Review

Cajal: Lessons on brain development

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\textbf{ABSTRACT}

In 1906, Santiago Ramón y Cajal was awarded the Nobel Prize in Physiology or Medicine in recognition of his work on the structure of the nervous system. At that time, almost all of Cajal’s work was carried out using the Golgi method, a technique devised by the Italian scientist Camillo Golgi, with whom he shared this prize. Cajal introduced several modifications to the method developed by Golgi and, to avoid the problems encountered in staining myelinated neurons, part of his studies were carried out on embryos and very young animals (the “ontogenetic method”). In this way, Cajal begin to describe aspects of the development of the nervous system. Here, we review some of his wonderful discoveries (for example, the description of the axonal growth cone) from which he derived some of his main theories on the anatomy and physiology of the nervous system: the chemotactic hypothesis and the neuron doctrine.

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1. Introduction

For most neuroscientists, Santiago Ramón y Cajal is the founder of modern Neurobiology. His systematic studies of all the structures in the central nervous system (CNS) represent the first major step towards unravelling the fine organization of this organ. In the words of the renowned neurophysiologist Rodolfo Llinás, “Ramón y Cajal personifies, above all, the belief that we actually can understand the nervous system, which represents, more than anything else, the very nature of what we are” (Llinás, 2003). The CNS is a precise and complex structure that enables living beings to carry out a panoply of functions. From an ontogenetic point of view, the CNS emerges from a series of coordinated and complex events that occur during embryonic and postnatal development. One of the brilliant intuitions of Cajal was to analyze embryonic animals in order to obtain from them the basic schemes of the more complex organization that is attained in the adult nervous system (Tello, 1935). In the present manuscript, we will focus on some of Santiago Ramón y Cajal important works related to brain development. Many of which were produced during what has been designated as “the golden period” of Cajal’s scientific activity, between 1887 and 1903 (Sotelo, 2003), which includes the year 1888 that in Cajal’s own words was, “my greatest year, my year of fortune” (Ramón y Cajal, 1917). This period commenced when Cajal was first introduced to the Golgi method and terminated with the publication of the reduced silver method and the reception of several of the most relevant distinctions of his career. The fruits of this period were his opera omnia, the classic but still remarkably fresh Histological texts included in: “Textura del Sistema Nervioso del Hombre y de los Vertebrados” (Ramón y Cajal, 1899).

2. The Golgi method

In February 1905, the Royal Academy of Science of Berlin awarded Cajal with the Helmholtz gold medal for his discoveries and the following year, he was awarded the Nobel Prize in Physiology or Medicine in recognition of his work on the structure of the nervous system. He shared the Nobel prize with the Italian Camillo Golgi, who discovered a histological method that was able to impregnate the totality of different types of nervous cells with a fine brown precipitate. Cajal was made aware of the existence of this technique by the Spanish psychiatrist Luis Simarro in 1887, while still a professor at the University of Valencia. Having seen the results of this staining, Cajal immediately put it into practice in his own laboratory. He rapidly became aware how useful this method was to explore the structure of the nervous system, but it presented an important defect: the Golgi technique was too capricious and unpredictable. Cajal realized that this method needed to be further elaborated and modified to be truly useful. The essence of the Golgi method consists in submersing small pieces of nervous tissue in an osmium-bichromic solution for several days, following which the pieces of tissue must be left in a fresh solution of silver nitrate for a few more days (Valverde, 1970). The result is that some cells become filled with a fine silver-chromate precipitate that makes them visible against a translucent yellow background (Fig. 1). In order to improve and to make the method more reproducible, Cajal introduced an important modification: the double impregnation, in other words repeating the same impregnation process twice. Furthermore, Cajal added some other more subtle but effective changes to the Golgi method, varying the times of tissue immersion in the osmium-bichromic solution according to the structure that he wished to study, or the age and species of the

Fig. 1 – Photomicrographs taken from some of Cajal’s original preparations impregnated with the Golgi method. (A) Pyramidal cells in the Ammon horn of the adult rabbit (10×). (B) Pericellular nest formed around a Purkinje cell (empty) by a climbing fiber in the newborn kitten cerebellum (20×).
animal to be studied. However, he rapidly realized that the impregnation of a cell finished at the exact point where the myelin sheaths start to wrap around the axonal fibers. This is the reason why Cajal started to use the Golgi method in very young animals, and even in embryos, before the onset of myelination. With this modification, he was able to obtain the impregnation of entire axons, from the soma to their targets, either within the same CNS structure or at a distance from where the soma could be found. This last modification constitutes his ontogenetic or embryological method that was to yield wonderful discoveries.

3. The histogenesis of the cerebellum confirms the individuality of neurons

The histological preparations that Cajal obtained with the Golgi method when he applied the modifications indicated above, and particularly with his ontogenetic method, convinced him that the fine structure of the nervous system was not as his fellow scientists believed at that time. Cajal was able to impregnate developing nerve cells while axonal and dendritic processes were still growing. In this way, he described how a large quantity of nerve cells developed and he elaborated extremely detailed drawings that faithfully reflected what he observed through the microscope. He identified different stages in the development of a neuron, completing the earlier descriptions of His. The first stage of the process occurs when the cell still has an ovoid or round soma (germinal cell) although soon after, a couple of polar expansions emerge (bipolar stage). The most robust of these, the leading process, generally ends in a lamellar structure that Cajal named the growth cone (see below). The opposite expansion, which is shorter and much thinner, is known as the trailing process. In the following stage (neuroblast) the trailing process is lost, and the cell then begins to develop the dendritic tree and the collateral branches of both the axon and the dendrites (Fig. 2A). Everyone knows that Cajal studied all the structures of the nervous system with the Golgi method, but if we focus on the first structure that he studied, the cerebellum, we get a good and clear impression of how he meticulously described the entire development of a specific type of neuron. This can be seen for the formation of the axon and the

Fig. 2 – A summary of cerebellar histogenesis from Cajal’s original drawings. (A) Phases in the development of the cell and nerve fiber: primary embryonic nerve cell (A); bipolar phase (B), showing the beginning of the growth cone; neuroblast (C); development of dendrites (D); formation of the collateral and terminal axon branches (E). (B) Successive phases in the development of the branching of the Purkinje cell: temporary dendrites (a); axon collaterals (b, c). (C) Migration and transformation of the granule cells of the cerebellum: primary embryonic cell (1); beginning of polar outgrowths (2, 3); formation of a horizontal bipolar cell (4); start of descending outgrowth (5, 6); phase of vertical bipolarity (7, 8); production of provisional dendrites (9, 10); pruning and refinement of the definitive processes (11, 12). (D) Development of the climbing fibers in the cerebellum: phase of the pericellular nets over the Purkinje cell body (a); climbing fiber along the dendrites of a Purkinje cell (b, c).
dendritic tree of Purkinje cells (Fig. 2B) or the complete formation of the granule cells and their axons, the parallel fibers (Fig. 2C). Cajal also identified and described the afferents that enter the cerebellum, the mossy and climbing fibers. These latter fibers make pericellular contacts with the soma of the Purkinje cells before climbing along the dendrites (Fig. 2D). Cajal observed the axons of the commissural neurons, a group of neurons localized more dorsal to the bulk of the motoneurons, without deviation or error, as if guided by an intelligent force” (Ramón y Cajal, 1892). In fact, the original drawings of Cajal (Figs. 3B, D) accurately reproduced the aspect of the growth cone stained with the Golgi method in the spinal cord of E2–E4 chick embryos (Figs. 3A, C, E). The Golgi-stained growth cones resemble (with an incredible precision) the images obtained with a variety of more modern impregnation techniques. In fact, more than one century after the original description by Cajal, the physiology of the axonal growth cones is one of the more active research fields in modern Neurobiology (interesting recent reviews are Henley and Poo, 2004; Chilton, 2006; Wen and Zheng, 2006), with relevant implications in neuro-repair and regeneration (reviewed in Niclou et al., 2006; Wieloch and Nikolich, 2006).

The growth cone is the distal edge of the elongating axon, acquiring a hand-like structure with filopodia (the fingers of the hand) and lamelipodia (the interdigital spaces). Cajal observed and represented the different morphologies that he observed with the Golgi method (Fig. 3D). Because no two were alike, he thought that he was observing the static image of a dynamic process and consequently, he imagined the growth cone “[...] like a living battering-ram, soft and flexible, which advances mechanically, pushing aside the obstacles that it finds in its way until it reaches its peripheral destination [...]” (Ramón y Cajal, 1917). In his Histology, Cajal wrote that “[...] from the functional point of view, one might say that the growth cone is like a club endowed with exquisite chemical sensitivity [...]” (Ramón y Cajal, 1899). This interpretation enabled Cajal to formulate, not much later, his neurotropic theory (see below). Today, it is accepted that the growth cone permits the growing neuron to receive and integrate the variety of physico-chemical signals present along its pathway, these signals being produced by intermediate targets and the surrounding tissue. Such cues will guide each individual axon to its final target, where it will establish synapses with one or more neurons (Mueller, 1999; Song and Poo, 1999). As predicted by Cajal in 1890, growth cones have been demonstrated to be structures that undergo continuous remodeling (expansion and retraction) until they reach their final targets, as was originally proven in vitro by Harrison among others (Harrison, 1910; Harrison, 1935); see De Castro, 1934 for a historical summary of the scientists confirming the predictions of Cajal), and more recently, in vivo as well (Tashiro et al., 2003). It seems remarkable that, in the original works of Cajal and those of one of his most relevant pupils, Tello, the morphological observations in reference to the functional implications of the growth cone were confirmed several decades later. For example, the observations that the neurofibrillar chassis is relatively small when compared to the total volume of the growth cone. This chassis is even smaller when the axons are growing faster but, on the contrary, it hypertrophies when the growth cone stalls at those places (commisures, decusations, etc.), which Cajal and Tello identified as presenting “difficulties for the advance [of the growth cone]” (Tello, 1935) and that are today identified as decision points, where axons may alter their growth towards the next target (Bovolenta and Mason, 1987; Godement et al., 1994). The observations that the advancing growth cones stop frequently were capital for Cajal and Tello to fight against the “hypothesis...
of the pre-established pathways” (supported mainly by Hensen, Bethe and Held) that was invoked to explain axon growth and nerve formation (Tello, 1935).

5. The chemotactic or neurotropic hypothesis

Cajal wondered as to the nature of the signals that would guide the growth cones towards their targets. In 1892, a mere two years after his discovery, Cajal applied the knowledge available from other scientific disciplines to launch his own hypothesis of how growth cones might be guided: his famous chemotactic or (in his own words) “neurotropic hypothesis” (Ramón y Cajal, 1892). Taking advantage of his knowledge of frontier research into infectious diseases, Cajal suggested that fluxes of chemoattractive substances might be responsible of guiding the growth cones towards their final targets: “[…] While not denying that the mechanical influences proposed by His may play an important role, we believe that one should also acknowledge the possible involvement of factors analogous to those associated with the phenomenon referred to by Pfeffer as chemotaxis, whose existence in leukocytes was highlighted by Massart and Bordet, Gabritchewsky, Büchner and Metchnikoff and that was later attributed to the unique joining of growth points in embryonic blood. […] If one admits that neuroblasts are endowed with chemotactic properties, then one might also imagine that they are capable of undergoing amoeboid movements, initiated by factors secreted from epithelial, neural or mesodermal elements. As a result, their processes may become orientated in the direction of chemical gradients and thus, they will be guided to the cells that secrete these cues” (Ramón y Cajal, 1892).

From the past two decades of research in the field of Developmental Neurobiology, we are forced to admit that Cajal’s hypothesis, although brilliant, was not completely correct. In fact, chemorepellents, such as secreted Semaphorins or Slits, have been described that appear to be as relevant as the chemoattractants (like Netrins; Tessier-Lavigne and Goodman, 1996; De Castro, 2003). Moreover, depending either on the battery of receptors expressed by a neuron or on the metabolic state of the neuron, the same molecule can attract or repel the growth cone (Song and Poo, 1999; De Castro, 2003). Indeed, we now know that not only do secreted molecules play a relevant role in guiding axons during outgrowth, but contact mechanisms are also involved in creating exclusion regions or in contrast, the highways that allow growth cones to progress rapidly towards their
objective (Tessier-Lavigne and Goodman, 1996; Klein, 2004). Whatever, the “chemotactic hypothesis” presented by Cajal was on the whole correct (later known as the “neurotropism” or “chemotropic hypothesis”). This could not be said about the other hypothesis that years later were proposed to explain the orientation of axonal outgrowth during development, like “stereotropism” (based in mechanic causes) or “galvanotropism” (based in the electric influences) for example (Weiss, 1934, 1941; Ariëns-Kappers et al., 1936).

But the “chemotactic hypothesis” was not readily recognized and it remained forgotten, even though the results of Langley’s experiments on the reinervation of the autonomic nervous system coincided with Cajal’s ideas a few years after they were originally proposed (Langley, 1895). Some seventy years later, Sperry dusted off Cajal’s hypothesis presenting the famous “chemoaffinity hypothesis”, based on the intercurrence of at least two gradients to produce differential chemical attraction: “The establishment and maintenance of synaptic associations [is] conceived to be regulated by highly specific cytochemical affinities that arise systematically between the different types of neurons involved via self-differentiation, induction through terminal contacts, and embryonic gradient effect” (Sperry, 1963). As brilliantly exposed by Constantino Sotelo (2003), Cajal wrote in 1892 that chemotaxic mechanisms might also be involved in the migratory process of postmitotic neurons (cerebellar granule cells or sensory ganglion cells, for example). Indeed he temporarily included in his “neurotropic hypothesis” both “positive (providing attraction towards the final destinations) and negative chemotaxis” (providing repulsion), although the dichotomy attraction/repulsion disappeared from his book on Histology where only attractive chemotropic influences were proposed (Ramón y Cajal, 1899). The exclusive contribution of chemotraction was sanctified later: “nothing indicated the intervention of negative neurotropic substances” (Ramón y Cajal, 1913). We also coincide with Sotelo in highlighting what is maybe the weakest point of the initial formulation of Cajal’s hypothesis. For Cajal, axon growth cones seemed to be guided exclusively by signals produced by the final targets (other neurons in the CNS, muscles and other target cells in the peripheral nervous system; Ramón y Cajal, 1909, 1911), which implies the extension of molecular gradients to extremes considered excessively large today. Years later, Cajal admitted the need for local cues as well (Ramón y Cajal, 1913), which would also include what we know as intermediate targets, a subject of increasing interest for modern Neurobiology (an example of this is reviewed in Carel and Rubenstein, 2004). Each one of these intermediate targets supposes an authentic crossroads in the path followed by the growth cone towards its destination. Each axonal population uses a specific combination of signals (diffusible molecules) present in their environment, which are totally different from those used by the growth cones of other neurons, even in those cases where the cells have a very similar embryonic origin (Jacob and Guthrie, 2000).

The first experimental confirmation of the “neurotropic hypothesis” came from the studies on the regeneration of the peripheral nerves. The studies of Forssmann (Forssmann, 1898), Ramón y Cajal (Ramón y Cajal, 1913), and Ariëns-Kappers (Ariëns-Kappers, 1920) demonstrated that living Schwann cells were needed for the proper regeneration of sectioned axons, suggesting that chemical substances produced by these myelinating cells are necessary for the axons to reinnervate the distal stump. However, the first clear experimental evidence regarding the molecular mechanisms involved in axonal guidance began to emerge in the 1980s, starting with the discovery of the cell adhesion molecules or CAMs (Edelman, 1983). The other big molecular breakthrough in this field was made by Marc Tessier-Lavigne when studying (perhaps not by chance…), the same structure in which Cajal described the growth cones: the developing spinal cord of the chick embryo (Tessier-Lavigne et al., 1988). The work of Tessier-Lavigne showed that the axons of the commissural neurons were attracted towards the ventral midline of the tube by a substance secreted by the floor plate, the structure in the midline of the neural tube. A few years later, his group identified the family of Netrins as the first secreted molecules involved in axonal guidance (Kennedy et al., 1994; Serafini et al., 1994). Curiously, and more or less in parallel, the first chemorepellents were also identified (Kolodkin et al., 1993; Pini, 1993; Messersmith et al., 1995).

One century after the concession of the Nobel Prize in Physiology or Medicine to Santiago Ramón y Cajal and Camillo Golgi, virtually every neuroscientist accepts that Cajal, with his “neurotropic hypothesis”, was the first to shed light on the correct explanation for a developmental process as complicated as the navigation of axons to form synapses.

6. Neurotropic action of the epithelia

Cajal thought that the neuroblast and its processes could be influenced by factors secreted from epithelial, neural, or mesodermal elements. Accordingly, he studied the neurotropic action of the epithelia and he published his results in 1919. First of all, through studying the development of the short axon neurons in the retina, Cajal observed an initial disorientation of the spongioblast and horizontal cells. He observed that these cells do not respond to neurotropic stimulus at the beginning of their development, but they simply adapt to their mechanical environment (Ramón y Cajal, 1919a). In a much more extensive work, Cajal checked whether the epithelia of the skin and mucosa (and its derived structures, such us glands and hair follicles) exerted a tropic action on the embryonic fiber. Usually, the axons are attracted by the structures that they must innervate but on other occasions, when the nerve fibers are very young, they grow and ramify well before the target structure develops. At that moment, Cajal again suggested the existence of a negative chemotaxis that would exert a repulsive influence, similarly, he thought, to that found in the fertilized ovule. It was in this way that he explained why a ciliated acoustic element does not receive more than one single nerve terminal calyx (Ramón y Cajal, 1919b).

Likewise, it has recently been reported that the brain membranes (meninges), although not an epithelial but rather a connective tissue, are implicated in the attraction and repulsion of cells and nerve fibers during development. Specifically, during cerebral cortex development, secretion of stromal-derived factor-1 by the leptomeninges of the brain is...
necessary for the attraction and maintenance of the Cajal-Retzius cells in the most external layer of the neocortical neuroepithelium (Borrell and Marín, 2006; Paredes et al., 2006).

7. Cell migration

Immature, developing neurons migrate from the sites where they are generated to specific positions that they will occupy in their mature condition, sometimes over long distances. As mentioned above, Cajal pointed out in his 1892 paper (Ramón y Cajal, 1892) that chemotactic mechanisms could also be involved in the migration of postmitotic cells: “[...] If one admits that neuroblasts are endowed with chemotactic properties, then one might also imagine that they are capable of amoeboid movements, initiated by factors secreted from epithelial, neural or mesodermal elements[...].” However, Cajal only really paid attention to the phenomenon of cell migration when he applied the ontogenetic method (see above) and he began to study the development of different cell types in several brain areas. For example, Cajal described the development of granule cells in his 1890 paper on the cerebellum (Fig. 2C), from a spherical morphology without processes they generate polar processes, transiently displaying a bipolar morphology and adopting a horizontal disposition (Ramón y Cajal, 1890a). Today, it is known that any nervous cell can be generated in any part of the ventricular/ subventricular zone of the developing brain, and that they may ascend or move to reach the preplate or more external layer of the brain. In this stratum, the cells adopt a horizontal disposition and generate two opposite processes, the so-called leading and trailing processes. In this disposition, cells can migrate tangentially and they can adopt a monopolar morphology loosing their trailing processes, as Cajal described (Ramón y Cajal, 1889). The tangential displacement to reach their definitive position has been recently reported in different systems, such as the olfactory system (rostral migratory systems, such as the olfactory system (rostral migratory

8. Conclusions

We understand different aspects of the development of the nervous system thanks to the pioneering work of Cajal. As mentioned here, Cajal studied the development of different neurons in several structures of the CNS, work that led to the discovery of the individuality of neurons and subsequently, to the proposal of the neuron theory, the chemotactic hypothesis, and that of the dynamic polarization. After his work on neurogenesis, Cajal studied the adult CNS profoundly in healthy, sick, or damaged animals. However, all his work on neurogenesis was compiled and published in 1929 (Ramón y Cajal, 1929). Although we should not forget that the most important objective for Cajal was the definitive recognition of the neuron theory, and that his final works were devoted to the defense of the neuron theory against the reticular theory (Ramón y Cajal, 1933). We cannot find a better way to conclude this essay than with the own words of Cajal about the relevance of the different developmental events on the morphology of the mature neuron:

“The innumerable processes and intercellular connections offered by the adult nervous system can be interpreted as the morphological expression of the infinite routes traced in space by currents of inducing or positive chemotropic substances during the entire developmental period. Thus, the total arborisation of a neuron represents the graphic history of conflicts suffered during its developmental life” (Ramón y Cajal, 1899).

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All the Cajal’s original histological preparations (except the used for photomicrographs in Fig. 3, which belongs to the Archivo De Castro) and drawings used to illustrate this essay are conserved at the Cajal Museum, in the Instituto Cajal (CSIC).