PERSPECTIVE

Staining neurons with Golgi techniques in degenerative diseases of the brain

A detailed morphological study of neurons in healthy and pathological conditions requires reasonably a number of special techniques, which may visualize the majority of neurons in a thick three-dimensional arrangement. A detailed visualization of neurons must include the cell body, most of the dendritic arbor, the dendritic spines, the axon, the axonal collaterals and the synapses. An ideal morphological technique for the study of degeneration and regeneration processes of the central nervous system must also visualize clearly the long and short neuronal circuits, as well as the dendritic and axonal bands and tracks.

It is also of substantial necessity that the histological techniques must enable the precise and accurate morphometric estimation of the dendritic spines, which is of great value for the study of aging, and the processes of neuronal degenerations and regeneration. The neuroscientist endeavors to study and follow precisely all the spatiotemporal morphological alterations, which occur during the various degenerative processes continuously, resulting in neuronal death and in serious degradation of the neuronal networks.

In histological preparations and in tissue cultures, degenerated and dead neurons may be detected by application of various techniques based on the different staining properties of living and dead cells. Among them, silver impregnation technique may be characterized as instrumental for the precise visualization and estimation of the morphological alterations of neurons and neuronal circuits in various pathological conditions.

Silver staining was introduced in histology by Camillo Golgi in 1873, more than 140 years ago, as 'reazione nera' (black reaction) (Golgi, 1873). The philosophy of this revolutionary technique was based on the gradual impregnation of the nerve cells and their processes by silver chromate, which could give them a deep dark, almost black appearance, on a yellow background. The technical procedure consists basically in the immersion of the formalin fixed brain in a solution of potassium chromate and potassium dichromate, followed by impregnation with silver nitrate, resulting in the formation of silver chromate eventually.

For many years, Golgi technique and its numerous versions and modifications remain ideal methods enabling to obtain clear insight in the morphological profile and the morphometric characters of the dendritic arbor, the dendritic branches, the spines and the estimation of the spine density in thick paraffin sections. The technique was extensively utilized and evaluated by Santiago Ramon y Cajal, in his unique description of the histology of the nervous system and in the validation and eventual establishment of the 'neuronal doctrine', under his authorship, concerning the morphological and functional organization of the nervous system (Cajal, 1909, 1954).

During the years, Golgi technique has undergone many modifications, enhancements and refinements, in order to attain the maximal visualization of neurons and neuronal processes, the minimal precipitations and the reasonable abbreviation of the total time that required for the procedure (Zhang et al., 2003). Among the many published versions, the most frequently utilized in current clinical and experimental neuropathology, designated frequently as "Neo Golgi" techniques, are the modified Golgi stain, the rapid Golgi technique, the modified Golgi-Cox method, the Golgi-Braitenberg method (Scheibel and Scheibel, 1978) and others, which are characterized as 'gold-toning' techniques, whenever silver is replaced by gold for precise visualization of special structures of the brain (Friedland et al., 2006).

All those techniques have greatly enabled to optimizing the quality of the impregnation of neurons and glial cells by metals, maximizing, at the same time, the visualization of the neurons. Under those techniques, the neurons are seen dispersed evenly and distributed uniformly, less patchy, stained richer, faster, easier, with bright background and sharp contrast. In addition, the excess staining solution is removed during embedding, a fact which makes the optimal and most detailed appearance of the neurons to be efficient and facilitates the detailed study of the neuronal circuits and the glial cells. However, all those techniques are very delicate and sensitive and it is important to perform any detail of them carefully and diligently, in order to obtain the maximal visualization of the neuronal population.

In the endeavor for a clear and accurate visualization of the sequential stages of the neuronal degeneration and death, silver impregnation techniques have played a prominent role for many years, enabling the study of degenerating axons, dendrites, spines, synaptic boutons and axonal terminals. Those techniques stain also the population of the reactive astrocytes, enabling the estimation of the neuronal, astrocytic ratio in degenerative conditions. In addition, silver techniques may also provide an insight in the pathogenesis of neurological disorders and clarify some of the mechanisms, which are involved in the remodeling of neuronal networks in the recovering brain, either on the basis of neuronal plasticity or under the possible therapeutic interventions. Thus, the modern versions of the techniques contribute substantially to the accumulation of valuable data, obtained from the detailed morphological analysis of the autopsy material in debilitating diseases, revealing 'Dark' neurons, dendritic alterations, neurofibrillary degeneration, neuritic plaques, microglial cell proliferation and alterations of the brain capillaries (Gallyas et al.,1993). They may be helpful in assessing and staging neurotoxicity and in understanding the role that the reactive astrocytes play in the degenerating processes. Whenever the neuroscientist applies these techniques may insert further in the pathways of neuronal disintegration, apoptosis and death, which may occur under various conditions and causative factors (Baloyannis, 2009).

For many years, we have applied the semi rapid Golgi method in our laboratory (Baloyannis, 2015) in an attempt to study the morphological and morphometric alterations of dendrites and dendritic spines in dementias, including vascular dementia, Parkinson's disease associated with dementia, frontal dementia, frontotemporal dementia and Alzheimer's disease, at any stage.

From the technical point of view, the brain at autopsy is immediately immersed in freshly prepared formalin solution 10%, a volume ten times of the brain's volume, and remains suspended for 30 days at room temperature, in the dark. Then samples of the brain ($3 \times 3 \times 3$ cm^3) are immersed in freshly

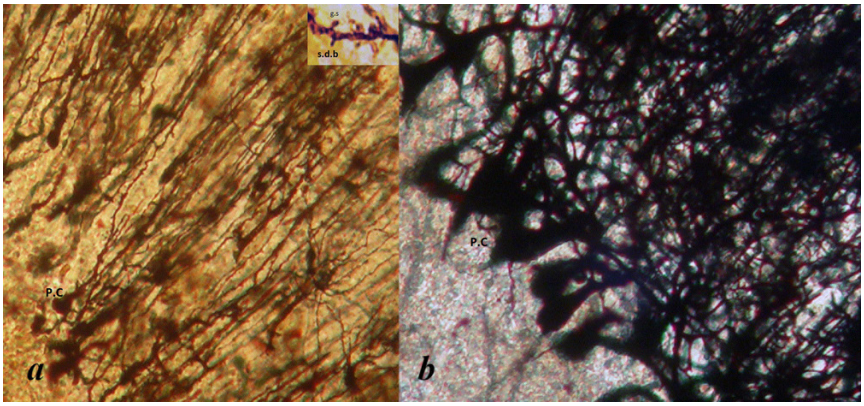

Figure 1 Dendritic pathology in Alzheimer's disease.
(a) Purkinje cells (PCs) from the vermis of the cerebellum of a case of Alzheimer's disease aged 69 years. The loss of the majority of the secondary dendritic spines is obvious in the molecular layer. There is also a tremendous loss of dendritic spines. (Rapid Golgi staining, × 1,200). Insert Giant spines (g.s.) on a secondary dendritic branch (s.d.b.) of a PC of the same case (× 3,200). (b) PC of the cerebellum of a normal control brain.

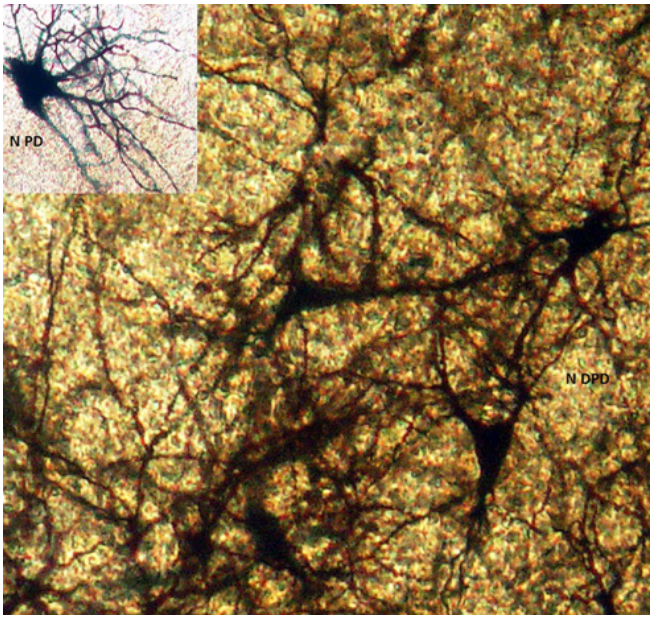

Figure 2 Neurons of the locus coeruleus of a case of Parkinson's disease associated with dementia aged 62 years (N DPD).
The loss of dendritic spines and the decrease of spine density is obvious: neuron of the locus coeruleus of a patient aged 65 years who suffered from Parkinson's disease, without dementia (N PD) (Rapid Golgi staining, × 2,000).

prepared potassium dichromate (7 g potassium dichromate in 300 mL of cold distilled water), remaining immersed for 10 days in the dark, at room temperature. Then the specimens are immersed in freshly prepared 1% silver nitrate (1 g of silver nitrate, dissolved in 100 mL distilled water) and remained in the dark, been agitated continuously for 10 days, at 16°C. According to our version, we enhance the staining by adding few grans of copper in the silver nitrate (Baloyannis, 2015). Then, the specimens are placed in a flat glass and the deposits of silver are removed gently. After a rapid dehydration in absolute alcohol, the specimens are embedded in melting paraffin. However, according to some new modified techniques the brain tissue is embedded in gel-albumin for sectioning on a vibratome. Sectioning is usually performed in a sliding microtome. Sections of 100 μm and 25 μm are cut alternatively and they are collected in a glass dish containing distilled alcohol. According to a version of Golgi-Nissl method, some of the sections are further post-stained with methylene blue, for a clear visualization of the neuronal population. Then the sections are cleared carefully with xylene. As soon as the sections become translucent, they are mounted between two large cover slips and are left to dry. The stained sections are studied in photomicroscope, placed on a titling table, in an attempt to achieve a three dimensional visualization of neurons and dendritic arbor.

At any stage of degenerative conditions, modern silver impregnation techniques may reveal the majority of the morphological alterations of dendrites, dendritic branches and spines, which are the smallest, most sensitive and vulnerable morphological parts of the neuron. The alteration of the size and shape of the dendritic spines and the decrease of their density on the dendritic branches is an initial phenomenon in dementias, observed in the cortex and the subcortical neuronal circuits, which provokes serious consequences in the mental faculties and the psychological homeostasis of the patients.

By the application of our version of semi rapid silver impregnation, we attempted to study and describe the majority of neurons and neuronal networks, in any area of the brain cortex, in the cerebellum and the subcortical centers. We described the soma, the shape and size of the dendritic arbor, the ramification of the apical and basal dendrites, the primary dendritic branches and the secondary ones. In addition, we described the type of angles between primary and secondary dendritic branches, the tertiary and terminal dendritic branches and the type of bifurcation of the branches. We also measured the length and the diameter of the dendritic segments and we calculated the ratio between primary, secondary and tertiary dendritic branches. In general, it must be emphasized that semi-rapid Golgi method is instrumental in revealing and quantitating dendritic pathology.

In Alzheimer's disease, Golgi techniques have demonstrated that Cajal-Retzius neurons of the layer I of the cortex of the brain hemispheres are among the earliest cells which degenerate, a fact that might play a substantial role in the disruption of the microcolumnar arrangement of the neurons in the cortex, affecting also the synapses, given that Cajal-Retzius cells play a crucial role in synaptogenesis. The same method shows clearly the substantial neuronal loss and the dendritic alterations in the hippocampus, proving also the reduced density of thorny excrescences on the apical and basilar dendrites of pyramidal neurons of the CA3 area. In the acoustic cortex, in Alzheimer's disease, a tremendous decrease in spine density is observed, associated with marked

morphological alteration of the dendritic arbor. Giant dendritic spines are frequently seen in neurons of the layers III and V layers, intermixed with small triangular, round or fusiform spines. In addition, the morphological study of the superior, middle, and inferior temporal gyri revealed a marked decrease of the number of Cajal-Retzius cells in the anterior parts of the gyri.

In the visual cortex, the semi-rapid Golgi method applied in early cases of Alzheimer's disease revealed extensive dendritic pathology, distortion of spines, numerous giant spines and substantial decrease of spine density (Baloyannis, 2005).

The application of the rapid Golgi and Golgi-Nissl methods for the study of the medial geniculate bodies and the inferior colliculi in Alzheimer's disease revealed marked neuronal loss. In addition, large polyhedral or round neurons showed substantial poverty of secondary and tertiary dendritic branches. Pathology of the spines is also prominent. Abnormal spine protrusion, spine distortion and giant spines are frequently observed in the context of a substantial decrease of spine density.

In the cerebellum, which usually demonstrates minimal Alzheimer's pathology, the semi-rapid Golgi method revealed tremendous loss of dendritic branches and dendritic spines and impressive decrease in spine density in the majority of Purkinje cells in the nodule of the vermis (**Figure 1**), the flocculus and the hemispheres (Baloyannis et al., 2000). The interneurons, basket and stellate cells are also decreased. Marked morphological and morphometric alterations of climbing and mossy fibers are also observed even at the early stages of Alzheimer's disease.

Golgi staining may also underline the important role of the vascular factor in the pathogenesis of Alzheimer's disease (Baloyannis and Baloyannis, 2012).

In vascular dementia, Golgi staining revealed tremendous decrease of the Cajal-Retzius cells in the molecular or plexiform layer, which was morphometric estimated of the range of 80–90% in comparison with normal controls. Decrease in spine density was noticed in the majority of the neurons of the acoustic cortex, affecting mostly the triangular and round neurons of the layers II, II and V. In addition, in the cerebellar cortex, marked loss of climbing fibers in the molecular layer is observed, associated with considerable loss of the tertiary dendritic branches of Purkinje cells (Baloyannis et al., 2000).

Semi-rapid Golgi staining revealed substantial loss of dendritic branches, dendritic spines and axonal retrograde collaterals in the locus coeruleus in cases of Parkinson's disease, associated with dementia (**Figure 2**), in comparison with parkinsonian patients without dementia.

Utilizing our version of Golgi technique in frontal dementia, frontotemporal dementia, Pick's disease and in primary progressive aphasia we noticed marked dendritic pathology, tremendous loss of dendritic spines and abnormal spines, and a fact which underlines the importance of the integrity of spines for a normal cognition.

Some modern methods have been introduced in the neuropathological investigation for detecting neuronal degeneration and death. Some of them include propidium iodide uptake, staining by the neurodegenerative marker Fluoro-Jade, TUNEL staining for labeling DNA fragmentation during apoptosis, immunohistochemical staining for microtubule-associated protein 2, and Timm sulphide silver staining for heavy metal alterations. However, not uncommonly an excessive neuronal death is hardly detected, given that dead neurons might be removed at the early stages of the degeneration by activated microglia.

In conclusion, the morphological and morphometric alterations of the dendritic spines, which the phenomena of substantial importance in ageing, dementias and psychiatric disorders are precisely visualized by Golgi staining, even at the early stages of the neuronal degeneration. In addition, the correlation of dendritic and spine pathology with the clinical phenomena in dementia may also be helpful in planning new therapeutic strategies aiming in protection or regeneration of dendritic branches and synaptic components.

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Baloyannis SJ (2015) Staining neurons with Golgi techniques in degenerative diseases of the brain. Neural Regen Res 10(5):693-695.

References

Baloyannis SJ, Manolidis SL, Manolidis LS (2000) Synaptic alterations in the vestibulocerebellar system in Alzheimer's disease – a Golgi and electron microscope study. Acta Otolaryngol 120;247-250.

Baloyannis SJ (2005) Morphological and morphometric alterations of Cajal-Retzius cells in early cases of Alzheimer's disease: a Golgi and electron microscope study. Intern J Neurosci 115:965-980.

Baloyannis SJ (2009) Dendritic pathology in Alzheimer's disease. J Neurol Sci 283:153-157.

Baloyannis SJ (2015) Staining of dead neurons by the Golgi method in autopsy material. Methods Mol Biol 1254:167-179.

Baloyannis SJ, Baloyannis JS (2012) The vascular factor in Alzheimer's disease: a study in Golgi technique and electron microscopy. J Neurol Sci 322:117-121.

Friedland DR, Los JG, Ryugoa DK (2006) A modified Golgi staining protocol for use in the human brain stem and cerebellum. J Neurosci Methods 150:90-95.

Gallyas G, Hsu M, Buzsaki G (1993) Four modified silver methods for thick sections of formaldehyde-fixed mammalian central nervous tissue: 'Dark' neurons, perikarya of all neurons, microglial cells and capillaries. J Neurosci Methods 50:159-164.

Golgi C (1873) Sulla struttura della sostanza grigia del cervello. Gazz Med Ita Lombarda 33:244-246.

Ramon y Cajal S (1909) Histologie du Systeme Nerveux de l'Homme et des Vertebres. Madrid, Instituto Ramon y Cajal, pp.774-838.

Ramon y Cajal S (1954) Neuron Theory or Reticular Theory? Objective Evidence of the Anatomical Unity of the Nerve Cells. Consejo Superior de Investigaciones Cientificas, Madrid, Spain.

Scheibel ME, Scheibel AB (1978) The methods of Golgi. In: Neuroanatomical Research Techniques (Robertson RT, ed.), pp90-114. NY, USA: Academic Press.

Zhang H, Weng SJ, Hutsler JJ (2003) Does microwaving enhance the Golgi methods? A quantitative analysis of disparate staining patterns in the cerebral cortex. J Neurosci Methods 124:145-155.