while SPs stained with GAL were limited to those with neuritic reactions. On hippocampal sections from PBD, CS clearly visualized PBs (Fig. 1C, E). In contrast, argyrophilia by GAL (Fig. 1D, F) was nearly absent or, if detectable, very weak (asterisks in Fig. 1D).

This contrast was further confirmed on mirror section pairs. Fluorolabeling of a mirror section pair from AD brains with AT8 and TR visualized NFTs and NTs (Fig. 2A, a) that were clearly stained with TR (red) or both TR and AT8 (yellow, Fig. 2A, a, arrowhead), dependent on their evolutionary stage [29]. One of the section pair was subsequently stained with CS (Fig. 2B, C), and the other with GAL (Fig. 2b, c). Each of the CS-positive structures was identifiable on the corresponding mirror section stained with GAL, and vice versa. These structures positive for both CS and GAL (asterisks and arrowheads in Fig. 2B, b, C, c) were also positive for TR or for both TR and AT8 (asterisks and arrowheads in Fig. 2A, a). Another section pair from PBD brains, initially fluorolabeled (Fig. 3A, a) and subsequently silver-stained either with CS (Fig. 3B, C) or GAL (Fig. 3b, c), demonstrated that each of AT8-positive PBs (asterisks and arrowheads in Fig. 3A, a) was stained with CS (Fig. 3B, C, asterisk and arrowhead). GAL, however, failed to label these PBs (Fig. 3b, c). Affinity of TR to these PBs was less evident (Fig. 3A, a) compared with that of NFTs (Fig. 2A, a).

Discussion

A number of methods of silver staining have been developed to delineate pathological lesions related to

Table 1 Demographic data of the cases (*PBD* Pick body disease, *AD* Alzheimer's disease)

Pathological diagnosis	Brain weight (g)	Age at death (years)	Duration (years)	Gender
PBD	1,090	56	1.5	M
PBD	1,080	64	5	F
PBD	1,120	67	10	M
PBD	890	83	?	F
AD	755	62	15	F
AD	1,250	65	5	M
AD	1,070	66	10	M
AD	780	72	15	F

Fig. 1 Discrepant argyrophilia in PBs. **A**, **B** AD; **C–F** PB disease. Serial sections from pyramidal layer of hippocampus. **A**, **C**, **E** CS; **B**, **D**, **F** GAL. NFTs in AD brain exhibit argyrophilia after staining with CS (**A**) and with GAL (**B**) methods. PBs similarly exhibit argyrophilia after staining with CS (**C**, **E**) but their argyrophilia after staining with GAL is, at most, weak (*asterisks* in **D**, **F**). *Arrows* in **A** and **B** and *arrowheads* in **C** and **D** indicate the same blood vessels. **E**, **F** Higher magnification of squared area in **C** and **D**, respectively. *Asterisks* in **E** and **F** indicate the same neuron harboring a PB (*PB* Pick body, *AD* Alzheimer's disease, *CS* Campbell-Switzer stain, *GAL* Gallyas-Braak stain, *NFT* neurofibrillary tangle). *Bars* **A–D**100 μm, **E**, **F** 25 μm
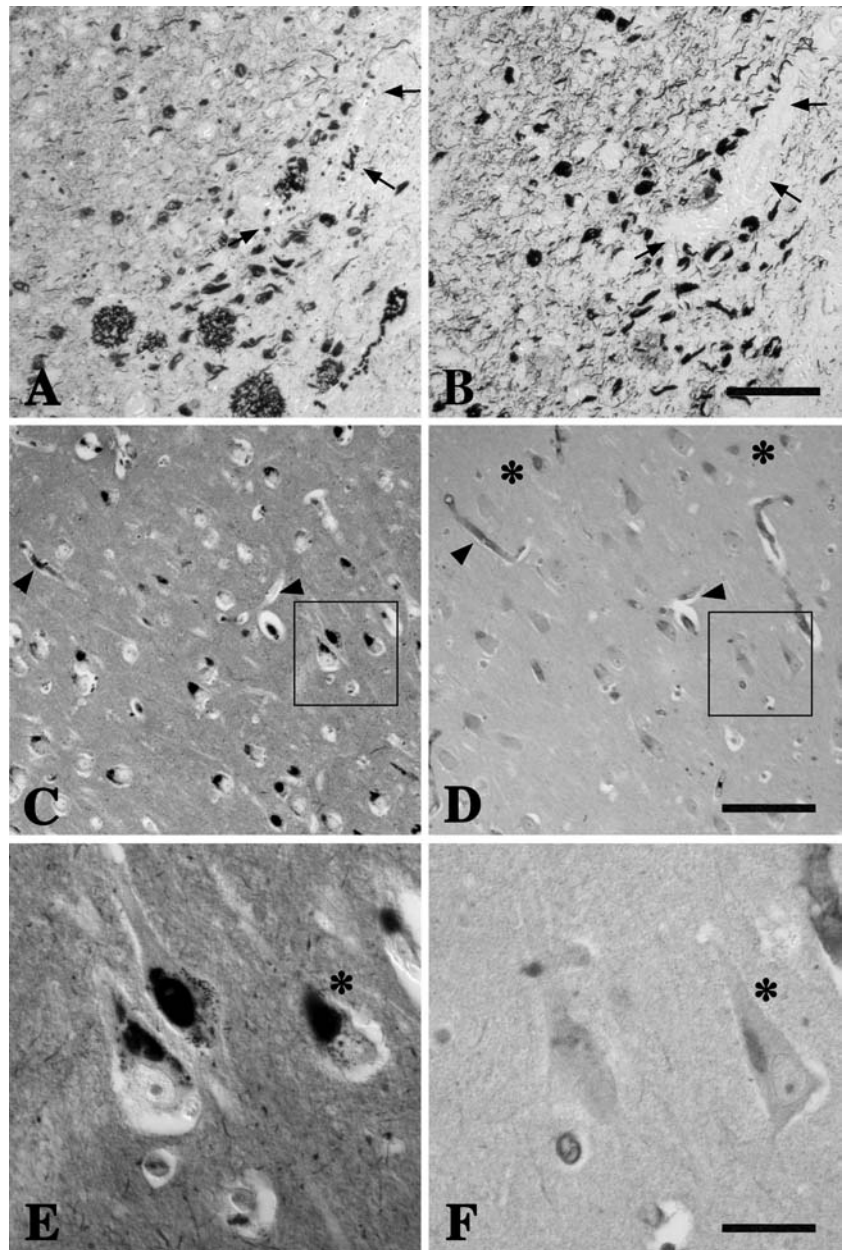
neurodegeneration [1, 2, 7, 9, 12, 14, 15, 22, 36]. Although some of them are highly useful in diagnosis and research, their utility is based merely on empirical relevance because little is known about the mechanism of how argyrophilic properties are engendered [13, 16, 17, 25]. For AD-related lesions, the number (quantity) of visualized lesions is dependent on the silver staining method used [8, 11, 18, 23, 33, 34, 35, 37]. This gives an impression that the sensitivity for visualizing AD-related lesions is dependent on the silver staining method. Our previous study [32], however, demonstrated that tau-positive lesions in CBD/PSP lacked argyrophilia when they were stained with CS, while they were clearly visualized with GAL. This contrast indicates that each staining method exhibits argyrophilia in disease-dependent manner. In other words, possible difference be-

tween silver staining methods is related not only to their sensitivity to detect lesions (quantitative difference) but also to their ability to distinguish lesions in disease-dependent manner (qualitative difference). In the present study, we expanded this approach to PBD, and found that the argyrophilia of PBs was detectable with CS but not with GAL. This staining profile was absolutely complimentary to that observed for tau-positive-lesions in CBD/PSP, which exhibit argyrophilia with GAL but not with CS. Although we have no direct explanation for this empirical distinction, one hypothesis is that argyrophilia with CS is related to tau deposits composed of the 3R species, as we observed in PBD, whereas that with GAL is linked to tau deposits mainly composed of the 4R counterpart, as reported in PSP/CBD [32]. NFTs of Alzheimer type, containing both the 3R and 4R

Fig. 2 NFTs exhibit equivalent argyrophilia with either silver staining method. Mirror section pair from pyramidal layer of hippocampus of AD brain initially fluorolabeled (**A**, **a**) with anti-PHF tau (AT8, *green*) and thiazin red (TR, *red*) and subsequently silver-stained with CS (**B**) or GAL (**b**). AT8-positive neurons are also stained with TR to yield *yellow color*, indicating that they are composed of rigid fibrillary structure. Fibrillary structures stained as *red* are extraneuronal NFTs that lost immunoreactivity to AT8. Both AT8-positive NFTs (*yellow*) and AT8-negative NFTs (*red*) exhibit argyrophilia with either CS or GAL method. **C**, **c** Higher magnification of the area indicated in **B**, **b**, respectively. *Arrows* (**A**, **B**, **a**, **b**) indicate the same blood vessel. *Arrowheads* and *asterisks* (**A**–**C**, **a**–**c**) indicate corresponding NFTs. *Bars* **A**, **B**, **a**, **b** 100 μm; **C**, **c** 30 μm
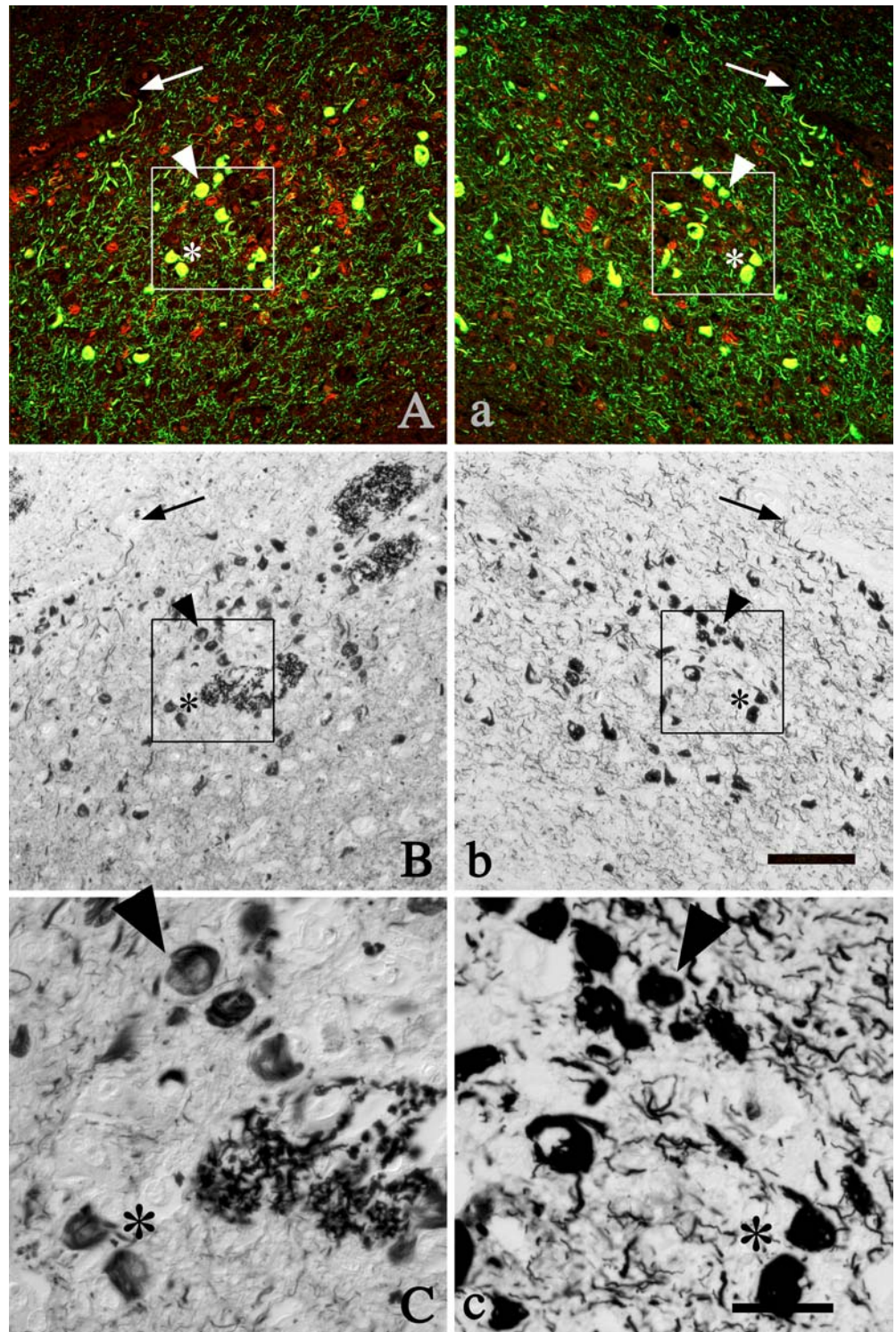
species, exhibit argyrophilia with either CS or GAL (Fig. 4).

Despite this sharp contrast, the staining procedures for GAL and CS are quite similar. Alkaline silver iodide is used after pretreatment with lantanium nitrate for GAL [4], while pyridine-silver is the initial step for CS [7]. Because subsequent visualization of silver particles is essentially the same for GAL and CS, this difference of initial silver reagents (alkaline silver iodide versus pyridine-silver) is one of the major factors responsible for this contrast. However, we do not yet know whether this distinction is based directly on the difference in tau isoforms or related to more complex composition after tau deposition. Another difference between PBs and NFTs depends on their affinity to TR, a fluorochrome that labels fibrillary structures

Fig. 3 Argyrophilia of PBs is dependent on the silver staining method. Mirror section pair from pyramidal layer of hippocampus of PBD brain initially fluorolabeled (**A**, **a**) with AT8 (*green*) and TR (*red*) and subsequently silver-stained with CS (**B**) or GAL (**b**). AT8-positive neurons are not stained with TR, indicating that they are composed of less fibrillary structure. **C**, **c** Higher magnification of the area indicated in **B** and **b**, respectively. *Arrows* (**B**, **b**) indicate the same blood vessel. *Arrowheads* and *asterisks* (**A**–**C**, **a**–**c**) indicate corresponding PBs (*PBD* Pick body disease). *Bars* **A**, **B**, **a**, **b** 100 μm; **C**, **c** 30 μm
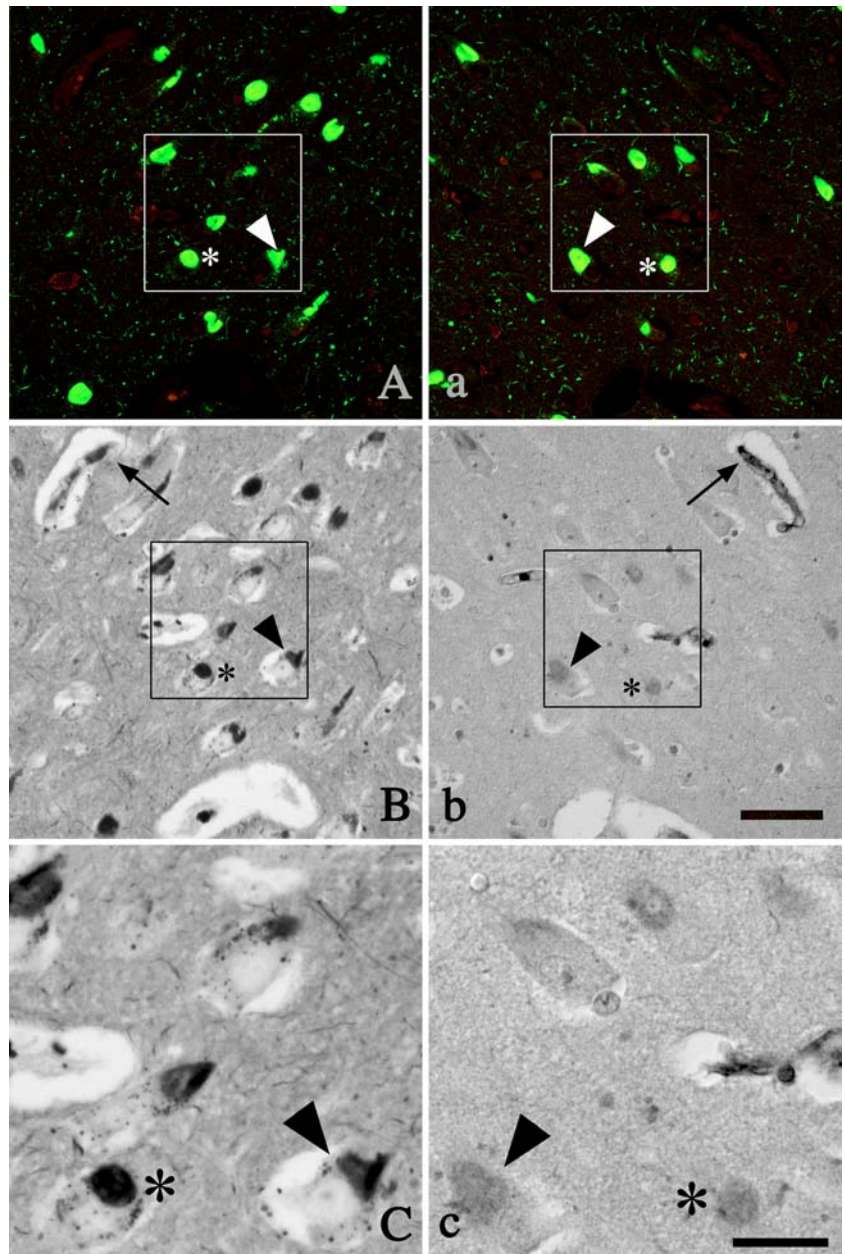
such as NFTs [30] or Lewy bodies [24]. This difference possibly represents a different density of fibrillary structures or qualitative difference in fibrillary composition, as demonstrated previously [29, 30]. The lack of GAL staining, with abundant CS staining, in PBs may be related to this difference. A mixture of 3R and 4R tau, as seen in NFTs, may lead to a more solid organization of fibrils recognized as NFTs [29], also identified clearly with TR. In contrast, deposition of 3R tau not accompanied by its 4R counterpart is organized into different, probably less fibrillary, structures that escape detection with TR or GAL. Our previous study demonstrated that PBs, composed predominantly of the 3R isoform of tau, are preferentially stained with Bodian method (BOD) but not with GAL [31]. Conversely, tau-positive structures in CBD are scarcely argyrophilic with BOD [28]. These data are compatible with the hypothesis that tau deposits, composed of 3R tau, exhibit selective argyrophilia with BOD and CS.

Through these studies, we became aware that each method of silver staining exhibits preferential affinity to tau-positive deposits in an isoform-dependent manner (Fig. 4). Although this correlation is empirical at present, and molecular mechanism to explain how argyrophilic properties specific for disease or tau isoform are engendered still remains to be elucidated, awareness of these differences will help in clearly recognizing and diagnosing specific pathological cascades, which are probably distinct from each other.
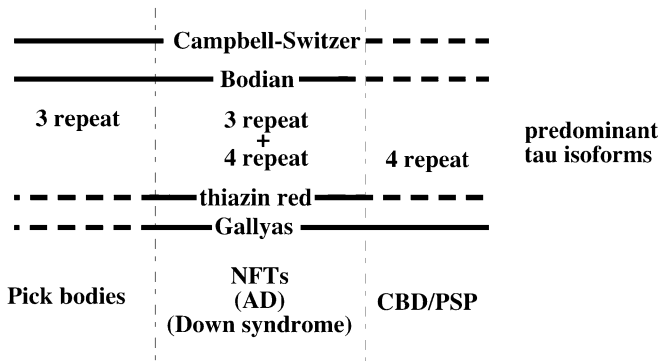
Fig. 4 Argyrophilic properties of tau-positive deposits are dependent on staining methods and on tau isoforms. Diseases are classified according to their predominant tau isoforms. *Solid lines* indicate positive argyrophilia and *interrupted lines* indicate negative argyrophilia (*CBD/PSP* corticobasal degeneration/progressive supranuclear palsy)

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