Histol Histopathol (2025) 40: 453-465

Histology and Histopathology
From Cell Biology to Tissue Engineering

REVIEW Open Access

The Golgi method. A historical through contemporary view

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Summary. Knowledge regarding the biology of the nervous system and its functions has gone through various theoretical, methodological, and interpretative stages throughout history, depending largely on technical advances that have allowed us not only to approach old questions from new perspectives but also to address new ones. One advance that constituted a watershed in the history of neuroscience was the appearance of a chrome-silver staining technique called the *Golgi method* that allowed the complete, three-dimensional observation of nerve cells. Discovered by Camilo Golgi and, later, modified significantly and employed by Santiago Ramón y Cajal, Golgi's method was crucial in demonstrating the veracity of the Neuronal Theory over the earlier Reticular Theory, and in revealing numerous findings related to the human brain and those of many other animal species, which continue to be analyzed today. Despite a period of scientific recession in the first half of the 20th century, the use of the Golgi method prevailed and even expanded in the second half of that century and into the 21st, as researchers continued to use it in its original or modified form and in combination with emerging methodologies. Currently, there are no signs of any decline in its use.

Key words: Golgi method, Ramón y Cajal, Nervous system, Neuroplasticity, Neurons, Dendritic spines

Introduction

The term 'neuroscience' was introduced in the mid-1960s to refer generically to the multidisciplinary, scientific study of the nervous system that seeks to understand the biological basis of behavior, however, this area of study is, in reality, much older. As in other scientific disciplines, advances in our knowledge about neuroscience have depended on technical and technological progress that aid in clarifying emerging issues and dilemmas. Over the years, successive generations of technical resources have become increasingly complex, so much so that today people believe that the more sophisticated the technology, the greater the specificity of the knowledge derived from applying it. While this is true, some technical resources that are not as complex as conceptual inertia suggests have been so important that they continue to provide relevant information today, for both clinical medicine and basic scientific knowledge. This is the case of histological staining techniques -like the Pap test- and metallic impregnation methods, respectively.

More than 150 years after its original design by Camilo Golgi, metallic impregnation-based techniques continue to provide valuable information on the biology of nervous system cells. Of course, this is based on posing scientific questions that are oriented to, and consistent with, the kinds of information each technique can provide. The *Golgi method* and its many related variants are used by numerous research groups in neurobiology in diverse countries, whether of Anglo-Saxon or Latin epistemic and interpretive orientation. This virtue is clearly attributable to the classic assertive and accurate pioneering works of Santigo Ramón y Cajal, who is considered the father of modern -even contemporary- neuroscience. Ramón y Cajal's works permeated all types of scientific cultures worldwide, a fact that objectively reflects the significance of the theoretical derivations that have and continue to foster the enhancement of scientific knowledge in the field of the neurosciences.

From a historical perspective, this review analyzes the development of neuroscience based on the knowledge acquired through the use of Golgi's method.

Neurocytology in the 19th century

According to the *Edwin Smith Surgical Papyrus* found in 1862, but not translated until 1930, the influential Egyptian personage Imhotep was the first man to use the term 'brain' to refer to the organ responsible for certain behavioral disorders. His was also the first allusion to a naturalistic and objective (though

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www.hh.um.es. DOI: 10.14670/HH-18-821

ultimately erroneous) perspective on brain function (González-Tapia and González-Burgos, 2024). However, it was left to Greek philosophers to forge the first broad historical movement that applied reasoning in attempts to explain the natural world. Moving ahead to the 17th century, René Descartes postulated a logical-rational method of obtaining knowledge that sowed the seeds of the scientific method. Empiricists opposed to Descartes, like Francis Bacon, maintained that knowledge derives from sensory experience as they laid the foundations of the positivist approach in the experimental sciences. These two visions (radical rationalism and empiricism) were 'reconciled' by the ideas of Emmanuel Kant, who argued that experience would be completely subjective were it not part of a synthesis -with pure reason- and that using reason without contrasting it to experience would inevitably lead to theoretical illusions (González-Burgos, 2024). This series of philosophical and epistemic postulates set the stage for later attempts to objectively explain the relation between the structure and function of the brain.

In the late 18th century, the first outlines of an objective vision of brain functions, specifically cortical ones, with the appearance of the theoretical-methodological current called localizationism. Later, in the 19th century, Franz Gall proposed that behavior was a product of brain activity in the framework of the so-called phrenological method, a model that assigned 35 moral-behavioral attributes to specific regions of the cerebral cortex which, it was believed, increased their volume until they became detectable as bulges in the skull, in this way establishing an individual's personality. Marie Jean Pierre Flourens, among others, strongly criticized Gall's phrenology arguing, based on experimental studies (mainly with birds), that the cerebral cortex could not be divided into functional units but, rather, was equipotential. One might say that Gall was correct in theory (structure-function) but wrong in terms of method (observation), while Flourens had the correct method (experimental) but erred in theory (functional non-specificity) (Finger, 1994). This controversy was clarified in the mid-1800s when neurosurgical and electrophysiological studies confirmed the existence of a cortical region for motor and speech and verified that the cerebral cortex had more-or-less circumscribed zones of tissue with distinct functional representations. By that time, the 'what?' had been accepted but the 'how?' remained unclear. That dilemma was addressed through various studies of cortical cytoarchitecture that eventually led to a fundamental historical controversy.

The technological advance represented by the invention and utilization of the optical microscope brought a hitherto unsuspected advance in the late 1830s that gave rise to the formulation of Cell Theory, though the term 'cell' had been coined much earlier, by Robert Hooke in 1665 (Turner, 1890). Around 1850, Albert von Kölliker stated that progress in understanding the interrelation of nerve cells was limited by the study techniques available, and maintained that without more extensive staining resources, no significant advances in the knowledge of neurohistology could be achieved beyond the elementary cytological descriptions of the cerebral cortex that Kölliker himself published in 1852. Yet, Karl Deiters and Max Schultze reported in 1865 that nerve cells consisted of a cell body, an "axis cylinder", and several "protoplasmic processes" that William His named dendrites in 1889 (from the Greek *déndron*, 'tree') (Finger, 1994; De Felipe, 2010).

In that period, studies of nerve tissue were performed using two main techniques. One consisted of fixing, embedding, and cutting tissues, then staining the sections obtained with hematoxylin or carmine, the two colorants most often employed. However, those histological staining techniques were unsatisfactory and insufficient for studying the structure of the brain due to the complexity and peculiar organization of that organ compared with other tissues (Bentivoglio, 1998), and because the images of nerve cells produced were incomplete. In most cases, only the nucleus, a narrow rim of cytoplasm, the perikaryon, and the initial segments of dendrites were visible. Hematoxylin or carmine staining revealed very little of the small, densely-packed nerve cells found in certain regions of the brain, generally referred to as "granules" (Pannese, 1996). Then Joseph von Gerlach introduced neuro-histological staining methods that used ammoniated carmine and gold chloride. Studies using these techniques provided support for the notion that axons do not fuse by anastomosis but, rather, coexist in some kind of independence, as Deiters and Kölliker had proposed earlier (Finger, 1994). In 1842, Benedict Stilling developed a new method for obtaining thin, transparent sections that consisted in hardening nerve tissue by freezing or with chemical reagents. Later, Deiters improved that technique (Deiters, 1865). The latter procedure involved immersing blocks of nerve tissue in chromic acid or potassium dichromate solutions as indurant agents that served to both fix and harden the tissue. That allowed researchers to mechanically isolate individual nerve cells under the microscope using dissection needles. That approach proved to be useful, though only the largest nerve cells could be isolated, and they were rarely complete because the fine terminal segments of their processes were often broken in the process and had to be reconstructed artificially (Pannese, 1996). This meant that the inferences derived from these procedures were strictly limited by technical factors, the precise brain region studied, and the nerve cell lineages that could be characterized. This summarizes the methodological framework that existed when a turning point occurred in the field of neuroscience.

The 'arrival' of the Golgi method

Camillo Golgi (Fig. 1) was born on July 7, 1843, in Corteno, an Italian community in the province of Brescia, in the Lombard Alps. In 1956, the name of the

town was changed to Corteno Golgi, in honor of the famous Italian scientist. As a medical student at the University of Pavia, he attended classes at the Institute of Psychiatry and worked in the experimental pathology laboratory, where he became convinced that theories had to be supported by concrete facts. He soon abandoned psychiatry to focus on the experimental study of the structure of the nervous system. In 1872, while living in precarious economic circumstances, Golgi accepted the post of Chief Medical Officer at the Hospital for the Chronically Ill in Abbiategrasso, near Milan. Later, he held several positions at the University of Pavia, before retiring in 1918. He died on January 21, 1926 (Bentivoglio, 1998).

Golgi was a prolific researcher. Among his scientific contributions, far too numerous to mention here, he provided the first complete descriptions of the axon and its collaterals, of the morphological features of glial cells (impregnated by his method), of the relation between glial cell processes and blood vessels, and of key features of glial tumors. He also described 'Golgi type I' nerve cells, known today as 'projection neurons', and 'Golgi type II' cells, characterized by axons that ramify in the vicinity of the cell body that today are called 'interneurons' (Bentivoglio, 1998). His contributions to the knowledge of cell biology include the discovery of the intracellular membrane system that bears his name: the 'Golgi apparatus'. Due to his transcendental contributions, Golgi is considered the 'father' of cellular neuroanatomy (Bentivoglio et al., 2011).

In the mid-19th century, knowledge of the structure of the brain was conditioned by the limited scope of the study techniques available. The need for more conclusive histological techniques was satisfied with the discovery of a new kind of stain that allowed anatomists to examine elements of the nervous system with much greater precision. Golgi proposed this new technique in 1873, and it came to be known as the *Golgi method*. This discovery took place in a kitchen at the Abbiategrasso hospital, which Golgi had converted into a rustic laboratory with a few instruments, where he worked at night by candlelight. Controversy surrounds the origin of the method, for Golgi never explained how he conceived the procedures that led him to formulate the complete technique (Finger, 1994). For this reason, the arguments of those who have dared to posit theories range from shrewd planning to pure serendipity, though all their hypotheses are highly speculative. One version holds that the discovery occurred relatively by accident thanks to the bactericidal effect of silver (López-Goñi, 2023) when Golgi attempted to stain pia mater with silver salts (Álvarez-Leefmans, 1994) and added silver nitrate to some older tissue samples, perhaps to prevent contamination and putrefaction. An apparently more realistic version emphasizes that Gustav Retzius had developed a silver staining technique to observe pia mater. In his autobiography, Retzius narrated the story he was told by one of Golgi's assistants concerning the discovery of the technique. In that account, Golgi was testing Retzius' silver stain on samples of brain tissue previously placed in potassium dichromate to analyze the pia mater (Torres-Fernández, 2006). Upon observing the adjacent brain tissue, Golgi perceived images of structures stained dark brown or black on a yellow background that he identified as nervous system cells. It seemed that the chemical 'combination' of potassium dichromate and silver nitrate had reacted with cells in the tissue. From that day forward, neuroscience entered a new stage and turned in a revolutionary direction, not only in the objective visualization of nerve cells but also in the interpretive approach that developed.

Fig. 1. Camilo Golgi (1843-1926).

According to Golgi's notes, the new technique had two variants: one slow, the other rapid.

Golgi described the slow method as follows: "Small organ fragments are placed in Muller's solution or a 3% potassium dichromate solution, then the concentration is increased to 5%. The fixative and pieces are kept in the dark. After 4-6 weeks, the first test is conducted with the material by removing a small piece and rinsing it for 24 hours in this same liquid. If we still do not find signs of impregnation in the slices on this piece made with the knife, we repeat the test after 8 days, and so on". In the years following the discovery of his original impregnation method, Golgi introduced important modifications, one of which was to add osmium tetroxide to the potassium dichromate (Golgi, 1898, 1989). This technique is still known as the rapid Golgi method. Golgi characterized the rapid method in these words: "We begin by placing small pieces of material as fresh as possible in a mixture of 8 parts of 2.5%

potassium dichromate solution and one part of 1% osmic acid solution, kept in the dark. From day three to seven, we take a few pieces every day, wipe them with filter paper, and immerse them in a 0.5-1% silver nitrate solution that soon turns yellow and must be refreshed immediately. After 24 hours the pieces are washed in 40% alcohol. Among the pieces examined, we will surely find some where impregnation has been successful" (Romeis, 1928). Those blocks were then dehydrated and cut but not embedded. Then sections (usually quite thick, 100 µm or more) were cleared in turpentine, placed on slides, and covered with damar gum without coverslips. In one variant, Golgi mounted sections on a coverslip that he then placed over the aperture of a hollowed-out wooden slide to observe them from both sides. That allowed him to observe the complete structure of nervous system cells as they had never been seen before. The randomness of the reaction made it possible to observe the cell body, axon, and branched dendrites of the cells as integral, tridimensional black structures immersed at a very low proportion (1-5%) in a translucent yellowish background (Scheibel and Scheibel, 1970; Valverde, 1970; Pasternak and Woolsey, 1975). It appeared that the cells were isolated one from another, though that affirmation was hotly debated at the time (Pannese, 1996, 1999) (Fig. 2).

Technically speaking, Golgi's method is a metallic impregnation technique. It is important to specify that these methods are considered special histological techniques because of the nature of the treatment applied to the tissue samples and the results obtained. This treatment lies outside the context of histological staining techniques, which are based on the affinity of various subcellular and cellular structures for the dyes used, based on their corresponding pH. Metallic impregnation techniques, in contrast, rely on the chemical modification of both the tissue elements impregnated and the reagents used (Ramón y Cajal and Tello y Muñoz, 1955; Ramón y Cajal and de Castro, 1972). The contrast to what usual staining using colorants showed was radical. Golgi called this reaction reazione nera, and wrote:

"*Using a method I developed that makes it possible to stain the elements of the brain in black, a staining procedure that requires the prolonged immersion of the pieces, previously fixed in potassium or ammonia dichromate in a 0.5-1.0% solution of silver nitrate, I could discover some facts concerning the structure of the grey matter of the brain that I think are worthy of being reported*" (Golgi, 1873).

In addition to his pioneering work on the cerebellar cortex, from 1874 to 1881 Golgi published a number of studies on the cerebellum, olfactory bulb, and spinal

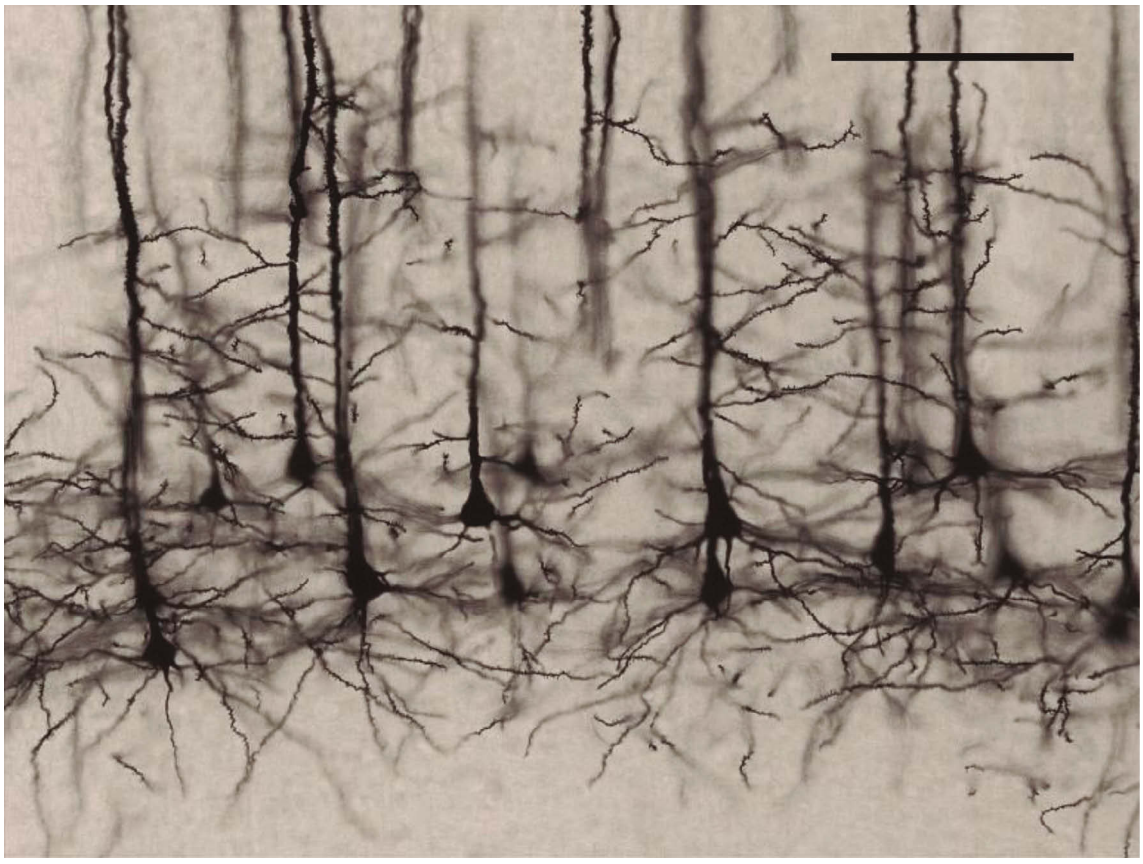

Fig. 2. Photomicrograph of some pyramidal neurons in the II/III layer of the prefrontal cortex, impregnated with a modification of the Golgi method (González-Burgos et al., 1992). Scale bar: 200 μm.

cord and, in 1884, a collection of neurohistological findings obtained with his method (Golgi, 1884).

Though some contemporary scientists used the Golgi method in their research, it was often disparaged as being "haphazard" in its results. In fact, many scientists of the time dismissed it, viewing it with great skepticism. As a result, Golgi's neurocytological findings were widely ignored by the scientific community, not only in Italy but also in other European countries. That situation prevailed until 1887, when Kölliker, a very well-respected neuroscientist of the period, declared:

"*...we know of no other procedure that reveals with such perfection the nerve cells of the grey matter and the neuroglial elements*" (Pannese, 1999).

The Reticular and Neuronal Theories

In the second half of the 19th century, controversy raged over the nature of the organization of nervous system tissues. On one side, a theoretical current called the Reticular Theory proposed the anatomical continuity of the components of nerve cells in a kind of diffuse network. On the other, supporters of the Neuronal Theory posited that nerve cells were anatomically independent of each other. It is important to note that the term 'neuron' was not coined until late in that century - 1891- by Wilhelm von Waldeyer-Hartz.

In the 1860s, Deiters and Kölliker thought that some neural cell processes (dendrites) formed a continuous network, though the latter eventually changed his position in favor of the Neuronal Theory based on new evidence. Joseph von Gerlach is generally recognized as the founder of Reticular Theory (Gerlach, 1872). At that time, neurophysiology required neuroanatomical foundations to explain how messages were transmitted in the brain. Reticularism proposed that this was based on the supposed structural continuities of nerve cells. Gerlach's Reticular Theory held that the arborizations of nerve cells were continuous through anastomoses, and that this structure formed an unbroken network throughout the nervous system. Golgi modified this concept by proposing that dendrites did not participate in nerve conduction since he had shown that they terminated freely without contributing to the nerve network (his "diffuse nerve network"), which he believed was the true conducting element. Given the challenges involved in applying a new method of study, especially the one of his own creation, in his initial report in 1873 -and even in the ensuing years- Golgi affirmed that the role of dendrites was related to nutrition. Based on those precepts, he claimed to support the holistic approach to brain function, as opposed to the idea of functional localization (Golgi, 1903); thus, he emerged as a supporter of Reticularism. He firmly believed that his own observations of ramified nerve elements supported that theory, so he could be considered its most notable defender.

Meanwhile, the theory that the nervous system, like other tissues, was composed of independent cells, was garnering increasing support from studies in other laboratories that, ironically, used Golgi's new impregnating method. Among the prominent scientists who supported Neuronal Theory, we can include Kölliker, Retzius, van Gehuchten, Athias, Duval, Marinesco, Wilhelm His, and August-Henri Forel. His and Forel's observations of embryonic tissues, for example, revealed that nerve extensions do not anastomose; hence they proposed that each nerve cell constituted a basic, independent unit that, together, formed the nervous system (De Felipe, 2010). However, their criticisms were insufficient to displace the Reticularist current, despite the internal contradictions that defenders of that posture confronted (Ramón y Cajal, 1952). Furthermore, no conclusive evidence demonstrated the incorporation of the brain structure into cellular theory and, therefore, a foundation for neurophysiology. That work would be left to another key supporter of Neuronal Theory: Santiago Ramón y Cajal (Fig. 3).

Cajal was born in Petilla de Aragón, province of Navarra, Spain, on May 1st, 1852, and died on October 17th, 1934. Among the many influential academic and scientific positions he held in Spain, we can mention his appointments as the Interim Assistant of Anatomy at the University of Zaragoza (1875), Director of the Anatomical Museum of Zaragoza, Chair of General Anatomy at the University of Valencia (1883), and teaching positions at the Universities of Barcelona (1887) and Madrid (1892). During his lifetime, his work

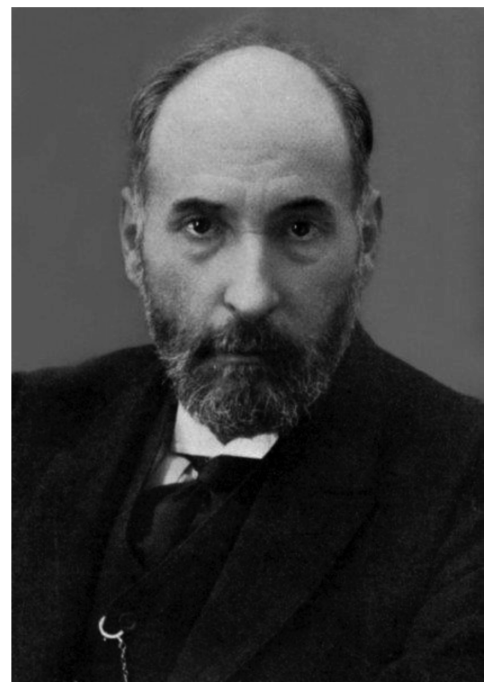

Fig. 3. Santiago Ramón y Cajal (1852-1934).

received many recognitions, perhaps most significantly the Nobel Prize he shared with Golgi in 1906. Just as Golgi is considered the precursor of cellular neuroanatomy, Cajal is recognized as the scientist who led classical histologists to provide the cellular basis for the emergence of modern neuroscience (Bentivoglio et al., 2011).

Cajal emerged as the most important supporter of Neuronal Theory, which correctly interpreted the nervous system as one composed of distinct units, for he helped demonstrate that neurons were anatomically and functionally independent polarized cells. Cajal was the most notable user of Golgi's method, which was introduced into Spain by Luis Simarro. Upon observing a histological slide prepared with this method, Cajal was astonished. Years later, he wrote:

"*Such an unexpected vision! Against a perfectly translucid yellow background there appeared, dispersed here and there, smooth, thin, black filaments [others] spiny and thick, black bodies in the form of triangles, stars, and tubes. One might say they were drawings done in India ink on Japanese paper. Eyes accustomed to the inextricable tangles of sections [stained by] carmine and hematoxylin -which forced the brain to make enormous efforts of interpretation and critique- were now quite consternated, for everything here is so simple and clear, with no confusion. There is nothing more to interpret; one need only observe this cell with its multiple, intricate branches, covered in rime, embracing an extraordinarily broad space with its oscillations; this smooth uniform thread that emerges from the cell travels an enormous distance, then suddenly unfolds in a bouquet of countless budding strands. One's eye is stunned, unable to pull away from such contemplation. The technical dream has become a reality! Metallic impregnation has generated such an unexpected image. This is the Golgi method*".

Like several other researchers, Cajal modified the method in various ways to improve its reliability and achieve unambiguous impregnations that would allow more objective interpretations of the organization of brain tissue (Ramón y Cajal and de Castro, 1972; Alonso, 1994; Dall'Oglio et al., 2010). Cajal worked with many animal species, and based many of his conclusions on studies of embryos and newborns. In addition to his detailed descriptions of various neuronal lineages, their extensions and connections with others, and the regional relations that exist between certain brain structures, among many other neurobiological features recorded in his monumental work *Histology of the Nervous System of Man and Vertebrates* (Ramón y Cajal, 1909), he proposed numerous theoretical, phenomenological, and conceptual postulates that literally revolutionized the understanding of biology and the function of the nervous system. In addition to consolidating Neuronal Theory (from which Charles Sherrington would coin the term 'synapse' in 1897), he inferred many anatomical-functional relations in the brain. He proposed, for example, that dendrites participate in the reception and propagation of electrical signals, postulated the principle of divergence ("conduction avalanche", in his words), discovered and named the "growth cone" in embryonic tissues, and posited that the learning process is accompanied by long-lasting modifications in the structure and function of synaptic connections between neurons, and that these changes are the neurobiological basis of memory (Álvarez-Leefmans, 1994). This latter proposal, in particular, is still solidly supported by an enormous amount of experimental evidence and continues to be studied intensely due to its many important implications.

Clearly, Cajal's scientific work and his legacy to neuroscience surpass the already transcendent demonstration of Neuronal Theory, which constitutes the basis upon which all the subsequent inferences and conclusions that he and many other classical and contemporary scientists have proposed.

The 20th century. An era of uncertainty surmounted

In the first quarter of the 20th century, the Golgi technique generated a wealth of information on the structure of the nervous system and neuronal circuits. For almost 30 years, that "black reaction" was widely employed in laboratories worldwide. However, that "golden age" of neurocytology seemed to come to an end with Cajal's death. In the period between the two World Wars, the method fell into disuse, indeed it almost seemed to have been forgotten. To further complicate matters, the Spanish Civil War significantly overshadowed the scientific merits of the Spanish school of neurohistological research (Torres-Fernández, 2006) which, without question, had permeated European scientific culture until the onset of World War II. Most of Cajal's disciples and their research groups were forced to flee the country, with many emigrating to countries in the Americas. Mexico stands out as the principal receiving country, as numerous distinguished disciples of Cajal chose to settle there and, indeed, went on to create important research teams in neuroscience and other scientific and clinical disciplines (Fernández-Guardiola, 1997; Dosil-Mancilla, 2009). At the same time, Pío del Río Hortega, another notable follower of Cajal (Río Hortega, 2015), finally emigrated to Argentina (Dosil-Mancilla, 2009). One of Cajal's last disciples, Rafael Lorente de Nó, settled in the United States, where he continued his neurohistological studies for some years using the Golgi technique. In fact, the first diagram of the microcircuits of the neocortex was a famous contribution by Lorente de Nó based exclusively on that method (Lorente de Nó, 1938).

The work carried out with this technique in the post-Cajal era and the first half of the last century was of relative importance (Jones, 1984). However, it has been suggested that the publication of the Sholl method (Sholl, 1953) for quantifying dendritic arborization was a relevant contribution, along with the modification to the Golgi-Kopsch method -which replaced osmium tetroxide with formaldehyde (Riley, 1979)- that

ultimately made it possible to prepare samples prepared by the Golgi method for analysis under electron microscopy (EM) (Millhouse, 1981; Fairén et al., 1996). This was significant because, despite its high-resolution power, EM alone does not make it possible to clearly recognize which axonal or dendritic processes belong to a certain type of neuron, so combining that technique with prior preparation by Golgi's method led to the conclusive confirmation of neuronal individuality and synaptic contacts between neurons (Gray, 1959a).

By the 1970s, observation of samples processed by the modified Golgi method under EM allowed a significant re-evaluation, which was soon complemented by the development of other methodological disciplines - neurophysiology, for example- in correlation with experimental behavioral studies. With that, Golgi's method regained its importance by giving rise to new theoretical approaches and fostering the advance from the descriptive to the experimental stage (Scheibel and Scheibel, 1970; Jones, 1984). Stell (1965) and Blackstad (1965) were the first researchers to combine this method with EM. After additional modifications, the group led by Fairén (Fairén et al., 1977, 1996) used EM to demonstrate the direct connection between two neurons, identified by optical microscopy. The Golgi-EM combination also formed the 'gateway' that allowed the application of histochemical and immunocytochemical techniques to correlate neuronal morphology and ultrastructure based on biochemical properties. To accomplish this, it was necessary to adapt Golgi's method for use with tissue sections, a procedure that can be combined with other methods, such as pathway retrograde tracing and various electrophysiological techniques (Fairén et al., 1977; Freund and Somogyi, 1983, 1989; Frotscher, 1992; Freund, 1993). In this regard, Maxwell Cowan, a renowned researcher in the use of neural pathway tracing techniques, has stated that the validity and importance of Golgi's method lies in the fact that what we know about neuronal morphology is due, in large part, to the material analyzed using that method, and that even though intracellular labeling methods reveal a greater complexity of axonal and dendritic arborizations, they cannot generate the images that a good Golgi preparation can provide (Valverde, 1998). Proof of this is that, although its revival as a major research tool in neurocytology was due to its association with EM, today the "black reaction" is once again being used autonomously. In studies of the evolution and ontogenetic development of the nervous system, for example, Golgi's method is currently a key approach in quantitative analyses of the branching pattern of dendritic trees at the light microscope level (Pannese, 1996).

In summary, it can be argued that, although 150 years have passed since its discovery, Golgi's method is still the most appropriate one for studies designed to analyze the complete neuronal architecture of any brain region, a claim that very few other techniques can make. Of course, this has been enhanced by other technological advances. According to reports in the neuroscientific literature, the Golgi method is essential for most neurocytological studies, as proven by the plethora of recent publications that cite this technique and modified versions in research in diverse experimental models and human neuropathology. The validity of Golgi´s silver impregnation method is further evidenced by the fact that modifications are still being published (González-Burgos et al., 1992; Rosoklija et al., 2003; Zhang et al., 2003; Moss and Whetsell, 2004; Friedland et al., 2006; Baloyanis, 2015; Ignell and Hill, 2022).

The chemistry of silver impregnation

Based on the implementation of the metallic impregnation method that Golgi devised, it was assumed that the chemical reaction that revealed the *reazione* nera consisted in the formation of silver chromate due to exposing nerve tissue to the mixture of potassium dichromate and silver nitrate. Nevertheless, that supposition was not demonstrated conclusively until 1971 in an X-ray diffraction study, which showed that the diffraction pattern of the reagent in neurons impregnated by Golgi's method did not reveal any other compounds containing chromium or silver (Fregerslev et al., 1971). Later studies showed that silver nitrate diffuses into tissue indurated by potassium dichromate to the point of saturation, and that certain regions become supersaturated with the ions of both compounds to initiate the 'nucleation' of the chemical product of the reaction in crystallized form. The initial impregnation that occurs inside the neurons that are to be impregnated is progressive (Chan-Palay and Palay, 1972), does not cross membrane limits, diffuses in every direction throughout the interior of the neuron (Špaček, 1989, 1992), and impregnates all intracellular structures, except the nucleus and mitochondria (Blackstad, 1965; Stell, 1965).

Initially, a complex endocellular network of dense, branched fibrils forms, accompanied by small, dense, randomly scattered granules. As impregnation continues, this network becomes thicker, and the granules enlarge to form 'nucleation centers'. Along the way, this impregnation may 'encounter' other nucleation centers, which follow the same growth pattern. Evidence suggests that the progression of impregnation from those nucleation centers occurs in structures represented by lipoprotein membranous systems to form lipoprotein-chromo-silver complexes (Valverde, 1970). When impregnation occurs along the outer edge of the neuronal membrane, a kind of extracellular mesh forms by granules similar to those that exist inside the neuron (Blackstad, 1965; Stell, 1965) that may (or may not) encompass neighboring cells. In principle, this suggests impregnation of the entire nerve tissue, but it has been suggested that the concentration gradient of ions decreases as one moves away from the nucleation centers and impregnated structures, inhibiting both the impregnation of adjacent cells and the spatial

distribution of the impregnated neurons (Špaček, 1989). This possibility concurs with the hypothesis that the onset of cell impregnation is a limiting factor for the possible impregnation of adjacent cells (Ramón-Moliner, 1970).

Several authors have proposed the influence of various intrinsic and extrinsic factors on nerve tissue to explain both the low proportion of impregnated neurons and the quality of the impregnation process. These include the physical trauma that tissues may suffer during handling before fixing, the fixation pathway (fresh or by intracardiac perfusion), the dose of anesthesia applied, the metabolic and/or electrical state of the neurons at the time of fixation, the quality of the reagents used, the cleanliness of the materials, processing times, the time elapsed between the sacrifice of the study subject and the onset of fixation, the type of chemical fixative used, the quality and degree of purity of the reagents used, the effect of light on the reagents, temperature, the internal pH of the neurons, the addition of other reagents to the indurating or fixing solutions, and the concentration of the reagents in solution (which could oversaturate the nucleation centers and produce nonspecific precipitates such as neurons that likely fail to fully impregnate) (Fox et al., 1951; Bertram and Ihrig, 1957; Bertram and Sheppard, 1964; Morest and Morest, 1966; Špaček, 1989; González-Burgos et al., 1992; Pannese, 1999).

According to the scientific literature available, it appears that the study of the chemistry of metallic impregnation is now an exhausted subject. However, certain questions have not been answered with any significant degree of certainty, including: why do some brain structures have greater difficulty than others in successfully impregnating constituent neurons?; why must distinct processing times be applied to different brain regions?; and what factors influence why very young, or very old, animals are less prone to neuronal impregnation than young or mature adults? Golgi's silver impregnation method and its variants continue to provide scientific information in a wide variety of experimental models and human neuropathology, however, knowledge of the chemistry of impregnation is still limited and should receive much greater attention from researchers. If more information were obtained in this regard, we could develop alternatives that take even greater advantage of this technique that, given its obvious potential, can provide a great deal of information on the neurobiology of the nervous system.

The Golgi method and dendritic spines

In addition to the historical controversy between the Reticular and Neuronal theories (Ramón y Cajal, 1952), the existence of dendritic spines endured as a dilemma until their existence was demonstrated objectively by Cajal (Ramón y Cajal, 1888). Golgi had already observed spines, but he did not recognize them as real cellular structures probably because he considered them artifacts.

The initial verification was performed by Cajal based on a voluminous amount of work in which spines were observed consistently. Cajal's innumerable illustrations of the histology and cytology of the nervous system (DeFelipe, 2010, 2018) attest to their objective existence. In this regard, he affirmed:

"*When protoplasmic expansions are studied using the Golgi method, certain morphological details can be observed that should be made known, for over time they may come to have physiological significance. One is the presence of certain short appendages or collateral spines that emerge at a right angle to the contour of the dendritic expansions and end in a rounded or ellipsoid thickening*". Further on he added: "*... At first, we thought these eminences were the result of a tumultuous precipitation of the silver, but the constancy of their existence and presence, even in preparations in which the reaction appears to be very delicate in the remaining elements, incline us to believe this to be a normal*

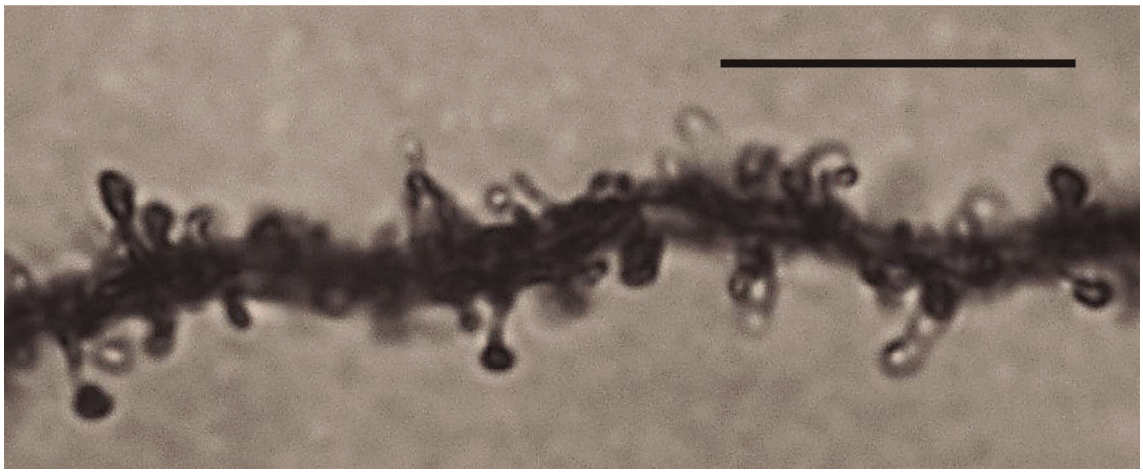

Fig. 4. Dendritic segment of a neuron in which a dense population of spines can be seen. Modification of the Golgi method (González-Burgos et al., 1992). Scale bar: 20 μm.

condition" (Ramón y Cajal, 1909).

Cajal's scientific conviction concerning these neural structures was clearly reflected in a letter he wrote to one of his last and most valued disciples, Lorente de Nó, two days before his death.

The Golgi method proved to be the most effective for visualizing spines in the light microscope, though their existence as cytoplasmic extensions of neurons was confirmed unequivocally in the 1950s with the advent of the electron microscope (Gray, 1959b). In addition, Gray conclusively demonstrated that spines were postsynaptic structures. Later, spines were seen in the soma (Mugnaini et al., 1967) and the axon hillock of neurons (Westrum, 1970). However, because most spines are found in dendrites, they are generically known as "dendritic spines" (Fig. 4).

Dendritic spines have now been widely studied using Golgi's method. They are cytoplasmic projections that clearly emerge from the dendrites of neurons and constitute 80% of excitatory synaptic contact sites. Measuring 0.1-2 μm in length (Harris and Stevens, 1989) and with only slight angular variations, they are arranged perpendicular to the longitudinal axis of the parent dendrite. Morphologically, the geometric structure of most spines is characterized by two regions: the head and neck (Harris and Kater, 1994), an

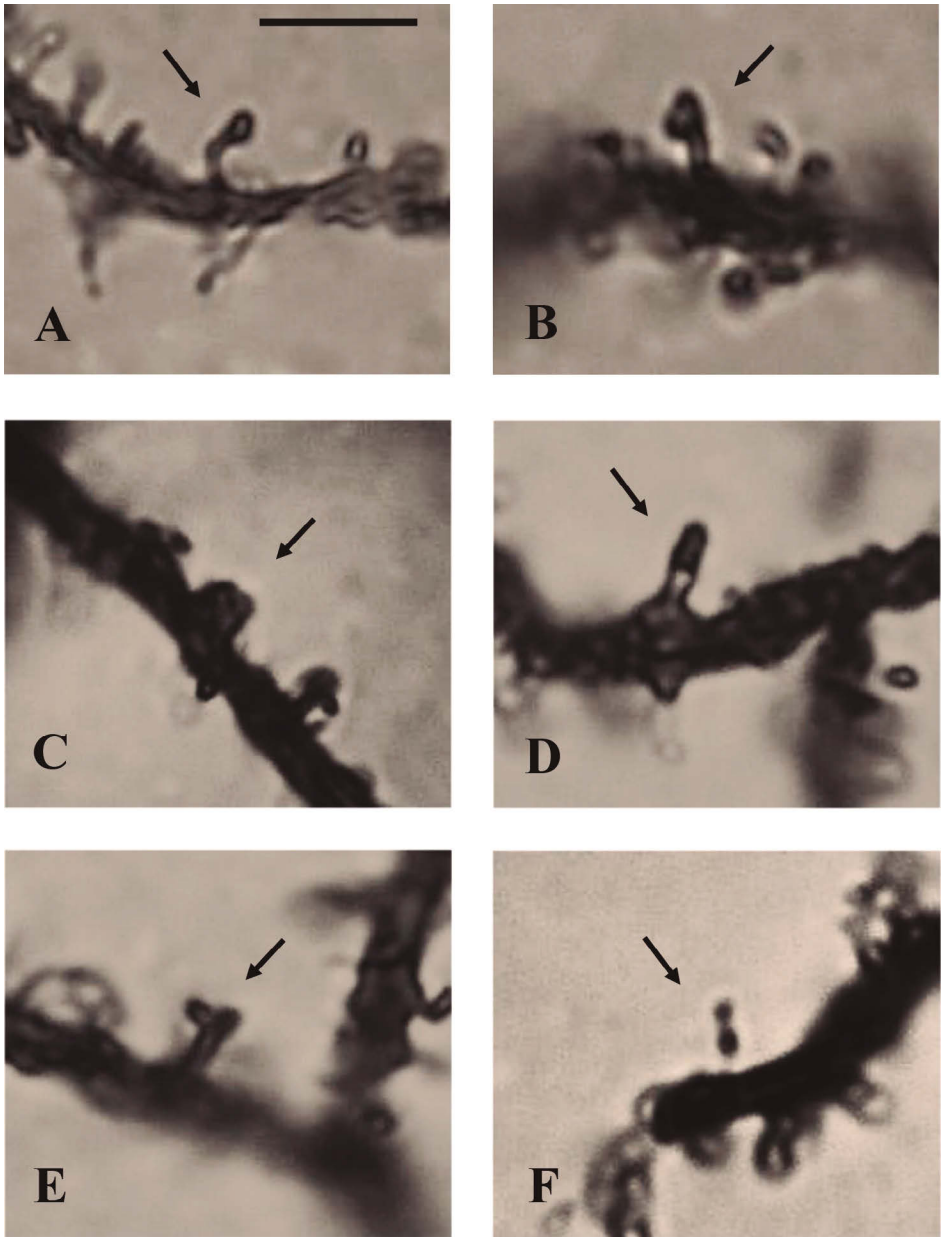

Fig. 5. Photomicrographs of a thin **(A)**, mushroom **(B)**, stubby **(C)**, wide **(D)**, branched **(E)**, and double **(F)** spines (arrows), impregnated with a modification of the Golgi method (González-Burgos et al., 1992). Scale bar: 5 μm.

anatomical property that confers distinct functional properties (González-Burgos, 2009, 2012, 2022; González-Burgos et al., 2015; González-Burgos and Velázquez-Zamora, 2023). The spine`s neck varies in length, but its diameter lies within a relatively small range. When a synaptic impulse is transmitted, the neck participates in regulating the passage of current mediated by calcium ions, thus modulating the function of the synaptic currents related to the excitatory afferent information transmitted to neurons (Koch et al., 1992; Koch and Zador, 1993). There are reports that the necks function as a kind of "pass valve" for the current that heads toward the parent dendrite and, eventually, the soma (Gulledge, 2023; Zagrebelski, 2023).

After the discovery of the relation of dendritic spines to the transmission of excitatory impulses (Gray, 1959a) mediated by glutamate (Johnson, 1972), subsequent studies suggested that, in addition to their density and the proximity between them (Harris and Kater, 1994), the transduction of excitatory messages could be influenced by their geometry (Peters and Kaiserman-Abramof, 1970). Based on that work, and to facilitate the interpretation of the functional activity of the spines, it was determined that they could be classified by the features of their head and neck. Based on Golgi's method and ultrastructural studies, they were initially categorized as thin, mushroom, stubby (Peters and Kaiserman-Abramof, 1970; Harris and Stevens, 1989), and branched types (Chicurel and Harris, 1992; Harris et al., 1992); however, additional, extensive experimental research has identified two more types: wide and double spines (González-Burgos, 2009, 2012, 2022; González-Burgos et al., 2015; González-Burgos and Velázquez-Zamora, 2023). Thin spines are characterized by a neck that is longer than the length of the head and a diameter slightly narrower than that of the head. The diameter of the neck of mushroom spines is very small in relation to that of the head, and short in comparison to the length of the head. Stubby spines show no distinction between the neck and head. Their total length is practically equal to, or may be less than, their diameter. Wide spines show geometric characteristics similar to the stubby type in that they do not have a clearly separable head and neck; however, their length is greater than their diameter. The features of the necks of branched spines are similar to those of the mushroom type, except that the head of this kind of spine is double. The two heads are of similar proportions but are separated by an invagination. Like branched spines, the double type has two heads. The difference is that the heads of double spines are aligned longitudinally and separated by a small-diameter neck similar to the one that separates the lowest head from the parent dendrite (Fig. 5). The proportional density of each type of spine varies according to the neuronal lineage, but the evidence available today suggests that thin spines are the most abundant ones in projection neurons, followed by mushroom, stubby, wide, branched, and, finally, double types (González-Tapia et al., 2015; González-Burgos and Velázquez-Zamora, 2023).

Dendritic spines are not immutable entities in terms of their geometric structure. Quite the contrary, they can present structural interconversions with a dynamism that operates in the order of minutes (Muller et al., 2000), a fact that has been elucidated by this and many other exhaustive, Golgi-based studies. The Golgi method has helped characterize some of the mechanisms and processes through which dendritic spines present plastic modifications, including *de novo* spinogenesis, redistribution along the dendritic trunk, reabsorption or pruning, and interconversion of one type to another, all mediated by signaling pathways and conformational adaptations of the molecules associated with the cytoskeleton (González-Tapia and Flores-Soto, 2023). These events are related to the processing of information with cognitive and/or non-cognitive content under normal or pathological conditions. Hence, based on the characteristics of the synaptic inputs they receive, these spines differentially process afferent information to postsynaptic neurons, depending on the characteristic functional activity of their various geometric shapes (González-Burgos and Vázquez-Hernández, 2023).

Conclusions and perspectives

The large number of studies carried out using the Golgi method and its variants in the years after its discovery have allowed us to learn a great deal about the biology of neurons and the functional organization of the nervous system. The work of many pioneering groups stands out in this field, led by researchers who have provided cutting-edge evidence and strengthened the methodological legacy left by Camilo Golgi and Santiago Ramón y Cajal for the training of new generations of scientists who have consistently moved this methodological line of scientific study forward. However, naming all the working groups that have contributed to the arduous task of elucidating the intricacies posed by the psychobiology of brain function would require more space than what is available in this text.

What we cannot fail to mention is that the studies conducted in recent decades using Golgi's method number in the thousands and do not seem to be decreasing with the passage of time. Furthermore, the methodological variants with which this silver chrome technique has diversified show a clear tendency to generate highly sophisticated technological resources that may well guarantee the survival of the award-winning Golgi hod for many years to come. In this regard, it is important to remember Cajal's words in the conclusions of his book, ¿*Neuronismo o reticularismo*? (Ramón y Cajal, 1952): "*And let us not fear future technical inventions, because if the facts have been well observed, they will endure, even if the interpretations change*".

Acknowledgements. The author thanks Paul C. Kersey for his support with the copy-editing of the manuscript.
Authors' contributions. IGB: conceptualization, writing, and editing of the manuscript, and supervision of the entire project.
Funding. This Review received no specific funding.
Conflict of interests. The author declares that he has no conflicts of interest.

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Accepted September 25, 2024