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Microscopic Investigations in Neurodegenerative Diseases

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Microscopy is the foundation of our understanding of the pathology of neurodegenerative diseases. It is the microscopic lesions associated with clinical disease processes which establish the clinicopathological entity. Initial advances in neurodegenerative disease pathology came in the form of silver impregnation techniques, which first elucidated neuronal microscopic anatomy and highlighted microscopic lesions in disease that were poorly visible using routine dyes. Enhanced understanding of the fine structure of disease-associated inclusions was obtained through electron microscopy. However, the current mainstay for the characterization of neurodegenerative disease is immunohistochemistry, and prominent among the probes are antibodies to amyloid-β, phospho-tau, α-synuclein, and ubiquitin. Overall, our understanding of the basic pathology of inclusions is considerable, and may have outpaced our understanding of the pathogenesis given that effective inclusion-targeted therapies has been disappointing to date.

Keywords amyloid; Bielschowsky; Bodian; Gallyas; immunohistochemistry; pathology; tau phosphorylation; α-synuclein, ubiquitin

1. Introduction

Since the original description of Alzheimer's disease (AD), the neuropathological interpretation of neurodegenerative diseases has been one of association, between a clinical phenotype on the one hand (e.g., cognitive decline, movement disorder, brainstem and cerebellar signs) and microscopic findings on the other. The combination of the two, the neurological findings and the microscopic lesions, define the disease, rather than one or the other in isolation. Thus, AD is the combination of progressive dementia clinically, and numerous senile plaques and neurofibrillary tangles microscopically. The differential diagnosis of progressive dementia alone is considerable, while numerous plaques and tangles can be seen in cognitively intact elderly as well as in other neurodegenerative conditions.

In spite of the lack of a diagnostic marker of AD microscopically, the tendency among pathologists and other scientists over the years has been to view microscopic lesions as having etiologic and pathogenic importance. Indeed, this mindset continues to the present, where numerous scientific papers, expensive clinical trials, and federal and other grant expenditures contain the precept that amyloid-β, discovered through isolation of senile plaques (a marker of advanced aged as much as disease), causes AD. The unfortunate consequence of this mindset has been to slow progress toward effective therapy in AD [1, 2].

On the other hand, a clear benefit of this perseveration on microscopic lesions in AD and other neurodegenerative diseases has led to detailed characterization of such lesions, and the impetus to develop novel techniques that uncover structures, epitopes, and adducts not apparent by routine microscopy. Thus, we now have a number of techniques and reagents available, in most cases, for routine diagnostic use. This can only help to facilitate understanding of these diseases overall and refine the association between clinical disease and pathological changes within the brain.

In this chapter, we will review some basic microscopic techniques used to characterize pathological lesions in neurodegenerative disease.

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2. Silver impregnation

Silver techniques represented a major advance in the study of microscopic neuroanatomy, and it is somewhat fitting that in 1906 when Alzheimer described the microscopic pathology of a patient who suffered cognitive decline using the Bielschowsky silver technique, Ramon y Cajal and Camillo Golgi were awarded the Nobel Prize for their pioneering neuroanatomical studies that featured prominently the Golgi silver technique. A recent, comprehensive review of silver impregnation techniques is available in the literature [3].

While the various silver impregnation protocols provided structural details at the light microscopic level that were not discernible using routine dyes (Hematoxylin and eosin, cresyl violet, luxol-fast blue), the regular use of various silver staining protocols has been hampered over the years by the capriciousness of the techniques and lack of standardization of methods, as well as insular nature of small laboratory sections and lack of sharing of essential details. Indeed, some have likened Bielschowsky silver staining to “witchcraft” when a given lab or technician is able to obtain quality results on a regular basis (W.J. Kupsky, personal communication). Nevertheless, silver staining methods have been the subject of continued effort and modification, and new and improved modifications of the basic technique will likely continue to appear in the literature.

2.1 Basic concepts of silver staining

Microscopic neuropathology would be of little use without the ability to provide contrast to tissue. One look at an unstained paraffin section makes this point very clear. Conventional dyes comprise the simplest approach to provide contrast by virtue of differential affinity for chemical elements of the tissue for the dyes being used; thus, nucleic acid and associated material (and therefore nuclei) attract hematoylin, while cytoplasmic proteins and organelles, and most extracellular matrix material attract eosin. The precise chemical basis for this is less important than the results, as the combination of the two dyes, or even a single dye, provides enough contrast in tissue sections to facilitate the identification of normal structures, and departure of normal (i.e., pathology) (Figure 1A). In contrast to silver staining, it is important to note that dyeing occurs in a simple, single step and does not involve chemical alteration of the dye.

Silver stains, on the other hand, first attach with differential affinity to various target elements as silver ions or silver salts, and then require a second reducing step, or chemical transformation, in order for metallic silver to be visualized microscopically. Thus, silver stains involve site-directed chemical transformation that, at least in theory, offers potential for specific labeling not otherwise inherent in conventional dyes [3]. Moreover, the fact that i) there is a time lag between the initial attachment step and the chemical transformation step, and ii) the two steps are independently affected by pH, temperature, and concentration of the reagents, the system is amenable to manipulation and experimentation with different developing technique; hence the proliferation of silver impregnation techniques, modifications of techniques, and chemical as well as physical developing techniques [4].

Attempts to identify molecular structures that bind silver using a given technique have been limited. Gambetti et al. [5] noted an association between Bodian silver staining and neurofilament species, while Iqbal et al. [6] found an association between PHF-tau and Gallyas silver staining. For practical purposes, however, neurodegenerative disease interpretation and classification using silver methods is almost entirely an empirical exercise. Emphasis is therefore placed on the ability of the staining protocol to demonstrate specific inclusions rather than the molecular target *per se*, so that clinicopathological correlation, the basic exercise in neurodegenerative disease diagnosis, can be refined.

Other issues to consider when using silver impregnation include size of the final silver particles vs. quantity of silver being deposited, as well as packing density of the target structure [3, 7]. Both issues are unresolved in terms of their effect on final staining intensity, emphasizing again the empirical nature of silver techniques over their ability to label specific molecules.

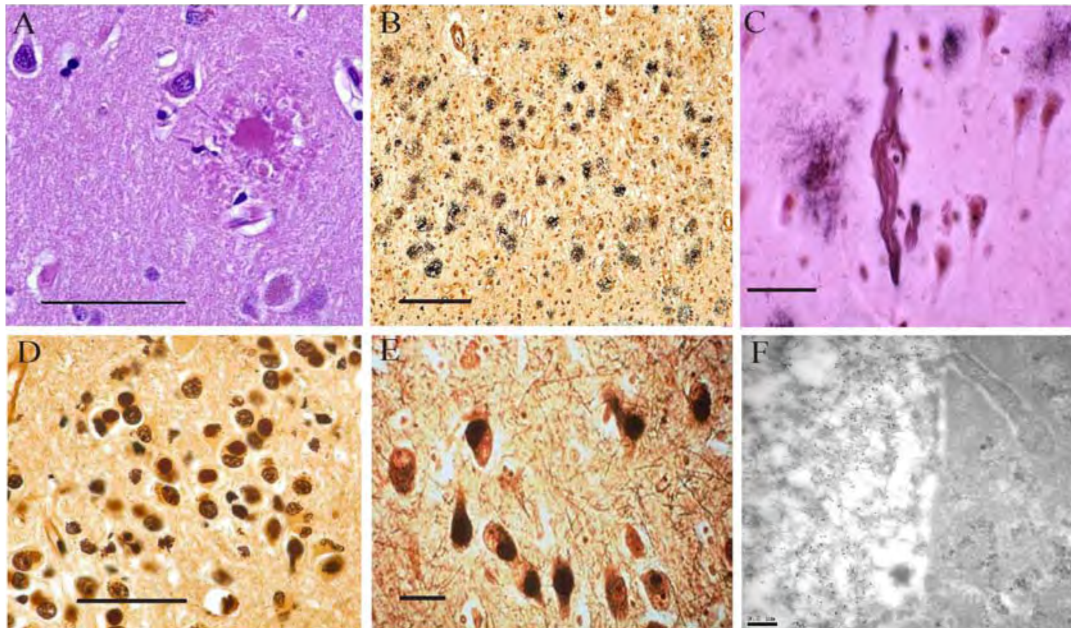

Figure 1. (**A**) Paraffin section of brain from a patient with AD stained with hematoxylin and eosin. Note the senile plaque with an amyloid core. Scale bar = 50 µm. **(B)** Bielschowsky silver impregnation of an AD brain demonstrating argyrophilic senile plaques. Scale bar = 100 µm. **(C)** Bielschowsky silver impregnation of an AD brain demonstrating an argyrophilic neurofibrillary tangle and diffuse plaques. Scale bar = 30 µm. **(D)** Bielschowsky silver impregnation of a patient with Pick's disease, demonstrating Pick bodies. Scale bar = 50 µm. **(E)** Bodian silver impregnation of a patient with Pick's disease. Note the normal axons in the background, typical of the Bodian preparation. Scale bar = 20 µm. **(F)** Immunogold electron microscopy of a Pick body showing straight filaments and labeling with phospho-tau antibody phospho-Thr 231. Image courtesy of Sabrina Mosaheb.

2.2 Specific silver techniques

The Golgi silver technique, while rarely used today, represented a major step in understanding of structural neuroanatomy. In the original technique, formalin fixed (or paraformaldehyde or glutaraldehyde perfused) tissue blocks are immersed in an aqueous solution of potassium dichromate. The block is then dried and immersed in another aqueous solution of silver nitrate. Sections are then cut (20 microns to 100 microns), dehydrated, and cleared. The technique has since been refined by substituting the silver precipitate with gold by immersion in gold chloride then oxalic acid, followed by removal of the silver by sodium thiosulphate. This produces a strong reaction in a cleaner background, and also preserves a greater degree of fine structure. If done properly, a subset of neurons and their dendritic arborizations will be demonstrated in fine detail.

The microscopic neuroanatomy of the neuron was elucidated essentially entirely by the Golgi technique, with no significant additional details being added since the original description by Ramon y Cajal. Indeed, the application of the Golgi technique to brain tissue established Cajal's neuron doctrine; that neurons are discrete cells (not connected in a meshwork); that neurons are genetically and metabolically distinct units; that they have cell bodies, axons, and dendrites; and that neural transmission goes only in one direction, from dendrites toward axons.

Bielschowsky methods have been the subject of numerous and repeated modifications over the years, testifying to the capriciousness and poor reproducibility of the technique [8]. Nevertheless, its application to dementia brains by Alzheimer proved to be a seminal contribution to neurodegenerative disease diagnosis and research (Figure 1B,C). Bielschowsky silver staining has its origin in the production of mirrors based on its use of the silver diamine complex that precipitates on the interface

between a glass surface and the solution. The initial application to tissue was performed by Fajersztajn, but improved by Bielschowsky by incubating formalin fixed tissues in silver nitrate prior to the silver diamine, along with pretreatment with pyridine. The most commonly used and arguably most reproducible modification of the Bielschowsky technique is that proposed by Yamamoto and Hirano [9], which employs the usual silver nitrate-ammoniacal silver steps, followed by another ammoniacal silver step in the developer. If done properly, Bielschowsky silver methods highlight senile plaques and neurofibrillary tangles in AD and Pick bodies in Pick disease (Figure 1D) while normal tissues remain minimally stained or unstained.

In contrast to Bielschowsky methods, Bodian silver impregnation has better reproducibility but also stains normal structures – axons and neurofibrils – per its original design [3] (Figure 1F). As such, it is less suitable for neurodegenerative disease interpretation, but quite useful in demonstrating normal axons and axonal alternations. Among its practical uses are the demonstration of relative axonal preservation in demyelinating conditions, and axonal swellings characteristics of acute to subacute diffuse axonal injury. Bodian silver involves silver proteinate, along with metallic copper to enhance the intensity of the staining since copper is more readily ionizable than silver. The reaction is developed in 1% hydroquinone and 5% sodium sulfite.

The Gallyas silver method exploits the principals of photography and "physical development," which provides reducing agents to form silver metal which have already been activated by exposure to light [10, 11]. In the Gallyas method, pretreatment of tissue sections with potassium permanganate, oxalic acid, add lanthanum nitrate/sodium acetate, is followed by alkaline silver iodate, and physical development that includes silver nitrate. The Gallyas method is superior to the Bielschowsky method in its reproducibility and ability to visualize abundant deposits [12] and also tends not to react with normal neural parenchyma. It is useful in demonstrating neurofibrillary tangles, as well as glial cytoplasmic inclusions of multiple system atrophy, while Lewy bodies, Pick bodies, and the apparent amyloid component of senile plaques generally show no labeling. The Gallyas method is recognized as the superior silver technique for demonstrating argyrophilic grains in so-called argyrophilic grain dementia [13].

In the Campbell-Switzer method, pyridine is present in the initial impregnation solution that also contains silver nitrate and potassium carbonate; this is followed by physical development similar to the Gallyas method [3]. The Campbell-Switzer method is also highly stable and reproducible. In spite of its many similarities with the Gallyas method, the Campbell-Switzer method labels Pick bodies, and generally does not label inclusions associated with progressive supranuclear palsy, corticobasal degeneration, and argyrophilic grain dementia. The basis for this is unclear.

2.3 Silver stains and dementia brain interpretation

Since the modified Bielschowsky silver method effectively labels both senile plaques and neurofibrillary tangles (as opposed to Gallyas, for example, which labels neurofibrillary tangles well, but labels senile plaques less well and fails to detect diffuse plaques), this method has generally been used as the standard technique for AD diagnosis [14-16]. In light of the methodological and reproducibility issues surrounding the Bielschowsky method, attempts at quantitation of histological lesions is a dubious exercise, and it is not surprising that histological diagnosis of AD on this basis suffers from low specificity and overlap with the cognitively intact elderly. Even more dubious is the correlation between Bielschowsky silver staining results and mild cognitive impairment. Such efforts nevertheless continue to appear in the literature [17].

The comparison of silver methods on the basis of neuropathological phenotype is a subject of some interest (Table 1). Since the Gallyas silver method does not label Pick bodies (a 3-repeat tauopathy [18]) and instead labels lesions of progressive supranuclear palsy, corticobasal degeneration, and argyrophilic grain dementia (considered 4-repeat tauopathies [19,20]), it has been suggested that Gallyas silver has an affinity for specific abnormal tau species. Indeed, some have suggested that inclusions be defined on the basis of their silver reactions [21]. Pick bodies and Lewy bodies, for example, should be Bielschowsky-

positive and Gallyas-negative. Lesions of progressive supranuclear palsy should be Campbell-Switzer-negative and Gallyas-positive. And so on. On the other hand, all silver techniques in current use variably stain both α-synuclein-positive and phospho-tau positive inclusions, again indicating an incomplete understanding of the molecular basis of silver impregnation [3]. As many inclusions are distinguishable on the basis of routine dyes (e.g. neurofibrillary tangles, Pick bodies, Lewy bodies), to say nothing of immunohistochemistry (see below), we believe that silver methods are best used to *highlight* inclusions, rather than define them.

Table 1 Labeling of neurodegenerative inclusions with Bielschowsky, Gallyas, and Campbell-Switzer methods (modified from [3]).

	Bielschowsky	**Gallyas**	**Campbell-Switzer**
Senile plaque	+++	++	+++
Diffuse plaque	+++	-	+++
Neurofibrillary tangles	++	+++	+++
Pick body	+++	-	+++
CBD/PSP/AG	-	+++	+
Lewy body	-	++	++
GCI	++	++	++

CBD/PSP/AG = lesions associated with corticobasal degeneration, progressive supranunclear palsy, and argyrophilic grain dementia; GCI = glial cytoplasmic inclusions of multiple system atrophy

3. Electron microscopy

3.1 Alzheimer's disease

Similar to electron microscopic studies across the range of disease processes, ultrastructural analysis of AD changes began in the 1960's, when it was noted that paired helical filaments (PHFs) were characteristic of neurofibrillary tangles [22]. Ultrastructural examination showed that PHFs have a maximum diameter of 20 nm and display a periodic narrowing to 10 nm every 80 nm. In addition to the PHFs, straight filaments have been described in AD, with no twisting and no variations in width along its length. These filaments are referred to as straight filaments, and typically measure approximately 15 nm in width. Straight filaments make up a small (about 5%) percentage of the abnormal filaments found in neurofibrillary tangles in AD, although they are considered the predominant ultrastructural feature of neurofibrillary tangles associated with progressive supranuclear palsy [23]. Biochemical analysis showed that the PHFs are primarily comprised of hyperphosphorylated tau protein, which has been substantiated by immunogold electron microscopy.

Neuritic plaques are composed of an extracellular core of filaments that measure 5-10 nm in diameter and are surrounded by dystrophic neurites and other debris, as well as microglial cytoplasmic processes and astrocytic processes containing glial filaments. Dense bodies, autophagic vacuoles and other membranous debris are common. As neuritic plaques are defined by the presence of dystrophic neurites, it is not surprising that PHFs are variably observed in neuritic plaques at the ultrastructural level.

The kinetics of plaque formation is among the many unknown questions in AD pathogenesis, although some have speculated that plaques progress from diffuse amyloid-β deposits to well developed senile plaques as described above. Not surprisingly, progression to varying stages of maturation has been described at the ultrastructural level, although it is important to emphasize that any detailed morphological analysis represents a "snapshot" at a single point time, such that any discussion about kinetics is highly conjectural.

The amyloid component of senile plaques - 5 to 10 nm non-branching fibrils typical of amyloid - was sought and described early on in electron microscopic studies, as an amyloid component to senile plaques was known on a histochemical basis since the description by Divry in 1927. Immunogold electron microscopy has since confirmed the presence of amyloid-β epitopes within the fibrils, substantiating the biochemical detection of amyloid-β [24].

3.2 Parkinson's disease

First described by Lewy in 1912, Lewy bodies are neuronal inclusions that are considered the pathological hallmark of Parkinson's disease. On light microscopic examination, Lewy bodies are eosinophilic, roughly spherical neuronal inclusions, often containing a pale peripheral halo. Electron microscopy of Lewy bodies reveals an amorphous electron-dense core surrounded by radiating filaments, initially thought to be comprised mainly of neurofilaments, but now known to contain α-synuclein as a principal protein component. The α-synuclein component has been verified by immunogold electron microscopy [25]. Lewy bodies also contain ubiquitin and other proteins involved in cytosolic proteolysis [26]. Granules of lipofuscin, dense-core vesicles, and degenerative changes are variably present.

3.3 Pick's disease

Electron microscopy of Pick bodies shows that they are primarily comprised of a mixture of filaments and microtubules [27]. Randomly arranged straight filaments with a diameter from 14 to 16 nm, intermediate filaments, and PHFs with a 22 to 24 nm diameter and a half period of 120 to 160 nm have all been described which are comprised principally of phosphorylated tau protein (see below) (Figure 1F). Degenerated organelles and entrapped vesicular and membraneous material are often trapped within the inclusion. Although Pick bodies appear well demarcated on light microscopic examination, they lack a limiting membrane.

4. Immunohistochemistry

The application of immunohistochemistry to brain tissue affected by neurodegenerative disease has rendered silver impregnation and electron microscopy obsolete in terms of molecular characterization of diseases. Indeed, in our laboratory, silver stains are used for historical purposes only, and electron microscopy is performed only for specific research projects. It is important to keep in mind, however, that immunohistochemistry requires a known target protein; in many cases that target protein was only discovered after detection of a lesion by other means (dyes, silver impregnation) and subsequent purification (e.g., amyloid-β, phospho-tau).

4.1 Amyloid-β

Following isolation and purification of senile plaques plaques, amyloid-β, a metabolic product of the amyloid-β protein precursor (AβPP), was found to be a major protein component of senile plaques, as well as the amyloid species that affects blood vessels in cerebral amyloid angiopathy. The presence of AβPP on chromosome 21, the early affliction of Down's syndrome patients by AD pathology, and the existence of kindreds with familial dementia and mutations in and around amyloid-β component of AβPP, set the Amyloid Cascade Hypothesis in motion, which continues to the present with only minor modifications [1].

Amyloid-β highlights amyloid deposits within the brain with a high degree of sensitivity and specificity, superior to silver impregnation techniques on both accounts. Diffuse plaques are readily identified, as are amyloid-β deposits within blood vessels (Figure 2A). The technique thus allows semiquantitative assessment of amyloid-β deposits in demented subjects at autopsy, and establishment of

the range of amyloid-β deposition that may be seen in individuals who are not demented. It further has facilitated characterization of experimental models of AD. As such, amyloid-β immunohistochemistry is an indispensable tool in both research and diagnosis.

The identification of different amyloid-β isoforms, with potentially different distributions and toxicities (e.g., Aβ1-40, Aβ1-42), has fostered development of isoform specific monoclonal antibodies as a further refinement in the study of amyloid-β pathophysiology within the brain.

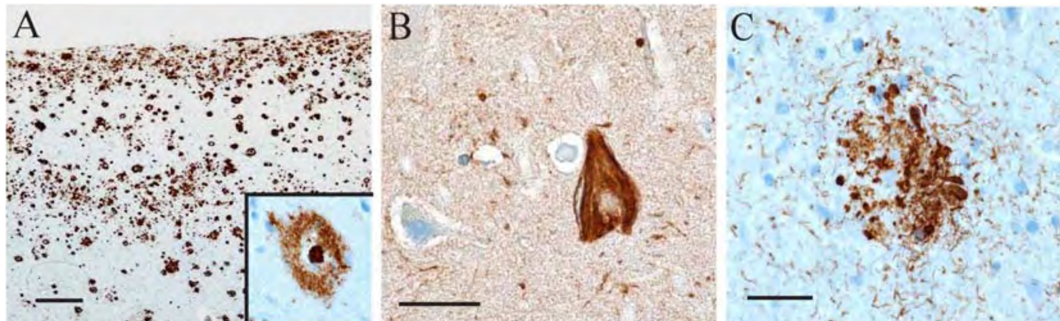

Figure 2. **(A)** Low magnification photomicrograph of cerebral cortex in a patient with AD, demonstrating numerous cored and diffuse plaques, as well as cerebral amyloid angiopathy, using amyloid-β immunohistochemistry (antibody 4G8). Scale bar = 100 µm. **(A, inset)** Senile plaque with amyloid core stained using amyloid-β (4G8) immunohistochemistry. Scaler bar = 40 µm. **(B)** Immunohistochemical stain for phospho-tau (AT8) in a patient with AD, demonstrating a phospho-tau-positive neurofibrillary tangle. Scale bar = 30 µm. **(C)** Immunohistochemical stain for phospho-tau (AT8) in a patient with AD, demonstrating a phospho-tau-positive neuritic plaque. Note the dystrophic neurites and neuropil threads, with absence of staining of the amyloid core. Scale bar = 30 µm.

4.2 Phospho-tau

Tau protein, a low molecular weight microtubule associated protein, is present in all nucleated cells. It is particularly abundant within neurons, and its role in maintaining microtubule stability within neurons appears particularly vital. In neurodegenerative diseases characterized by tau accumulation (Table 2), tau protein becomes hyperphosphorylated, and phosphorylated at abnormal residues, as an apparent imbalance between phosphatase and kinase activity [28]. Antibodies thus used to characterize neurodegenerative disease via immunohistochemistry recognize abnormal, disease associated epitopes (e.g., Alz-50, PHF-tau, AT8).

Similar to amyloid-β, phospho-tau immunohistochemistry highlights “neurofibrillary pathology” with a high degree of sensitivity and specificity. In addition to demonstrating neurofibrillary tangles in tissue sections (Figure 2B), many of which are subtle or undetectable with routine dyes, phospho-tau immunohistochemistry demonstrates related inclusions such as neuropil threads, dystrophic neurites (Figure 2C), and diffuse “pre-tangle” tau accumulations.

Clinicopathological studies have demonstrated that phospho-tau accumulations correspond more closely to clinical signs compared to amyloid-β accumulations, and progress in a stepwise fashion from transentorhinal, to limbic, to isocortical areas, forming the basis for AD staging [1,29].

Tau pathology is not limited to AD, but is characteristic of other disease processes including progressive supranuclear palsy, Pick disease (Figure 3A), corticobasal degeneration (Figure 3B), frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), post-encephalitic Parkinsonism, ALS/dementia/Parkinsonism complex of Guam, and argyrophilic grain dementia. Application of phospho-tau immunohistochemistry to various neurodegenerative diseases has fostered the description of a number of tau-positive inclusions. The conditions, in turn, have been examined biochemically for tau isoforms that have either three (3R tau) or four (4R tau) microtubule binding repeats. Table 2 contains a list of tauopathies, associated inclusions, and preferential tau isoforms expressed.

Table 2 Tauopathies

Tauopathy subtype	Tau-immunoreactive microscopic lesions	Preferential tau isoform
Alzheimer's Disease	Neurofibrillary tangles (NFT) Neuropil threads Dystrophic neurites	3R/4R
Pick's disease	Pick bodies Pick cells (focal, weak staining)	3R
Progressive supranuclear palsy	NFT - globose and flame shaped Pick body like inclusions Neuropil threads Tufted astrocytes Coiled bodies (oligodendroglial)	4R
Corticobasal degeneration	Corticobasal bodies (globose NFT) Pick body like inclusions Neuropil threads Astrocytic plaques Coiled bodies (oligodendroglial)	4R
FTDP-17	NFT* Pretangles Pick bodies Neuropil threads Astrocytic plaques Tufted astrocytes Coiled bodies	3R/4R
ALS/PDC-Guam	NFT Neuropil threads	3R/4R
Post-encephalitis Parkinsonism	NFT Neuropil threads	3R/4R
Argyrophilic grain disease	Argyrophilic grains	4R

FTDP-17 = Frontotemporal dementia with Parkinsonism linked to chromosome 17
ALS/PDC-Guam = amyotrophic lateral sclerosis/dementia/Parkinsonism complex of guam
3R = three repeat tau isoform
4R = four repeat tau isoform

4.3 α-Synuclein

α-synuclein has long been recognized as an important protein involved in cell cycle control and differentiation in lower animals, including annual forebrain reorganization in songbirds [30]. A potential role in neurodegenerative diseases had not been predicted until mid 1990's, when a kindred of Parkinson's disease subjects were examined by linkage analysis and found to have an α-synuclein mutation [31]. Since then, α-synuclein has been determined to be the major protein component in Lewy bodies (Figure 3C), as well as Lewy neurites, and has been the subject of intensive investigations. α-synuclein has also been linked to the glial cytoplasmic inclusions in multiple system atrophy (Figure 3D), and in various inclusions in neurodegeneration with brain iron accumulation type 1 (previously known as Hallervorden-Spatz disease). α-synucleinopathies along with associated α-synuclein positive inclusions are listed in Table 3.

Table 3 Synyucleinopathies

Synucleinopathy subtype	**Inclusions demonstrates by alpha-synuclein immunohistochemistry**
Parkinson's disease	Lewy bodies Lewy neurites
Lewy body dementia	Lewy bodies Lewy neurites
Multiple system atrophy	Glial cytoplasmic inclusions Neuronal cytoplasmic inclusions Neuronal intranuclear inclusions Neurites
NBIA-1	Lewy bodies Lewy neurites Axonal spheroids

NBIA-1 = neurodegeneration with brain iron accumulation type 1

4.4 Ubiquitin immunohistochemistry

Ubiquitin immunohistochemistry was among the earlier immunohistochemical methods for characterization of neurodegenerative diseases. As ubiquitin functions to tag abnormally accumulating proteins for proteolysis by lysosomal and non-lysosomal pathways, it is perhaps not surprising that ubiquitin immunohistochemistry would highlight various inclusions [32]. Ubiquitin immunohistochemistry, however, is not uniformly and strongly reactive across the range of inclusions; it labels neurofibrillary tangles less well than does phospho-tau, and does not appreciably label senile plaques. Ubiquitin immunostaining does, however, label Lewy bodies. Indeed, prior to the availability of α-synuclein immunohistochemistry, ubiquitin immunohistochemistry was the method of choice for identifying neocortical Lewy bodies.

Occasional inclusions label with neither phospho-tau, nor α-synuclein, but react with ubiquitin. In this respect, ubiquitin immunohistochemistry remains a necessary technique in the interpretation and characterization of the spectrum of neurodegenerative processes. Among such inclusions are small intracytoplasmic inclusions seen in a subset of frontotemporal dementia cases (so called motor neuron disease or MND-type inclusions (Figure 3F), Lewy body-like inclusions of amyotrophic lateral sclerosis (Figure 3F), and dystrophic neurites in Huntington's disease [33].

5. Antigen retrieval

Proteinaceous inclusions among the various neurodegenerative diseases share in common the property of protease-resistance and insolubility. Thus, in order to enhance immunoreactivity, sections are typically subjected to one or more "antigen retrieval" methods. The chemical basis for antigen retrieval has not been completely elucidated, although it is reasonable to speculate that epitopes of interest are buried in more soluble molecules, such that by exposing the tissue to some denaturant or chemical modification, the less soluble and more abundant epitope is exposed (e.g., phospho-tau, amyloid-β, protease-resistant prion protein), facilitating a stronger overall reaction.

Formic acid, citrate-EDTA, and proteinase K are among the chemical treatments used for purposed of antigen retrieval. Microwaves, steamers, and autoclaves may also be used to facilitate the retrieval by increasing molecular motion. Perhaps the most aggressive antigen retrieval regimen that exists involves prion protein immunohistochemistry (typically using monoclonal antibody clone 3F4). The currently used method [34], which is a modification of the original method by Kitamoto et al. [35], involves pretreatment in 99% formic acid for one hour, followed by autoclaving for 20 minutes in the presence of 1.5 mMol HCl. By using such drastic pretreatment, the soluble, proteinase K sensitive prion protein is degraded, leaving behind only the proteinase K resistant (pathogenic) prion protein (Figure 3G).

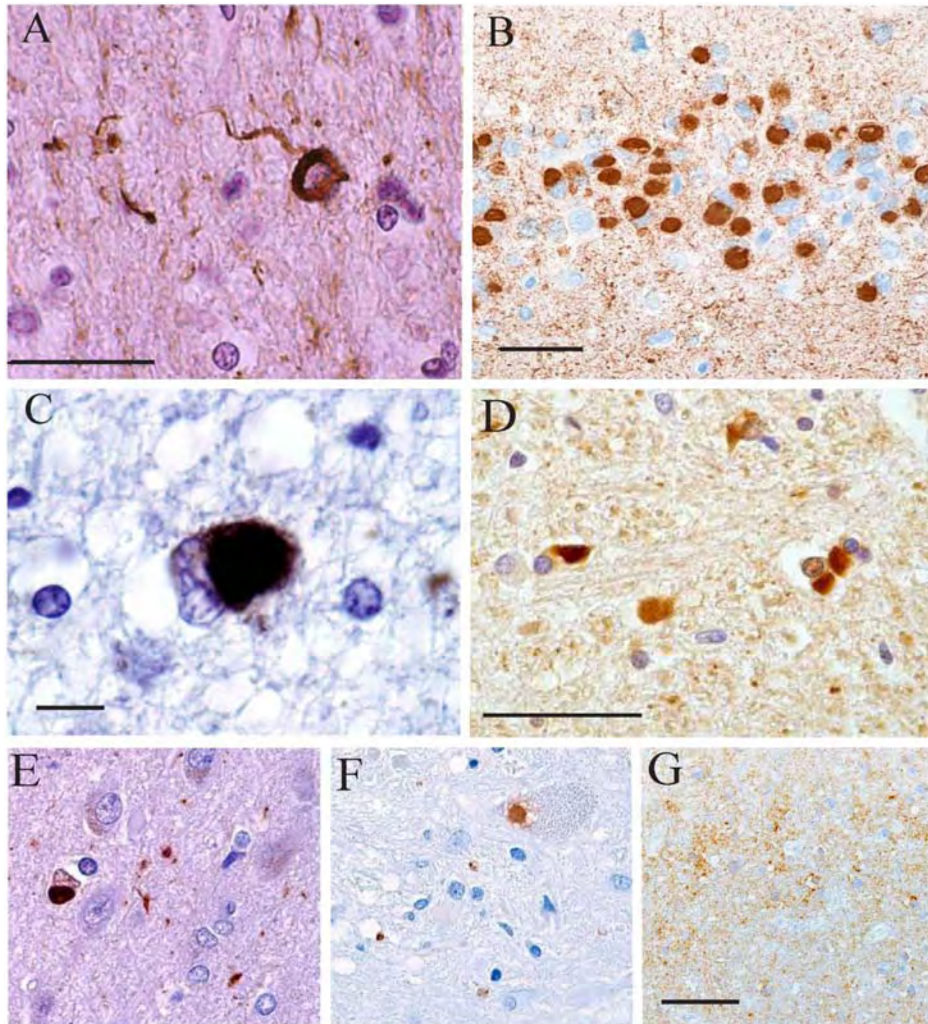

Figure 3. **(A)** Immunohistochemical stain for phospho-tau in white matter of a patient with corticobasal degeneration, demonstrated a phospho-tau positive coiled body surrounding an oligodendrocyte nucleus. Scale bar = 30 µm. **(B)** Immunohistochemical stain for phospho-tau in the granular layer of the fascia dentata, demonstrating numerous Pick bodies. Scale bar = 40 µm. **(C)** Neocortical Lewy body demonstrated by alpha-synuclein immunohistochemistry. Scale bar = 10 µm. **(D)** α-synuclein immunohistochemistry demonstrating glial cytoplasmic inclusions in a patient with multiple system atrophy. Scale bar = 50 µm. **(E)** Ubiquitin immunohistochemistry in patient with frontotemporal dementia, demonstrating an ubiquitin-positive, MND-type inclusion involving a layer 2 neuron of the frontal neocortex. Scale bar = µm. **(F)** Ubiquitin immunohistochemistry in a patient with amyotrophic lateral sclerosis, demonstrating an ubiquitin-positive, Lewy body like inclusion, involving an anterior horn cells. Scale bar = µm. **(G)** Prion protein (3F4) immunohistochemistry in a patient with the classic form of sporadic Creutzfeldt-Jakob disease (sCJD), following formic acid pretreatment, and autoclaving in the presence of HCL. The immunostain in this case demonstrates the so-called synaptic pattern of immunoreactivity in gray matter, typically seen in the classic form of sCJD.

It should also be noted that the most commonly used fixatives in histopathology processing form protein cross-links by forming methyelene bridges between reactive groups. Since some pathological

processes involve adduct formation on amino groups of proteins (e.g. advanced glycation end product formation), it is advantageous to use a primary fixative that lacks a cross-linking property. Smith et al. [36], thus studied AD brains using methacarn (60% methanol. 30% chloroform, 10% glacial acetic acid) and were able to elucidate a number of adducts that occurred in parallel with AD pathology. Among these included advanced glycation modifications pentosidine, pyralline, and carboxymethyl-lysine, and nitrotyrosine [36-39]. Lipid peroxidation adducts hydroxynonenal and malondialdehyde, whose identification in tissue is also hampered by cross-linking (formalin) fixation, have been identified using methacarn as the primary fixative. Some reports have further suggested that methacarn fixation enhances genomic DNA analysis in paraffin embedded tissues [40].

6. Summary

Microscopic analysis of the human brain affected by neurodegenerative disease has progressed over the last century and a half from simple dyes, to silver impregnations and their numerous modifications, to electron microscopy, and finally to immunohistochemistry and immunoelectron microscopy. For diagnostic purposes, routine dyes and immunohistochemistry are all that are required to characterize in detail the various neurodegenerative processes. Silver impregnation may have a slight benefit of lower cost compared to immunohistochemistry, but still suffers in terms of lack of specificity and capricious methodology. Electron microscopy, including immunoelectron microscopy, and other in-situ techniques (e.g., *in situ* hybridization, *in situ* PCR) are research techniques reserved for specific hypotheses and have no significant diagnostic utility.

In general, the various modalities focus principally on inclusions, and while it is true that many of these inclusions may be epiphenomenal [1], significant advances both technically and in terms of understanding of disease pathogenesis have been made with microscopy as the foundation. The hope is that these prodigious efforts will someday soon translate into effective therapy and we will thus have more to show for our efforts than copious descriptions of microscopic and submicroscopic lesions.

Acknowledgements Work in the authors' laboratories is supported by the National Institutes of Health, the Alzheimer's Association, and Philip Morris USA Inc. and Philip Morris International.

References

[1] R.J. Castellani, H.G. Lee, X. Zhu, A. Nunomura, G. Perry and M.A. Smith, Acta Neuropathol (Berl) **111**, 503 (2006).
[2] R.J. Castellani, X. Zhu, H.G. Lee, P.I. Moreira, G. Perry and M.A. Smith, Expert Rev Neurother **7**, 473 (2007).
[3] T. Uchihara, Acta Neuropathol (Berl) **113**, 483 (2007).
[4] F. Gallyas, Histochemistry **64**, 97 (1979).
[5] P. Gambetti, L. Autilio Gambetti and S.C. Papasozomenos, Science **213**, 1521 (1981).
[6] K. Iqbal, E. Braak, H. Braak, T. Zaidi and I. Grundke-Iqbal, Neurobiol Aging **12**, 357 (1991).
[7] I. Feigin and J. Naoumenko, J Neuropathol Exp Neurol **35**, 495 (1976).
[8] H.S. Garven and F.W. Gairns, Q J Exp Physiol Cogn Med Sci **37**, 131 (1952).
[9] T. Yamamoto and A. Hirano, Neuropathol Appl Neurobiol **12**, 3 (1986).
[10] G.R. Newman and B. Jasani, J Pathol **186**, 119 (1998).
[11] F. Gallyas, Acta Morphol Acad Sci Hung **19**, 57 (1971).
[12] H. Braak, E. Braak, T. Ohm and J. Bohl, Stain Technol **63**, 197 (1988).
[13] M. Tolnay, M.G. Spillantini, M. Goedert, J. Ulrich, D. Langui and A. Probst, Acta Neuropathol (Berl) **93**, 477 (1997).
[14] S.S. Mirra, A. Heyman, D. McKeel, S.M. Sumi, B.J. Crain, L.M. Brownlee, F.S. Vogel, J.P. Hughes, G. van Belle and L. Berg, Neurology **41**, 479 (1991).
[15] Z.S. Khachaturian, Arch Neurol **42**, 1097 (1985).
[16] Neurobiol Aging **18**, S1 (1997).
[17] D.A. Bennett, J.A. Schneider, J.L. Bienias, D.A. Evans and R.S. Wilson, Neurology **64**, 834 (2005).

[18] S. Taniguchi, A.M. McDonagh, S.M. Pickering-Brown, Y. Umeda, T. Iwatsubo, M. Hasegawa and D.M. Mann, Neuropathol Appl Neurobiol **30**, 1 (2004).
[19] G.A. Jicha, R.C. Petersen, D.S. Knopman, B.F. Boeve, G.E. Smith, Y.E. Geda, K.A. Johnson, R. Cha, M.W. Delucia, H. Braak, D.W. Dickson and J.E. Parisi, J Neuropathol Exp Neurol **65**, 602 (2006).
[20] D.W. Dickson, J Neurol **246 Suppl 2**, II6 (1999).
[21] R. de Silva, T. Lashley, C. Strand, A.M. Shiarli, J. Shi, J. Tian, K.L. Bailey, P. Davies, E.H. Bigio, K. Arima, E. Iseki, S. Murayama, H. Kretzschmar, M. Neumann, C. Lippa, G. Halliday, J. MacKenzie, R. Ravid, D. Dickson, Z. Wszolek, T. Iwatsubo, S.M. Pickering-Brown, J. Holton, A. Lees, T. Revesz and D.M. Mann, Acta Neuropathol (Berl) **111**, 329 (2006).
[22] M. Kidd, Nature **197**, 192 (1963).
[23] O. Bugiani, G.L. Mancardi, A. Brusa and A. Ederli, Acta Neuropathol (Berl) **45**, 147 (1979).
[24] P.D. Gorevic, F. Goni, B. Pons-Estel, F. Alvarez, N.S. Peress and B. Frangione, J Neuropathol Exp Neurol **45**, 647 (1986).
[25] M.G. Spillantini, R.A. Crowther, R. Jakes, M. Hasegawa and M. Goedert, Proc Natl Acad Sci U S A **95**, 6469 (1998).
[26] S. Kuzuhara, H. Mori, N. Izumiyama, M. Yoshimura and Y. Ihara, Acta Neuropathol (Berl) **75**, 345 (1988).
[27] S. Murayama, H. Mori, Y. Ihara and M. Tomonaga, Ann Neurol **27**, 394 (1990).
[28] I. Churcher, Curr Top Med Chem **6**, 579 (2006).
[29] H. Braak and E. Braak, Acta Neuropathol (Berl) **82**, 239 (1991).
[30] P.J. Kahle, M. Neumann, L. Ozmen and C. Haass, Ann N Y Acad Sci **920**, 33 (2000).
[31] M.H. Polymeropoulos, C. Lavedan, E. Leroy, S.E. Ide, A. Dehejia, A. Dutra, B. Pike, H. Root, J. Rubenstein, R. Boyer, E.S. Stenroos, S. Chandrasekharappa, A. Athanassiadou, T. Papapetropoulos, W.G. Johnson, A.M. Lazzarini, R.C. Duvoisin, G. Di Iorio, L.I. Golbe and R.L. Nussbaum, Science **276**, 2045 (1997).
[32] V. Manetto, G. Perry, M. Tabaton, P. Mulvihill, V.A. Fried, H.T. Smith, P. Gambetti and L. Autilio-Gambetti, Proc Natl Acad Sci U S A **85**, 4501 (1988).
[33] J.Q. Trojanowski and D. Dickson, J Neuropathol Exp Neurol **60**, 1123 (2001).
[34] R. Castellani, P. Parchi, J. Stahl, S. Capellari, M. Cohen and P. Gambetti, Neurology **46**, 1690 (1996).
[35] T. Kitamoto, R.W. Shin, K. Doh-ura, N. Tomokane, M. Miyazono, T. Muramoto and J. Tateishi, Am J Pathol **140**, 1285 (1992).
[36] M.A. Smith, S. Taneda, P.L. Richey, S. Miyata, S.D. Yan, D. Stern, L.M. Sayre, V.M. Monnier and G. Perry, Proc Natl Acad Sci U S A **91**, 5710 (1994).
[37] M.A. Smith, P.L. Richey Harris, L.M. Sayre, J.S. Beckman and G. Perry, J Neurosci **17**, 2653 (1997).
[38] R.J. Castellani, P.L. Harris, L.M. Sayre, J. Fujii, N. Taniguchi, M.P. Vitek, H. Founds, C.S. Atwood, G. Perry and M.A. Smith, Free Radic Biol Med **31**, 175 (2001).
[39] L.M. Sayre, D.A. Zelasko, P.L. Harris, G. Perry, R.G. Salomon and M.A. Smith, J Neurochem **68**, 2092 (1997).
[40] C. Uneyama, M. Shibutani, N. Masutomi, H. Takagi and M. Hirose, J Histochem Cytochem **50**, 1237 (2002).