THE MAJOR DISCOVERIES OF CAJAL AND HIS DISCIPLES: CONSOLIDATED MILESTONES FOR THE NEUROSCIENCE OF THE XXIst CENTURY

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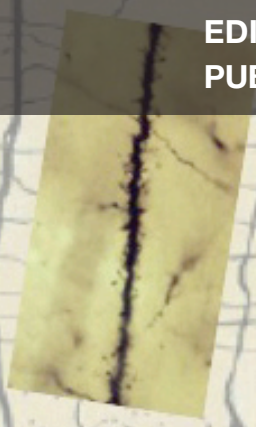

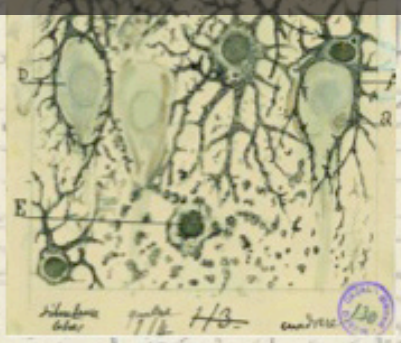



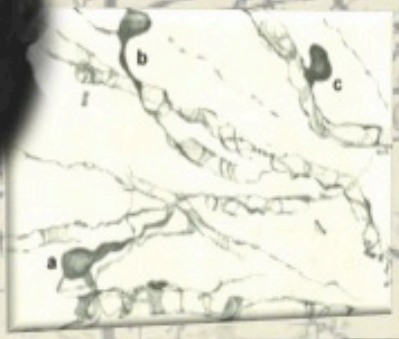
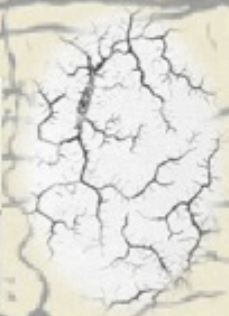

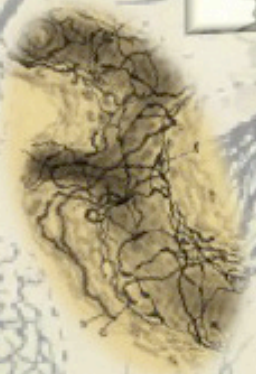

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THE MAJOR DISCOVERIES OF CAJAL AND HIS DISCIPLES: CONSOLIDATED MILESTONES FOR THE NEUROSCIENCE OF THE XXIst CENTURY

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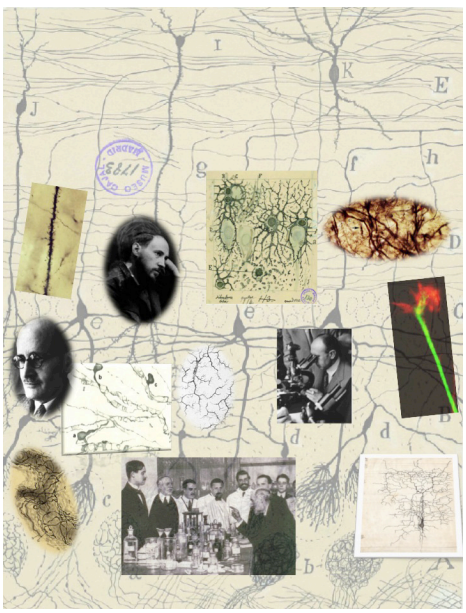

Cajal and the main of his disciples: a recreation over an original drawing of Santiago Ramón y Cajal illustrating the synaptic organization of the mammalian olfactory bulb. First row of insets: dendritic spines of a piramidal neuron stained with Golgi´s method (left), self-portrait of Santiago Ramón y Cajal in front of a blackboard with a profile in chalkwork (center-left); original drawing of Santiago Ramón y Cajal representing neuroglial cells and including a couple of newly-divided astrocytes (center-right); detail of microglial cells (arrow in the right inset). Second row insets: portrait of Pío del Río-Hortega (left); original drawing of Pío del Río-Hortega illustrating oligodendrocytes (center-left); detail of microglial cells by Pío del Río-Hortega (center); portrait of Fernando de Castro at the microscope in 1950 (center-right); neuronal growth cone stained with current immunofluorescent methods (right). Third row of insets: detail of an original drawing of Fernando de Castro illustrating the innervation of the carotid region (left); photograph of Cajal teaching to different members of the Spanish Neurohistological School, like Gonzalo R. Lafora, Domingo Sánchez, Jorge Francisco Tello and Nicolás Achúcarro (center); detail of the sketch of a cortical neuron by Rafael Lorente de Nó.
All the images have been taken from the articles composing this Ebook.

When Santiago Ramón y Cajal started to unravel the fine structure of the nervous system in the last decades of the XIXth century maybe only his unbeatable soul of brave Spaniard imagined that most of the descriptions were scientific truths that lasted to date. Simple histological stainings, curiosity to ameliorate these, monocular microscopes, patience for drawing his observations and a rich imaginative open mind: this is the recipy for Cajal success. His descriptions of connectivity in the nervous system, compiled in Cajal´s opus magna published in 1904 ("Textura del sistema nervioso del hombre y los vertebrados") and 1911 ("Histologie du systeme nerveux"), have been corroborated by modern techniques decade after decade. Even more, the main hypothesis that Cajal raised are universally recognised as biological laws, today: the neuron theory, the law on the dynamic polarization of the neuron and the chemotropic hypothesis. That is: the nervous system is not a sincitial network but is formed by individual cells; the transmission of the nerve impulses follow a main direction within a given neuron; the axons are guided by chemical substances in a chemotropic way, till form synapses with their targets.

Attracted by Cajal´s strong personality and scientific success, a number of medical students and doctors join him in the crusade to explore the nervous system. And the seed planted by the universal savant was really successful: Francisco Tello described interesting aspects of the regeneration of peripheral nerves which are very useful for neuroscientist currently working in this topic; Nicolás Achúcarro significantly contributed to study neuroglia and future microglia; Pío del Río-Hortega identified two out of the four main nervous cell types, the oligodendrocytes and microglia, and proposed an almost still valid classification for the CNS tumours; Fernando de Castro made was the first description of arterial chemoreceptors in the carotid body; Rafael Lorente de Nó was a dominant figure of Neuroscience for decades after the IInd World War, first describing the columnar organization of the cerebral cortex well before Mountcastle, Hubbel and Wiesel. Even less recognised co-workers and disciples of Cajal (his brother Pedro Ramón y Cajal, Domingo Sánchez, the neurologist Rodríguez-Lafora... protagonised discoveries that are consolidated scientific truths today).

Altogether, it is difficult (if not impossible) to find a school in biology contributing in such a fundamental and variated way to the common acervo like the collectively known as Cajal School or Spanish Neurological School. Although the particular way to work of the Maestro, selecting a pleiade of brilliant collaborators with whom accomplish such a titanic feat, giving them freedom for their studies, has been recognised and confronted to antagonic systems followed by other relevant scientists and scientific schools, the general recognition of such a significant major milestones for Neuroscience and their vigency in the well-marched XXIst century is not: this is the purpose of this Ebook, to remind all these examples of how successful can be the scientific work when it is minutious, constant and performed by brilliant, imaginative and skilled scientists with a minimal conditions supporting their efforts.

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EDITORIAL
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Editorial: The Major Discoveries of Cajal and His Disciples: Consolidated Milestones for the Neuroscience of the XXIst Century

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Keywords: History of Neuroscience, History of Science, neuron theory, neuronism, Cajal, synapse, dendritic spine, glia

The Editorial on the Research Topic

The Major Discoveries of Cajal and His Disciples: Consolidated Milestones for the Neuroscience of the XXIst Century

When Santiago Ramón y Cajal (1904; 1911) began to understand the fine structure of the nervous system in the last decades of the XIXth century, he was almost certainly alone in his conviction that his descriptions were scientific truths, many of which have outlived him and remain relevant. Simple histological staining, a monocular microscope, an unabated curiosity, patience and a special talent to represent his observations in sketches, and drawings, as well as a rich imaginative and open mind, all elements that together account for Cajal's success. His descriptions of the connectivity in the nervous system were compiled into an opus magna that was first published in 1904 ("Textura del sistema nervioso del hombre y los vertebrados," in Spanish) and subsequently translated into French in 1911 ("Histologie du systeme nerveux"). As the decades have passed, one by one all his theories have been corroborated using modern techniques, and the main hypotheses that Cajal postulated have become universally recognized as biological laws: The neuron theory; the law of the dynamic polarization of the neuron and the principle of connectional specificity.

Attracted by Cajal's strong personality, a number of medical students and doctors joined him in his crusade to explore the nervous system, and the seeds planted by the many faceted savant proved to be truly fructiferous. As such, among the successes of his disciples: Francisco Tello described interesting aspects of the regeneration of peripheral nerves, which still prove to be useful to neuroscientists currently working in this field; Nicolás Achúcarro contributed significantly to the study of neuroglia and subsequently, microglia; Pío del Río-Hortega identified two of the four main types of nervous cell, the oligodendrocytes and microglia, and his proposed classification for CNS tumors remains valid today; Fernando de Castro was the first person to describe the existence of arterial chemoreceptors to control blood composition, and who place them in the carotid body; Rafael Lorente de Nó was a dominant figure in Neuroscience for decades after the IInd World War, first describing the columnar organization of the cerebral cortex, well before the confirmatory works of Mountcastle, Hubbel, and Wiesel. In addition, several of the less well recognized co-workers and disciples of Cajal made discoveries that have been consolidated and are considered scientific truths today, including his brother Pedro Ramón y Cajal, Domingo Sánchez, and the neurologist Rodríguez-Lafora.

As a whole, it is difficult to find a scientific school that has contributed in such a fundamental and varied way to our common cultural heritage as that of Cajal. Cajal's particular way of working

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involved sharing his enthusiasm with select group of promising and proven collaborators, and giving them freedom to carry out their studies. This format has been recognized and compared to other systems established by relevant contemporary scientists and scientific schools, although maybe no other has marked such significant milestones in Biomedical Sciences that remain relevant well into the XXIst century. The main purpose of this Special Research Topic is to remind us of all these examples of how successful scientific study may become when attention is paid to detail, and brilliant, imaginative and skilled scientists work with unwavering commitment despite receiving minimal support.

An analysis of the scientific legacy of Cajal and his disciples (collectively known as the Spanish Neurohistological School, or more colloquially, Cajal's School) is tantamount to studying the fundamentals of the organization and function of the nervous system. In this Research Topic, the main contributions of the Spanish Neurohistological School are discussed in depth and in the light of the current dogma in Neuroscience. In this sense we would like to emphasize that the *corpus doctrinae* upon which this Research Topic is founded constitutes a solid conceptual guide for future generations of neuroscientists. The contributions focus on the most relevant areas in which Cajal and his disciples influenced neuroscience today. In this issue, the outstanding and experienced panel of contributors offer new perspectives on Cajal's work and theories, allowing the reader to obtain many relevant new insights into how the brain and mind works.

Dr. Delgado-García's takes a view of Cajal's work centered on the basic principles of cortical and nuclear organization, as well as addressing the old and new meanings of neural plasticity. Many interesting questions and hypotheses appear in his contribution, following on from a deep conceptual incursion in the world of the mind of Cajal. Very interesting and original papers from Dr. Blanco and Drs. Rozo and Rodríguez Moreno, respectively, analyse the historical and international context of the period in which Cajal was active, drawing comparisons with another great contemporary scientist, Ivan Pavlov. Addressing the parallels between their Nobel prizes in relation to Darwin's theory of Evolution, the reasons why Cajal extensively analyzed comparative anatomy become clear in this article, a search for the principles of organization and specificity of the connections in the nervous system. A complete overview of the influence of Darwin's ideas, and of the contribution of Cajal's theories on neuronal network plasticity and learning is presented by Ferreira et al. This article can be considered as one of the most relevant contributions to understand the true added value of Cajal's ideas to modern Neuroscience.

The paper dealing with the discovery of growth cones in the developing and lessioned nervous system by Drs. Tamaríz and Varela-Echevarría is a key piece of work in this issue, critical to fully understanding how Cajal derives his hypothesize about the dynamic reorganization of the nervous system. This paper presents a well-balanced combination of historical and recent data on growth cones and axon guidance, allowing the reader to obtain a clear idea about the chemotropic postulate in the development and regeneration of the nervous system. Like growth cones, dendritic spines were for many of Cajal's contemporary scientists simply artifacts of staining and/or fixation. In this issue a paper from Dr. Yuste comprehensively describes how Cajal concluded that dendritic spines are not only active structures on neurons but, more importantly, active elements relevant for central nervous system plasticity and connectivity. These two papers highlight the impact today of two of Cajal's more relevant milestones (growth cones and spines) on our understanding of the nervous system's capacity to change and adapt.

The Cajal-Retzius cells that lie in layer I of the brain cortex represent one of Cajal's more enigmatic findings, and these cells have been analyzed in depth in this Research Topic from three converging points of view. Dr. Marín-Padilla, one of Cajal's most enthusiastic followers, contributes a manuscript that adopts the perspective of Cajal as a pioneer in analyzing this type of neuron in depth, and of an expert in applying silver techniques to human brain sections. This paper is unique in this Research Topic, as far from simply extending a bridge from the historical to the modern perspective, it offers us insight to understand Cajal's way of thinking. Both, the historical peripeteia that the discovery of Cajal-Retzius cells represents and the current perspective of the role of these neurons in the developing cortex are analyzed extensively in two important papers by Drs. Gil et al. and by Drs. Martínez-Cerdeño and Noctor.

As an example of Cajal's descriptions of the sensory system, the well written article about the olfactory system by Drs. Figueres-Oñate et al. deserves particular mention. This article is peppered with beautiful images that are analyzed meticulously, and it presents Cajal's complete anatomical descriptions of olfactory bulb cells and the connectivity of central pathways. A very interesting paper by Dr. Navarrete and Araque demonstrates that our current ideas about the active role of astrocytes in regulating neuronal activity were implicit in several pieces of Cajal's work and that of some of his disciples. Their splendid analysis highlights once more the current validity of the hypotheses postulated by Cajal.

The most outstanding contributions to neuroscience of Pío del Río-Hortega can be found in a set of three papers in this Research Topic. A masterful overview of his contribution to the discovery of microglial cells, and that of his mentor Nicolás Achúcarro, together with a novel insight into the future of functional neuroanatomy is presented by Drs. Tremblay et al. A mini-review by Drs. Pérez-Cerdá et al. also includes a summarized sketch of Río-Hortega's biography and the basis of the silver carbonate method that he developed for glial cell staining. In addition, their article includes a very interesting comparison of Río-Hortega's classification of oligodendrocytes with that currently accepted for glial subtypes. Finally, by focusing on the role of the Spanish Neurohistological School's contribution in pathology, Dr. Ramón y Cajal Agüeras offers a very interesting view about del Río-Hortega's pioneer analysis and classification of brain tumors.

The figure of Rafael Lorente de Nó, one of Cajal's disciples who had the greatest impact on modern Neurophysiology, is carefully analyzed and discussed in a paper written by Dr. Larriva-Sahd. This paper thoroughly describes Lorente's concept of the cortical columnar organization of the brain and of input

segregation at the dendritic trees of pyramidal cells. It also addresses Lorente's main general theories, such as the neural basis of the unidirectional chain of neurons in reflexes, as well as the creative and outstanding functional models of subthreshold synaptic stimulation or temporo-spatial decoding in neuronal networks.

Finally, Dr. de Castro undertakes to explain the relevance of his grandfather, providing an extensive description, along with a beautiful collection of images from his personal historical files, he successfully outlines the scientific stature of Fernando de Castro and his contribution to our understanding of the peripheral nervous system, as well as his repercussions on current Neuroscience. Similarly, a magisterial analysis of Fernando de Castro's work on chemo- and baroreceptors is included by Dr Constancio Gonzalez et al.[1] which definitively shows de Castro's central role in establishing the basis of the nervous system's regulation of blood pressure.

Unfortunately, we were unable to find authors to offer a suitable contribution on maybe the first of Cajal's disciples, Jorge Francisco Tello, even though the regeneration of the nervous system is currently is field of significant interest. Similarly, no-one was found to contribute a chapter on Gonzalo R. Lafora, the renowned neuropathologist that described progressive myoclonic epilepsy or Lafora's disease. This was a pity but time constraints impeded us from covering these topics.

To close the Special Research Topic, there is an eloquent epilog by Drs. Lerma and De Carlos that focuses on the dimension of Cajal's work, not only in the field of Neuroscience but also, as a leader in promoting modern Spanish science. This is a very well written historical sketch that allows the readers to obtain a more complete understanding of the wider dimension of Cajal and of his ability to train brilliant students and collaborators.

In conjunction, we think that this Research Topic represents an outstanding work on the History of Neuroscience and perhaps, one of the most complete analytical discussion of Cajal's life and legacy, and that of the Spanish Neurohistological School, published in English to date[2]. The subject matter well merits the effort that has been made, and we feel we have achieved our main objective: To show how pioneering, vast and valid were the discoveries of this group of scientists, and how they influenced, and continue to influence the evolution of Neuroscience as a discipline even today.

We would like to deeply acknowledge all contributors to this Research Topic for their effort and excellent papers.

AUTHOR CONTRIBUTIONS

MM and FC have co-written this editorial.

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[1]Unfortunately, the great Professor of Physiology and our beloved friend Constancio González could not view this Special Research Topic completed because he died at the begining of the summer 2015. His was one of the first contributions received for this Special Research Topic.

[2]In Spanish, we should remark that postumously published by Fernando de Castro, the last direct disciple of Santiago Ramón y Cajal (de Castro, 1981).

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Cajal, Retzius, and Cajal–Retzius cells

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The marginal zone (MZ) of the prenatal cerebral cortex plays a crucial role in cellular migration and laminar patterning in the developing neocortex and its equivalent in the adult brain – layer I, participates in cortical circuitry integration within the adult neocortex. The MZ/layer I, which has also been called the plexiform layer and cell-poor zone of Meynert, among others, is home to several cell populations including glia, neurons, and Cajal–Retzius (CR) cells. Cajal once said that the MZ is one of the oldest formations in the phylogenetic series, and that the characteristics of layer I in human are similar in all vertebrates except fish (Ramon y Cajal, 1899). Despite the presence of CR cells in the MZ/layer I of all developing and adult vertebrate brains, and more than one hundred years of research, the phenotype and function of layer I cells have still not been clearly defined. Recent technological advances have yielded significant progress in functional and developmental studies, but much remains to be understood about neurons in MZ/layer I. Since the time of Retzius and Cajal, and continuing with modern era research from the likes of Marín-Padilla, the study of CR cells has been based on their morphological characteristics in Golgi staining. However, since Cajal's initial description, the term "CR cell" has been applied differently and now is often used to indicate reelin (Reln)-positive cells in MZ/layer I. Here we review the history of work by Cajal, Retzius, and others pertaining to CR cells. We will establish a link between original descriptions of CR cell morphology by Cajal, Retzius, and others, and current understandings of the cell populations that reside in MZ/layer I based on the use of cellular markers. We propose to use the term "CR cell" for the class of neurons that express Reln in the MZ/layer I in both prenatal, developing and adult cerebral cortex.

Keywords: Ramón y Cajal, Cajal–Retzius cells, marginal zone, layer I, horizontal cells, short axon cells, Reelin

POSTNATAL MAMMALIAN LAYER I BY CAJAL

Neurons in layer I were first described by Ramon y Cajal (1890). After several years of work using a variety of staining protocols including the Golgi method, Cajal concluded that there were two types of cells in layer I of the postnatal mammalian cerebral cortex: large horizontally oriented neurons, and neurons with a short axon [also called "Golgi corpuscle cells" by Ramon y Cajal (1897)]. Cajal also noted that other cellular structures were present in layer I including terminal dendritic bouquets, ascending axonal arborizations, and glial cells. This review will focus on neurons that have their soma within layer I: Cajal's horizontal cells, and cells with a short axon. We will contrast the description of these cells by Cajal and his colleagues more than one hundred years ago with current information.

In 1890, Cajal described a new type of large fusiform neuron in layer I of neonatal rabbit cerebral cortex that had a horizontal orientation. He collected additional data from the neonatal cerebral cortex of cat, rabbit, rat, and dog to show that in each species these cells were scarce and positioned at different levels in layer I, particularly deeper portions of layer I and superficial portions of layer II. These horizontal cells possessed processes that were parallel to the pia and from which originated thin branches, one of which was an axon. The axonal process of these cells was very long and thick with ascending collaterals that extended at right or obtuse angles to terminate exclusively within layer I (Ramon y Cajal, 1895).

In 1895, Cajal described a second distinct population of layer I neurons that possessed a short axon. These cells were distributed equally at different levels within layer I – superficial, intermediate and deeper portions – in neonatal rabbit and rat. The short axon cells exhibited several different morphological shapes and sizes, but tended to be larger in deeper portions of layer I, and gave rise to a large number of dendrites extending from the perikarion in all directions, but primarily toward the pial surface (Ramon y Cajal, 1895). The dendrites of short axon cells were usually very short, branched near the soma, and did not exit layer I (Ramon y Cajal, 1899). Based on morphology, he described at least two subtypes of short axon cells in mammals and at least four principle subtypes in human. He used the terms common type or medium-sized, large type, small type, and neurogliaform type to distinguish these cell subtypes. He determined that the medium-sized cells were the most abundant of the short axon cells in layer I and were positioned within intermediate and deep portions of layer I. The medium-sized cells were characterized by ascending dendrites and

an axon that coursed parallel to the pial surface of the cortex. The large type possessed long, thick dendrites that descended into layers II and III, and a thick axon that ran parallel to the pia within layer I. The small cell type had small oval or piriform soma and a thin axon that ramified near the cell body. Some of the small cells were positioned very close to the pia. Finally, Ramon y Cajal (1895) described the neurogliaform type as dwarf cells in the deepest portion of layer I that elaborated very complex and dense terminal arborizations.

PRENATAL HUMAN MARGINAL ZONE BY RETZIUS

In 1891, one year after Cajal described horizontal cells in layer I of the postnatal rabbit cortex, Retzius described cells located within the outer layer of the cerebral cortex in human fetuses at six to eight months of gestation. In his writings, Retzius commented that layer I cells had previously been noted by many other neuroanatomists using Golgi and other techniques, including Meynert, Henle, Schwalbe, Krause, Ranvier, Toldt, Kahler, and Obersteiner. However, due to the difficulty of impregnating these cells with the Golgi technique, and since some of these researchers thought that Golgi-labeled structures in layer I were artifacts, and other thought that they were glial cells, these layer I cells were not described in any detail. Therefore, it was Cajal and then Retzius who first dedicated time to study these complex layer I cells (Retzius, 1893).

Retzius first studied the MZ in the developing cerebral cortex using Golgi stained material from late gestation fetal human, and later in prenatal mouse, rat, cat, and dog. He noted that it was difficult to obtain good results using the Golgi method, that he was only able to stain a small proportion of MZ cells in third-trimester human cortex, and that he was not able to stain these cells in fetal human cortex younger than 6 months of gestation, nor in the brains from young children. His first opinion was that the cells he stained within the human MZ were very different both in form and orientation from the cells described one year earlier by Cajal in postnatal rabbit. Retzius initially thought that these cells were glial cells, but later agreed with Cajal that they were neurons, and also concluded that the human cells shared much in common and were potentially related to those described by Cajal. He initially referred to these cells as carrots cells since its body resembled a carrot, but later suggested the name "Cajal cells." Therefore, it was Retzius who first used the term "Cajal cells" for cells of layer I in postnatal animals and fetal human. He described how the "Cajal cells" in humans exhibited soma with different morphological shapes and had cellular processes that were more numerous and elaborate than in other mammals. It is important to note that the cells first described by Cajal had a precisely positioned horizontal soma, but this was not the case for the cells described by Retzius (1893). Furthermore, if we were to stain the cells described by Cajal and by Retzius with somatic markers available today, such as Reln, they may not appear to be related cell types based on morphology.

Retzius described human fetal Cajal cells as having round, oval, carrot-like, polygonal, or unevenly shaped soma of different sizes positioned at different levels within layer I, in some cases displaying a parallel orientation and in other cases a perpendicular orientation with respect to the pia (see **Figure 1A**). Retzius showed that these cells had several processes that emanated in various directions from the soma: superficially toward the pial surface, deep into the underlying cortex, or horizontally via small thick processes that often lead toward the surface. They had a total of 6–8 horizontal branches, each elaborating multiple processes extending to the surface and forming a characteristic mesh pattern (Retzius, 1893; Koelliker, 1896).

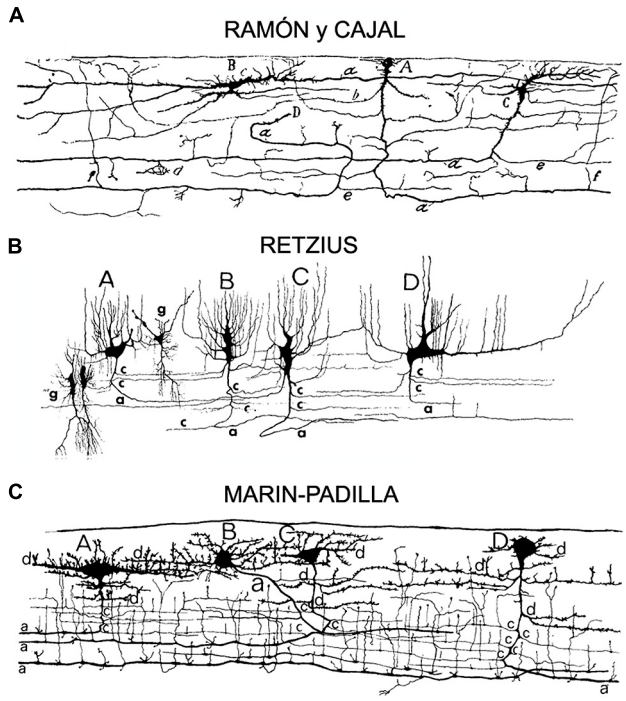

FIGURE 1 | Adapted from Marin-Padilla (1990). Original drawings of Cajal–Retzius cells from the motor cortex of a **(A)** 2-month-old infant (Ramon y Cajal, 1911), **(B)** a 26-week-old fetus (Retzius, 1894), **(C)** a newborn (Marin-Padilla and Marin-Padilla, 1982), sagittal perspective of layer I. Pyriform or triangular (Cajal A; Retzius A,B,C; Marin-Padilla C,D), horizontal monopolar or bipolar (Cajal B; Retzius D; Marin-Padilla A), and stellate like (Marin-Padilla B) cell types.

In a later publication, Cajal again described his two types of layer I cells – the horizontal and short axon cells – and also referred to a third type as "special cells of the cortex named by Retzius as Cajal cells." This emphasizes that, at that point in history, Ramon y Cajal (1897) did not think that his horizontal cells and the layer I cells described by Retzius were homologous. Soon after Veratti proposed the idea of including the "Cajal cells" described by Retzius within Cajal's horizontal cell category. Cajal came to accept this proposal, saying "with time I feel less repugnancy toward this idea" (Ramon y Cajal, 1897). In his later publications, Cajal referred to his horizontal cells and to the "Cajal cells" that were described by Retzius as one and the same.

CAJAL–RETZIUS CELLS BY KOELLIKER

While Retzius was not able to label Cajal cells with the Golgi method in cortical tissue obtained from children, Koelliker (1896) used Golgi and clearly detected such cells in cortical tissue from both children and adults and agreed with Retzius' descriptions.

Koelliker (1896) thought it difficult to discern the function of Cajal cells (in postnatal animals) and Retzius cells (in prenatal human). His opinion was that both cell types were to be kept separate and for this purpose he named them differently. He wrote that Cajal cells were much simpler and that there were fewer Cajal cells than Retzius cells in the MZ/layer I. The characteristic features of Cajal cells were so similar to neurons that he assumed they were indeed nerve cells. In contrast, he believed that the Retzius cells were glial by nature, and he suggested that they were "special glial transitory" cells. He stated that if Retzius cells were nerve cells, he could not explain the function of the multiple extensions ending at the surface, and the function/meaning of the entire structure would be in question. He reasoned that all of the superficial extensions of the Retzius cells ended in the upper surface glial layer of the brain, which is located above the stratum zonale that does not contain any nerve fibers (Koelliker, 1896). Nonetheless, some researchers have suggested that it was Koelliker who coined the term CR cells to describe the layer I cells described by Cajal in neonatal brain, and by Retzius in fetal brain (Huntley and Jones, 1990).

PRENATAL MARGINAL ZONE AND POSTNATAL LAYER I IN HUMAN BY CAJAL

Cajal followed his discoveries and those of Retzius by carefully studying the morphological similarities and differences between horizontal neurons in fetal MZ and neonatal and adult layer I in human. He concluded that horizontal cells were more abundant and larger in human. Cajal used the Golgi method to examine layer I cells in tissue obtained from seven- to nine-month human fetuses, and reported that horizontal cells were distributed through the entire thickness of the MZ, but were more numerous in close proximity to the pia (Ramon y Cajal, 1899). Agreeing with the descriptions by Retzius, he reported that the morphology of fetal horizontal cells varied, and included cells with fusiform, triangular, stellar, and piriform morphologies. The most characteristic features of these cells were horizontal processes that branched, yielding an extraordinary number of long, varicose horizontal fibers from which sprung at right angles innumerable ascending processes that terminated in rounded knobs near the surface of the cortex. After giving rise to a large number of ascending processes, the horizontal processes became thinner and finally subdivided into terminal branches that extended under the pia or in the most superficial region of the MZ (Ramon y Cajal, 1899). Cajal compared the data he obtained from humans with that obtained from rabbit, cat and other animal species. He concluded that in non-human mammals, fetal horizontal cells give rise to a comparatively smaller number of horizontal branches and that these extended shorter distances than those in human. Cajal compared the MZ in several cortical areas and came to the conclusion that it was populated by the same elements across cortical areas. The only potential difference he noted was that in some areas, for example motor cortex, the MZ was thicker than in others, and Ramon y Cajal (1899) proposed that this resulted from an increased number of pyramidal neuron dendritic terminations and horizontal cells. Cajal also compared the morphological features of cortical CR cells to those in the MZ of cerebellum, hippocampus and dentate gyrus and found them to be similar (Ramon y Cajal, 1899).

After examining neonatal layer I in tissue obtained from 15- to 30-day-old human brains, Cajal reported that most of the ascending branches described by Retzius in the fetal brain were no longer present after birth, and that the remaining fibers changed directions and ramified within layer I (see **Figure 1**). He determined that layer I cell bodies were smaller in the neonatal brain, and that horizontal projections were thinner in diameter but notably longer when compared to fetal cells (Ramon y Cajal, 1899). These long horizontal branches formed a system of parallel fibers that retained their original orientation. They had a thick myelinated axon that ran parallel to the pial surface for long distances, and issued collateral processes that ramified around the soma of short axon cells in layer I (Ramon y Cajal, 1895). Basing his work on Retzius' description of horizontal cells in the human fetus, Cajal identified three main morphologies of adult CR cells, unipolar or marginal cells, bipolar cells, and stellate or triangular cells. Cajal was not able to stain adult layer I cells with Golgi but used other techniques, including methylene blue, to describe the presence of short axon cells in adult layer I ("cilindro-eje" cells in Spanish). Cells exhibiting a morphology equivalent to Cajal's large horizontal cells have since been reported in vertebrates including crocodiles, lizards, non-primate mammals, non-human primates, and human (see **Figure 2**; Ramon y Cajal, 1896; Bernier et al., 1999; Goffinet et al., 1999; Perez-Garcia et al., 2001; Martinez-Cerdeno and Clasca, 2002; Martinez-Cerdeno et al., 2002, 2003; Tissir et al., 2003; Molnar et al., 2006; Ramos-Moreno et al., 2006; Meyer, 2010).

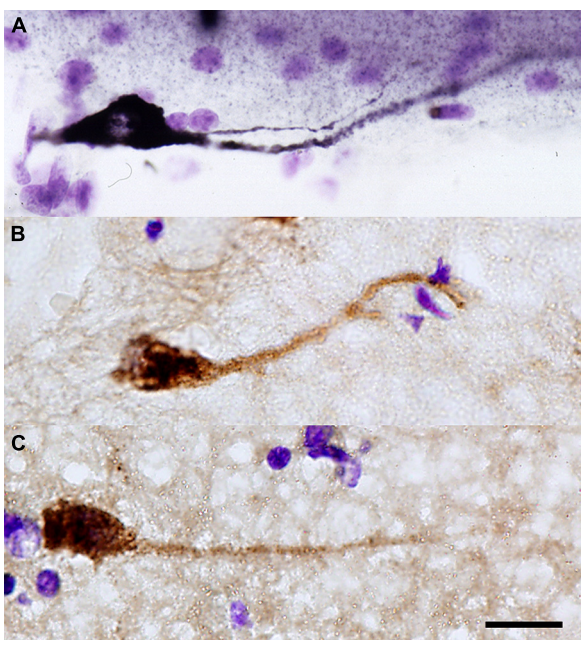

FIGURE 2 | Reln+ horizontal cells in the temporal cortex of adult macaque (A) and human (B,C). Scale bar: 10 μm.

Cajal hypothesized that the function of layer I cells was probably to establish connections between the terminal arborizations of Martinotti cell axons and/or association fibers coming from the white matter, with the terminal branches of the pyramidal neuron apical dendrites. He further proposed that the function of the large horizontal cells was to establish connections between layer I elements that were separated by considerable distances, while short axon cells performed the same function for elements separated by short or moderate distances (Ramon y Cajal, 1899).

ADULT HUMAN LAYER I BY MARÍN-PADILLA

Neither Retzius nor Cajal were able to Golgi-stain layer I cells in the adult cortex. However, one century later Marín-Padilla successfully used the Golgi method to perform detailed characterizations of cells within layer I of human neocortex (Marin-Padilla and Marin-Padilla, 1982). Marín-Padilla described the presence of cells in layer I of infants (eight-month-old), children (eight-year-old), young adults (16-year-old), and adult (26-year-old; Marin-Padilla, 1990; see **Figure 1B**). Until Marín-Padilla demonstrated the presence of both horizontal and short axons cells in adult layer I, the concept that these cells do not exist in the adult human brain had become widely accepted. The idea that CR cells are not present in the adult may have been initially proposed by Connel (1941, 1947, 1951). This hypothesis was supported by work that showed degenerating CR cells in the MZ of both rodents and humans (Derer and Derer, 1990; Meyer and Gonzalez-Hernandez, 1993; Super et al., 1998; Zecevic and Rakic, 2001; Abraham and Meyer, 2003; Tissir et al., 2009). But, recent work has shown that layer I horizontal neurons that express Reln (CR cells) persist in the adult layer I (see **Figure 2**; Pesold et al., 1999; Martinez-Cerdeno and Clasca, 2002; Martinez-Cerdeno et al., 2002, 2003; Chowdhury et al., 2010). Several factors may have contributed to the concept that CR cells were absent from layer I in adult human neocortex. First, the progressive postnatal expansion of the cerebral cortex dilutes the CR cell population within layer I (Marin-Padilla, 1990). Marin-Padilla studied adult CR cells in detail and concluded that the cortical expansion that occurs during postnatal development impacts the location of CR cell soma and principal dendrites, but does not alter the distribution of the long horizontal axonal processes. As a result, CR cell bodies are only found in specific locations, while the axonal processes are distributed throughout layer I in the entire cerebral cortex (Marin-Padilla, 1990). Furthermore, Marin-Padilla demonstrated that the axonal processes in layer I of any given cortical area represent those of all the CR cells in that area. Therefore, CR cell numbers are low in some cortical regions and the study of many consecutive sections is required to encounter CR cell bodies and principle dendrites. Despite the apparent change in layer I cell density, Marin-Padilla (1990) emphasized that CR cells persist in the adult cerebral cortex with essentially unchanged morphological properties, especially of axonal processes. Second, additional cell populations are present in high numbers in the MZ of the prenatal and developing brain, but are absent or reduced in the adult, contributing to the progressive cell loss in the MZ/layer I. Many cortical interneurons migrate through the MZ en route from the ganglionic eminences to the dorsal neocortex, but leave the MZ to enter the underlying cortical plate (Kriegstein and Noctor, 2004). Cortical microglia are also concentrated in the pial meninges and MZ during development, but achieve and even distribution throughout cortical tissue in the mature brain (Cunningham et al., 2013). Both migrating cortical interneurons and microglia can present with horizontal profiles in the MZ, temporarily adding to the overall population of horizontally oriented cells in the MZ/layer I during development.

MODERN ERA CAJAL–RETZIUS CELL RESEARCH

The first neurochemical characterization of CR cells was performed by Huntley and Jones (1990), who showed that CR cells express calcium-binding proteins such as calbindin and parvalbumin. More recent work has shown that the expression of calcium binding proteins within layer I cell populations is heterogeneous and depends on the species and stage of development (Martinez-Galan et al., 2014). CR cells are glutamatergic (Imamoto et al., 1994; Alcantara et al., 1998; Marin-Padilla, 1998; Meyer et al., 1998; Ina et al., 2007) and best known for their expression of Reln and a series of transcription factors including Tbr1, Tbr2, and P73 (Hevner et al., 2001, 2003; Meyer et al., 2002; Hodge et al., 2013). Reln is synthesized and secreted into the extracellular matrix of the MZ, diffusing through the developing cortex. Reln binds to the ApoR2 and VLDLR receptors expressed by radial glial cells and newborn neurons migrating to the cortical plate (Rice et al., 1998; D'Arcangelo et al., 1999; Rice and Curran, 2001; Tissir and Goffinet, 2003). Reln activates the adapter protein Dad1, and PIK3, leading to serine 3 phosphorylation of cofilin, an actin-depolymerizing protein that promotes the disassembly of F-actin. Phosphorylation at serine 3 renders cofilin unable to depolymerize F-actin, thereby stabilizing the cytoskeleton (Frotscher et al., 2009). CR cells are believed to be crucial for both the development and the evolution of a laminated pattern in the pallium (Trommsdorff et al., 1999; Howell et al., 2000; Kuo et al., 2005; Abellan et al., 2010). In addition, it has been suggested that CR cells signal to ventricular zone progenitors and function as modulators of early cortical patterning (Griveau et al., 2010). For a comprehensive review on the function of Reln protein, see Frotscher et al. (2009).

Recent studies have determined that layer I cells are derived from extracortical sites, including the cortical hem, septum, retrobulbar area, and thalamic eminence (Grove et al., 1998; Meyer and Wahle, 1999; Meyer et al., 1999; Meyer et al., 2002; Abraham et al., 2004; Takiguchi-Hayashi et al., 2004; Bielle et al., 2005; Yoshida et al., 2006; Cabrera-Socorro et al., 2007; Ceci et al., 2010; Gu et al., 2011). The newly generated CR cells migrate tangentially within layer I to reach their destination. Some evidence suggests that the CR cells generated at different sites populate different cortical regions and may thus play distinct, region-specific roles and function in neocortical development (Bielle et al., 2005; Gu et al., 2011). On the other hand, CR cells of different ontogenic origins display comparable functional properties in the early postnatal cortex and may therefore perform similar functions within the transient neuronal networks of the developing cortex (Sava et al., 2010). The distinct origins of CR cells are likely an additional factor that contributes to the heterogeneity

of layer I cell types. For more detail on CR cell origins, see Meyer (2010).

Recent studies have also expanded our knowledge of CR cell physiology. CR cells, together with GABAergic interneurons, form a dense network in layer I throughout various neocortical areas, exhibit characteristic membrane patterns and firing patterns, and receive both GABAergic and non-GABAergic input (Anstotz et al., 2013). Modern studies propose, as Cajal did one century earlier, that the source of CR cell inputs include layer I-targeting Martinotti-like interneurons, which express functional group I mGluRs (Cosgrove and Maccaferri, 2012). This suggests that enhanced glutamate release is critical for the establishment of an mGlu1α-dependent micro-circuit, which leads to the activation of CR cells (Cosgrove and Maccaferri, 2012). It has also been shown that GABAergic subplate neurons innervate CR cells. These synaptic connections are thought to be transient and therefore important for neocortical development (Myakhar et al., 2011). For more details, see Luhmann et al. (2014).

DeFelipe together with other 42 experts in cortical cytoarchitechture recently classified cortical interneurons using the "gardener" method for classification and found high consensus for some terms such as Chandellier cell and Martinotti cell. However, this approach found low consensus among experts for the term CR cell (DeFelipe et al., 2013), demonstrating that defining CR cells is not consistent across the field. It is clear that MZ/layer I cells are heterogeneous in morphology, size, location, age, molecular expression, origin, and species. This apparent heterogeneity presents challenges and can potentially seed confusion in reporting results. This is not a new problem as the term "CR cell" has been applied differently for over the last 100 years. For example, CR cells may refer to horizontal cells present only during fetal development (Bradford et al., 1977), or to short axon cells in embryos and adult (Marin-Padilla, 1990), or MZ/layer I cells that express AChE but not GABA (Huntley and Jones, 1990; Soda et al., 2003), or more commonly as the MZ cells that express Reelin (Meyer and Goffinet, 1998). Cell classification can be approached as lumping or splitting. We suggest a definition of CR cells that is more inclusive. In considering the most basic question: what is a Cajal Retzius cell? It is useful to take into account how difficult it is to arrive at an answer:

(1) Cajal, Retzius, Koelliker, Verati, and others disagreed on the identity of CR cells and changed their opinion on this matter.
(2) Cajal first described horizontal cells in layer I of postnatal animals, while Retzius described horizontal cells in human fetuses.
(3) Not all the cells described by Cajal or Retzius were horizontal.
(4) Cajal described multiple morphological categories and subcategories and classifications for cells in layer I (not included in this manuscript).
(5) The cells described by Cajal and Retzius in their original work could be viewed as similar or different, based on morphological criteria.
(6) Original studies used the Golgi method as a principal approach for cell labeling, while current research often uses immunohistochemistry.
(7) Only the Golgi method, rarely used today, labels the entire dendritic arbor of MZ/layer I cells.
(8) The Golgi method may not label all cell types in the MZ or layer I.
(9) Horizontally oriented cells in the MZ of the developing brain will include migrating interneurons originating from the ganglionic eminences, and microglia. Therefore the termination of interneuron migration, and the dispersal of microglia contribute to the decreased cell number in layer I during the postnatal period. These normal developmental processes are distinct from the cell death of CR cells.
(10) Neurons in MZ/layer I originate from a variety of anatomical regions.
(11) The adult cerebral cortex also has Reln-expressing horizontally oriented neurons.
(12) CR cells express pallial and subpallial markers.
(13) Reln is the main protein used to label CR cells. However, Reln does not label all horizontal neurons in layer I, while at the same time labels cells with a morphology matching that of the short axon cells described by Cajal.
(14) The function of adult layer I cells has not been completely determined.
(15) Cajal proposed that both horizontal cells and short axon cells serve similar connectional functions.

To avoid discrepancy due to the heterogeneous nature of cell types in layer I based on measures such as morphology, we propose to use the term "CR cell" to describe a *class* of cells, rather than a single cell type. In the same way that the term "pyramidal neuron" refers to a class of cortical neurons that includes distinct subtypes, we propose to use the term 'CR cell' to refer to a class of neurons that includes multiple subtypes based on specific cell morphology, location, age, origin, and marker expression. In this scheme, the term "CR cell" will describe any Reln+ neuron present in the developing MZ and the postnatal/adult layer I of the cerebral cortex. The term "CR cell" will not include the pioneer-neurons of Fairén that emit the earliest descending axonal projection from the cerebral cortex to the subpallium (Morante-Oria et al., 2003), nor will it include migrating interneurons derived from the ganglionic eminences. This approach will create a single class of neurons, CR cells, that comprise multiple heterogeneous subtypes based on molecular and morphological considerations.

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Historical first descriptions of Cajal–Retzius cells: from pioneer studies to current knowledge

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Santiago Ramón y Cajal developed a great body of scientific research during the last decade of 19th century, mainly between 1888 and 1892, when he published more than 30 manuscripts. The neuronal theory, the structure of dendrites and spines, and fine microscopic descriptions of numerous neural circuits are among these studies. In addition, numerous cell types (neuronal and glial) were described by Ramón y Cajal during this time using this "reazione nera" or Golgi method. Among these neurons were the *special* cells of the molecular layer of the neocortex. These cells were also termed *Cajal* cells or *Retzius* cells by other colleagues. Today these cells are known as Cajal–Retzius cells. From the earliest description, several biological aspects of these fascinating cells have been analyzed (e.g., cell morphology, physiological properties, origin and cellular fate, putative function during cortical development, etc). In this review we will summarize in a temporal basis the emerging knowledge concerning this cell population with specific attention the pioneer studies of Santiago Ramón y Cajal.

Keywords: neocortical development, pioneer neurons, radial glia, cortical hem, reelin, calretinin

INTRODUCTION

Today it is generally accepted that Santiago Ramón y Cajal's (1852–1934) studies, in particular the neuronal theory, should be considered the beginning of modern neurobiology (DeFelipe, 2002; Lopez-Munoz et al., 2006; De Carlos and Borrell, 2007). Thus, numerous aspects of Cajal's activities, from a point of view of both scientific and academic, have been largely described in several manuscripts, reviews and books. Some of them focused on determining the relevance of Cajal's technological advance to current neurobiology (DeFelipe and Jones, 1992; Jones, 2007). Today, when we characterize "translational research" as a robust pillar of an appropriate scientific strategy we should note, if we look at Cajal's notes, that this vision is not new. In fact, it was also developed by Cajal, among others, during his scientific career. In science, the development of new methods and their implementation as translational tools for research is one of the mandatory in the face of new challenging issues regarding how to obtain relevant scientific data. As indicated, this vision was one of the greatest and most relevant contributions of Cajal to the "scientific method," expanding the descriptive aspects of the method to a more deductive approach, as clearly demonstrated by his drawings. Indeed, this may be seen in the first stage of his research, between 1877 and 1887, and previous to the discovery of the "reazione nera" or Golgi method (Camillo Golgi, 1843–1926), in a visit to the private laboratory of Luis Simarro (1851–1921). Cajal equipped the Anatomy Department (Medical School) of the University of Valencia (1883) and Barcelona (1887) with optical microscopes. These pioneer microscopy units were the result of the privileged microscopic observations of the histological preparations of Aureliano Maestre de San Juan (1828–1890). In fact, we cannot describe the advances of Cajal without making a mention to the microscopic drawings and microphotographs, most of them developed at high magnification and using various histological methods, which meant a challenging issue at that time. However, we should not underestimate his deductive potential since in the hands of Cajal, the Golgi method showed a different neuronal organization from that described by Golgi and other scientists using the same method (Golgi, 1873). Another relevant aspect of Cajal's studies was the description of the neuronal architecture by analyzing the development and then degeneration of the nervous system. Thus, during the period from 1887 to 1903, Cajal carried out intense and productive scientific activity, with the help of the Golgi method, in many descriptive aspects not only of mature nervous tissue but also of its development. In this review we would like to present some of the data that Cajal and colleagues published concerning a specific cell type located in the superficial layer of the developing cerebral cortex: the Cajal–Retzius cell. In addition we would like also to consider these results in light of current knowledge of this cell population.

FIRST DESCRIPTIONS OF CAJAL–RETZIUS CELLS: FROM THE *CAJAL* CELLS OF RETZIUS TO THE HUMAN *RETZIUS* CELLS OF KÖLLIKER THROUGH THE *SPECIAL* CELLS OF CAJAL

Cajal–Retzius cells have been extensively analyzed since Cajal first described them in 1890 (Ramón y Cajal, 1890). At that time, he was intrigued by the existence of a dense axonal plexus of nerve fibers that run horizontally to the surface of the cerebral cortex in the

molecular layer. Some contemporary neuroanatomists described that these fibers were myelinated and suggested a putative origin for them. For example, Carlo Martinotti (1859–1918) suggested that they originated from the branches of pyramidal axons of the second and third cortical layer (Martinotti, 1890). However, the exact origin of them was unknown due mainly to the limitations of the histological techniques. Moreover, other scientists working on the structure of the neocortex described the presence of cells in layer I as well as the lamination of the human cortex using methylene blue staining without specific descriptions of these cells (Meynert, 1867). Taking advantage of the Golgi method, Cajal studied the composition of the marginal layer in newborn small mammals such as rabbit, cat, dog and rat (Ramón y Cajal, 1890). He observed that these fibers, in contrast to what was contained in Martinotti's theory, arose mostly from two different cell types present in the same molecular layer: *polyhedral* and *fusiform* cells. The first were of medium size with four or five rough dendrite branches that extended in all directions, the axons of which ramified profusely in the most superficial part of the molecular layer. The second neuronal type was thinner and very elongated, with a smooth contour and with an ovoid soma and two opposed branches that extended horizontally over a considerable distance and finally bent and ascended to the cerebral surface. In their horizontal trajectory, their processes produced collateral processes or appendages which terminated in the upper portion of the molecular layer (**Figure 1**). But surprisingly, under the analysis of Cajal, these cells frequently showed two or three axons that came off the dendritic branches at a great distance from the cell body and then ran opposed and horizontally until they ramified in ascendant collaterals which afterwards turned so as to run horizontally, populating the entire marginal layer. This characteristic led Cajal to refer to them as *special cells*. Apart from this histological description (Ramón y Cajal, 1890), he took the risk of attributing to them a functional role and considered they might serve as a connection between pyramidal cells from distinct areas of the cortex. Thus, the arborizations of their nerve fibers contacted the apical dendrites of pyramidal cells; for this reason he also conferred upon them the name of *superficial cells of association* (Ramón y Cajal, 1890, 1891b).

Gustaf Retzius (1842–1919) identified these cells in embryos of diverse species (rabbit, cat, and dog) and called them *Cajal* cells (Cajal'sche Zellen; Retzius, 1893). The first description of these Cajal cells by Retzius was in parallel with the study of another cell type identified by Cajal as "interstitial" cells of the cortical white matter of dogs (Ramón y Cajal, 1891a, 1893). Indeed, Retzius described, in plate I of this publication of 1893, the presence of horizontally fusiform cells similar to those reported by Cajal. However, he failed to identify the same cell type in human fetuses. This led to Rudolph Albert von Kölliker's (1817–1905) reserving the name of *Cajal* cells for mammals and employing the term *Retzius* cells for their human fetal homologues (Kölliker, 1896).

Some years later, the axon-like appearance of the majority of the cellular processes in these cells led Cajal to modify his previous opinion and to consider that these cells lacked a differentiation of processes into axons and dendrites and that they therefore shared the same morphological significance (Ramón y Cajal, 1897). However, the observations of Retzius in human

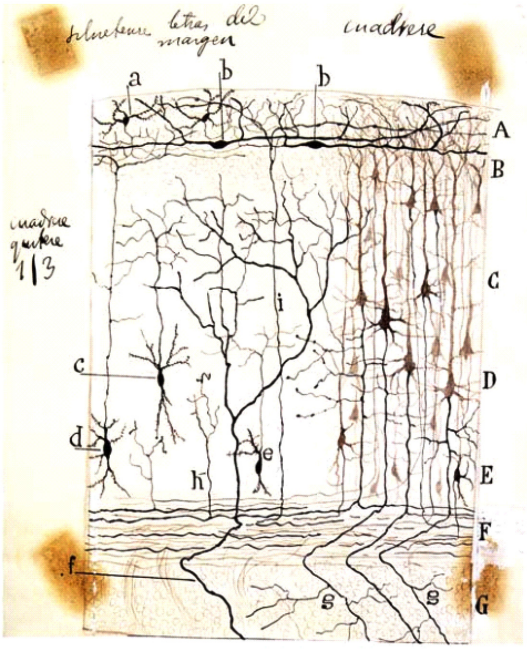

FIGURE 1 | Schematic drawing by Cajal of a Golgi-impregnated preparation of the cerebral cortex. In this illustration, Cajal compiled some of his findings from small mammals (rabbit, mouse, etc.) reported between 1890 and 1891. Note both the presence of the polyhedral (or stellate) cells *(a)* and the horizontal fusiform cells *(b)* in the plexiform layer. **A**, plexiform layer; **B**, small pyramidal cell layer; **C**, medium pyramidal cell layer; **D**, giant pyramidal cell layer; **E**, polymorphic cell layer; **F**, white matter; **G**, striatum. Reproduced, with permission of the Inheritors of Santiago Ramón y Cajal, from Reference (Ramón y Cajal, 1923).

fetuses and of Emilio Veratti (1872–1967) in rabbits (Veratti, 1897), in addition to his own observations obtained with new techniques (methylene blue and reduced silver nitrate methods), led him to give up the notion that these *special cells* possessed multiple axons and that only one behaved like a legitimate nerve fiber (Ramón y Cajal, 1904). Furthermore, due to the great morphological differences observed between cells from newborn children and fetuses, he concluded that in humans these *special cells* showed two stages: the fetal and the adult form. According to this theory, most of the fine ascending processes present in the fetal form were destined to atrophy in the days following birth, becoming almost completely absent in post-natal periods and therefore conferring upon these cells their characteristic adult form.

Cajal continued to study these *special* cells throughout his life in different areas of the human cortex such as visual, motor, olfactory, and acoustic areas, and also in different mammal species, birds, and reptiles, thereby performing the first comparative analysis of them (Ramón y Cajal, 1904, 1911; DeFelipe and Jones, 1988).

A SECOND PHASE OF CAJAL–RETZIUS CELL ANALYSIS: CORTICAL LAMINATION STUDIES PREVAIL OVER CAJAL–RETZIUS CELL DESCRIPTIONS

Although Cajal and Retzius exhaustively characterized these cells, the great morphological complexity that they show in different

species and in different developmental stages in addition to the random results obtained by the Golgi method have caused great confusion. Numerous studies have been directed to determining other aspects of the developing cortex instead of analyzing in greater detail the biology of Cajal–Retzius cells. For example, when analyzing descriptions of the white matter cells, intermediate zone/subventricular zone, and subplate, Cajal–Retzius cells appear in the published data as being cited but not studied in detail (Hatai, 1902; Von Economo and Koskinas, 1925; Aström, 1967). Even the appropriate name has become a matter of controversy, and they have received different names such as Cajal cells, Retzius cells and Retzius–Cajal cells (Fox and Inman, 1966; Konig, 1978). While the debate continued until recent years (Meyer et al., 1999; Fairen et al., 2002), the most widely accepted term today is Cajal–Retzius cells (henceforth CR cells).

THE THIRD PHASE OF CAJAL–RETZIUS CELL STUDIES: GROWING INTEREST IN THE 1970s BECAUSE OF THE INFLUENCE OF THE BOULDER COMMITTEE

In 1970, a group of neurobiologists published a seminal review in Anatomical Record describing the basic principles of the development of the central nervous system (Boulder Committee, 1970). Although lacking some information (e.g., the subplate was not identified as a developmental layer in the manuscript and CR cells, although mentioned, were not included in the general scheme), the effort at summarizing most of the information obtained during several studies (mainly in humans) was very positive. In fact, the committee assigned the molecular layer a relevant role during cortical development. Following the publication of the manuscript, several studies analyzed the birthdates as well as the ultrastructure of the neuronal and glial populations described in the manuscript (Konig et al., 1977; Rickmann et al., 1977; Shoukimas and Hinds, 1978; Konig and Marty, 1981; Sievers and Raedler, 1981). But especially, special attention should be paid to the numerous studies developed by Miguel Marín-Padilla (1930) concerning the structure and development of the primitive plexiform layer/layer I in cats, hamsters, and humans (Marin-Padilla, 1971, 1988; Marin-Padilla and Marin-Padilla, 1982). In accordance with the original descriptions of Cajal and Retzius, Marin-Padilla, a follower in the trail laid down by Cajal, described the molecular layer as the first cortical lamina to develop during corticogenesis, characterized by the presence of a horizontal plexus of fibers with scattered primitive neurons. More relevantly, Marin-Padilla determined that both fibers and neurons are further split into the superficial (layer I) and deeper layers (layer VII, in human) by the appearance of the cortical plate (Marin-Padilla, 1978). Marin-Padilla's descriptions of CR cells are based on the use of the Golgi method and are very similar to those reported by Cajal. He described CR cells in the fetal stage as cells with triangular, inverted pyramidal or fusiform cell bodies with two horizontal dendrites with ascending fine branches. The axon of the CR cells bifurcates in layer I and form the tangential fibers of Retzius (Marin-Padilla, 1988).

The controversy about the different morphological features of these cells seems to be resolving gradually due to recent studies employing advanced techniques as *in vivo* two-photon imaging (Chowdhury et al., 2010) and the use of several markers such as Acetyl cholinesterase, Calretinin, Reelin, p73, CxCR4, etc. (Cabrera-Socorro et al., 2007; Anstötz et al., 2013; Ma et al., 2013; **Figure 2**). Nevertheless, the fact that these markers are not really specific for CR implies a huge disadvantage for studying these cells in detail, and therefore the characterization of a real specific marker should be one of the main goals of the researchers in this area.

ORIGIN AND MIGRATORY ROUTES OF CR CELLS: A "ROAD RUNNER" IN THE DEVELOPING CORTEX

From the earliest studies, CR cells were thought to be generated in the ventricular region of the pallium (Meyer et al., 1998), while in the classical studies cited above they were described in different cortical regions. Classical descriptions do not discriminate between CR cell morphology and characteristics with respect to their origin. However, by using using Golgi impregnation (Marin-Padilla and Marin-Padilla, 1982) or other techniques such as tritiated thymidine (Parnavelas and Edmunds, 1983), it has been shown how CR cells undergo morphological changes to be transformed into resident layer I cells during cortical development in cats (Luskin and Shatz, 1985) and primates, including humans (Marin-Padilla, 1988). Thus, the incorporation of CR cells in the preplate came to be considered the result of radial migration during corticogenesis (Boulder Committee, 1970) and this was confirmed by recent studies (Gorski et al., 2002). But,

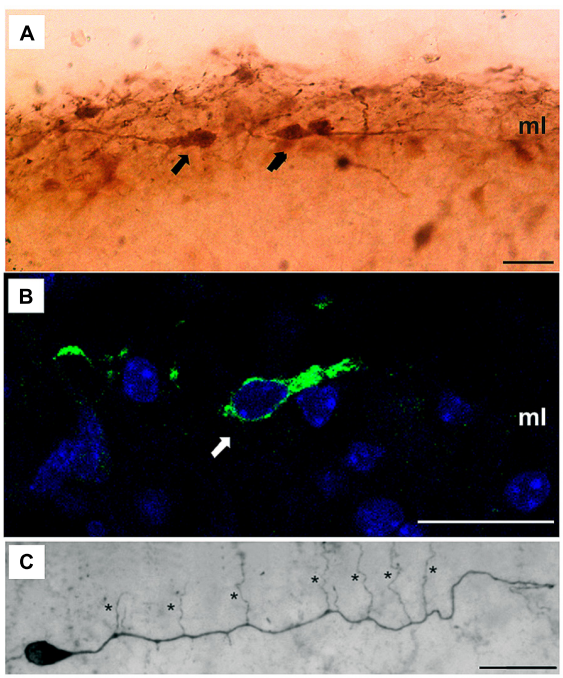

FIGURE 2 | Dendritic morphology of CR cells. (A,B) High magnification microphotographs illustrating several CR cells (arrows) immuno-labeled with different specific markers such as Calretinin **(A)** or Reelin **(B)** in the molecular layer (ml); **(C)** Light microscopic image of a biocytin-filled CR cell from a P11 old CxCR4–eGFP mouse showing a single thick stem dendrite with vertically oriented side branches (marked by asterisks). Scale bars in **(A–C)** are 20 μm. Reproduced, with permission, from Anstötz et al. (2013).

recent evidences indicate that rodent CR cells are generated in different neuroproliferative regions of the telencephalon (see below) and that from these sources they migrate tangentially to completely populate the pallium. In summary, we can note that a small percentage of CR cells originate in the neuroepithelium while the majority of them originate outside the pallium. In 1998, an study focused in the subpial granular layer (SGL) in human fetus revealed that one population of Reelin-negative granule neurons from, after arriving in the marginal zone (MZ), differentiates into Reelin-producing neurons with CR morphology (Meyer and Goffinet, 1998). Some years later, analyzing the development and organization of layer I in macaque monkey, it was confirmed that SGL might be a site of origin for late-generated CR cells. Moreover, it was hypothesized that the olfactory primordium gave rise to the SGL in monkey, thereby putting this zone forward as another source of CR cells (Zecevic and Rakic, 2001). An additional source of CR cells was identified on the basis of p73 expression in 2002. In human fetus, at eight gestational weeks, a mediolateral gradient in the density of p73/Reelin-positive neurons in the neocortical MZ suggests that a subset of CR cells migrates tangentially from the cortical hem and taenia tecta (Meyer et al., 2002). The cortical hem as a source for CR in mouse was demonstrated using the IG17 transgenic mouse and *in utero* electroporation (Takiguchi-Hayashi et al., 2004). Recent studies have reported that cortical hem-derived CR cells mainly populate the dorso-medial regions of the cortex (Gu et al., 2011). Furthermore, in mice in which the cortical hem has been ablated, the MZ contains very few or no cells (neither CR nor other cell types) as well as very low levels of Reelin (Yoshida et al., 2006). In 2005, it was proved the existence of two previously unknown sites of origin for two distinct subsets of CR cells: the ventral pallium at the pallial–subpallial boundary (PSB) and the septum (Bielle et al., 2005). Using a knock-in strategy, combined with DiI labeling, they followed the fate of the progeny of *Dbx1*-derived cells through their entire lifespan. These cells gave rise to Reelin-positive neurons and became CR cells in the postnatal cortex with different characteristics (Bielle et al., 2005). The generation of *Dbx1*-derived cells seems to occur a bit earlier in the septum than in PSB. Moreover, cells migrated from the septum to the medial and piriform cortex and did not express Calretinin, whereas from the PSB they migrated to the dorso-lateral and piriform cortex and expressed Calretinin. More recently, the thalamic eminence (TE) (Takiguchi-Hayashi et al., 2004; Cabrera-Socorro et al., 2007; Meyer, 2010) and the "amygdalar hem" have been proposed as additional putative sites of origin (Meyer, 2010). In fact, in rodent TE-generated cells express markers of CR cells such as Calretinin (Abbott and Jacobowitz, 1999), DeltaNp73 (Tissir et al., 2009), and low levels of Reelin (Meyer, 2010). Although their final destination is unknown, they may reach the di-telencephalic sulcus and amygdala. Immediately caudal to the TE, there is another putative source of CR, the "amygdalar hem," a small triangular area of neuroepithelium that connects the corticomedial amygdala to the choroid plexus at E12 in mice and which is also known as the "strionuclear neuroepithelium" (Altman and Bayer, 1995); cells originating in this area express high levels of Reelin when they are near the pial surface and therefore might represent CR cells whose final destinations are the amygdala and entorhinal cortex (Meyer, 2010). In summary, the cortical hem is the main source for CR cells but they are also produced in several sites such as SGL, taenia tecta, PSB, septum, TE, and the amygdalar hem.

Nowadays, we may affirm that CR cells originate in various focal sources in the developing brain, although we may not rule out the possibility that additional origin sites might exist. This multi-zonal production of CR may guarantee complete coverage of the cerebral cortex. Moreover, the various subtypes of CR cells generated at different sites intermingle in the cortex, in a way that cortical areas present a different proportion of distinct CR subtypes. This might contribute to determining area-specific properties (Bielle et al., 2005). At this point, two recent specific discoveries should also be noted: first, the distribution of CR cells in layer I depends on inhibitory cell-cell mechanisms (Villar-Cervino et al., 2013), and second, the distribution of CR cells is largely associated with their interaction with radial glia (Kwon et al., 2011). Finally, we would also like to remark that, probably, all the controversy generated around CR cells since they were first described by Cajal is due to the fact that they come from different sites what produces different CR subpopulations with specific morphological and physiological characteristics. Therefore, in order to achieve a thorough understanding of CR cells, researchers need to develop strategies that combine birth-dating and tracing studies with specific markers.

ROLE OF CAJAL–RETZIUS CELLS IN RADIAL NEURONAL MIGRATION

The mammalian neocortex is a highly ordered structure in which different types of neurons are arranged by tangential and radial migration during embryonic development to form the final laminated organization. This elaborate assembly is accomplished in distinct steps. The first step is the formation of the preplate, composed of a superficial plexus of corticopetal nerve fibers and a heterogeneous population of post-migratory cells, including CR cells, interneurons, and future subplate neurons (Bielas et al., 2004; Bystron et al., 2008). In the second step, waves of post-mitotic neurons exit the ventricular zone and move in a radial direction toward the pial surface, where upon they split the preplate into the MZ (above), which would contain the CR cells, and the subplate (below), thus establishing another cellular band known as the cortical plate. This new layer of cells contributes to layers II-VI of the cortex in rodents (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1972). In 1995, it was described how CR cells are responsible for the correct lamination of the neocortex through the secretion of an extracellular protein called Reelin (D'Arcangelo et al., 1995; Ogawa et al., 1995). In mice lacking Reelin the preplate does not split properly into the MZ and subplate. Consequently, this structure constitutes a "superplate" in the most superficial region of the cortex. Cortical plate neurons accumulate beneath the superplate; young neurons cannot migrate outward by passing across pre-existing cell layers. This results in an inverted pattern of neuronal positioning in all laminated structures (neocortex, cerebellum and hippocampus) as well as in subcortical structures such as the olfactory bulb, inferior olivary complex, and facial nucleus (Caviness and Sidman, 1973; Caviness, 1982). The list of molecular partners of the Reelin pathway is continuously increasing and it has been shown that the integrity of the Reelin-signaling cascade is essential for the correct positioning of cortical plate neurons

and disruption of any of its components leads to failure of radial migration (Gao and Godbout, 2013).

Recently, the comparison of Reelin patterns between amniote species showing some degree of cortical lamination (mammals and lizards) and those with no obvious pallial cytoarchitectonic condensation at all (turtles and birds) led to a "Reelin hypothesis" for cortical developmental evolution, with the condensation of Reelin-expressing cells being a key feature of the establishment of a sophisticated laminated pattern. In fact, the relevance of developing layer I during cortical evolution was hypotethized some years earlier (Marin-Padilla, 1978). These comparative data point up the importance of the Reelin pathway, and hence of CR cells, in the morphogenesis and cytoarchitecture of pallial structures (Abellan et al., 2010). However, we should take into account that Reelin is not uniquely expressed by CR cells but also by interneurons (Alcantara et al., 1998), and therefore we should not confer the main role in orquestrating the radial neuronal migration to CR cells, even though Reelin-positive interneurons have only been detected at post-natal stages (Alcantara et al., 1998; Ma et al., 2013).

Moreover, different strategies addressed to eliminating the presence of CR cells have questioned its importance in cell migration. These methods highlight the relevance of CR cells in radial glia maintenance and function (Soriano and del Rio, 2005). For example, local application of a toxic agent to newborn mouse cortex ablates CR cells and disrupts cell migration to layers II/III, causing radial glia to change to astroglia (Super et al., 2000). And in mutants for p73 and Emx1/Emx2 in which there is an absence of CR cells, the cortical pattern is altered although preplate partition and cortical plate formation are not disturbed (Shinozaki et al., 2002; Meyer et al., 2004). In contrast, the ablation of the cortical hem, the predominant source of CR cells, did not produce the inverted lamination observed in the *reeler* mutant (Yoshida et al., 2006). In line with this unexpected result, some studies have suggested that, rather than CR cells, it is the integrity of the pial basement membrane and meningeal cells that is crucial for correct cortical histogenesis (Halfter et al., 2002; Beggs et al., 2003). Nevertheless, another link has been recently reported between CR cells and radial migration through the immunoglobulin-like adhesion molecule Nectin1. In this study, authors described that these cells express Nectin1 which interacts with Nectin3, present in projection neurons, and that this interaction is critical for radial migration (Gil-Sanz et al., 2013). Interestingly, these molecules belong to the Reelin signaling pathway which indicates, once again, a role of CR cells and Reelin in this process.

POST-NATAL FATE OF CR CELLS: RODENTS VS. PRIMATES

Some studies have suggested that CR cells undergo a morphological change in order to become resident interneurons of layer I in adult neocortex (Parnavelas and Edmunds, 1983). Others have proposed that the decrease in CR neuron density is caused by dilution from the expansion of the cortex during development, without a clear morphological transformation (Marin-Padilla, 1990). However, the most widely accepted theory is that most of the cells are destined to disappear by adulthood and undergo cell death (Derer and Derer, 1990). In rodents, signs of CR neuron degeneration begin in the second post-natal week, as evidenced by retraction of thin appendages from their main dendrite, swelling of the endoplasmic reticulum, and darkening of the cytoplasm (Derer and Derer, 1990; Soriano et al., 1994; del Rio et al., 1995). This CR cell loss has also been reported to occur directly after migration in monkeys and humans (Zecevic and Rakic, 2001; Abraham and Meyer, 2003). In these previous studies it was already reported that in rodents nearly 95% of CR cells present at birth may disappear before P10–P14 (del Rio et al., 1995; Soda et al., 2003) and these data have recently been recalculated analyzing identified CR neurons that express the green fluorescent protein under the control of different promoters such as the early B-cell factor 2 (Ebf2) or CxCr4 promoters (Chowdhury et al., 2010; Anstötz et al., 2013). Researchers concluded that most CR cells die progressively by apoptosis from P7 onwards and only a small fraction (3–4%) present at birth survive into adulthood (Chowdhury et al., 2010; Anstötz et al., 2013; Ma et al., 2013). However, in contrast to the nearly complete elimination of CR cells in the post-natal neocortex, 25% of CR cells appear to survive in the hippocampus of adult animals (Super et al., 1998; Anstötz et al., 2013), reflecting a differential role between neocortical and hippocampal CR cells in the adulthood. Therefore, neocortical CR cells appear at early stages of embryonic life, they increase their density at the first post-natal week and decrease from there until reaching minimum levels.

CR CELLS DURING POST-NATAL LIFE

There is no plausible explanation for the presence of CR cells during embryonic life and their atrophy and eventual disappearance shortly after birth. Historically, as we have indicated above, researchers have focused their efforts in studying the role of CR cells in cortical migration at the prenatal period. However, it would be interesting to think about a putative function of CR cells during early post-natal stages, when CR cells reach their highest density (between P3 and P7) (Anstötz et al., 2013) and the cortical lamination has been already completed. The first post-natal week is a critical period for the maturation of interneurons and pyramidal cells in the neocortex and for the establishment of their final connections. Several studies have described that CR cells show spontaneous activity and that they could belong to an early cortical network that controls the maturation of the cerebral cortex (Marin-Padilla, 1998; Aguilo et al., 1999; Radnikow et al., 2002). On the other hand, it was already demonstrated that this cell population was one the earliest functional neurons in the developing human brain when a study in 1968 described the presence of acetylcholinesterase activity in their cytoplasm in 4-month-old human fetuses (Duckett and Pearse, 1968). Moreover, they are related to interneurons and neurons via input and output connections, receiving GABAergic, serotonergic, glutamatergic, and noradrenergic inputs and sending glutamatergic information (**Figure 3**; del Rio et al., 1995; Radnikow et al., 2002; Janusonis et al., 2004; Chowdhury et al., 2010; Myakhar et al., 2011; Anstötz et al., 2013). The morphological (long-range horizontal axonal projection) and electrical features of the CR cells and their synaptic input–output relationship at this period would facilitate the stabilization of interneurons and pyramidal dendritic trees suggesting that CR cells can integrate information in layer I and send projections to target neurons to facilitate the formation of the neocortical network during very

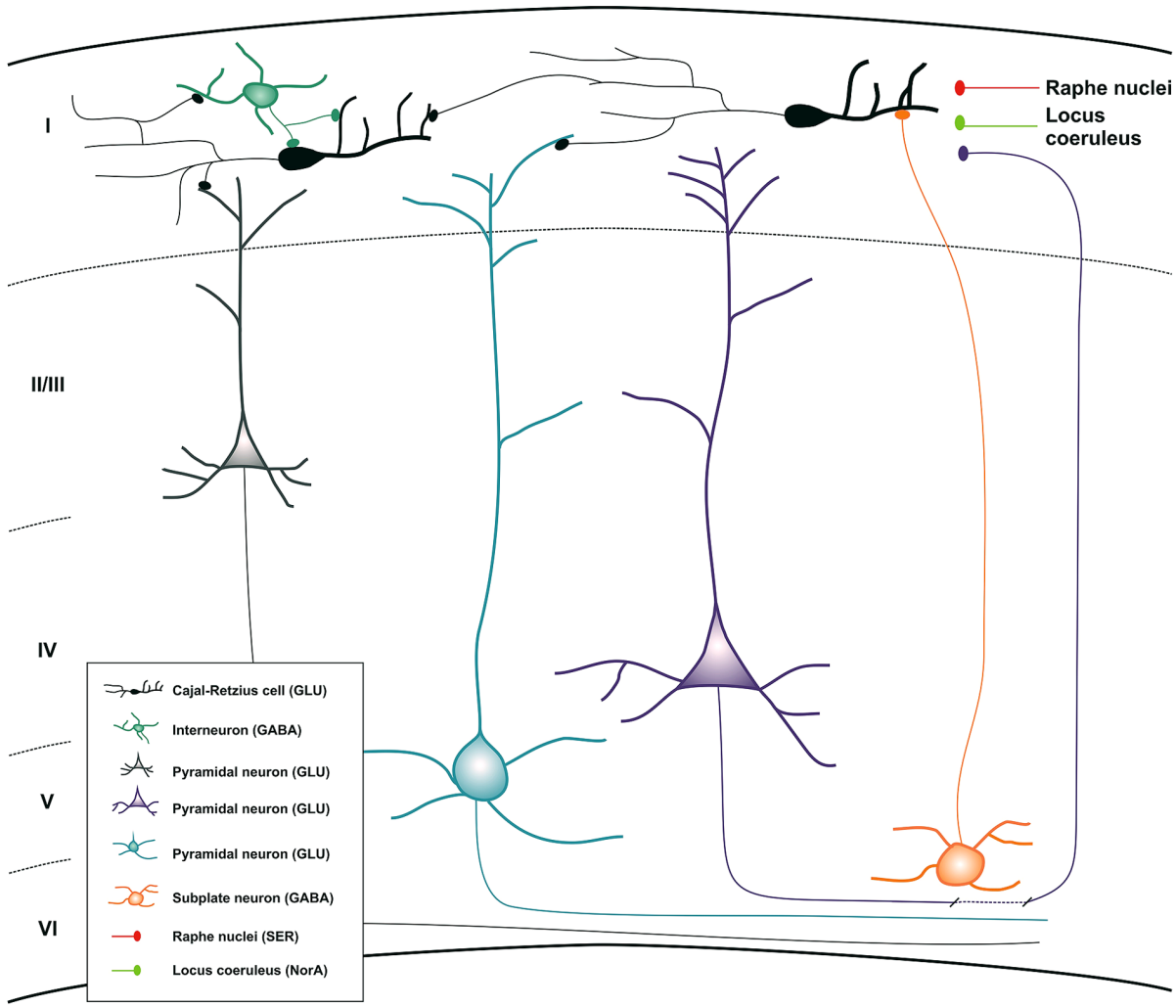

FIGURE 3 | Schematic drawing summarizing all the inputs and outputs connections of CR cells during neocortical development. Inset map shows the distinct cell populations involved in the process with their principal neurotransmitters. Abbreviations: GLU, glutamate; GABA, gamma-aminobutyric acid; NorA, noradrenalin; SER, serotonin.

early stages of development, lending support to Cajal's associational theory (Molliver et al., 1973; Konig et al., 1975; Bradford et al., 1977; Konig and Marty, 1981; Rickmann and Wolff, 1981). After the early neocortical network is established, most of the CR cells would degenerate and die. Furthermore, no functional role of the small population that remains in the adulthood has been offered.

CR CELLS THROUGHOUT EVOLUTION

As indicated above, Cajal and Retzius described, for the first time, CR cells in the MZ of the fetal and early post-natal neocortex in humans, small mammals (rabbit, cat, dog, rat, and mouse), birds, and reptiles. Since then, CR cells have been described in mammalian and non-mammalian vertebrates (Tissir et al., 2003; Cabrera-Socorro et al., 2007; Abellan et al., 2010). Although CR cells are present in all amniotes, the Reelin signal in CR cells increases in mammals and is even higher in primates including humans (Meyer and Goffinet, 1998). In mammals, it has been proposed that the increasing proportion of p73-positive CR cells may contribute to the evolutionary amplification of Reelin-signal in the MZ during development (Cabrera-Socorro et al., 2007). Moreover, p73 expression seems to be related to the prolonged survival and increased differentiation of dendritic and axonal processes of CR. Observing CR cells in lizard, mouse and human, it was proposed that p73 may play a role in the acquisition of complexity in CR cells during evolution. In fact, CR cells are rudimentary in lizards, relatively simple in mice, and more complex in primates (Cabrera-Socorro et al., 2007).

The abundance of CR cells seems to correlate with the size of the cortical hem, which has been demonstrated to be small in sauropsids such as crocodiles (Tissir et al., 2003), lizards (Goffinet et al., 1999) and chicks (Cabrera-Socorro et al., 2007) and maximal in humans (Cabrera-Socorro et al., 2007; Villar-Cervino and Marin, 2012). The varying size of the cortical hem influencing

the number of CR cells could be understood as relevant factors in the evolution of cortical regions across vertebrates. An additional difference between primates and other vertebrates is that in the former at least two types of Reelin-producing cells have been described: large CR cells and at later developmental stages smaller SGL cells (Zecevic and Rakic, 2001). This second cellular type is thought to compensate for the progressive loss of CR cells during the long period of corticogenesis in primates (Zecevic and Rakic, 2001). In another study, it was reported a differential expression of LIM-homeodomain (LIM-hd) factors in primates, birds, and rodents (Abellan et al., 2010). They proposed that the expression of a larger repertoire of LIM-hd transcription factors in CR cells may correlate with their diversification and morphological complexity. These factors are assumed to convey higher molecular diversity and the possibility of promoting the emergence of novelties. Indeed CR cells in primates express at least four LIM-hd factors while in rodents the figure is only two. In chicks, none of these factors were found with the exception of Lhx5 in a small zone of the cortical hem (Abellan et al., 2010). In summary, we can conclude that the different abundance and complexity of the different CR cells subpopulations, which are characterized by both their specific origin and molecular profile, is involved in the level of complexity of the neocortical structure.

CONCLUDING REMARKS

Numerous efforts have been targeted to understand the biology of the CR cells population since it was first described by Cajal in 1890. Paradoxically, when we analyze carefully all the studies reported during more than a century, we realize that the most important features of these cells (morphological and physiological properties) were already indicated by Cajal by employing very rudimentary methodological techniques. This fact points out the importance of his work for the current Neurobiology knowledge. Nowadays, their morphology and electrical properties are better known and we can also specify that they come from several origin sites, although the cortical hem is the most important source. Apart from this, evidence shows that CR cells exert different functions throughout the distinct periods of development, thus regulating the radial neuronal migration during prenatal life and possibly facilitating the cortical network assembly in the post-natal stage. However, to fully understand the exact role of the CR cells in the building of the cerebral cortex, new strategies that may allow the characterization of the different CR cells subsets are needed.

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Human cerebral cortex Cajal-Retzius neuron: development, structure and function. A Golgi study

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The development, morphology and possible functional activity of the Cajal-Retzius cell of the developing human cerebral cortex are explored herein. The C-RC, of extracortical origin, is the essential neuron of the neocortex first lamina. It receives inputs from afferent fibers that reach the first lamina early in development. Although the origin and function of these original afferent fibers remain unknown, their target is the first lamina sole neuron: the C-RC. This neuron orchestrates the arrival, size and stratification of all pyramidal neurons (of ependymal origin) of the neocortex gray matter. Its axonic terminals spread radially and horizontally throughout the entirety of the first lamina establishing contacts with the dendritic terminals of all gray matter pyramidal cells regardless of size, location and/or eventual functional roles. While the neuron axonic terminals spread radially and horizontally throughout the first lamina, the neuronal' body undergoes progressive developmental dilution and locating any of them in the adult brain become quite difficult. The neuron bodies are probably retained in the older regions of the neocortex while their axonic collaterals will spread throughout its more recent ones and eventually will extend to great majority of the cortical surface. The neocortex first lamina evolution and composition and that of the C-RC are intertwined and mutually interdependent. It is not possible to understand the C-RC evolving morphology without understanding that of the first lamina. The first lamina composition and its structural and functional organizations obtained with different staining methods may be utterly different. These differences have added unnecessary confusion about its nature. The essential emptiness observed in hematoxylin and eosin preparations (most commonly used) contrast sharply with the concentration of dendrites (the cortex' largest) obtained using special (MAP-2) stain for dendrites. Only Golgi preparations demonstrate the numerous dendritic and axonic terminals that compose the first lamina basic structure. High power microscopic views of Golgi preparations demonstrate the intimate anatomical and functional interrelationships among dendritic and axonic terminals as well as synaptic contacts between them. The C-RC' essential morphology does not changes but it is progressively modified by the first lamina increase in thickness and in number of terminal dendrites and their subsequent maturation. This neuron variable morphologic appearance has been the source of controversy. Its morphology depends on the first lamina thickness that may be quite variable among different mammals. In rodents (most commonly used experimental mammal), the first lamina thickness, number and horizontal expansion of dendrites is but a fraction of those in humans. This differences are reflected in the C-RC' morphology among mammals (including humans) and should not be thought as representing new types of neurons.

Keywords: cerebral cortex, Cajal-Retzius cell, development, structure, function

INTRODUCTION

The so-called Cajal-Retzius (von Koelliker, 1996) of the neocortex first lamina has been the source of continued controversy since originally described by Cajal (1891) and (Retzius (1893, 1894)). Its morphology, types, biochemistry, persistence vs. disappearance and role continue to be debated (Cajal, 1911; Derer and Derer, 1990; Meyer and González-Hernández, 1993; Meyer et al., 1999; Soriano and Del Río, 2005; Myakhar et al., 2011; Gil-Sanz et al., 2013; Martínez-Cerdeño and Noctor, 2014).

Because of this neuron controversies have only contributed additional confusion, we still lack, more than a century of its original description, a clear understanding of this neuron nature, morphology and functional role. I will describe the human cerebral cortex Cajal-Retzius cell developmental morphology and that of the first lamina, which I consider to be inseparable and mutually codependent.

Most descriptions about this neuron have focused on its variable morphology, although few explored the reasons why is

that. What is a C-RC? Why is it morphology variable? Does it persist or does it disappear? How many first lamina neurons deserve the designation of C-RC? What is its functional target? What is its interrelationship to the neocortex first lamina? It is present in the adult brain? And, more important, what is its functional role in the developing vs. the adult brain? These and other questions about this essential neuron of the neocortex first lamina are explored herein using the rapid Golgi procedure.

The paper honors Camilo Golgi "reazione near" (Golgi, 1873) and it countless contributions to Neurosciences and laments that nowadays it is seldom used remaining essentially ignored by young neuroscientists. Cajal improved the Golgi procedure, used it throughout his life and his contributions constitute the foundations of modern Neurosciences. His classic Golgi studies of the newborn cerebral cortex remain essential and unsurpassed (Cajal, 1899a,b, 1900, 1901).

OBSERVATIONS

Concerning the C-RCs controversies the following commentaries may be pertinent. The neocortex first lamina thickness among mammals is quite variable and must be considered when describing and/or comparing the C-RC' variable morphology among them. In most mammals (specially in rodents) the first lamina thickness, the number of pyramidal neurons reaching it and the functional maturation of their terminal dendritic branches represent but small fractions of those observed in the human brain. These differences will be reflected in the structure and thickness of both the first lamina and the C-RCs for each mammalian species. While the C-RC morphology among mammals may be quite variable, it could simply reflect developmental adaptations and should not be thought as representing different types of neurons.

Moreover, the morphology of any C-RC depends on the plane of view. Originally, Cajal, Retzius and myself described three different morphologic types (pear shape, monopolar and bipolar) without realizing that they represented different views (planes of sections) of the same multipolar tangential neuron (Marín-Padilla, 1990). Therefore, any perpendicular view of the neuron body and dendrites represents an incomplete view of its actual three-dimensional morphology. Hence, single perpendicular views of this neuron' body and dendrites could be quite variable without representing different types of neurons. On the other hand, the C-RCs axonic terminals should be similar in perpendicular and parallel sections of the first lamina. Therefore, perpendicular, parallel and tangentially cut sections of the cortex first lamina are required to understand this neuron three-dimensional morphology (Marín-Padilla, 1990). Comparison of human and other mammals' data could be misleading and often erroneous.

The generally accepted idea that the C-RC represents a transient neuron that will eventually disappear is supported by the fact that it is quite difficult to visualize any of them in the adult brain. It must be understood that the difficulty only applies to the location the neuronal' body, since its axonic terminals are recognized throughout the entire first lamina associated to the pyramidal cell dendrites (Marin-Padilla and Marin-Padilla, 1982; Marín-Padilla, 1984, 1990). Since the actual number C-RCs, is established early in development, the location of their body will undergoes progressive dilution during brain prenatal and postnatal developments. Since first recognized in 6-w-o (weeks of age) embryos, the human brain expansion is extraordinary as its surface area increases from 19 cm^2 at 14-week-gestation to 700 cm^2 at birth, to 1166 cm^2 in the adult brain (Blinkov and Glezer, 1968). Moreover, the first lamina expansion throughout the cortex complex gyral patterns will be by far the greatest. The difficulty in locating a neuron body will increase exponentially during the brain extraordinary surface expansion. In the adult brain, the study of ten of thousand of sections would be necessary to localize a single C-RC body. During more than 50 years studying the human brain, I have identified C-RCs bodies, as well as their axonic terminals, throughout prenatal development, in many newborn infants, in few young children and, by serendipity, in a 4-year-old child, in a 22-year-old, a 72-year-old and a 86-year-old men and in a 36-year-old woman (Marín-Padilla, 1990, 2011; Martín et al., 1999). Considering the brain surface area and the relative small number of sections studied, these few observations will support the C-RCs' persistence in the adult brain. A presume body of a C-RC has been identified in adult rats striate cortex of one hemisphere by injections of a retrograde label on the opposite one (Martínez-García et al., 1994). Others agree with the persistence of C-RCs in the adult brain (Meyer et al., 1999). A developmental "dilution" of the neuron body is a better explanation than their so-called disappearance in the adult brain.

A C-RC early function is the secretion of *Reelin* that will orchestrate the ascending migration of neuroblasts from the ependymal layer to the first lamina (using radial glia as guides), their transformation into pyramidal neurons, the order of migration, the neuron size and the eventual stratification of the gray matter (Marín-Padilla, 1992, 2014; Ogawa et al., 1995; Meyer et al., 1999; Soriano and Del Río, 2005). Since the number of pyramidal cells terminal dendrites reaching the first lamina increases progressively, the C-RC axonic terminal branches must elongate (*developmental horizontalization*) to reach all pyramidal cells throughout the cortex' expanding surface, while the neuron body will remain on its original location. During late prenatal and postnatal cortical maturations, the dendrites undergo additional functional expansions compelling further extension (horizontalization) of C-RCs axonic terminals and, hence, increasing their body's isolation and consequently, increasing the difficulty in locating any of them. While the C-RCs bodies may be difficult to locate in the adult brain, their long horizontal axonic terminals (Retzius tangential fibers) are recognized throughout the first lamina, in both perpendicular and tangentially cut sections (Marín-Padilla, 1984, 1990). In my opinion, the C-RC bodies are probably retained in the cortex older regions (primary motor, sensory, visual and acoustic cortices) while their axonic terminals continue to spread throughout more recent ones (associative regions) that eventually will represent the great majority of the cortex surface.

The first lamina has other types of neurons, mostly incorporated during late prenatal development. They have different morphologies, biochemical compositions and local functional roles and should not be confused with C-RCs. In my opinion, unique developmental, morphological and functional

features characterize the C-RCs of the human developing and adult neocortex. C-RCs are also recognized in the cerebral cortex of amphibian and reptiles, attesting to their ancient participation in the cortex developmental structure and function (Cajal, 1911; Marín-Padilla, 1998).

It could be stated that most of the controversies about C-RCs do not seem to be applicable to the human cerebral cortex. Also that the neuron' nature, development, morphology and possible function cannot be understood and/or separated from those of the neocortex first lamina.

C-RC ORIGIN

The C-RC is the first neuron recognized in the developing neocortex of humans and other vertebrates (Marin-Padilla, 1971; Marín-Padilla, 1990, 1998, 2011). Its origin seems to be extracortical as other early subplate neurons (Marin-Padilla, 1971). C-RCs enter tangentially into developing cortex and are recognized under the pial surface as a large horizontal neuron. They are first recognized in 20-day-old cat and 6-week-old human embryos. Together with early pyramidal-like neurons and Martinotti cells, they constitute the mammals' primordial cortical organization that resembles the primitive cortex of amphibian and reptiles (Marin-Padilla, 1971; Marín-Padilla, 1998). The subsequent incorporation of pyramidal neurons (of ependymal origin) establishes, simultaneously, the first lamina, above it, and the subplate zone below denoting the mammalian new cortex (neocortex) dual origin (Marin-Padilla, 1971; Marín-Padilla, 1998).

In humans, the ascending migration of pyramidal neurons toward the first lamina, start around 8-week-gestation and is nearly completed by 16-w-g (Marín-Padilla, 2011, 2014). At this age, all pyramidal neurons of the gray matter have terminal dendrites within the first lamina. The gray matter deeper and older pyramidal cells start their ascending functional maturation at this age. Concomitantly, the microvascularization, the first protoplasmic astrocytes and the first inhibitory neurons (of extracortical origin) are also recognized throughout the gray matter deeper and older region (Marín-Padilla, 2011, 2012). The pyramidal neuron is a mammalian innovation characterized by distinct developmental, morphological and functional features and by its permanent functional attachment to C-RCs axonic terminals and the first lamina (Marín-Padilla, 2014). C-RCs axonic terminals must elongate horizontally to contact all pyramidal cells terminal dendrites arriving to the first lamina throughout the developing cortex, while their bodies remain on their original location and become progressively "diluted".

Neocortical neurons without these features, regardless of the pyramidal shape of their bodies, should not be considered and/or labeled as pyramidal neurons.

C-RC DEVELOPMENTAL ROLE

By secreting *Reelin*, the C-RC orchestrates the ascending migration, arrival, size and eventual stratification of the gray matter pyramidal neurons (Marín-Padilla, 1992, 2014). It establishes and maintains structural and functional interrelationships with the terminal dendrites of all pyramidal neurons reaching the first lamina. During late prenatal maturation of, the cortex, the terminal dendrites continue to expand adding new branches and the first lamina thickness increases accordingly as well as the horizontal spreading of C-RCs axonic terminals. The terminal dendrites functional growth and branching will continue during the neocortex postnatal maturation increasing the first lamina thickness, the further elongation C-RCs axonic terminals and the concomitant progressive "dilution" of their bodies (Marín-Padilla, 2011).

From the start of cortical development, afferent fibers without collaterals ascend from the white matter, reach the first lamina and become long horizontal fibers through its upper half (Cajal, 1911; Marin-Padilla, 1971; Marin-Padilla and Marin-Padilla, 1982). During early neocortical development, afferent fibers from the subplate reach the first lamina and hence the C-RCs (Marin-Padilla, 1971). Their function remains unknown, although a possible Gabaergic nature has been recently proposed (Myakhar et al., 2011). The C-RCs and these afferent fibers axonic terminals intermingle with pyramidal cells terminal dendrite throughout the first lamina. These afferent fibers functional target must also be the C-RCs since no other neurons are recognized in the first lamina during early development. It is possible that early subcortical centers of the developing brain could send inputs to the developing neocortex prior to the arrival of the more specific thalamic, callosal and cortico-cortical ones. The functional role of these early afferent fibers remains unknown.

C-RC DEVELOPMENTAL MORPHOLOGY

The neurons original descriptions by Cajal, Retzius and myself coincide in all features (**Figure 1**). In perpendicular sections, the C-RC' soma and dendrites are located within the lamina upper region and their morphology may take a pear shape, monopolar and/or bipolar (**Figure 1**). The neuron descending axon gives off several horizontal collaterals distributed throughout the lamina middle region and terminates into a horizontal axonic fiber that runs through its lower region (**Figures 1**, **2**, **3A–D**). During late prenatal development, the number of C-RCs horizontal axonic collaterals increases paralleling the functional expansion of the pyramidal neurons terminal dendrites (**Figure 2**). Consequently, the first lamina and the C-RCs thickness increase concomitantly (**Figure 2**). Although, the C-RC essential morphology remains basically unchanged during the cortex subsequent maturation, it will be progressively modified (Marín-Padilla, 2014).

The C-RCs' axon and its collaterals have numerous ascending and fewer descending thinner branches, considered to represents its functional terminals (**Figures 1**, **2**, **3A–D**). Since the terminal dendrites of pyramidal neurons are the only receptive elements within the first lamina they are considered to represent the C-RC functional targets (Marín-Padilla, 1984).

The functional target of the original afferent fibers that reach the first lamina (Cajal, 1911, 1933; Marin-Padilla, 1971) must be the C-RCs since they are the only first lamina neurons during early cortical development. Eventually they could also target the terminal dendrites of arriving pyramidal cells. These afferent fibers terminals intermingle with C-RCs dendrites and with the pyramidal terminal dendrites throughout the first lamina, suggesting a functional interaction among them (Marin-Padilla and Marin-Padilla, 1982; Marín-Padilla, 1984). C-RCs'

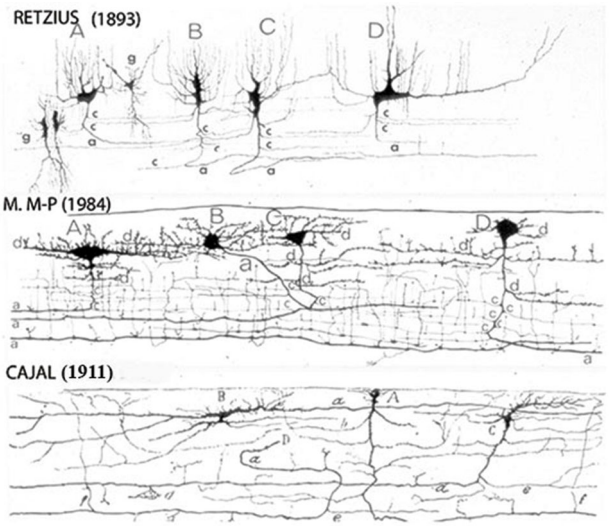

FIGURE 1 | Reproductions of Camera lucida drawings from the original Golgi studies of the human cerebral cortex of Retzius, Cajal and myself illustrating our agreement on the C-RCs morphological features. The neuron' body and dendrites are located in the lamina upper region and may be pear shape, monopolar and/or bipolar, its descending axon have long horizontal axonic collaterals through its middle region and its terminal axon becomes a long horizontal fiber through its lower region. Both, its axonic collaterals and terminal axon have numerous ascending and descending branchlets considered to represent the neuron functional outlets. The pyramidal cells terminal dendrites represent the first lamina main receptive elements. To reach all dendritic terminals, the C-RC axonic collaterals and terminal axonic fiber must elongate radially throughout the first lamina while its body remains on its original location and will undergo developmental dilution. The first lamina thickness in the newborn motor cortex is roughly 200 micrometers. (From: Marín-Padilla, 1990).

evolving morphology and first lamina structural organization, composition and thickness cannot be separated, as they are mutually interdependent (**Figure 2**).

Retzius, Cajal and myself pointed out that the neuron long horizontal axonic terminals (tangential fibers of Retzius) acquire myelin sheath during early postnatal life. In humans, their myelination starts around the fourth and fifth postnatal years (Kaes, 1907; Langworthy, 1933; Yakolev and Lecours, 1967; Rocker and Riggs, 1969; Retzius, 1894; Brody et al., 1987). They are the earliest components of the gray matter to undergo myelination, emphasizing their functional relevance. Perpendicular sections of myelin stained (Klüver-Barrera method) preparations of children and adult motor cortices show myelinated fibers running through the first lamina lower region (Marín-Padilla, 1990). While in tangential section these myelinated fibers crisscross each other throughout first lamina.

During late prenatal development, small neurons and the terminals of specific thalamic, interhemispheric and cortico-cortical fibers are also incorporated in the first lamina (Marín-Padilla, 1984). While C-RCs' axonal terminals and those of the afferent fibers could establish contacts with all pyramidal cells terminals dendrites throughout the first lamina, these fiber incorporated later only establish contacts with the terminal dendrites of regional pyramidal cells. These late incorporated

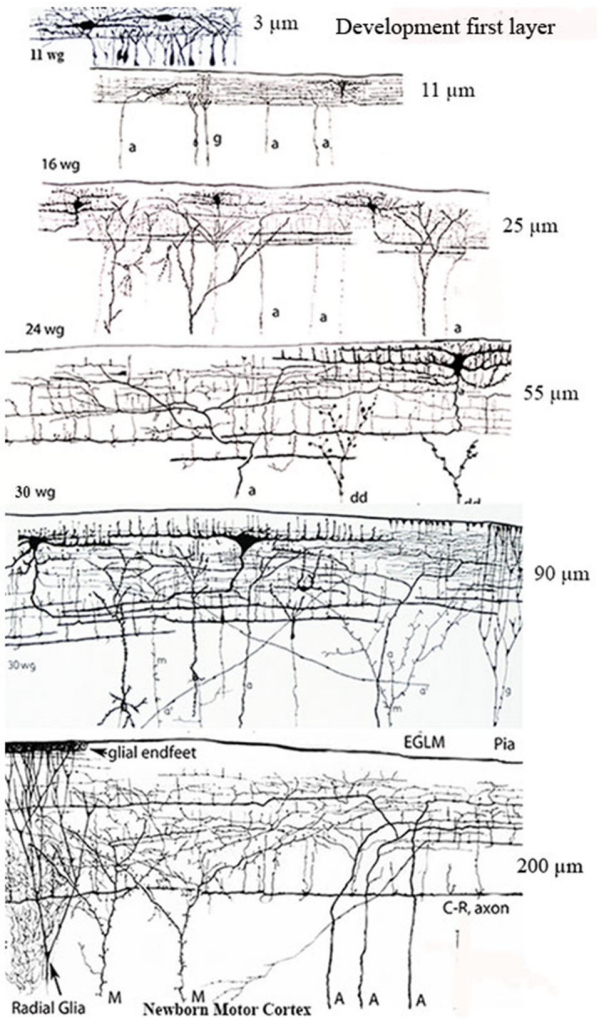

FIGURE 2 | Camera lucida drawings reproductions of rapid Golgi preparations of the developing human motor cortex showing the progressive structural and functional maturations of the first lamina, of its C-RCs and of its original afferent fibers, through 11-, 16-, 24- and 30-week-old fetuses and newborns infants. The first lamina increasing thickness is expressed in micrometers on the right of each gestational age. The lamina increasing thickness results from the increasing number of pyramidal cells terminal dendrites reaching and maturing within it. The C-RCs essential morphology remains unchanged during the neocortex entire prenatal development. Original afferent fibers (a, A) from the white matter reach the developing first lamina and become long horizontal terminals through its upper region, where the C-RCs body and dendrites are located. The C-RCs descending axon gives off several thin horizontal axonic collaterals through the lamina middle region and become a long thick horizontal fiber through its lower region. The axonic terminals of Martinotti cells (M) also reach and branch locally within the first lamina. Numerous radial glia fibers reach the first lamina contributing glial endfeet to the neocortex external glial limiting membrane. Later in development, axonic terminals (a') from thalamic, interhemispheric and cortico-cortical fibers also reach the first lamina and are locally distributed. There are no reasons to think that the extraordinary concentration of fibers terminals within first lamina will disappear in the adult brain since none of its terminal dendrites do. See also **Figure 7B**. Key: a = afferent fibers terminals; g = radial glial cell terminals; dd = degenerating dendrites often recognized during mid-gestation; M = Martinotti cell axon terminals; C-R = C-RCs axonic terminals; Pia = pial surface. (From: Marín-Padilla, 1990).

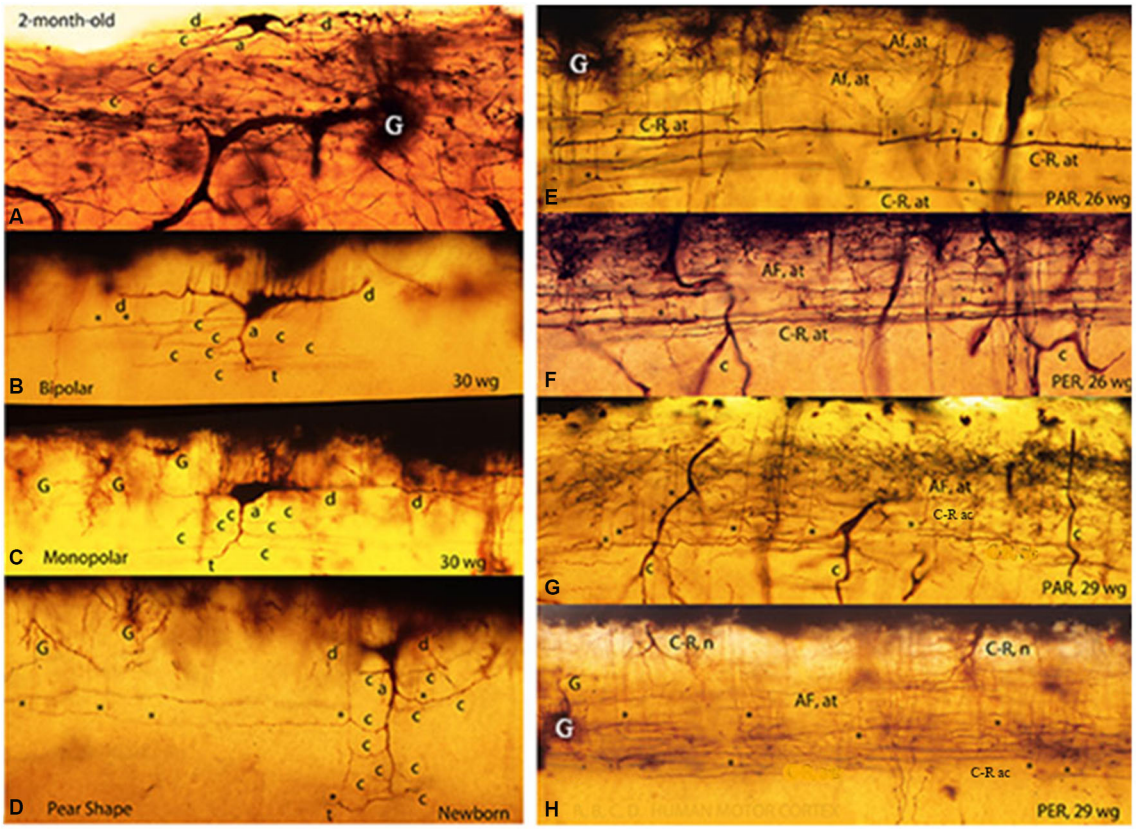

FIGURE 3 | Composite figure of photomicrographs of rapid Golgi preparations of the human motor cortex' first lamina showing the C-RC' body variable morphology, including irregular (A), bipolar (B), monopolar (C) and pear shapes (D). The middle location of the neuron thinner axonic collaterals (C-R ac) and the lower one of its thicker axonic terminals (C-R at) are shown in parallel **(E,G)** and perpendicular **(F,H)** cut sections of the motor cortex first lamina from 26- and 29-w-o fetuses. The first lamina structural organization is undistinguishable in parallel and perpendicularly cut preparations. Some C-RC' bodies **(H)** and the upper distribution of the terminals (AF. at) of original afferent fibers **(E–H)** are also shown. At this age, the first lamina thickness is roughly 90 micrometers (From: Marín-Padilla, 1990).

fibers are possibly related to the eventual motor, sensory, acoustic, visual and/or associate functions of regional pyramidal neurons.

The C-RCs body variable morphology (upper region), that f its thinner axonic collaterals (middle region) and that of its thicker axonic terminals (lower region) were essentially identical in both parallel and perpendicular cut. It might be a reason that could explain why Golgi preparations cut perpendicular and/or parallel to the precentral gyrus long axis are morphologically undistinguishable (**Figures 3E–H**). The study of tangentially cut Golgi preparations solve the paradox, roughly 100 years of the neuron original description (Marín-Padilla, 1990).

In tangentially cut Golgi preparations, the C-RC appears as a three-dimensional multipolar neuron with radiating dendrites and a descending axon with long radiating horizontal collaterals and terminates into a long horizontal fiber that may projects in any direction within the cortex (**Figures 4**, **5**, **6A,B**). The direction of any perpendicular section of the first lamina will determine the neuron' body pear shape, monopolar and/or bipolar morphologies (Marín-Padilla, 1990). Moreover, Cajal (1911) described and illustrated a large neuron with radiating dendrites in methylene blue preparation of the cat cortex first lamina as well as the crisscrossing of axonic terminals. He did not realize that it could have represented a tangential view of a C-RC.

Since Golgi preparations thickness range between 150 to 200 micrometers, is possible, in a single tangential cut, to visualize the first lamina entire thickness and structural organization (**Figures 6U,M,L**). Tangentially cut Golgi preparations of first lamina will show C-RCs bodies with variable morphologies through its upper region (**Figure 6U**), the crisscrossing of the neuron thinner axonic collaterals through its middle one (**Figure 6M**) and the crisscrossing of the neuron thicker axonic terminals through its lower one (**Figure 6L**). The fact that both the C-RCs' axonic collaterals and terminal axons crisscross each other within the first lamina, during mid gestation, was puzzling and also required clarification. The need to reach all the terminal dendrites throughout the expanding cortical surface will explain the radial expansion of C-RCs axonal collaterals and eventually the crisscrossing with those of neighboring neurons through the first lamina middle region. Similarly, the neurons axonic terminals will eventually crisscross each other through the lamina lower region as they project in all directions.

C-RC FUNCTION

Essentially, the C-RCs (including bodies, radial axonal collaterals and terminals axons), the original afferent fibers terminals and the pyramidal neurons terminal dendrites are the only components of the neocortex first lamina during prenatal development. These three elements intermingle with each other throughout the first lamina in both the developing and the adult cerebral cortex (Marin-Padilla and Marin-Padilla, 1982; Marín-Padilla, 1984, 1990). Their anatomical interrelations suggest functional ones as well as.

If pyramidal cells terminal dendrites, within the first lamina, are going to be the C-RC functional targets, a radial spread of its axonic collaterals should be expected. Since pyramidal cells terminal dendrites are incorporated progressively into the first lamina, the C-RCs axonal collaterals must elongate radially to meet them, throughout the neocortex first lamina. By its radiating axonal collaterals, each C-RC establishes a circular functional territory establishing contacts will all terminal dendrites within it (**Figures 4E**, **5**, **6A**). In the course of cortical development, each C-RC circular territory enlarges as new terminal dendrites are incorporated into it, and those already present expand functionally (**Figures 4A–E**, **5**, **6A,B**). The enlarging circular territories of neighboring CRCs will eventually merge with each other. The convergence of neighboring C-RCs functional territories will explain the crisscrossing of their respective axonal collaterals through the lamina middle region (**Figures 6M,L**). Hence, all dendritic terminals within the first lamina will be contacted by the radial expansion of the C-RCs' axonal collaterals.

Similarly, during development the neuron thicker axonic terminals of neighboring C-RCs will eventually crisscross each other throughout the lamina the lower region (**Figures 6B,L**). Tangential sections of myelin stained (Klüver Barrera stain) preparations of adult brains demonstrate the crisscrossing of myelinated axonal terminals within the first lamina (Marín-Padilla, 1990).

Since neither the C-RCs axonal terminals nor the pyramidal cells terminal dendrites disappear, they will continuously share anatomical and functional interrelationships in both developing as well as adult brains. One possible function of C-RCs might be a functional input shared by all pyramidal cell dendrites throughout the neocortex, regardless of their eventual functional role. The nature of this input remains unknown.

In conclusion, the C-RC evolving morphology and possible function responds to the maturations of the terminal dendrites of all pyramidal neurons throughout the first lamina of the neocortex. In conclusion, the C-RC evolving morphology and

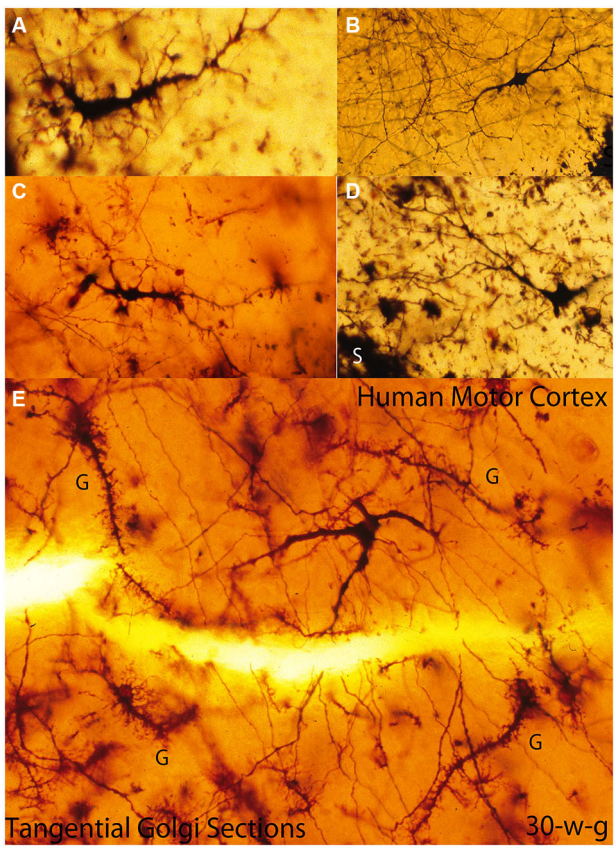

FIGURE 4 | Composite figure of photomicrographs of tangentially cut Golgi preparations of the motor cortex first lamina of 30-w-o fetuses showing the actual multipolar morphology of C-RCs' bodies (A-E), the first lamina special glial (g) often referred as comet cells (E) and the crisscrossing of fine axonic terminals (H) through the lamina upper region. (From: Marín-Padilla, 1990).

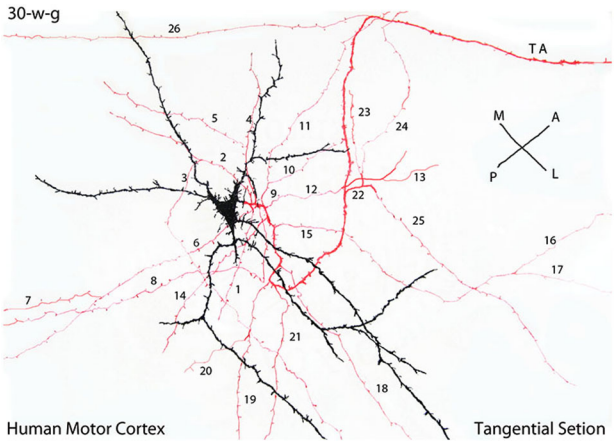

FIGURE 5 | Camera lucida drawing of a tangentially cut rapid Golgi preparation of the motor cortex of a 30-w-g fetus, illustrating the multipolar morphology of a C-RC with radiating dendrites (black), a descending axon with numerous (26) radiating horizontal collaterals (red) and the neuron axon (TA) terminal (red) that projects in an antero-lateral direction. The radial expansion of the axonic collaterals of each CRC establishes a functional circle making functional contacts with all pyramidal cells terminal dendrites within it. At 30-w-g, the diameter of the functional territory of this particular C-RC is already 14 mm. Since both the neuron axonic terminals and the pyramidal cells dendrites are already present in the first lamina their functional territory will continue to expand functionally during the neurons subsequent maturation. During cortical development, the diameter of C-RCs functional circles increases progressively, intermingle with neighboring ones eventually covering the entire first lamina and will contact all pyramidal cells terminal dendrites throughout the neocortex. The illustrated rectangular region measures roughly 380 × 150 micrometers. (From: Marín-Padilla, 1990).

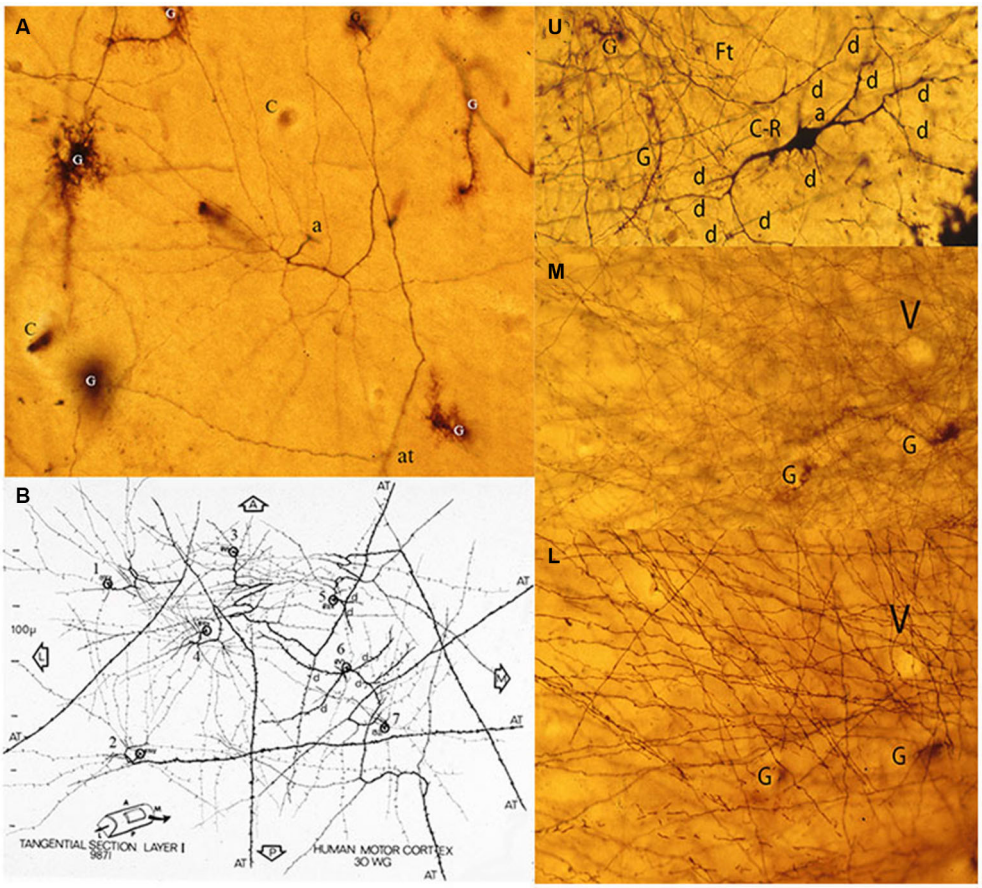

FIGURE 6 | Composite figure of photomicrographs and a camera lucida drawing (B) of tangentially cut rapid Golgi preparations of the motor cortex of a 30-w-g human fetus. **(A)** Tangential view of a C-RC axon (a) with numerous radiating collaterals and its axonic (at) terminal, first lamina special astrocytes (comet cells) and some capillaries (c). The neuron' radiating axonic collaterals establish an expanding functional circle contacting all pyramidal cells terminal dendrites within it. **(B)** Montage of camera lucida drawings of the axons from seven C-RCs with their radiating axonic collaterals and terminal axons projecting anteriorly, posteriorly and/or laterally within the first lamina. U, M, L. Three consecutive tangential views of first lamina, from a 30-w-o fetus, showing in the upper level **(U)** the neuron' multipolar morphology (d), a descending (a) axon and some astrocytes (g). The middle level **(M)** shows the crisscrossing of the neuron' fine axonic collaterals and a tangentially cut capillary (v). The lower **(L)** level shows the crisscrossing of the neuron thicker axonic fibers, the same capillary (v) and glial (g) cells. The thickness of a single Golgi preparation (ca. 150 μm) permits to visualize the first lamina structural organization in its entirety (From: Marín-Padilla, 1990).

possible function are intimately entwined with those of the first lamina and the maturing pyramidal cells terminal dendrites.

THE NEOCORTEX FIRST LAMINA COMPOSITION AND STRUCTURE

Three completely different views as well as conceptions of the neocortex first lamina composition and structural organization emerge from the use of various staining procedures (**Figure 7**). An essentially barren structure with very few neurons, scattered glial cells and, practically, nothing else, is evident in most routine staining procedures, such as hematoxylin and eosin (**Figure 7A**). It is not surprising since these methods failed to stain dendrites and/or axonic terminals that represent the first lamina more distinctive and abundant components. This vision of emptiness of the first lamina' composition and structure have persisted and consequently it has remained inadequately studied. When dendritic and axonic terminals are stained, using the Golgi method, the actual composition and structural complexity of the neocortex first lamina becomes apparent (**Figure 7B**). The majority of the axonic terminals within the first lamina are from C-RCs and, to a lesser degree, from original afferent fibers. Both fibers are recognized within the first lamina through the neocortex entire prenatal and postnatal maturations (**Figures 2–7B**). Moreover, when specific staining for dendrites is used (microtubule-associate protein-2), the concentration of dendrites within the first lamina is by far the greatest found throughout the cerebral cortex. This extraordinary dendritic concentration must reflect its functional relevance (**Figure 7C**). These different views and resulting conceptions of the neocortex first lamina have persisted and have been an additional source of confusions and controversies.

During late prenatal development, terminals from specific thalamic, interhemispheric and cortico-cortical fibers also reach

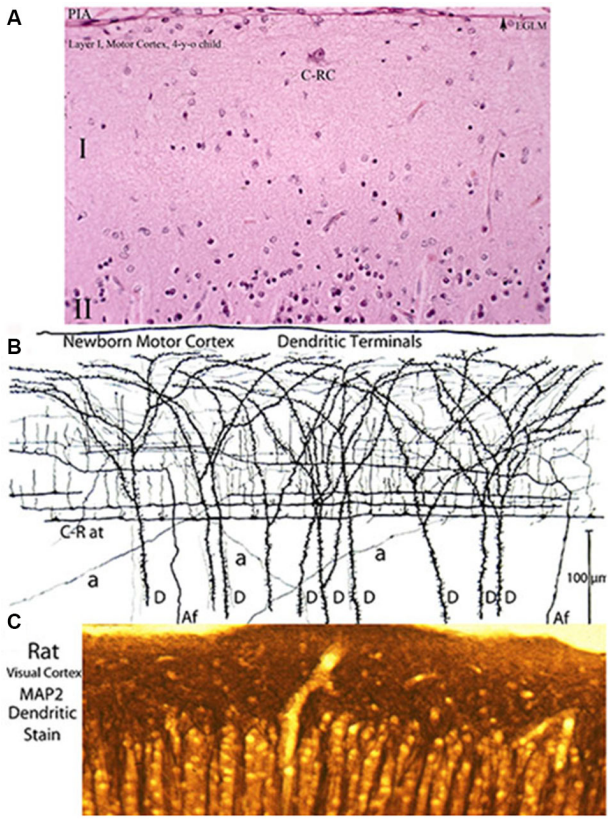

FIGURE 7 | Three entirely different views of the neocortex first lamina' composition and structural organization obtained with various staining methods. **(A)** Reproduces its essentially empty appearance in hematoxylin and eosin preparations (the most commonly used staining procedure) of a 4-year-old child motor cortex showing a large C-RC (C-RC), several small glial cells and, practically, nothing else. The lamina pial surface external glial limiting membrane (arrow) and the upper edge of the second (II) lamina are also illustrated. **(B)** Montage of camera lucida drawings from rapid Golgi preparation of a newborn' motor cortex showing numerous dendritic and axonic terminals that constitute its essential components. The pyramidal cells terminal dendrites **(D)** represent the first lamina main receptive elements. Most of the fibers are from C-RCs' axons. Its thicker axonic terminals (C-R at) run through the lamina lower zone and its thinner axonic collaterals through its middle zone. Both axonic terminals have numerous ascending and fewer descending branchlets that represent the neuron' functional elements. While C-RCs axonic terminals are recognized throughout the first lamina, the neuron' bodies are quite difficult to locate because of their developmental dilution. During early development, the first lamina also receives afferent fibers (Af) from the white matter that are distributed through the lamina middle and upper zones. During late development, the first lamina also receives additional terminals (a) from thalamic, interhemispheric and cortico-cortical fibers that are distributed locally. **(C)** Reproduces a view an adult rat visual cortex stained with MAP2 (microtubules associated protein-2) a specific stain for dendrites, demonstrating that the concentration of dendrites within the first lamina is by far the greater throughout the neocortex (Donated by Professor Alan Peters, Boston University School of Medicine). These morphological discrepancies about the neocortex first lamina composition and structural organization have also been a source of controversy. Of the three staining procedures, the classic Golgi method conveys the most accurate account of the first lamina composition and structural and functional organizations. The rectangular sections illustrated **(A,B)** measure roughly 230 × 150 micrometers and **(C)** 150 × 60 micrometers. See also **Figure 2**.

the first lamina (Marín-Padilla, 1984). Their distribution and hence their functional influence within the neocortex first lamina is essentially regional. The functional targets of these late incorporated afferent terminals are the terminal dendrites of regional pyramidal neurons (**Figure 7Ba**). These late incorporated afferent fibers must be related to the specific functional activity of pyramidal cell contacted by them.

To best visualize and demonstrate the structural and functional complexities of the neocortex first lamina high power microscopic views of Golgi preparations are needed (**Figure 8**). The rectangular area illustrated in **Figure 8**, measures roughly 160 × 60 µm and includes the lamina' upper and middle zones. These preparations demonstrate that the neocortex first lamina is essentially composed of axonic and dendritic terminals that intermingle with each other (**Figure 8**). Moreover, synaptic contacts between fibers and dendritic spines are often observed in these reparations (**Figure 8** arrows). The first lamina structural and functional complexity disclosed in Golgi preparations contract sharply with its apparent emptiness observed with routine staining procedures (Compare **Figures 6A**, **8**).

The functional relevance of the neocortex first lamina is undeniable considering that the terminal dendrites of all pyramidal neurons throughout the neocortex, regardless of size, location or eventual functional role, are represented in it as well as the numerous axonic terminals of C-RCs. The neurons' soma is difficult to locate because, in the course of neocortical maturation, they have been progressively diluted.

Neither the neocortex first lamina nor the C-RCs functional roles are known. It is time to abandon the controversies and to start investigating the roles they play in overall functional organization of the neocortex. The present account on the structural and functional organizations of the neocortex first lamina and that of its essential neuron, the C-RC, hopefully will stimulate renew interest on both structures, as well as in the need for using the classic Golgi staining procedure.

CONCLUSIONS AND FUTURE DIRECTIONS

The C-RC is the essential neuron of the neocortex first lamina. It receives inputs from subcortical afferent fibers that reach the first lamina during early in development. The C-RC neuron orchestrates the arrival, size and stratification of all new pyramidal neurons (of ependymal origin) of the neocortex gray matter. Its axonic terminals spread radially and horizontally throughout the entire first lamina establishing contacts with the dendritic terminals of all pyramidal neurons regardless of size, location and/or eventual functional roles. While the neuron axonic terminals spread throughout the entire first lamina targeting the terminal dendrites of pyramidal neurons, its body undergoes progressive developmental "dilution" and it becomes quite difficult to locate any of them in the adult brain. The C-RCs bodies are probably retained in the developing neocortex older cortical regions (such as the primary motor, somatosensory, visual and acoustic regions) while their axonal collaterals will spread throughout its more recent ones (associative areas) that will represent the great majority of the brain surface. This will explain the progressive "dilution" of C-RCs bodies throughout the neocortex.

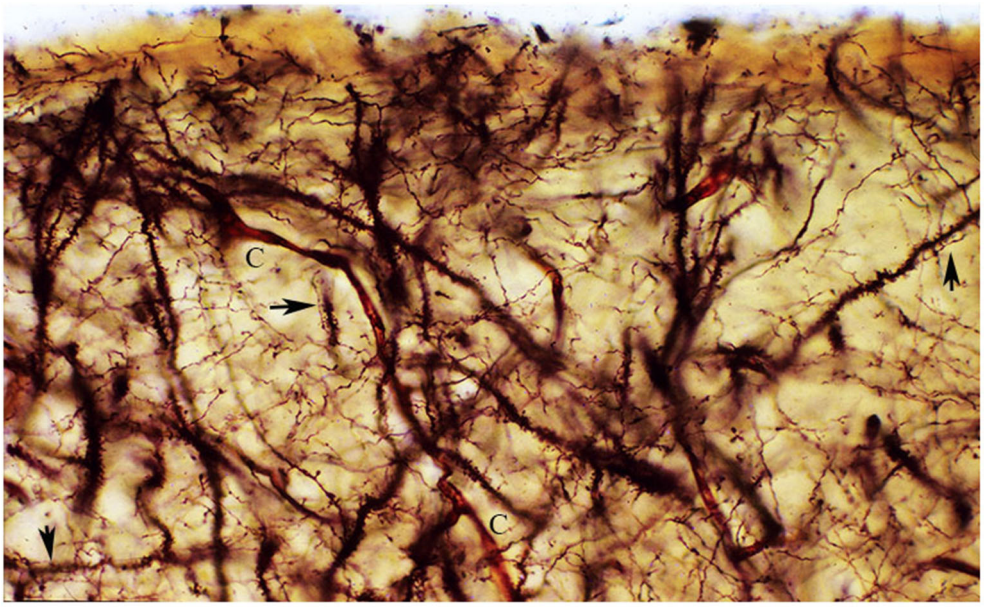

FIGURE 8 | The figure reproduces a rectangular area of the first lamina upper and middle regions from a newborn motor cortex, measuring roughly 140 × 60 micrometers of a rapid Golgi preparation, the pial surface is at top. That is essentially composed of axonic and dendritic terminals intermingle with each other. Moreover, synaptic contacts (arrows) between axonic terminals and dendritic spines are often recognized (arrows). To these two elements, blood capillaries (C), special astrocytes and the axonic terminals of the original afferent fibers should be added. Rapid Golgi preparations of the first lamina best and faithfully illustrate its essential components and their structural and functional organizations.

The human cerebral cortex C-RC is distinguished by distinctive developmental, morphological and possible functional features and by long axonic processes that extend and crisscross throughout the first lamina in both the developing and the adult brains. Therefore, most of the controversies expressed about the C-RCs are not applicable to those in the human cerebral cortex.

Furthermore, the C-RCs' morphology, nature and possible function cannot be understood without neocortex first lamina where they resided. Their evolutions are concomitant, inseparable and codependent.

The terminal dendrites of all pyramidal neurons of the neocortex gray matter represent the first lamina main receptive elements and the C-RC its main functional unit. The C-RCs axonal terminals reach and contact all pyramidal cells terminal dendrites, within the first lamina, regardless of their size, location and/or eventual functional activity. Moreover, the C-RC terminal axonic branchlets make synaptic contacts with the spines of pyramidal cells terminal dendrites.

The C-RC role within the neocortex overall functional activity remains unknown and should be investigated. It is difficult to comprehend than, more than a century of its discovery we still lack a clear understanding of the C-RC nature and function as well as that of the cortex first lamina. Most controversies about the C-RC and henceforth on the neocortex first lamina have contributed unnecessary confusions and should be left to rest.

Undoubtedly, additional studies are needed to elucidate the C-RC and the neocortex first lamina functions. The following suggestions may help guiding this overdue investigation: (a) the C-RC only known input is from afferent fibers that reach the first lamina early in neocortical development; (b) their origin and function remain unknown and must be among the first ones established in the developing neocortex; (b) it must be necessary to transform all arriving neuroblasts into pyramidal neurons; (c) it must be necessary for orchestrating the arrival, size and eventual stratification of all pyramidal neurons within the gray matter; (d) it must be necessary for maintaining all pyramidal neurons functionally active before they start receiving specific thalamic inputs that will determine their eventual function; (f) it must be limited to the neocortex first lamina; (g) it must reach all pyramidal cells terminal dendrites, within the first lamina, throughout the neocortex; (h) it must be shared by all pyramidal neurons throughout the neocortex, regardless of size, location and/or eventual functional role; (i) it must persists into the adult brain since all pyramidal neurons terminal dendrites also persist; and, (j) it must be distinguished from the specific functional roles of thalamic, interhemispheric and cortico-cortical fibers terminals on pyramidal neurons that are linked to their specific motor, sensory, acoustic, visual and/or associative functions.

By exposing the neocortex first lamina anatomical composition and functional complexity and that of its essential components, namely: the Cajal-Retzius neurons and the dendritic terminals of all pyramidal neurons, this paper hopes to stimulate renewed interest in them as well as in using the Golgi procedure in future brain studies.

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Cajal and the Conceptual Weakness of Neural Sciences

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The experimental and conceptual contributions of Santiago Ramón y Cajal remain almost as fresh and valuable as when his original proposals were published more than a century ago—a rare example, contrasting with other related sciences. His basic concepts on the neuron as the main building block of the central nervous system, the dynamic polarization principle as a way to understand how neurons deal with ongoing active processes, and brain local structural arrangements as a result of the functional specialization of selected neural circuits are concepts still surviving in present research papers dealing with brain function during the performance of cognitive and/or behavioral activities. What is more, the central dogma of the Neuroscience of today, i.e., brain plasticity as the morpho-functional substrate of memory and learning processes, was already proposed and documented with notable insights by Ramón y Cajal. From this background, I will try to discuss in this chapter which new functional and structural concepts have been introduced in contemporary Neuroscience and how we will be able to construct a set of basic principles underlying brain functions for the twenty-first century.

Keywords: Ramón y Cajal, neural sciences, neural functional laws and principles, neural plasticity and elasticity, higher brain functions

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A Man of Laws and Principles

> *"The reason of the unreason with which my reason is afflicted so weakens my reason that with reason I murmur . . ."*
>
> *—Miguel de Cervantes, Don Quixote of La Mancha*

There are two main ways to increase the size and scope of a given science, and from that perspective Neuroscience is no exception. The easier one is to develop a new instrument (electron microscope, patch-clamp, functional magnetic resonance imaging) capable of providing original data impossible to obtain with existing devices. More difficult is to provide new conceptual and fruitful insights aimed at illuminating the core of the selected science, opening new approaches and pathways to advance it to still-unknown regions. In accordance with the title of this short review of Santiago Ramón y Cajal's contributions to the development of modern Neuroscience, present-day neuroscientists are not particularly characterized by their ability to generate general principles capable of supporting and assimilating the large amount of experimental data collected during the past century (Delgado-García, 2006, 2011).

Besides his experimental contributions to the proper visualization and description of the cellular composition of the nervous tissue, Ramón y Cajal was a man of laws and principles, always ready to find and to propose the conceptual networks supporting and integrating his experimental findings. For example, Ramón y Cajal (1923) summarizes the four laws governing the morphology and connectivity of nerve cells as follows: (a) axons

terminate in free ends; (b) axons lean against somas or dendrites; (c) somas and dendrites also participate in nerve impulses; and (d) those impulses are transmitted across those protoplasmic contacts. Making a comparison with the electrical instrumentation available in his time, Ramón y Cajal imagines that neural transmission takes place in the same way as current transmitted in the connections of electric components by a sort of induction, as in the coils of the same name.

Impressively, the first chapter of his *Textura del sistema nervioso* (1899) is full of principles relevant to the general design and evolution of the nervous systems of invertebrates and vertebrates. In the fifth chapter of that book, Ramón y Cajal describes another three laws fundamental in the organization of all nervous systems: (a) *law of economy of time*, by which neural pathways are as short as possible, adopting imaginative geometric solutions to this aim; (b) *law of economy of matter*, explaining why some axons are originated from somas or principal dendrites depending on their optimal location with relation to their projecting sites; and (c) *law of economy of space*, according to which neuronal somas and their protrusions are arranged in such a way as to avoid empty spaces around them. Finally, in chapter XLVIII of the above-mentioned book, Ramón y Cajal describes a series of functional postulates implied in the organization of cortical centers and pathways: (a) *unity of spatial and tonal perception*, referring to the cognitive need to generate a single and integrated perception corresponding to the whole set of stimuli present in the external world; (b) *concentric symmetries*, predicting in some ways the existence of cortical maps, mainly for two important sensory (visual and auditory) modalities; and (c) the already described laws of space and protoplasmic savings.

An additional law proposed by Ramón y Cajal has, like many other of his proposed conceptual generalizations, important functional implications: the law of the nervous avalanche or volley (Llinás, 2003). The conduction of nerve impulses in the form of volleys allows the simultaneous activation of numerous neural populations from a single sensory stimulus; among other functional advantages, this enables the generation of appropriate functional responses from neural sites located far away from the stimulated site.

The Enigmatic Arrows in Ramón y Cajal's Drawings

From my modest point of view, the three fundamental contributions of Ramón y Cajal to past and present Neuroscience are his exhaustive description of the morphology and connectivity of the nerve cells, the proposal of neurons as the building blocks of every brain, and the *principle of dynamic polarization*. In this section I will address my comments to that functional principle. In his *Recuerdos* (1923), Ramón y Cajal writes that this dynamic polarization principle was developed initially in 1891. In accordance with additional findings and critical comments, the principle was further developed in the so-called *theory of axipetal* (orthodromic) *polarization*.

For Ramón y Cajal, the transmission of the nerve impulse takes place from the protoplasmic branches (i.e., the dendrites) to the neuronal body (i.e., the soma), and from this to the nervous expansion (i.e., to the axon). While dendrites and the soma represent a receptive device, the axon is the organ for transmission and distribution of neural messages. Ramón y Cajal developed his dynamic polarization principle from his detailed reconstructions of the (organized) cellular structure of the cerebellar cortex (**Figure 1**), but he successfully applied the same principle to many other neural circuits, such as, for example, to the interaction of sensory signals with motor commands in the cerebral cortex. He predicted that the centrifugal movement of voluntary motor commands transmitted across the two motor neurons (i.e., the projecting pyramidal cell and the motoneuron) to the skeletal muscles is originated in the dendrites of the pyramidal cells—that is, in the outer cortical layers, a cortical area receiving afferent sensitive, callosal, and other association fibers.

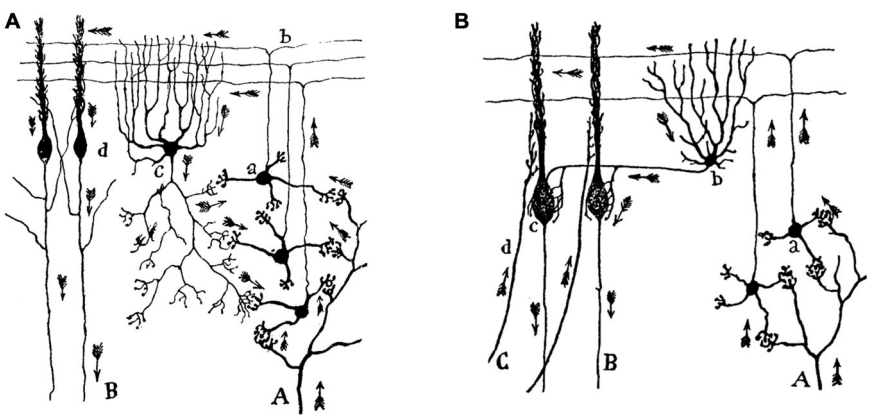

FIGURE 1 | Movement of the nerve impulse across the cerebellar circuit, according to Santiago Ramón y Cajal. (A) An illustration of the direction and sense of the nerve impulse arriving along mossy fibers **(A)** at the granule cells (a) and from these, along the parallel fibers (b), at the Golgi (c) and the Purkinje (d) cells. The nerve impulse leaves the cerebral cortical circuit along Purkinje cell axons **(B)**. **(B)** Another view of the cerebellar cortical circuit including basket cells (b) and climbing fibers **(C)**. Taken from Ramón y Cajal, *Textura del sistema nervioso del hombre y de los animales* (1899–1904).

More than one hundred and twenty years after the seminal proposal by Ramón y Cajal, it is still difficult to explain how he was able to imagine the correct arrangement (direction and sense) of arrows included in his reconstructions of cortical and subcortical circuits. Indeed, to generate a dynamic principle for a dead and fixed tissue is (besides a contradiction) a genial interpretation of what one can see. Even now, it is difficult to imagine this orderly functional organization when looking through a microscope at a Golgi preparation of the cerebellar or the hippocampal cortices. Interestingly, with his functional proposal Ramón y Cajal not only was close to explaining the unidirectional character of chemical synapses, but also offered the background for the proper interpretation of many neural circuits from a functional point of view.

This linear and two-dimensional model of neuronal organization and functioning still has many followers and still underlies our current concepts of brain activities during complex functions such as learning and memory processes. Furthermore, Ramón y Cajal makes important suggestions about the functional role played by cortical short-axon neurons in cognitive, emotional, and related functions as opposed to those played by long-axon (projecting) neurons more related to sensory perception or to the execution of motor commands. The concept of reverberant circuits put forward by Lorente de Nó (1938) can be considered another attempt at advancing the functional consequences of the dynamic polarization principle and overturn this interpretation of the brain as a garden of intertwining paths. Today, people studying complex and distributed cerebral functions such as sleep and awareness (Hobson and Friston, 2012), or neuronal functioning during the actual acquisition of new motor and cognitive abilities (Delgado-García and Gruart, 2006; Gruart et al., 2015), have realized that these functional cerebral states require an interpretation different to (although not necessarily more complex than!) those proposed by Ramón y Cajal many years ago.

On the basis of the seminal studies of Hamburger and Levi-Montalcini (1949), as well as on those carried out by so many others (see Hamburger, 1980; Levi-Montalcini et al., 1996), it is now possible to propose a (new) *trophic polarization principle*. This principle indicates the dependence of neurons in the adult mammalian brain on molecular (?) signals generated by their target neurons, and makes reference to the maintenance of neuronal connections and the survival of interconnected neurons in the adult brain (Delgado-García and Gruart, 2004). In this way, whereas the concept of dynamic polarization suggests a flux of nervous information from dendrites and soma towards axon terminals, the concept of trophic polarization means an antidromic flux of information from the neuronal target, across the axon, toward the soma of the innervating neuron.

Nuclei vs. Cortices

In the very first chapter of his *Textura* (1989), Ramón y Cajal explains that the evolution of the nervous system leads to a functional specialization, which is latterly transformed into a morphological redefinition. As an expected result of the laws of time-, space-, and matter-saving it is logical that neurons carrying out similar functions pack together, forming nuclei. A coherent consequence of this principle is that the firing rate (FR) of a motoneuron located in a brainstem motor center can be representative of the firing activities of the other thousands of neurons located in the same nucleus (Delgado-García, 2011). The mammal abducens (ABD) nucleus is a good example of this proposal. This nucleus contains motoneurons (Mns) and internuclear interneurons (Int)—the first projecting to the extraocular lateral rectus muscle and the second projecting to the contralateral medial rectus subdivision of the oculomotor nucleus. Anyone following the evolution of these two groups of neurons in vertebrates will notice that in parallel with the progressive displacement of the two eyes to a frontal position, enabling a binocular vision, the two types of neuron approach progressively their somas in the pons as already determined in different species of mammals (Cabrera et al., 1993). Finally, these two types of neuron are perfectly intermingled in primates, constituting the ABD nucleus, where they share vestibular, reticular, and many other afferents to the nucleus (**Figure 2**; Escudero and Delgado-García, 1988).

Thus, as a functional consequence of the above contentions, we can generalize a principle by which neurons that are together in a nucleus fire under similar physiological rules. But, can we say the same for neurons packed together in layers? In this case, it is more than possible that the FR of a given Purkinje cell, or of a pyramidal neuron, is generated with a functional pattern different to the one shown by nearby neurons. When dealing with cortices, the functional consequences of this characteristic neuronal organization are not so evident. Nevertheless, the main contribution to advance the physiological rules of cortical arrangement was probably the concept of functional cortical column put forward initially by Mountcastle (1957), and later followed by many others (see Hubel and Wiesel, 1977).

Ramón y Cajal suggested that the appearance of the pyramidal cell enables the proper storage of sensory perceptions recollected from the external world in the form of ideas and volitions. In fact, in some of his early writings, Ramón y Cajal qualified the pyramidal cell as the *psychic neuron*, a concept that he seems later to have abandoned (López-Piñero, 2000; Goldman-Rakic, 2002). In any case, thanks to this specialized type of neuron, the perceived sensory events do not need to be immediately (and automatically) transformed into a motor response, but can be retained indefinitely by pyramidal cells, mainly by those located in association areas of the cerebral cortex. This information, stored in the form of memories in cortical cells, can be used later in the presence of new physical and/or social contingencies. For Ramón y Cajal, cortical association areas play an interconnection role between the more-specialized primary sensory and motor areas. It is possible that the peculiar organization of cortical circuits explains the difficulties for the proper identification of the functional codes of specific cortical sites—i.e., functional codes are modified moment by moment in accordance with the ongoing internal functional needs depending on the external constraints. According to Ramón y Cajal, the extension and structural complexity of the cortical gray layer are intimately related with the hierarchical psychological range of every

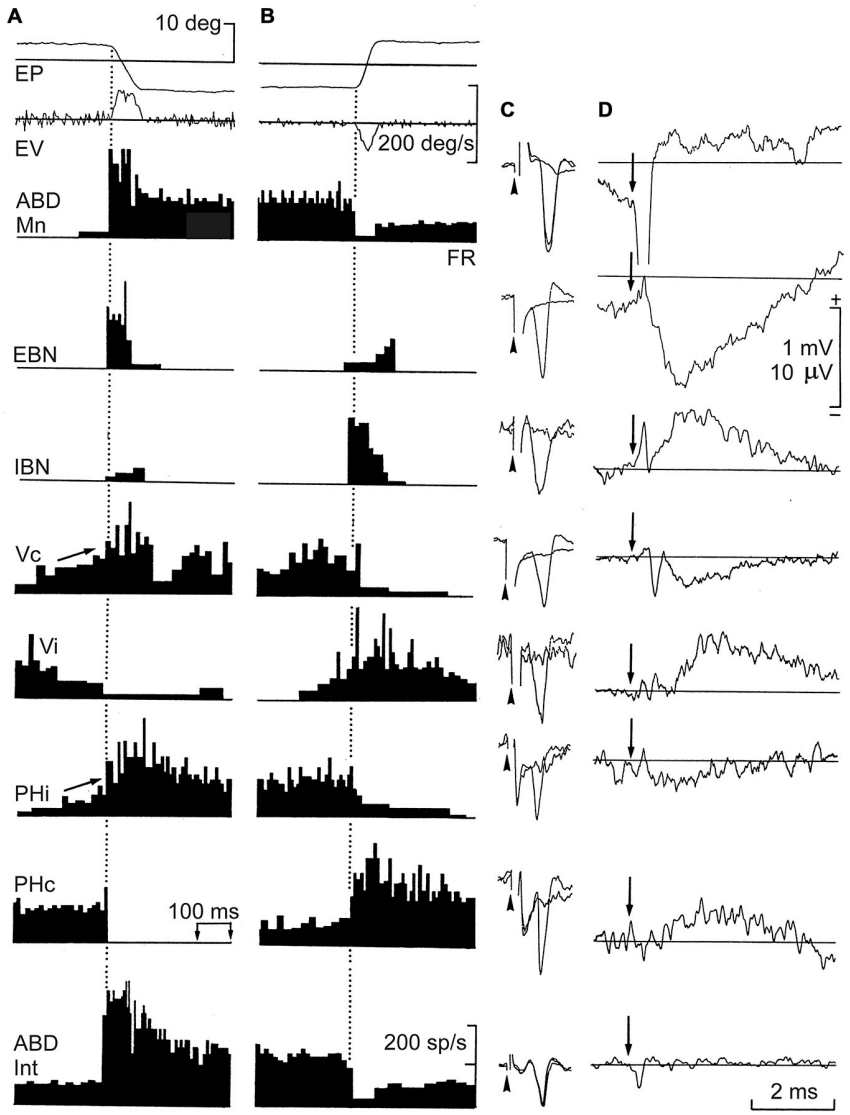

FIGURE 2 | Firing rate (FR), antidromic activation, and averaged field potentials induced by short-lead excitatory (EBN) and inhibitory burst neurons (IBN), and by ipsi- and contralateral vestibular (Vi, Vc) and prepositus (Phi, PHc) neurons projecting to the abducens (ABD) nucleus. Recordings were carried out in alert cats. **(A,B)** Neuronal activity recorded from the illustrated neurons during spontaneous saccades in the on- and off-direction, respectively. **(C)** Antidromic all-or-none activation of recorded neurons by microstimulation of the ABD nucleus. Arrow-heads indicate the beginning of the stimulus. **(D)** Average of field potentials recorded in the ABD nucleus triggered (arrow) by these identified neurons. Abbreviations: EP and EV, horizontal eye position and velocity; FR. The discharge rate, antidromic identification from their projecting sites, and field potential induced by ABD motoneurons (Mns) and internuclear neurons (Int) are also shown. Taken with permission from Escudero and Delgado-García (1988).

vertebrate. More comments on this point are offered in the following section.

Long Axons and Short Minds

Apparently, Ramón y Cajal did not like the distinction made by Golgi between sensory and motor neurons. In contrast, and as another example of his insightful generalizations, he divided nerve cells into those presenting short axons, branched and restricted inside the gray matter of the cortex, and those of long axons, constituting the white matter, bundles, and nerves (De Castro, 1981). Whereas it seemed clear for Ramón y Cajal that long-axon cells take care of rapidly sending sensory or motor messages far away in the peripheral and central nervous systems, he wondered about the reason for the presence of so many short-axon neurons interposed in, or collateral to the main neural pathways (**Figure 3**). Almost at the end of his *Recuerdos* (1923), Ramón y Cajal suggests that the functional excellence of the human brain is intimately related to the enormous abundance in number and in different structural displays of the so-called short-axon neurons. Indeed, short-axon neurons are mainly located in structures not immediately

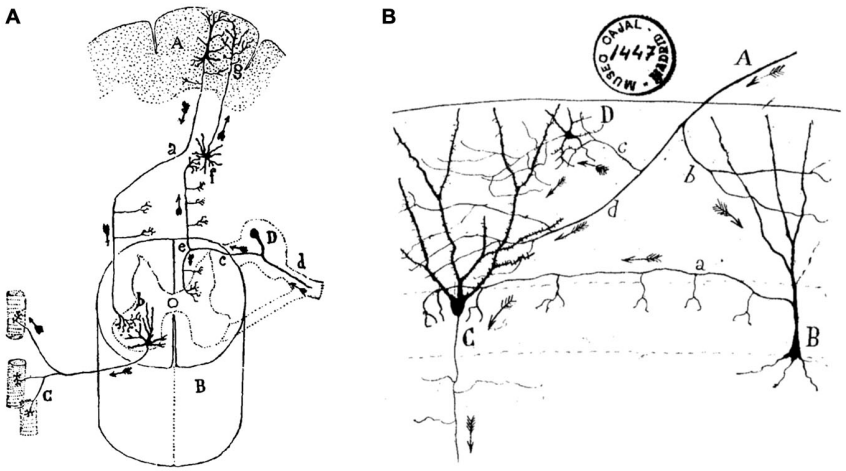

FIGURE 3 | Ramón y Cajal's diagrams illustrating long-axon and short-axon neurons. (A) Direction and sense of conscious motor commands and sensory signals in the nervous system. The diagram illustrates the cerebral cortical psychomotor region **(A)**, the spinal cord **(C)**, the muscle fibers **(C)**, and the spinal ganglion **(D)**. Motor commands descend by the pyramidal cell axons (a) which make contact with motoneuron dendrites (b). In turn, motoneuron axons terminate on the muscle fibers of their corresponding motor unit. Sensory signals arrive from peripheral receptors (d), and across the dorsal root (c) and the ascending tract (e) reach second-order neurons (f), which project to cerebral cortical neurons (g), where Ramón y Cajal assumes that they make contact with the protoplasmic branches (dendrites) of pyramidal cells. **(B)** An illustration of the twists and turns followed by axonal afferents to the dentate gyrus (DG) across short-axon neurons. **(A)** afferent fiber; **(B)**, short-axon neuron terminating around the somas of granule cells **(C)**; **(D)**, another type of short-axon neuron; b, c, d, different branches of the afferent fiber. Taken from Ramón y Cajal, Textura del sistema nervioso del hombre y de los animales (1899–1904).

related to the generation of reflex responses, such as the cerebral cortex, the striatum, the thalamus, or the optic lobes. These short-axon neurons would act (in a still-unknown way) as peculiar stores for the psychic energy, being responsible for higher functions such as memory, ideation, and decision-making. In contrast, long-axon neurons would take care of the more bureaucratic role of carrying sensory information to the corresponding cortical sites, or motor commands to peripheral effectors.

Throughout his writings, Ramón y Cajal appears to be conscious of the extreme difficulties in proposing an architectonic and dynamic plan of the cerebral cortex able to explain mental functions. As he says, the cerebral cortex is like a forest in which many researchers have got lost. Indeed, he expects that future neuronal engineers will be the ones finding the proper pathways in this dense forest, an expectation not yet fulfilled. At least Ramón y Cajal realized that complex cognitive functions should be the result of the combined actions of a large number of commemorative cortical primary and secondary areas. Indeed, the cortex receives a huge amount of selective sensory information, allowing not only an appropriate internal representation of the external world, but also the internal design of appropriate motor responses. Years later, Llinás (1988) describes the same ideas in some more-contemporary words: "the mind is a computational state of the brain generated by the interaction between the external world and internal set of reference frames". Never was knight so honored by his followers!

For his proposal of the putative roles of short-axon interneurons, Ramón y Cajal did not take into consideration the existence of inhibitory neurons, usually located in the circuits collateral to the main neuronal pathways that he drew with his habitual precision (Sotelo, 2003). Interestingly, some contemporaries of Ramón y Cajal were already aware of the presence of active inhibitory mechanisms in the brain of animals—as for example in the description by Pavlov (1927) of habituation as an active inhibitory process. Also, Freud (1895), in one of his early books (*Project for a scientific psychology*), proposes the concept of repression as an active inhibition of psychic energy, including a rather elementary scheme of brain circuitry: when this *psychic energy* is actively prevented from following a given neural pathway, it has to be redirected to other neural sites. In this way, the psychic energy is understood as a quantifiable entity. It is a pity that even today nobody has been able to put coefficients to this metaphoric form of energy, a fact contrasting with the remarks made in the following section.

A Still-Unstated Principle

In my opinion, there is an important part of cerebral functions that can currently be understood with no major difficulties. Indeed, motor functions can be reasonably explained with the help of I. Newton's second law (i.e., acceleration is produced when a force acts on a mass). This proposal was fruitfully developed in the past 50 years by Robinson (1981) and his followers, up to convincingly explaining the intrinsic neural organization of the extraocular motor system (Delgado-García, 2000). From an evolutionary point of view, it can be proposed that brains were originated by the need for movement at a certain speed in a three-dimensional world in which vertebrate bodies have to move against gravitational forces, and also have to deal with the viscous and elastic components of the external physical elements (see Llinás, 1988).

It is remarkable that all written contributions from Ramón y Cajal are always illuminated by the dynamic contributions of his cellular and histological findings (Sotelo, 2003). In this regard, and in the light of the many different morphological types of neuron described in his *Textura* (1899–1904), how is it possible that neither Ramón y Cajal nor any of his contemporary colleagues raised the issue of this huge morphological diversity? What is that for? For example, Purkinje cells are quite different from the other four (or five) types of neuron present in the cerebellar cortex. In turn, those cortical cerebellar cells are noticeably different in shape and dimension from those located in the cerebral cortex, in the thalamus, or in the brainstem. All of us can readily agree that every neuronal type exhibits specific physiognomic profiles (De Castro, 1981), but what about their functional peculiarities? What about their physiology? Today, we can describe without much difficulty the *physiology* of the motoneuron: a specialized type of neural cell encoding the current length of the innervated muscle and the speed with which this length is modified. Perhaps we can also explain the physiology of retinal rods and cones as their capability to transduce a given band of the electromagnetic spectrum into biopotentials. What is not so easy is to offer similar synthetic functional descriptions for most central nervous system cells. What could be the physiology of inferior olive cells, of reticular, thalamic or pyramidal neurons, and even of the most complex known neuron, the Purkinje cell? More experimental approaches, ranging from immunohistochemistry to patch-clamp and from the precise determination of expressed constitutive and functional proteins to the presence of selective membrane receptors and related molecular elements, do not necessarily contribute to a better understanding of the functional reason why a given neuronal type needs to be right where it appears.

In any case, it seems convenient to start the outline of a new functional principle that we could initially refer to as the *transformation principle*. It is clear that the different ionic conductances present in the available neuronal types confer on them quite diverse functional properties. The different neural types across a neural pathway (i.e., visual, auditory, motor, etc.) were classically considered passive relay points in the transmission of sensory percepts or motor commands. However, it seems reasonable to propose that every neural type present in a given pathway or circuit plays a specific and unique role. This uniqueness is dependent on their electrogenic and secretory properties, at the same time that the latter are dependent on the ionic conductances present in the plasmatic membrane and/or on the types of protein expressed inside the neuron. In this way, the nervous system cannot be considered a huge set of an enormous amount of neural elements capable of detecting sensory signals and of switching them into motor commands, but better as a distributed structure able to *transform* the incoming information from sensory receptors in an autonomous and highly organized internal world, not necessarily dependent on the external milieu. This internal milieu generated by the activity of hundreds of different types of neuron is capable of producing original decisions not necessarily contingent on external demands. It should be expected that a different functional state would correspond to each of the many different functional capabilities of the set of different neuronal types composing a brain (**Figure 4**). In accordance, each neuronal type described by Ramón y Cajal must have its own physiology. We need only to identify them.

The Game is Between Plastics and Elastics

Were the brain as plastic as many contemporary neuroscientists seem to think, it would not be necessary to revisit the *Textura* (1899–1904), because neurons illustrated in the text would no longer exist. Nevertheless, Ramón y Cajal (1905) might be included in the *plastic* group—for example when he proposes that interneuronal connectivity, besides its hereditary origins, is susceptible to being influenced and modified during youthful years by education and habits. In fact, Ramón y Cajal was certainly influenced by the proposals of Tanzi (1983) and many others, regarding the motility of dendritic protrusions, including their spines, and that of axon terminals. Ramón y Cajal suggests that such motility might be dependent on the use of the involved neuronal connections, a fact obviously related with the psychomotor activities (such as sleep, memory, and thinking) of the subject. These thought-provoking proposals still survive among contemporary neuroscientists (Kandel, 1998, 1999; Albright et al., 2000; Yuste and Bonhoeffer, 2001; Sotelo, 2003). In consequence, either sleep processes (in Ramón y Cajal's time) or learning and memory phenomena (in ours) are explained not on the basis of the intrinsic properties of neuronal circuitry, but on its capability of being modified. However, A. Von Kölliker (a contemporary of Ramón y Cajal) raised some concerns; he realized that while psychic processes are fairly stable in the same subject, amoeboid changes in neuronal connections are continuous and disorganized, mostly related to nutritional and thermal phenomena. Indeed, neurons and glial cells are capable of a certain motility (Ramón y Cajal, 1899–1904; Bonhoeffer and Yuste, 2002); the point is how to establish proper causal relationships between conformational changes in neuronal connectivity and adaptive modifications in the behavior and mental status of the subject. Ramón y Cajal envisages that some changes in neuronal connectivity (lazy connections, partial disconnections of commemorative systems) could be the substrate for selective neural pathologies. In other words, neural plastic changes will not always be beneficial for the supporting individual!

Assuming that plastic changes are those that remain for long periods of time, the concept of neural plasticity is not applicable to the regeneration of nervous tissue after a lesion. In such case, it would be better to talk of *elasticity*—i.e., a mechanism allowing the return to the preceding state. Peripheral nerve regrowth after a section is a well-known example of this notion. Another example is the retraction effect of both tetanus and botulinum neurotoxins on presynaptic axons terminating on the infected motoneuron (Pastor et al., 1997). Once the neurotoxic effects are over, all of the presynaptic terminals (a few thousand for a motoneuron!) return to the postsynaptic membrane in a similar proportion and with a similar functionality, allowing the complete recovery of the motoneuron physiology (Pastor

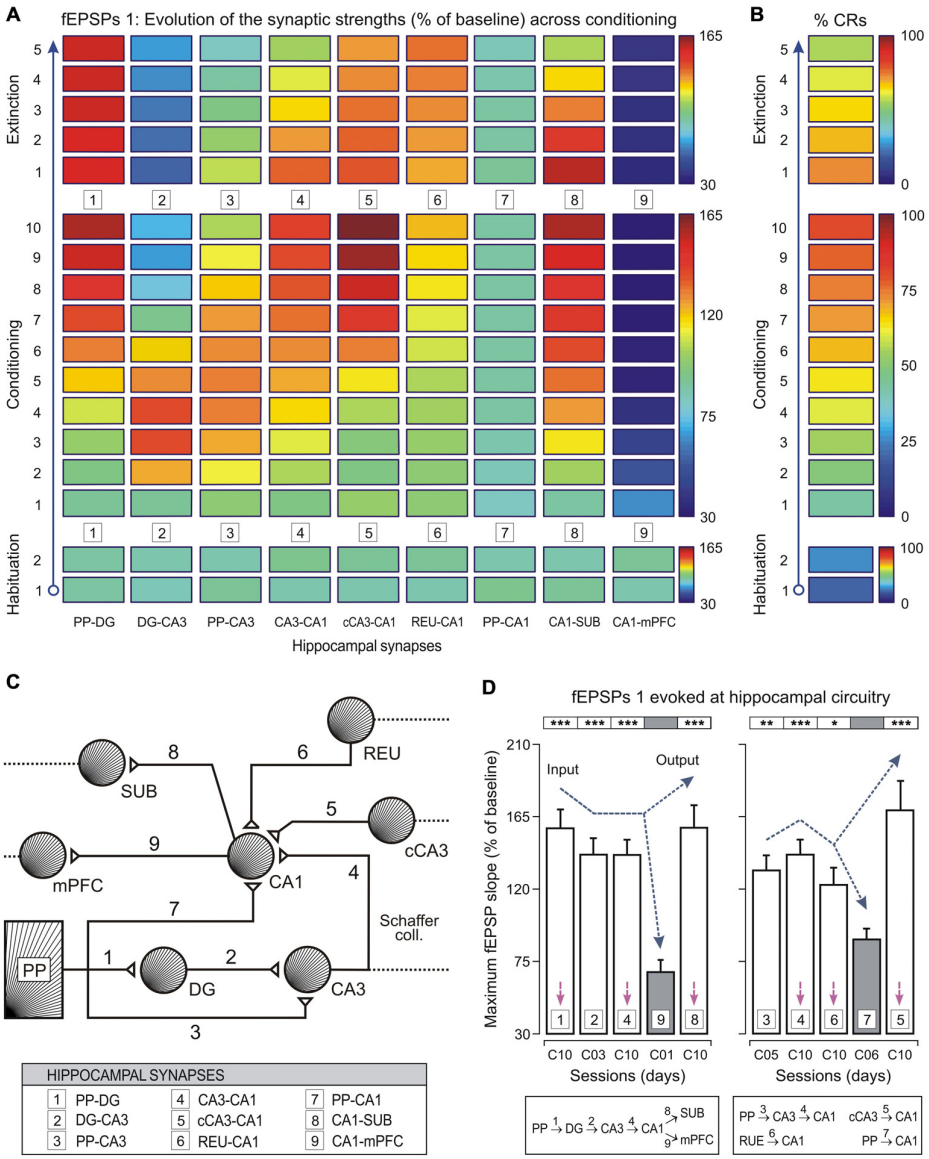

FIGURE 4 | Changes in strength of different hippocampal (and related) synapses during classical eyeblink conditioning of behaving mice. **(A,B)** Evolution of field excitatory post-synaptic potential (fEPSP) slopes (as % of baseline, see **(A)**) collected at 9 synapses, and percentage of conditioned responses (% CRs, see **(B)**) across the successive training sessions. Selected synapses were: 1, perforant pathway (PP)–DG; 2, DG–CA3; 3, PP–CA3; 4, CA3–CA1; 5, contralateral CA3 (cCA3)–CA1; 6, thalamic reuniens nucleus (REU)–CA1; 7, PP–CA1; 8, CA1–subiculum (SUB); and 9, CA1–medial prefrontal cortex (mPFC). Note that some synapses increased in learning-dependent strength across training (PP–DG, CA3–CA1, cCA3–CA1, REU–CA1, and CA1–SUB), whilst for others their strengths increased at the beginning of the conditioning sessions and decreased at the end (DG–CA3, and PP–CA3). Finally, some of them did not present any significant change (PP–CA1), or decreased across training (CA1–mPFC). See code color bars to the right of **(A,B)**. **(C)** A diagram of hippocampal synapses included in this study, indicating their main input and output connections. **(D)** Mean values of maximum synaptic strength across learning for the 9 synapses. According to the statistical analyses, 4 synapses (1, 2, 4, and 8) presented maximum synaptic strengths significantly different from those evoked by synapse 9 (left histogram). Note the potentiation of synapse 8 in contrast to the inhibition of synapse 9 during the acquisition process. A similar analysis (right histogram) showed that synapses 3, 4, 6, and 5 presented significantly different maximum synaptic strengths from those shown by synapse 7. However, synapses 1, 4, 5, and 8 presented similar synaptic strengths at the asymptotic level (session C10, see magenta arrows) of the acquisition process. Error bars represent SEM. Taken with permission from Gruart et al. (2015).

et al., 1997; Delgado-García and Gruart, 2004). Thus, the nervous system is not only capable of modifying its intrinsic connectivity in a sustained way (for learning to occur), but also of returning to its previous state after some types of neural lesion (for regenerative processes).

In accordance with the law of the morphological progress (Ramón y Cajal, 1923; De Castro, 1981), neurons would add new appendages to their terminals and would increase their connections with other neural cells as a result of the increasing functional adaptability. For Ramón y Cajal (at the Rome

Meeting, 1894), these plastic phenomena are more frequent and abundant in the cerebral cortex, in contrast to more-stable centers (evolution-ankylosed systems) such as the brainstem and the spinal cord. From an evolutionary perspective, it is reasonable to think that very old neural systems with a well-defined function are less susceptible to modification than those appearing more recently. An example of the former is the vestibular system, whose more-recent adaptive changes took place a hundred million years ago (Delgado-García, 2000), while a good example of the latter is the association cortex, very susceptible to modification precisely because of its functional indefinition (Gould, 1980).

To finish this section, and taking advantage of Kölliker's suggestions (see above), we should keep in mind that the brain also has a tendency to compensate for unwanted changes (as in the early stages of many neurodegenerative diseases). These *stability* functions are probably more evident in the older neural systems. For example, extraocular Mns can easily compensate for the experimental removal of a considerable percentage of presynaptic afferents, a phenomenon less readily observed in cortical pyramidal cells (Delgado-García, 2011).

Ready to Depart

Although from time to time we are presented (Bullock, 1959; Shepherd, 1972; Bennett, 2002; Bullock et al., 2005; Guillery, 2005) with (rather timid) criticisms of Ramón y Cajal's neuronal doctrine, the truth is that we still lack a really new conceptual framework of brain structure and functions capable of surpassing the preceding proposal. I should expect a big jump from Ramón y Cajal's arrows in a two-dimensional space to a three- or fourth-dimensional brain analyzed during its functional states: sleeping, dreaming, thinking, learning, and remembering. Apart from some recent attempts (for example, results collected using functional magnetic resonance imaging and related techniques; Fox and Raichle, 2007; Yao et al., 2015), the path followed was the opposite, and experimental neuroscientists mostly preferred to move toward the inside of the neuron (Kandel, 1998, 1999; Albright et al., 2000; Changeux, 2001) in search of the molecules that make possible learning, social interactions, or attentive phenomena. In the words of Santiago Ramón y Cajal, it seems that we are becoming followers of the religion of the small (trivial?). Perhaps in the reasonable curiosity to find at the lower integration level the explanation for a given functional process, we are at risk of forgetting the search for the origin of emergent properties emanating from the brain *in vivo*.

Figure 5 illustrates an example of emergent properties rather difficult to identify by a study of the intracellular properties and compositions of the involved neural elements. In contrast, the emergent resonant oscillatory property of the eyelids was obtained from physiological recordings carried out, for comparative purposes, in different species of mammals (Gruart et al., 2000).

Apart from the above considerations, at the time of explaining brain properties we are too prone to use metaphors originated from our scientific and cultural surroundings. For example, Descartes imagined brain function in the light of the pneumatic systems then available in the gardens at Versailles. Mendeleev attempted to explain human behavior following rules obtained from his famous periodic table. Pavlov, Freud, and Skinner tried to describe both behavior and mental states in accordance with their respective discoveries. Even Ramón y Cajal assumed that interneuronal communication took place similarly to the electrical devices (accumulators, induction coils) available in his time, and compared neuronal connectivity to the emerging telephonic system. We are still in a similar tessitura: "if we ever do find some powerful generalization that applies to brains in particular then it will probably not be about brains as a piece of biology but about brains as computing entities" (Guillery, 2005). Although it is possible that in the near future our industrial products look as similar to us as sons are similar to their fathers, it is evident that this metaphoric approach (in the absence of appropriate coefficients and quantifications) seems not very productive in the mid or long term.

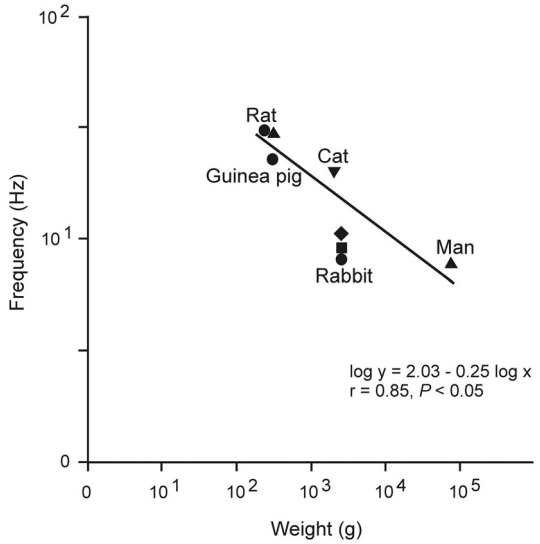

FIGURE 5 | Relationship between mean body weight and the dominant frequency of eyelid responses for different species. Plot illustrates data obtained from rats, guinea pigs, cats, rabbits, and humans. These data suggest that lid biomechanics is tuned to the weight and viscoelastic properties of the lid. Note that the slope of this relationship (−0.25) is the same as that characterizing heart rate and body mass (in kg) in mammals. Taken with permission from Gruart et al. (2000).

Have contemporary Neuroscience feet of clay? Parodying the title of Ramón y Cajal's major book, the brain is a text from which we know precisely its texture (thanks to his contributions, as well as to those of many other neuroscientists), but we are still incapable of understanding its semantics.

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frontiers in Neuroanatomy

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The discovery of dendritic spines by Cajal

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Dendritic spines were considered an artifact of the Golgi method until a brash Spanish histologist, Santiago Ramón y Cajal, bet his scientific career arguing that they were indeed real, correctly deducing their key role in mediating synaptic connectivity. This article reviews the historical context of the discovery of spines and the reasons behind Cajal's obsession with them, all the way till his deathbed.

Keywords: dendritic spines, Cajal, Golgi, cortex, cerebellum

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Our story starts the spring of 1888, in Barcelona. At that time, this progressive and international city was undergoing a febrile creative period in literature, arts, architecture and industrial development, taking the leadership in the creation of modern Spain. A similar revolution was occurring in the relative quiet and obscurity of the Department of Histology of its Medical School, in the laboratory of Santiago Ramón y Cajal, a newly arrived Professor of Histology and Pathological Anatomy, who was starting his scientific career after a relatively tumultuous youth.

One of Cajal's personality traits was his strength of character. Indeed, Cajal was Aragonese and, in the popular culture of Spain, Aragonese and other northern Spaniards are considered to be single-minded and persistent. This is captured in a tale of an Aragonese farmer ("baturro") riding his donkey on the train tracks and, when faced with an incoming train at full speed and blowing its whistle to warn him, tells the train that "blow as much as you want, but you are the one who needs to step out of the tracks." Single-mindedness and persistence were combined in Cajal with a superb intuition and observation capabilities. Cajal himself credited his scientific successes not to his intelligence, education or training, but instead to his "will power," combined with good experimental techniques, laboriousness and plain common sense (Ramón y Cajal, 1923).

On May 1st, precisely on the day of his 36th birthday, Cajal published a monograph entitled "Estructura de los centros nerviosos de las aves" (Structure of the Nervous Centers in Birds) in the first issue of a journal that he himself produced, edited, and financed (Ramón y Cajal, 1888). As he later wrote, the publication of this journal used up all his savings and prevented him and his wife from affording household help to care for their five children (Ramón y Cajal, 1923). In his monograph, a brief communication with two figures, Cajal described the application the Golgi stain to the cerebellum of birds. Cajal had just been taught the Golgi staining method by his friend Simarro in Madrid, who himself had recently learnt it from Ranvier in Paris, one of the premier neuroanatomists of the time (Fernandez and Breathnach, 2001). The Golgi impregnation enabled, for the first time, the relatively complete staining of the dendritic trees of neurons and is even today still widely used for the morphological analysis of dendrites. To a greater extent than any of his peers, Cajal had been struck by the power of the Golgi technique, particularly when applied to the developing nervous system, to reveal neuronal morphologies. In this brief article, Cajal noted that the surfaces of Purkinje cells were covered with small protrusions, which he called "espinas," (i.e., "spines," as in the spines of a rose, or "thorns"). In his own words: "…*Also, the surface of the Purkinje cells dendrites appear ruffled with thorns or short spines, which in the terminal dendrites look like light protrusions. Early on we thought that these eminences were the result of a tumultuous*

precipitation of the silver; but the constancy of their existence and its presence even in preparations where the staining appears with great delicacy in the remaining elements, incline us to consider them as a normal disposition." (Ramón y Cajal, 1888; translation by the author) (**Figures 1**, **2**).

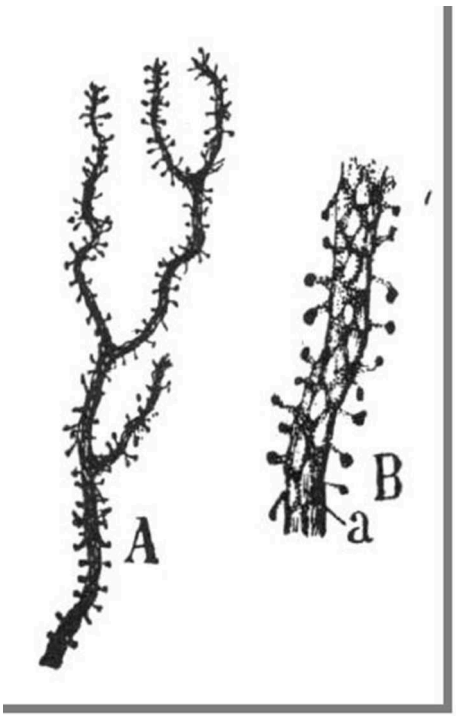

FIGURE 1 | Original illustrations from Cajal, displaying dendritic spines from a cerebellar Purkinje cell, as drawn from Golgi material (Ramón y Cajal, 1899c). Reproduced with permission from "Herederos de Santiago Ramón y Cajal."

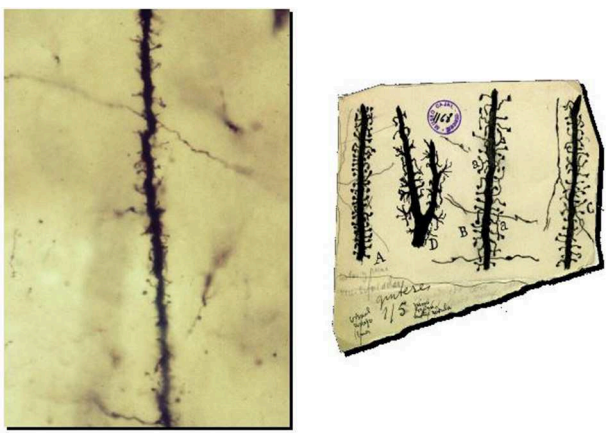

FIGURE 2 | Preparation and drawings of Cajal illustrating spines. Left: Photomicrograph of a dendrite of pyramidal neuron from one of Cajal's original preparations (Courtesy of Cajal Institute in Madrid). **Right:** Cajal drawings of spines from rabbit (A), 2 month old child (B), one month old cat (C) and cat spinal motoneuron (D). Reproduced with permission from "Herederos de Santiago Ramón y Cajal."

In this relatively brief communication, written in Spanish, a language not commonly used by international scientists, spines were described and named for the first time. In this same publication Cajal could not confirm the presence of anastomoses between axons and dendrites, hypothesized to exist by Golgi and other investigators, and proposed that neurons are independent units in the nervous system. This assertion, in agreement with ideas from other investigators (Bock, 2013), laid the basis of the "neuron doctrine," an opposing hypothesis to Golgi's established "reticular theory," in which neurons would form a continuous network of physically joined cells (see Shepherd, 1991). Thus, in the same publication, he changed the core of Neuroscience, with two fundamental and apparently unrelated observations: neurons are independent from each other and are covered with spines. In his later career he will proceed to link both facts into our modern conception of the brain.

To put this 1888 study in perspective, it should be noted that Cajal was not the first one to use the Golgi method and also not the first to observe spines. Other investigators, like Kolliker, Dogiel, Meyer and even Golgi himself, more established than Cajal and working in well-recognized centers of anatomical research at the time, had observed spines before him. However, these researchers regarded spines as fixation artifacts or silver precipitates outside the neurons, and in their scientific publications they drew neurons with smooth dendritic trees, devoid of spines. But even today, spines are still clearly visible in Camilo Golgi's original preparations (Purpura, pers. comm.), so Golgi must have to ignore them since he drew neurons with smooth surfaces. This was not such an unreasonable choice considering that the Golgi method is notoriously capricious and variable in results. Still today, it is poorly understood how exactly Golgi impregnations work. To make things more confusing, other observable structures on the surface of neurons, such as dendritic varicosities, were thought to be artifactual by Cajal himself (Ramón y Cajal, 1904). So it is understandable how Cajal's proposal that spines were real structures was met with skepticism.

Rather than buckle under the pressure of his contemporaries, and perhaps shielded from them due to his relative isolation, far from the centers of scientific inquiry of his time, Cajal pressed on in his studies on the structure of spines, in a flurry of publications that followed. Shortly afterwards, Cajal revealed that spines are not particular to birds but are also present in the dendrites of many neurons of the cerebral cortex of mammals (Ramón y Cajal, 1891b). Importantly, he speculated that spines must receive axonal inputs from other neurons, and thus serve as the main point of contact between axons and dendrites (Ramón y Cajal, 1894). This is the point where his neuronal doctrine came full circle: neurons are independent from each other but (at least those in the cerebral cortex) they connect to one another through their axons and spines.

Being curious and inquisitive, Cajal wondered what was the advantage of using spines as recipient sites for axonal connections, given that axons could in principle connect directly to the dendritic trunk. He proposed the idea that spines would greatly extend the surface of the dendrites, and therefore dramatically increase their capability to receive axons. This hypothesis was based on the comparison between spines and intestinal

villi, where a highly branched structure increases the surface area of the cell. In addition, Cajal proposed that physical changes in spines could be associated with neuronal function and learning (Ramón y Cajal, 1891a, 1893). Imagining that his histological preparations were still alive, he argued that, in the living animal, spines could move and change, growing with activity and retracting during inactivity or sleep. So physical movements of the spines could be capable of connecting or disconnecting neurons. As he put it "*Since it seems rather likely that the named spines represent points of charge or of current gathering, their retraction (which in this fashion would isolate them from the terminal nerve fibers, with which they are in contact) would give rise to the individualization or separation of neurons*" (Ramón y Cajal, 1899c). Indeed, one of the most exciting recent findings has been the discovery that spines are not stable structures, but are constantly moving and experience morphological plasticity *in vivo* and *in vitro* (**Figure 3**) (Fischer et al., 1998; Dunaevsky et al., 1999). Therefore, although Cajal's intuition that spines can connect and disconnect during the day cycle has not yet been demonstrated, the general idea that spines are morphologically plastic is still central to the study of the function of spines.

In 1896, partly to defend himself from attacks that his so-called spines were artifacts of the Golgi method and did not appear with other staining procedures, Cajal extended his Golgi observations of spines using a different method, the Ehrlich methylene-blue stain (Ramón y Cajal, 1896a,b). In this publication, he refined this technique and showed that it could also reveal spine morphologies, when properly used.

In subsequent years, Cajal described with great detail spines in motor, visual, auditory and olfactory human cortices (Ramón y Cajal, 1899a,b, 1900a,b). In 1899, he summarized many of his observations on his book "Histology of the Nervous System of Man and Vertebrates," where he restated his view that spines increase the surface area of dendrites and thus serve as site of contacts between dendrites and axons. In an additional effort to convince his colleagues, he collected together all his arguments that spines were not artifactual, because:

1. Spines are shown by different methods, like Golgi, Cox or methylene blue stains.
2. They always arise in the same position of the neuron, from the same regions of the brain.
3. Spines are never or rarely found in certain parts of the neuron (like the axon, soma or initial dendrites).
4. Spines do not resemble crystal deposits when viewed with higher power objectives.
5. Spine pedicles (necks) can be occasionally detected.
6. Spines can be stained by neurofibrillary methods.

Moreover, noting that cells from more highly evolved animals have more spines, he argued that spines were probably related to intelligence (Ramón y Cajal, 1899c, 1904).

Finally, in one of his last contributions to the problem, Cajal discussed which axons specifically contact spines (Ramón y Cajal, 1933). Cajal argues that spines can be contacted by different types of axons. According to him, in cortical pyramidal neurons, spines can be contacted by: (i) axonal collaterals from other pyramidal

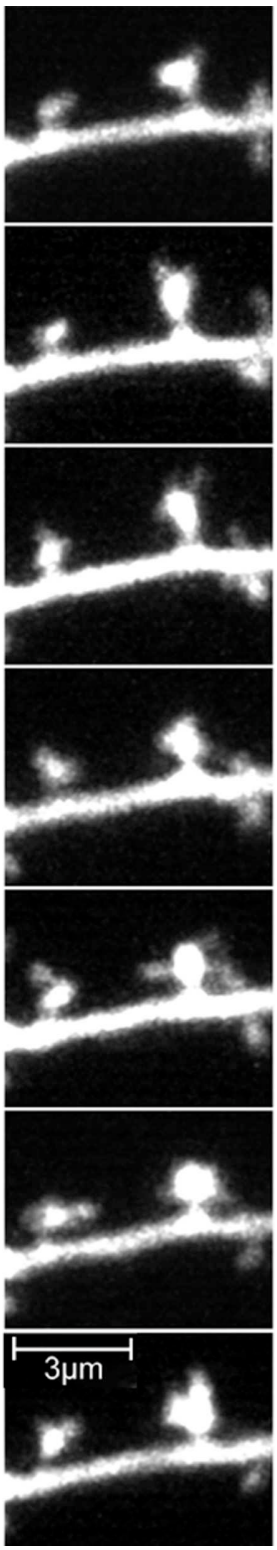

FIGURE 3 | Spine morphological plasticity in a pyramidal neuron. Frames from a two-photon movie of 8.5 min duration of GFP labeled pyramidal neurons of postnatal cortical brain slice from a postnatal mouse. Note the large morphological plasticity of the spines. Reprinted with permission from (Portera-Cailliau et al., 2003).

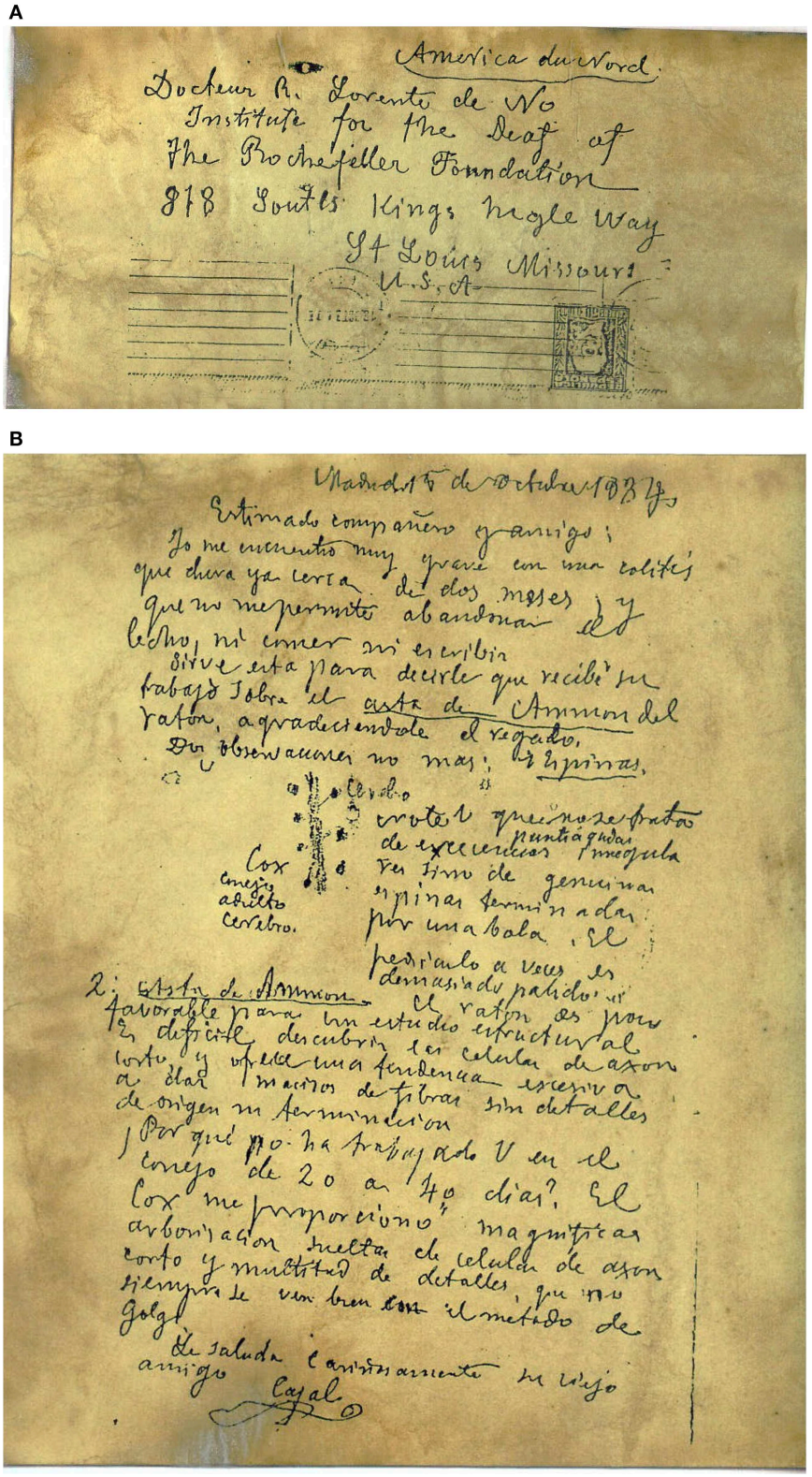

FIGURE 4 | Letter from Cajal to Lorente de Nó. (A) Envelope addressed to R. Lorente de Nó, Institute of the Deaf at The Rockefeller Foundation in St. Louis, Missouri, USA. **(B)** Manuscript letter. Note the drawing of dendritic spines. Paragraph is translated in the text. Reproduced with permission from "Herederos de Santiago Ramón y Cajal."

cells, (ii) axons from some interneurons (Golgi type II cells), and (iii) axons from other associative neurons.

Cajal was obsessed with spines, and he undertook a personal crusade, pretty much alone and till his deathbed, to convince his peers that spines were not only real, but also crucially important. Indeed, on his deathbed, Cajal was still arguing about spines. In a letter in shaky handwriting to his disciple Lorente de Nó on October 15th, 1934, 2 days before he died (**Figure 4**), after reporting that he is so sick that he cannot leave his bed or work anymore, he advises Lorente to pay close attention to spines. He writes: "*.....Note that spines are not irregular protrusions but instead genuine spines ending in a ball. The neck is sometimes too lightly stained …*" (Copy of autograph letter to Lorente, courtesy of Dr. Francisco Alvarez, Creighton University, translation by the author).

In spite of this string of arguments and the combined weight of his evidence, Cajal's conclusions were not readily accepted. Eventually, many of his contemporaries, such as Retzius, Schaffer, Edinger, Azolay, Berkley, Monti, and Stefanowska came to agree with him and confirm their appearance in their preparations.

At the same time, not much work was carried out on spines and Cajal's proposal of the role of spines in connecting axons and dendrites would have to wait till midcentury for its confirmation. This occurred by the introduction of a new technology, electron microscopy, which enabled the visualization of the fine structure of cells with unprecedented spatial resolution. Indeed, in the 1950s, De Robertis and Palay performed the first ultrastructural analysis of synapses (DeRobertis and Bennett, 1955; Palay, 1956) and shortly afterwards, synapses were demonstrated on spines (Gray, 1959a,b). Cajal was proven correct and spines became a bona-fide topic of interest for neurobiological studies.

Since the 1950's, each decade has brought along an increased number of studies of spines, with a recent acceleration of studies published since 1990. Nevertheless, the specific function of the spine, more than a hundred after their discovery, is still subject to great debate and many different hypotheses have been proposed (Peters and Kaiserman-Abramof, 1970; Swindale, 1981; Harris and Kater, 1994; Shepherd, 1996; Harris, 1999; Yuste et al., 2000; Yuste and Majewska, 2001; Alvarez and Sabatini, 2007; Bourne and Harris, 2008). Our knowledge of spine morphology, ultrastructure, biochemistry, development, dynamics, calcium compartmentalization, biophysical properties and electrophysiology, has exploded. This rich phenomenology has opened up many questions related to spines, indicating their importance. As Cajal wrote "the future will prove the great physiological role played by the spines" (Ramón y Cajal, 1904).

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frontiers in Neuroanatomy

REVIEW
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The discovery of the growth cone and its influence on the study of axon guidance

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For over a century, there has been a great deal of interest in understanding how neural connectivity is established during development and regeneration. Interest in the latter arises from the possibility that knowledge of this process can be used to re-establish lost connections after lesion or neurodegeneration. At the end of the XIX century, Santiago Ramón y Cajal discovered that the distal tip of growing axons contained a structure that he called the growth cone. He proposed that this structure enabled the axon's oriented growth in response to attractants, now known as chemotropic molecules. He further proposed that the physical properties of the surrounding tissues could influence the growth cone and the direction of growth. This seminal discovery afforded a plausible explanation for directed axonal growth and has led to the discovery of axon guidance mechanisms that include diffusible attractants and repellants and guidance cues anchored to cell membranes or extracellular matrix. In this review the major events in the development of this field are discussed.

Keywords: chemotropic, neurotropic, brain, development

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An Influential and Unsuspected Discovery

In the study of nature, great discoveries are often made in the context of heated debates regarding alternate explanations for biological phenomena. On other occasions, discoveries reveal hidden or previously unnoticed aspects of the systems under study. The latter is the case for the discovery of the growth cone, a structure that would strongly influence neuroscientific work, particularly neurodevelopmental research. This structure, whose existence was unsuspected, is located at the distal tips of growing axons. It was discovered by Santiago Ramón y Cajal in 1890 when he delved into the study of chick embryos in his search for evidence supporting the neuron doctrine, then at the center of a raging controversy among the most prominent neuroscientists. This discovery attests to the keen analytical eye of Cajal and shortly afterwards he put it into the context of the "neurotropic" hypothesis that played an instrumental role in settling the rival "neuronist" and "reticularist" theories for the organization of the nervous system (Guillery, 2007). The discovery of the growth cone prompted new lines of research, but the hypothesis exerted its most profound influence on neurodevelopmental research only about a hundred years later.

In this essay, we will review the major developments in the field of axon guidance into which the concept of the growth cone was inserted, and we will discuss its influence on the field. Various and very through historical accounts of the discovery of the growth cone have been written in recent years (Sotelo, 2002; de Castro et al., 2007; Garcia-Marin et al., 2009); here the focus will be on the development and evolution of the concept of growth cone and its influence on

neuroscientific thought and knowledge. The chronology of the major events, mainly related to axon guidance during embryonic development, will be maintained throughout this brief essay; parallel developments, however, will sometimes be described in consecutive sections.

Neuroscientific Research at the Time of the Discovery of the Growth Cone

The idea that axons were part of nerve cells and that these cells were the individual morphological and functional units that composed the nervous system, was put forward by His and Forel in 1887 (Cajal, 1907; Partsalis et al., 2013). His derived his ideas from developmental studies in which he observed the continuity from post-mitotic neurons to growing axons (Sotelo, 2002). Forel, in turn, observed atrophy of cranial motor neurons after severing their nerve roots (Sotelo, 2002). This notion contrasted with the prevalent view at the time that the nervous system consisted of a group of nerve cells connected in a "continuous network" which, in turn, derived from previous discoveries made in the context of the "cell theory", in which muscle cells were recognized as multinucleated cellular entities (Guillery, 2007).

The work of His, Forel, Gower, Kollicker, Retzius, Gehutchen, von Lenhossek, Nansen and Cajal himself contributed to the neuron doctrine which was formally enunciated by Waldeyer-Hartz in 1891 (Guillery, 2007). It viewed the neuron as the structural, ontogenetic, functional and trophic unit of nervous tissue and further stated that individual neurons communicated via contiguous interactions and not by fusion between them. This idea was later complemented by Cajal and Van Gehutchen's notion of dynamic polarization which recognized dendrites and cell bodies as the receptive portion of nerve cells and the axon as their effector component. In contrast, in the context of the reticularistic theory, the nervous system was viewed as a continuous system in which all nerve cells anastomosed into a single network (Guillery, 2007).

These opposing views of the structure and workings of the nervous system, confronted each other at both the morphological and functional levels, but they also needed adequate explanations at the ontogenetic level. That is, accurate descriptions of the structure of the nervous system required consistent mechanisms of the way in which it is formed during embryonic development. While the reticular theory invoked fusion of individual cellular units to generate a fully anatomosed nervous system (Hensen's "catenary" or "polygenic" theory) (Cajal, 1929a; Partsalis et al., 2013), the neuronist theory proposed that axons grew out of the neuronal cell bodies and reached other neurons but never fused with them. It was in this atmosphere that the discovery of the growth cone was made.

Enter the Growth Cone

The idea that axons grew out of nerve cells was generally accepted at that time and stemmed primarily from the influential work of His. However, there was neither a paradigm of directional growth nor any idea that the end of the growing axons could respond to any feature of the tissue it traversed. Cajal turned to the study of chick embryo preparations seeking evidence in support of what later would be called the "neuronistic" view of the organization of nerve tissue, as stated above. This was only possible by using the silver staining method of Golgi with improvements by Cajal (for a detailed account of the development of these methods see (Garcia-Marin et al., 2009)). Since it was found that myelination limited the reach of the Golgi method, Cajal worked with younger and younger embryos. This led him to study spinal cord preparations of three- and four-day old chick embryos in which he described the stereotypical behavior of growing axons (**Figure 1**). He soon observed that the distal tip of the axons of commissural neurons widened into a "cone-like lump with a peripheral base" decorated by triangular or short thorny processes (Cajal, 1890). This description refers to what is currently considered the main morphological feature of a growth cone, the widened end of a growing axon with lamellipodia and filopodia at its leading edge (**Figure 2**). Using complementary information obtained by two histological methods, the Golgi method and the reduced silver nitrate method, Cajal was able to identify with more detail the structure of the growth cone, thus describing it as containing a "neurofibrillar bundle", which is now known to be composed of actin filaments (AFs) and microtubules (**Figure 3**; Garcia-Marin et al., 2009).

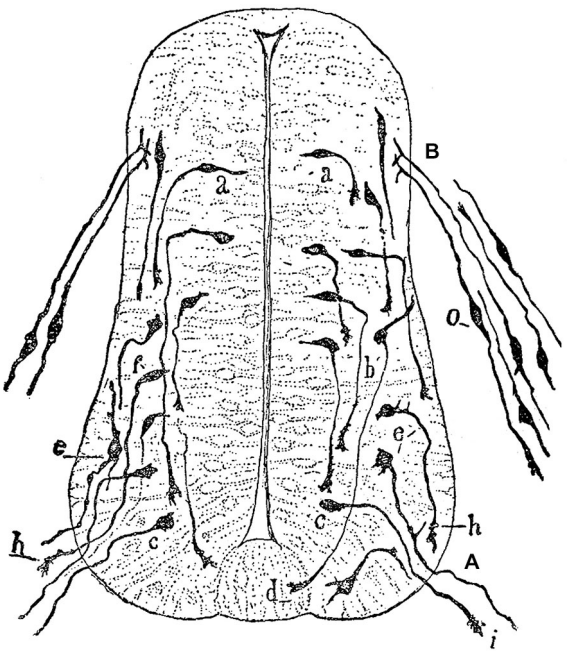

FIGURE 1 | Drawing by Cajal of a spinal cord section of a three-day old chick embryo. **(A)** Ventral nerve root; **(B)** Posterior nerve root; **(a)**, young nerve cells; **(b)**, more developed cells that probably correspond to commissural neurons; **(c)** piriform cell of the ventral nerve root; **(d)** growth cone of a commissural axon; **(e)** radicular cell with rudiments of protoplasmic branches; **(h,i)** growth cones of ventral nerve root axons; **(o)** dorsal root ganglion cells. Figure taken and legend translated from Cajal (1929b).

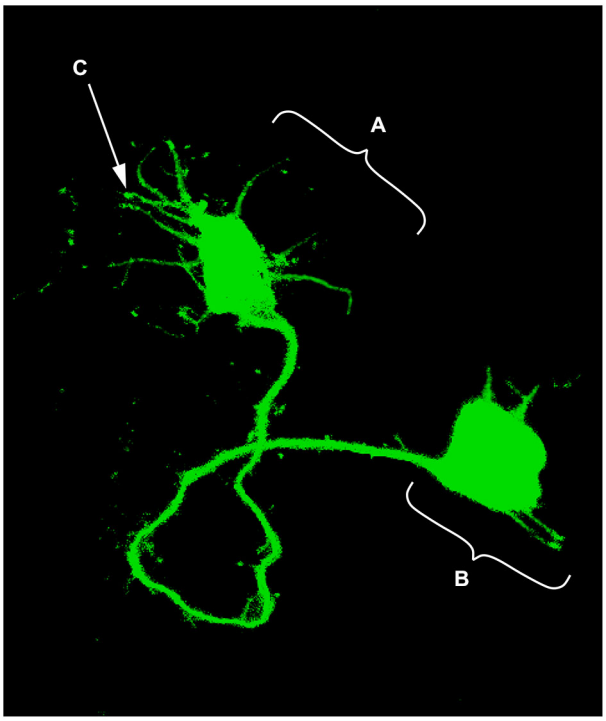

FIGURE 2 | Single neuron electroporated with a green fluorescent protein (GFP) vector in an embryonic chick hindbrain. **(A)** Growth cone; **(B)** Neuronal soma; **(C)** Filopodia (digitally enhanced for better visualization).

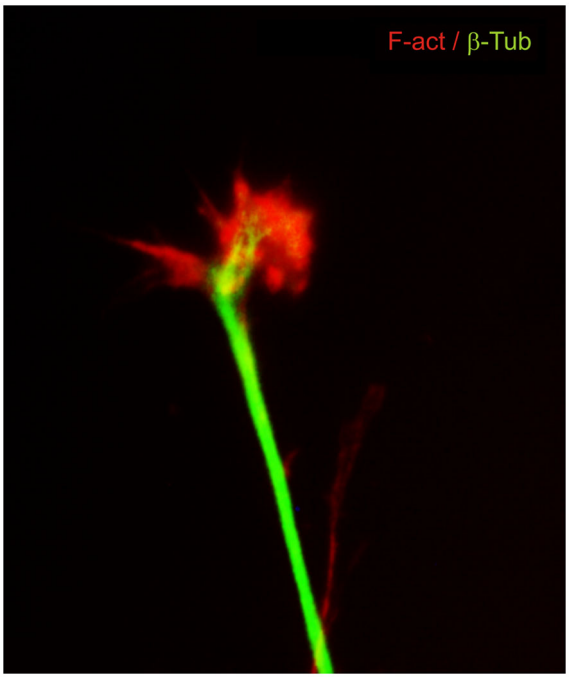

FIGURE 3 | Dorsal root ganglion neuron in culture immunostained for cytoskeletal components. β-III tubulin microtubules (green) are located mainly in the axon shaft and in the central part of the growth cone. F-actin filaments (red) are located at the leading edge and in the lamellipodia and filopodia.

It was thus that Cajal, walking the untrodden path of scientific inquiry in the central nervous system, identified a structure which he referred to using various names, eventually settling on the term "growth cone".

The Chemotactic or Neurotropic Hypothesis

Concomitant with the discovery of the growth cone, Cajal observed that the behavior of growing nerve fibers was reproducible but depended on their origin: commissural axons navigated ventrally from the dorsal aspect of the spinal cord and across to its contralateral side, axons of motor neurons exited ventrally, and fibers of neurons from the dorsal root ganglia entered into the dorsal spinal region (**Figure 1**). These observations led Cajal to consider that nerve fibers "adopt predetermined directions and establish connections with defined neural or extra neural elements... without deviations or errors, as if guided by an intelligent force" (Cajal, 1890).

On occasion of a cholera outbreak in Valencia in 1885 where he was then Professor at the University of Valencia, Cajal had a brief but fruitful excursion into microbiology which acquainted him with contemporary knowledge regarding the oriented movement of leucocytes toward bacteria guided by gradients of substances produced by the latter (Sotelo, 2002). This is thought to have strongly influenced and inspired the 1890 ideas of directed axonal growth in response to chemical signals that diffused from their targets that he would later include in formulating the neurotropic hypothesis. In the early stages of the formulation of his postulates regarding axon growth, he envisioned diffusible attractive and repulsive signals that impinged upon growth cones thus determining their direction of growth. In later and more mature descriptions, the repellants were not part of the hypothesis. Moreover, based on the observation of the morphological diversity of growth cones in different regions of the spinal cord, Cajal proposed that the growth cone was motile and responsive to the nature of the substrate, which also determined the route axons would take as they extended (Cajal, 1899; Garcia-Marin et al., 2009). Both components of his postulate, the response to graded, diffusible chemical signals and the interaction with the substrate, would, in time, prove to be very accurate.

At a scientific meeting in the same year that Cajal first described the growth cone, and also based on observations in embryonic chick tissue, Michael von Lenhossek proposed that the "free-end" of growing nerve cell fibers were endowed by a "miraculous energy" that allowed them to extend along their pathway (de Castro et al., 2007). Although no mention was made of a specialized structure at the fiber end, the general interpretation of its relevance in the directed growth of nerve cell fibers coincided with that of Cajal.

Cajal's remarkable ability to extract mechanistic explanations of the directed growth of axons based on snapshots of

developmental tissue preparations may be difficult to appreciate today. It is a great achievement of his acute observation ability. This hypothesis led Cajal and others to study the neurotropic mechanisms that were paramount in his postulate (de Castro et al., 2007). As embryonic tissue did not allow him to obtain the proof he wanted, he turned instead to the study of regenerating peripheral axons. In this work, Cajal succeeded in demonstrating that regenerating axons grew towards grafted nerves only when living Schwann cells were present and not when these nerves had been treated with chloroform, thus killing the cells (Sotelo, 2002). Although these findings provided support for the neurotropic hypothesis, the definitive proof would come only about one hundred years later.

Immediate Effects of the Neurotropic Hypothesis

The neurotropic hypothesis, with the growth cone at its vanguard, significantly strengthened Cajal's argument for the validity of the neuron theory. It not only provided a mechanistic view of how nerve connections are established between individual cellular units, but it also consolidated the view of the nerve cell, the neuron, as first named by Waldeyer, as the basic structural and functional unit of the nervous tissue. Although definitive proof of the neuron theory was only obtained half a century later by Estable and DeRobertis (Guillery, 2007), it was a conceptual leap that provided the neuron as a useful reductionist tool that persists in most areas of present day neuroscientific research. The neuron view, in a subsequent turn of the wheel, fueled developmental studies that addressed the behavior of growing fibers from individual nerve cells, which were by then considered to remain independent even as they established contacts with other neurons or muscles through structures which would later be named "synaptic contacts" by Sherrington (1906).

Study of the Growth Cone without the Knowledge of Chemotropic Effectors

After the original proposal, the neurotropic hypothesis languished as no evidence was produced which fully confirmed the notion of the "diffusible signals" envisioned by Cajal. The growth cone, however, continued to be a useful concept intimately associated with the observed behavior of growing axons. It was thus during this apparent impasse, that different neuroscientists initiated various lines of research into aspects of the growth cone, such as its behavior, morphology, ultrastructure, dynamics, molecular composition and interaction with the substrate.

Observation of neuron fiber outgrowth was not possible until the first decade of the twentieth century when Ross Harrison developed a culture method for neural tissue explants (Harrison, 1910). He observed that the elongation of processes from the leading edge of the growth cone is involved in the motility of the structure and described two phenomena in nerve development, "(a) the formation of the primitive nerve fiber through extension of the neuroblastic protoplasm into a filament—protoplasmic movement; (b) the formation of the neurofibrillae within the filament—tissue differentiation…" (Harrison, 1910; Keshishian, 2004). This evidence finally confirmed that axons elongate from a single cell body, further supporting the neuron doctrine of Cajal and setting aside the syncytial theory of Victor Hensen and that of the Schwann cell origin of the axons held by Theodor Schwann (Garcia-Marin et al., 2009). This secured the growth cone as the leading actor in axon outgrowth.

Since the methods used in the early studies with cultured nervous tissue only allowed the growth of axons on solid substrates while submerged in liquid medium, research on axon guidance went mostly into the analysis of the interaction of axons and growth cones with the substrate relegating the chemotropic hypothesis to the background for decades. This led Weiss in 1941 (Weiss, 1941) to propose the "contact guidance" hypothesis, which postulated the need for a contact surface with specific matching between the axons and their substrate situated in the intercellular matrix immediately adjoining cells but not at a distance. Several reports thereafter focused on describing the motility of the growth cones. Phase-contrast microscopy and time-lapse imaging improved the observation of neuron outgrowth (Pomerat, 1951), and *in vivo* observations suggested similarities of growth cone displacement with that of pseudopodia of amoeba and macrophages (Pomerat, 1951; Hughes, 1953; Nakai, 1956). Electron microscopy of projections from dorsal root ganglia entering the neural tube of rabbit embryos, allowed the filamentous contents of axons and growth cones to be observed, revealing large spindle-shaped varicosities containing smooth reticulum, mitochondria, dense bodies, neurofilaments and microfilaments (Tennyson, 1970). This description supported Cajal's findings, in Golgi silver-impregnated dorsal root ganglion, of neurons entering the spinal cord (Cajal, 1909).

Moreover, electron microscopy revealed the first detailed distribution of cytoskeletal components within the growth cone, with neurofilaments in its central area and in the microspikes or filopodia; microtubules were mainly found in the axon with a few protruding into the growth cone (Yamada et al., 1970). A fine filamentous network was also described in the lamelipodia and filopodia and occasionally thin microtubule (MT) filaments were observed invading the filopodia (Yamada et al., 1970; Yamada and Wessells, 1971). In the same reports, and using inhibitors of polymerization of cytoskeletal components such as cytochalasin B and colchicine, Yamada and co-workers showed that inhibition of AF polymerization induced retraction of growth cones while high concentrations of colchicine inhibited MT depolymerization and eventually induced axon retraction. This demonstrated for the first time the relevance of AF and MT polymerization in growth cone formation and neurite outgrowth (Yamada et al., 1970; Yamada and Wessells, 1971). These results led to the conclusion that AF are important for growth cone shape and that MT are essential for axon structure, thus opening the field to a molecular explanation of growth cone motility.

The Second Age of the Neurotropic Hypothesis and the Identification of Chemotropic Molecules

In parallel to the advances on the cytoskeletal dynamics of the growth cone, the neurotropic hypothesis has recently experienced a revival. The first major development that marked this revival was the finding by Andrew Lumsden and Alun Davies of a signal that elicited and attracted the growth of neurites from trigeminal sensory axons (Lumsden and Davies, 1986). In keeping with one of the original postulates of Cajal, the final target, in this case the whisker pad epithelium, exerted the described effect on the sensory neurons that innervate this tissue. Soon thereafter, technological advances including the great analytical power of biochemistry and molecular biology and the ability to manipulate embryonic tissue *in vitro* and *in vivo*, allowed the first truly chemotropic molecules for growing axons to be discovered. Using the same culture method devised by Lumsden and Davies, Tessier-Lavigne and collaborators detected, in the floor plate of chick embryos, diffusible signals that attracted commissural axons (Tessier-Lavigne et al., 1988). The attractive signal was identified a few years later as a member of a family of proteins named netrins by the same group (Kennedy et al., 1994; Serafini et al., 1994). Since then, more protein families have been identified that have chemotropic effects on many different axon types in invertebrates and vertebrates. These include semaphorins (the first family known to include chemorepellants), slits, and some proteins that were previously known to have other biological effects, such as Shh, FGF8, and HGF (Tessier-Lavigne and Goodman, 1996; Varela-Echavarría and Guthrie, 1997; Huber et al., 2003). The far-reaching findings that have been made from the study of these axon guidance molecules include: that among them there are both attractants and repellants, that the same molecule may have both effects on different axon types, that the same axon type responds specifically to different guidance cues and that its ability to respond to these cues changes during development. Moreover, for each family of guidance cues, the receptor complexes that allow the growth cone to detect the signals and the main components of the intracellular cascades involved in the axon response have been identified (Huber et al., 2003; Bashaw and Klein, 2010).

Interaction of the Growth Cone with Signals Anchored to the Substrate

Numerous studies have also revealed that responses to chemotropic guidance cues necessarily involve interaction of the growth cone with the substrate, thus confirming one of Cajal's ideas regarding nerve cell fiber growth. Families such as cell adhesion proteins of the immunoglobulin superfamily (IgCAMs), integrins, and extracellular matrix proteins such as fibronectins, laminins and thrombospondins mediate the anchoring of axons and growth cones to the substrate during their growth and elicit cytoskeletal responses (Neugebauer et al., 1991; Osterhout et al., 1992; Myers et al., 2011). For example, during growth cone movements, weak interactions with the substrate result in a slow, forward movement of cytoskeletal components, high retrograde flow, and no tension at the adhesion sites (Suter and Forscher, 2000). In contrast, strong adhesion mediated by NCAM, N-cadherin and integrins, translates the myosin-driven AF flow into forward growth cone movement which, in turn, attenuates retrograde flow and enhances the actomyosin-mediated traction force pulling the growth cone forward (Suter and Forscher, 2000; Suter and Miller, 2011). In this sense, the adhesion of the growth cone to the substrate also mediates the responses to guidance cues; repulsive effects of the neurotropic proteins Slit via their receptors Robo, inhibit N-cadherin-mediated cell adhesion (Rhee et al., 2002). On the other hand, several lines of evidence have shown that DCC, a netrin-1 receptor, interacts directly with kinases involved in regulating adhesion, such as FAK, Src and Fyn and that their disruption blocks axon attraction and turning in response to netrin1 (Liu et al., 2004; Ren et al., 2004). Other evidence shows a relationship between the adhesion proteins integrins and L1 with Plexins and Neuropilins, which are components of the receptor complexes of semaphorins (Castellani et al., 2002; Barberis et al., 2004; Valdembri et al., 2009; Seerapu et al., 2013).

A particular type of interaction between the growth cone and its target cells was envisioned by Sperry in 1963 as part of the "chemoaffinity hypothesis" that had been proposed earlier and had matured for over two decades (Sperry, 1963). He proposed that axons and neurons carry chemical tags that endow them with specific affinities for other neurons thus mediating selective "attachment" to them. Furthermore, the hypothesis also included gradients of chemical signals that explained the topographic projection of retinal axons into the tectum. A molecular explanation of the evidence supporting this hypothesis came with the discovery of ephrins and their receptors, both of which are membrane- anchored proteins that fit the definition for the chemical graded tags of Sperry's postulates (Cheng et al., 1995; Drescher et al., 1995).

From the Molecular Basis of Growth Cone Motility to Mechanistic Explanations of its Response to Guidance Cues

Several decades of research on the growth cone cytoskeleton have revealed the relevance of the dynamic interplay between AF and MT during axon growth. Immunofluorescent techniques and optical resolution methods have confirmed the differential distribution of cytoskeletal components at the growth cone and have provided exquisite details of the structure and function of the cytoskeleton during dynamic behavior (Gomez and Letourneau, 2014).

Based on these studies, three main regions with differential distribution of AF and MT are currently recognized in the growth cone and can be visualized by Differential Interference Contrast (DIC) microscopy. The peripheral region (P), composed of filopodia and lamellipodia, is characterized as an AF-rich region with filaments assembling at the filopodial tips and a meshwork-like array of AF in the lamellipodia. The transitional domain (T) contains arc-like AF structures delimiting the central domain (C) and some advancing MT overlapping with AF. The C region is rich in MT with their polymerization-plus ends facing the T zone

and with punctate F-actin distribution (Schaefer et al., 2002; Dent and Gertler, 2003; Suter and Miller, 2011). Abundant evidence has shown that interactions between AF and MT in the growth cones are essential for axon outgrowth, growth cone turning, and response to guidance cues for pathfinding and branching (Letourneau et al., 1987; Challacombe et al., 1996; Dent and Kalil, 2001; Buck and Zheng, 2002; Schaefer et al., 2002, 2008; Lee and Suter, 2008; Geraldo and Gordon-Weeks, 2009; Suter and Miller, 2011).

The studies on the dynamics of the growth cone cytoskeleton have also contributed to our understanding of the molecular mechanisms underlying the response to guidance cues. Early reports using Ti1 neurons of limb buds from embryonic grasshopper first described the role of actin polymerization for oriented projection of the growth cone (Bentley and Toroian-Raymond, 1986). One year later, Letourneau and co-workers published the seminal concept of the two forces exerted at the growth cone, the "push" of MT and the "pull" of AF, which has been instrumental in understanding growth cone motility and response to guidance cues (Letourneau et al., 1987). At present, it is known that the rate of F-actin polymerization and retrograde actin flow toward the base of the filopodia affects the rate of extension of filopodia and lamellipodia and therefore the translocation of the growth cone (Forscher and Smith, 1988; Lin and Forscher, 1995; Mallavarapu and Mitchison, 1999). ATP-actin monomers are assembled into filaments near the membrane in the distal P region of growth cones, and in a myosin motor-driven process, are transported rearward into the T region, where filaments are severed and ADP-actin monomers are recycled into new polymerizing filaments (Lin and Forscher, 1995; Dent and Gertler, 2003). The rearward transport is linked to interactions with the substrate via cell adhesion and extracellular matrix proteins (Dent and Gertler, 2003; Suter and Miller, 2011). When growth cones turn in response to positive guidance cues, MT selectively invade the axon branches that redirect toward the guidance signal, and a local rearrangement and advance of MT is observed in the filopodia that become stabilized towards the direction of turn (Sabry et al., 1991; Bentley and O'Connor, 1994; Lin and Forscher, 1995). The positive guidance cues polarize the formation of filopodia in the growth cone and stabilize their extensions by decreasing retrograde F-actin flow and depolymerization of AF. Conversely, negative guidance cues inhibit the protrusive activity of AF and MT polymerization at the growth cone (Gallo and Letourneau, 2004).

Unifying Views of Growth Cone Function and its Interaction with the Environment

The concept of growth cone, together with diverse technological and methodological developments in the field, has led to the identification of chemotropic molecules, components of the extracellular matrix, and integral membrane proteins that determine axon pathfinding. In a nutshell, diffusible guidance cues may act as attractants or repellants for axons by impinging upon growth cones, while signals anchored to the substrate may permit, inhibit, or enhance axon growth. Together, the response to these signals, mediated by membrane receptors on the growth cone, is accompanied by changes in cytoskeletal organization of the growth cone and its adhesion to the substrate as it courses through the developing brain (**Figure 4**). Current knowledge confirms that the instructive directional signals of both neurotropic proteins and contact guidance cues are integrated by growth cones, resulting in stereotypical axon route finding (Myers and Gomez, 2011; Bonanomi et al., 2012; Moore et al., 2012; Leung et al., 2013; Garcia-Peña et al., 2014).

Although this picture closely mirrors Cajal's chemotropic hypothesis in several respects, significant diferences between them exist; namely, Cajal proposed that the final targets were the source of attractive signals for growing axons and that "negative neurotropic substances" did not appear to participate in their growth, changing his initial view on the matter (de Castro et al., 2007). While final targets have been shown to exert attractive effects, intermediate targets also secrete chemotropic molecules with essential roles. Moreover, as stated above, chemotropic proteins include repellants as well as attractants.

Future Challenges

Complex axon guidance mechanisms involving several, simultaneous signals have been documented. Many challenges lie ahead, however, as the efforts to understand the control of axon pathfinding have been directed only to a handful of axon types in the developing nervous system. Formidable problems are posed by neuronal types such as noradrenergic neurons of the locus coeruleus whose fibers project into practically all major brain areas. Although the same general principles of axon guidance are likely to operate in the projection to each of these areas, understanding their coordinated projection will require a considerable effort. Moreover, the complexity and dynamics of the substrates traversed by growing axons has made this a more difficult issue to study. In this regard, important facts are that most axons appear to interact with other axons during their route finding (**Figures 4**, **5**), and that the response to chemotropic molecules is modulated by strong interactions with the substrate. For example, we have observed interaction between axons from opposite sides of the developing mouse hindbrain that is relevant for their decussation (Sandoval-Minero and Varela-Echavarría, 2008) and the anatomical coupling of sensory projections to motor axons influences sensory axon projection (Wang et al., 2011). Moreover, our group has recently shown that ascending midbrain dopaminergic axons grow in close apposition to descending GAD65-positive axons and that this interaction appears to participate in stereotypical dopaminergic projection (Garcia-Peña et al., 2014). We also observed that previously known misprojections of dopaminergic axons in embryos lacking the chemotropic molecules Slit1 and Slit2 or their receptors Robo1 and Robo2 (Dugan et al., 2011) were accompanied by severe alterations of the underlying GAD65 axon scaffold. From our results, it follows that the dopaminergic guidance defects in these embryos may be secondary to the alteration in the pre-existing GAD65 scaffold and that many such interactions may be taking place between other axon types throughout the developing brain. Hence, the conclusions of

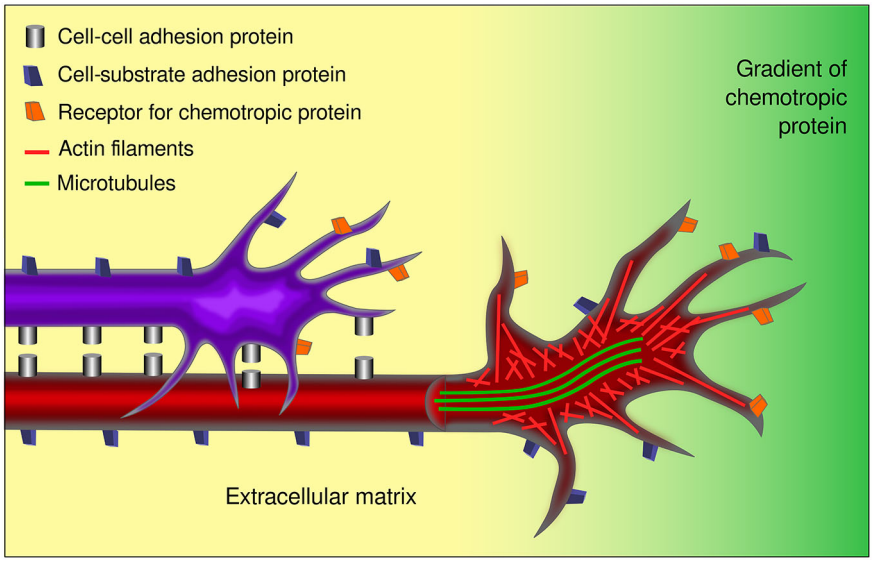

FIGURE 4 | Aspects involved in the response of growth cones to guidance cues. Growth cones respond to several external factors including chemotropic proteins and signals anchored to cell or axon membranes and to the extracellular matrix. Growth cones integrate the information conveyed by the receptors to the various guidance signals inducing the changes in the cytoskeleton associated to axon navigation. The cell-cell and cell-substrate interactions involved, make axon pathfinding a complex and multifactorial event. In the figure, a pioneer axon is shown (red) that interacts with its substrate (yellow) and responds to a chemotropic signal (green gradient). A follower axon (purple) interacts additionally with the pioneer axon. The differential distribution of microtubules and actin are indicated in the open view of the red axon.

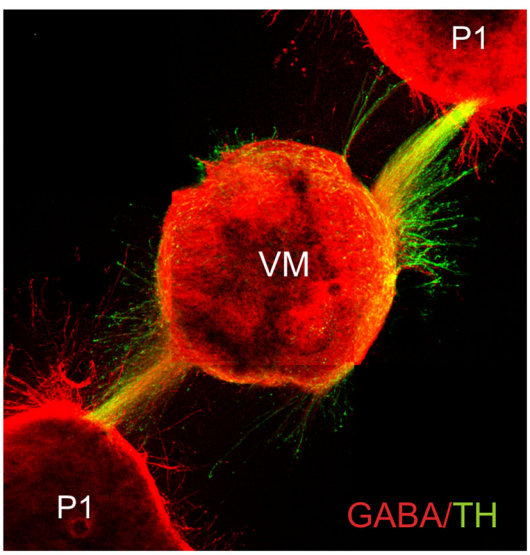

FIGURE 5 | Fasciculated projection of GABA$^+$ and dopaminergic (TH$^+$) axons in culture. E13.5 rat embryo pretectum (P1) explants exert an attractive effect upon axon GABA/TH fascicles growing from ventral midbrain explants (VM) in collagen gel cultures. Cultured gels were immunostained for GABA (red) and TH (green) (Garcia-Peña et al., 2014).

previous studies addressing the role of chemotropic guidance cues in the projection of dopaminergic and other axon types may require re-evaluation to incorporate the effect of the contact-mediated interaction with other axons or cells along their pathway.

On another matter, recent evidence suggests that signals that guide axons can be not only chemical but also physical properties of the substrate such as its nanotopography and stiffness, re-opening the field to what Weiss defined as "selective conduction" (Weiss, 1947). Modern micro- and nanofabrication techniques are contributing to an understanding of how physical cues are involved, since earlier experiments with aligned extracellular components or cells did not allow detection of chemical and physical influences on growth cones and axon growth (Ebendal, 1976; Hynes et al., 1986; Alexander et al., 2006). Substrate stiffness is also an element that impinges on growth cone behavior, as substrate rigidity can affect axon branching and extension (Flanagan et al., 2002; Kostic et al., 2007; Leach et al., 2007). Interestingly, the response to stiffness varies between neuronal types; while substrate stiffness modulates axon growth of DRG neurons, hippocampal neurons seem to be insensitive to it (Koch et al., 2012). How neurons sense rigidity, however, remains unknown and is under intense study (Hoffman-Kim et al., 2010; Moore and Sheetz, 2011; Suter and Miller, 2011; Dupin et al., 2013).

Concluding Remarks

The long history of the growth cone concept has led us to discover many mechanisms involved in axon pathfinding during development. Additional evidence reveals that regenerating axons respond to signals in their environment following the same general principles. This has prompted efforts to use the information derived from developmental studies to elicit and guide the growth of axons in adult models of human diseases in neuro-regenerative medicine (Díaz-Martínez et al., 2013). This

field is poised for important developments with many potential applications and a significant impact on human health.

The discovery of the growth cone and the formulation of the chemotropic hypothesis constitute two of the towering achievements of Cajal. Despite their shortcomings owing to the limited knowledge available at the time they were proposed, the concept of the growth cone and the chemotropic hypothesis, provided a framework to a complex type of processes in a way that took over a hundred years to fully understand.

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Unraveling Cajal's view of the olfactory system

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The olfactory system has a highly regular organization of interconnected synaptic circuits from the periphery. It is therefore an excellent model for understanding general principles about how the brain processes information. Cajal revealed the basic cell types and their interconnections at the end of the XIX century. Since his original descriptions, the observation and analysis of the olfactory system and its components represents a major topic in neuroscience studies, providing important insights into the neural mechanisms. In this review, we will highlight the importance of Cajal contributions and his legacy to the actual knowledge of the olfactory system.

Keywords: olfactory bulb, olfactory cortex, olfactory epithelium, glia, neuron

INTRODUCTION

Santiago Ramón y Cajal is called the father of modern neuroscience for our current understanding of the nervous system really began through his work. Cajal postulated the main principle of neuroscience, the Neuron Doctrine, which recognizes the neuron as the basic anatomical and functional unit of the nervous system (Ramón y Cajal, 1891). His view was opposed to the reticular theory developed by Golgi (1873). The principal advantage that Cajal had over his contemporaries was the better understanding of the Golgi method allowing thus its correct interpretation. With this edge Cajal was able to give a successful explanation to the static view of the sections impregnated by the Golgi method. He was even able to make predictions on physiological brain properties that are being demonstrated nowadays thanks to more sophisticated techniques. Based on observations done using the Golgi method Cajal concluded: "It happens sometimes that the reaction of Golgi runs from one fiber to another when two of them intersect, resembling branches or anastomotic examples. This error can only be avoided by using high magnifying lenses and not giving credit to other branches other than those that appear in the focal plane and on the level of those triangular thickenings that are never absent in cases of a legitimate branch" (Ramón y Cajal, 1890b). In birds' cerebellum, Cajal correctly described that the surface of Purkinje cells "appears bristling with thorns or short spines" (Ramón y Cajal, 1888); Golgi instead, rejected the existence of these spines, considering them artifacts of the silver staining technique. He also established the connections between neurons and drew the maps of the trajectory of nerve currents and impulses that led him to formulate the Law of Dynamic Polarization: "The protoplasmic expansions, dendrites, and the cellular body have axipetal conduction (i.e., toward the axon); whereas the axon has dendrifugal and somatofugal conduction (i.e., it comes from the dendrites or the cellular body)" (Ramón y Cajal, 1899). Furthermore, Cajal described the growth cone as a "concentration of protoplasm of conical form, endowed with amoeboid movements" (Ramón y Cajal, 1890a). Another of Cajal's contributions was the formulation of the Neurotropic Theory (Ramón y Cajal, 1892a); which shows how nerve cells find their way to their targets during development. In the formulation of these Laws explaining the morphological and functional organization of the nervous system, the analyses of the olfactory system was critical; this due to its accessibility, its orderly organization in layers and the easy identification of the main direction of the nervous message flow. Thus, the aim of this article is to give a brief outline of Cajal's main contributions to the knowledge of the olfactory system along with some key developments in our current understanding of this system.

OLFACTORY CIRCUIT

> "The flow of the nervous movement in the bulb would be the following: the olfactory imprint is collected in the mucosa by the peripheral expansion of the bipolar cells and is then transferred to the glomeruli where both the mitral corpuscles as well as the pyramidal or fusiform cells from the molecular layer collect said imprint to raise it to the brain. [...] In summary, there are two main junctions: one in the glomeruli and another one in the cortex of the olfactory lobe. In each one of these junctions the movement acquires more diffusion, partaking in its conduction an increasingly larger number of nervous corpuscles" (Ramón y Cajal, 1892b).

The olfactory system represents an excellent model of the cellular interaction between the periphery and the central nervous system. In the nasal cavity is located the olfactory epithelium (OE) where the olfactory sensory neurons (OSNs), in direct contact with the environment, are contained. OSNs project their axons, through the cribriform plate, to contact target cells in the olfactory bulb (OB). OB projection cells send the olfactory signal to the olfactory cortex (OC), which includes the olfactory

tubercle, piriform cortex, amygdala, and entorhinal cortex. The olfactory information is then further transmitted to the thalamus, hypothalamus, or hippocampus (**Figure 1A**). One of Cajal's most important contributions, the *Law of dynamic polarization*, was possible by the observation of the direction of the signal flow from one neuron to the next in this system. In particular, he used arrows to represent in his histological drawings the flow of information from the periphery (OE) to the OB in the brain and then onto the OC (**Figure 1B**): "Excitation is conducted at the glomeruli, where numerous olfactory fibers end. Here, the motion is transmitted along several currents directed along the path of the projection cells (mitral or superior, medial, and inferior tufted cells), from the intraglomerular tufts, to the axis-cylinders and their cerebral endpoints in the olfactory centers" (Ramón y Cajal, 1890b). Thus, even without a functional frame, Cajal proposed the direction of the information flow that was later corroborated by physiological studies (reviewed in Shepherd and Erulkar, 1997), although the presence of axonless granule cells in this system challenged the *Law of dynamic polarization* (Shepherd et al., 2007; Sassoè-Pognetto, 2011).

Cajal's detailed study of the olfactory system and its components (**Figure 2A**) (Ramón y Cajal, 1890b) laid the foundations for later contemporary studies (**Figures 2B,C**). In his book "*Recuerdos de mi vida*" (Ramón y Cajal, 1917), he defines the OB as an accessible and regular structure, comparable to the cerebellum and retina. In this system, once again, he evidenced the nerve propagation by contact and the important role of dendrites: "The history of the physiological interpretation of the structure of the olfactory bulb provides a typical case of the crippling influence of theoretical prejudices. Golgi had already discovered before us the most important facts of that structure, the singularly invaluable concurrency within the glomeruli of the olfactory fibers, on the one hand, and the dendritic tuft of mitral cells on the other; but his rigid conception of the diffuse nervous network did not allow him to recognize the great physiological scope of such provision" (Ramón y Cajal, 1917).

These characteristics are reinforced by the incorporation of new cellular elements not only during development, but also during adulthood (Altman, 1969; Lois and Alvarez-Buylla, 1994). The plastic process in the OB is the result of the combination of cellular contributions from either the telencephalic subventricular zone as a part of the central nervous system, and the olfactory placode/epithelium, which "represents a peripheral nervous center" (Ramón y Cajal, 1892b).

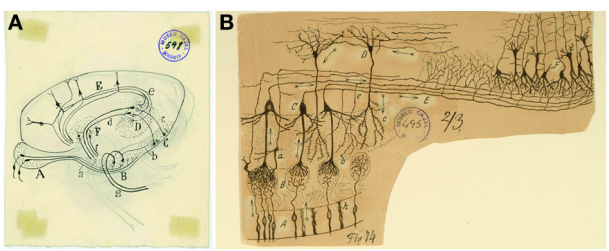

FIGURE 1 | Overall view of the olfactory circuit. (A) Unpublished original Cajal cartoon of the olfactory system. **(B)** Cajal's diagram outlining the circuitry and the trajectory of the nerve impulse (arrows). *(A)* Bipolar cells of the olfactory mucosa. *(B)* Olfactory glomeruli. *(C)* Mitral cells. *(D)* Grains. *(E)* External root (lateral olfactory tract). *(F)* Olfactory cortex. *(a)* Small tufted cell; *(b)* main dendrite of a mitral cell; *(c)* terminal branch of a grain; *(e)* recurrent collaterals of a mitral cell; *(g)* surface triangular cells of the olfactory cortex; *(h)* epithelial cells of the nasal mucosa (Ramón y Cajal, 1894). Cajal Legacy (Instituto Cajal, CSIC, Madrid, Spain).

OLFACTORY EPITHELIUM

> "The olfactory mucosa contains the nervous cells from where the olfactory fibers that reach the brain through the ethmoid's lamina cribrose; it thus represents a peripheral nervous center. [...] The bipolar or olfactory cell represents the real reception organ of the odorant impulse or stimulus" (Ramón y Cajal, 1892b).

The olfactory epithelium is the place where volatile odorant molecules are initially detected and it is composed by three cell types: OSNs, supporting cells and basal cells. OSNs (**Figure 3**) are bipolar cells located in the intermediate OE region, distributed between the supporting cells. Their apical processes end at the lumen in non-motile cilia, while the thinner descending axon "gives neuronal character to the bipolar cell" (Ramón y Cajal, 1892b) and transmits the impulse to the OB. The supporting

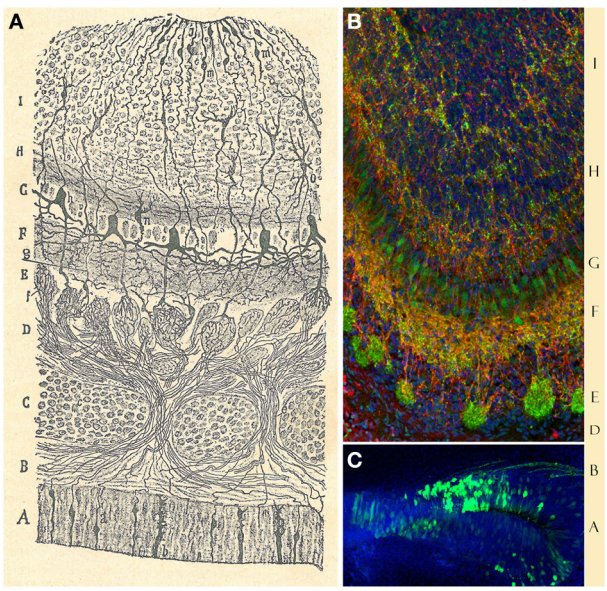

FIGURE 2 | Olfactory bulb and epithelium. (A) Original Cajal drawing showing the "antero-posterior view of the olfactory bulb and nasal mucosa of the newly-born mouse." *(A)* olfactory epithelium; *(a)* olfactory sensory neuron; *(b)* sustentacular cell. *(B)* Dermis. *(C)* Ethmoid bone. *(D)* Fibrillar layer. *(E)* Glomerular layer. *(F)* Inferior molecular layer. *(G)* Mitral cells layer. *(H)* Superior molecular region. *(I)* Granular zone. *(c)* Cartilage. *(e)* Olfactory nerve. *(f)* Arborization of the olfactory fibers within the glomeruli. *(g)* Central cyllinder-axis of an inferior tufted cell. *(h)* Mitral cell. *(i)* Grains. *(j)* Epithelial cells. *(n)* Lower grain. *(o)* Large stellated cell (Ramón y Cajal, 1890b). **(B)** Immunohistochemical staining for Dab1 protein (reelin signaling mediator, green) and Map2a,b protein (microtubule associated protein, red) on mouse olfactory bulb sections at P3. Nuclei counterstained with Hoechst (blue) (Martín-López et al., 2011). **(C)** Olfactory sensory neurons (green) at E17, labeled after *in utero* electroporation of an EGP-expressing plasmid into the olfactory placode at E11. Nuclei counterstained with Hoechst (blue). Sagittal mouse brain section.

or sustentacular cells exhibit an irregular morphology, but their nuclei are mostly located apically, thereby being narrower on their basal side (**Figures 3A,B**). The characteristic morphology of these cells offers "numerous facets or hollow molds in order to adapt to the bipolar corpuscles [...] and their mission appears to be no other than preventing any contact between them, avoiding any horizontal current communication" (Ramón y Cajal, 1892b). Basal cells, not described by Cajal, form a single cell layer in the basal lamina, near the underlying bone of the OE (Retzius, 1892). They have a constant turnover (Graziadei, 1973) being the precursors of OSNs (Suzuki et al., 2013).

One of the main advances in the study of this system was the cloning of the olfactory signal transduction molecules, in particular the odorant receptors (Buck and Axel, 1991) located on the OSNs cilia. In mice there are over five million OSNs, each expressing just one among the thousand odorant receptor genes (Zhang and Firestein, 2002). These chemosensory receptors are odorant-binding proteins with seven transmembrane domains coupled to G-proteins. Each receptor is codified by the allele of a single gene (Buck and Axel, 1991) and binds only odor molecules of a certain family. They are responsible of transforming the chemical information into electric signals in the olfactory circuit. Genetic tools reported that OSNs, expressing a given odorant receptor, are intermingled and randomly distributed within four large OE zones. These zones are symmetric in both sides of the nasal cavities and are divided based on the expression pattern of some odorant receptors (Ressler et al., 1993; Vassar et al., 1993). Furthermore, OSNs expressing the same receptor converge upon a stereotypical pair of glomeruli (Mombaerts et al., 1996). Nonetheless, the mechanisms by which a set of OSNs, expressing certain odorant receptor, innervates a discrete amount of glomeruli are not well-known; although it seems to be dependent on environmental cues, as well as on intrinsic OSN/odorant receptor factors (reviewed in Mombaerts, 2006; Blanchart and López-Mascaraque, 2011).

Cajal showed that OSNs axons ended into the glomeruli (**Figure 4A**): "This fibril goes through a part of the dermis indivisible and without anastomosing, then gathering with others in tight bundles, goes upwards later, always preserving its individuality, through the ethmoid's lamina cribrosa and assaults, finally, the olfactory bulb, ending arborizing in the thickness of one glomeruli of this central nervous system organ" (Ramón y Cajal, 1892b). Even when the past decades have seen enormous achievements by the implementation of new technologies, Cajal's morphological descriptions provided the basis for subsequent studies. *In situ* hybridization and immunohistochemistry revealed the molecular features of OSNs (**Figures 3C–E**). Later, the use of HRP, retrograde fluorescent markers (Fast Blue, Diamidino Yellow), biotinylated dextrans and lipophilic fluorescent tracers (e.g., DiI, DiO, DiA) confirmed the pathway of OSN axons from the periphery to the OB. At this respect, **Figure 4D** shows the path of retrogradely OSN labeled cells after a DiI injection into the OB. Furthermore, techniques such as *in utero* electroporation of an EGP-expressing plasmid used to study the olfactory pit cell migrations allowed also a further visualization of these nerve bundles (**Figure 4C**). These axons do not ramify until they reach the glomeruli (**Figure 4B**), where they will make contacts with the dendrites of the projection neurons. It is within these specialized structures where the information from the periphery is integrated and then conducted to the rest of the brain. Additionally, the development of the OB is not dependent on the presence of the OE or the synaptic input from the OSNs (López-Mascaraque et al., 1996; López-Mascaraque and De Castro, 2002), although OSN axons are critical during OB

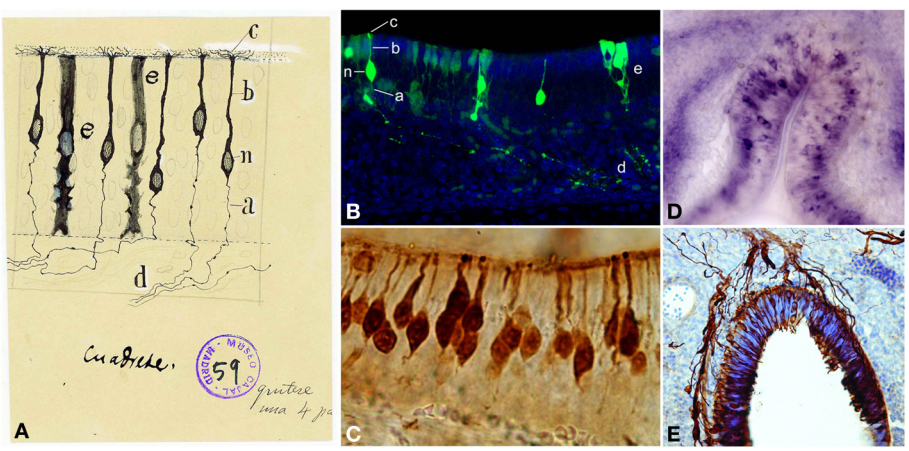

FIGURE 3 | Olfactory sensory neurons (OSNs). (A) Cajal drawing illustrating the cell types and their morphologies in the olfactory epithelium. OSN components: *(n)* nucleus; *(b)* dendrite; *(c)* apical cilia; *(a)* axon. *(d)* OSNs axons bundle. *(e)* supporting or sustentacular cell. The drawing shows the handwriting of Cajal with specific instructions for the required reduction publication factor in the margins (Ramón y Cajal, 1917). Cajal Legacy (Instituto Cajal, CSIC, Madrid, Spain). **(B)** Olfactory epithelium labeled E18 cells after *in utero* electroporation of an EGP-expressing plasmid injected into the olfactory placode at E14 (green). Hoechst (blue). Sagittal mouse section. *a–e, n* correspond to the counterpart structures labeled by Cajal in **(A)**. **(C)** Immunohistochemistry for the Tuj1 marker in mouse olfactory epithelium shows both mature and immature OSNs. **(D)**. *In situ* hybridization for *Nrp-II mRNA* in coronal sections of mouse olfactory epithelium at E14. **(E)** Immunohistochemistry for Tuj1 marker in a mouse olfactory placode coronal section at E11. Panels **(C–E)** were taken by Albert Blanchart.

layering in the final orientation of mitral cells (López-Mascaraque et al., 2005).

SPATIAL CELL ARRANGEMENTS IN THE OLFACTORY BULB

Next station in the olfactory pathway is the olfactory bulb: "the olfactory nerves, which bore into the cranium base through several holes in considerable numbers, and assault the olfactory bulb where they end" (Ramón y Cajal, 1890b). One of the most important of Cajal findings was the demonstration of the entire course of the olfactory fibers. Cajal made the real assumption that these fibers come from the mucosa (OE) and end into the glomerulus at the OB not as a network, as Golgi thought, but by free varicose arborizations.

The OB has a well-defined laminar structure and is formed by different cell populations divided into projection neurons (mitral cells and some tufted cells), interneurons (periglomerular cells, external tufted cells, short axon cells, granule cells, Van Gehuchten cells, and Blanes cells) and glial cells (astrocytes, oligodendrocytes, olfactory ensheathing cells, NG2, and microglia). The innermost part of the OB, the ependymal zone, contains progenitor cells.

Golgi considered the OB formed by three layers (Golgi, 1875) while Schwalbe (1881) proposed six layers. The definitive description of cell types and disposition in six layers was given by Cajal and his disciples (Ramón y Cajal, 1890b; Blanes, 1898). From the outside in, the OB is organized in the following layers: the olfactory nerve layer (ONL), glomerular layer (GL), the external plexiform layer (EPL), the mitral cell layer (MCL), the internal plexiform layer (IPL) and the granule cell layer (GcL) (**Figures 5A,B**). Cajal stated that the ONL was formed by unbranched *"nerve fibrils"* which preserve the same thickness along their trajectory from the OE. Besides, this layer contains an extremely interesting population restricted exclusively to the olfactory system regions, the olfactory ensheathing cells (Valverde and López-Mascaraque, 1991). During development, olfactory ensheathing cells coexist with astrocytes as part of the migratory mass (Doucette, 1990; De Carlos et al., 1996; Blanchart and López-Mascaraque, 2011; Blanchart et al., 2011). Olfactory ensheathing cells maintain certain progenitor characteristics (Schwarting et al., 2007) and are responsible, among other things, for the permissibility within the OB to OSNs axons growth during development and adulthood, thus being a key component of the ability of the OE to continually regenerate.

Next stratum, the GL, is defined by Cajal as the target of the fibrils coming from the OSNs: "Under the peripheral fibrillar

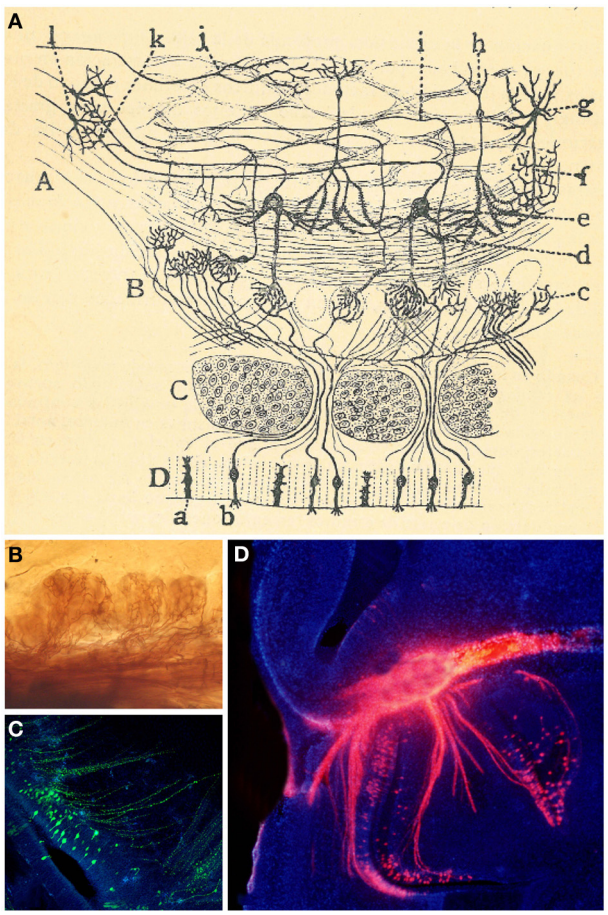

FIGURE 4 | Connection between the olfactory epithelium and the olfactory bulb. (A) Cajal schematic drawing of the mammal's olfactory system. *(A)* Olfactory lobe. *(B)* Olfactory bulb's glomerular layer. *(C)* Cribriform plate. *(D)* Olfactory epithelium or nasal mucosa; *(a)* supporting cells; *(b)* OSNs (Ramón y Cajal, 1892b). **(B)** Axonal arborizations of OSNs axons in several glomeruli stained with the Golgi method. **(C)** Olfactory sensory neurons (green) at E17, labeled after *in utero* electroporation of an EGP-expressing plasmid into the olfactory placode at E11. Nuclei counterstained with Hoechst (blue). Sagittal mouse section. **(D)** Retrogradely labeled OSN cells after a DiI crystal application into the OB (red). Hoechst (blue).

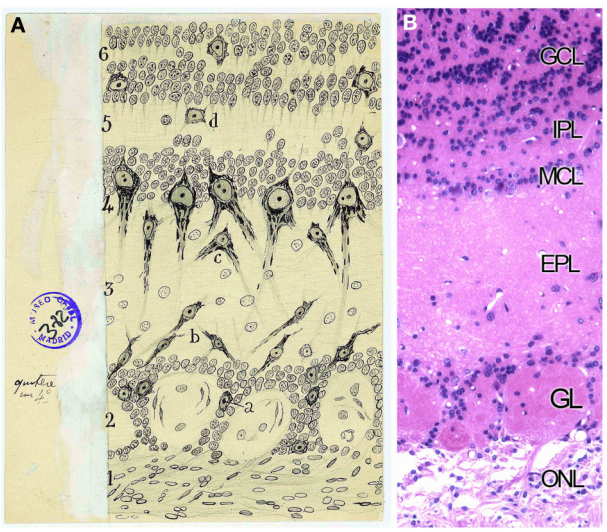

FIGURE 5 | Layers distribution of the Olfactory bulb. (A) Original Cajal drawing of the frontal section of the rabbit olfactory bulb (Ramón y Cajal, 1901). *(1)* Nerve layer (ONL). *(2)* Glomerular layer (GL). *(3)* External plexiform layer (EPL). *(4)* Mitral cells layer (MCL). *(5)* Inner plexiform layer (IPL). *(6)* Grains and white matter layer (GcL). *(a)* Peripheral tufted cells; *(b)* middle; *(c)* internal *(d)* short axon cells. Cajal Legacy (Instituto Cajal, CSIC, Madrid, Spain). **(B)** Olfactory bulb mouse coronal section of hematoxylin and eosin nuclei staining showing the different layers described by Cajal in **(A)**.

layer lays an irregular area of two or more rows of disordered ovoid masses called olfactory glomeruli. [...] They are composed of the terminal branches of olfactory fibers, the thick plume of dendrites arriving from deeper zones, certain tiny nerve corpuscles and, finally, some neuroglial elements" (Ramón y Cajal, 1890b). More than a century ago Cajal stated the exact input of the OSNs into the glomeruli, although Golgi reported the intraglomerular branching of the olfactory fibers (Golgi, 1875). In 1890 Cajal described the composition of each glomerulus (**Figure 6A**): the terminal arborization of the olfactory fibers, the thick apical dendrites from deeper regions, considerable tiny nervous corpuscles and several neuroglial elements (Ramón y Cajal, 1890b). Those tiny nervous corpuscles correspond to tufted or fusiform nerve cells, that collaborate in the formation of what he called intraglomerular plexus (**Figures 6B,C**), and external grains or short axon nerve cells, which branch within glomeruli, cells classified by Golgi as glial cells. Nowadays, a further characterization can be achieved either by the specific expression of different markers for each cell type presents or by the cell's physiological properties. Moreover, while Cajal studied the development of this system both in younger and/or phylogenetically less complex animals, nowadays we describe the cellular contributions, e.g., to the OB, after *in utero* viral infections (Blanchart et al., 2011) or by electroporation of different plasmids. In fact, a clonal analysis of glial cell populations can be performed with the Star Track approach (García-Marqués and López-Mascaraque, 2013). Moreover, a modification of this technique that uses an ubiquitous promoter (*UbC-Star Track*, (Figueres-Oñate and López-Mascaraque, 2013) allows a more comprehensive lineage study of all the cell populations (**Figure 6D**).

Since the apical dendrite of mitral cells and 2–3 dendrites of tufted cells penetrate into the territory of each glomerulus, Cajal noted that "the propagation of the nerve impulse is not individual, from a single neuron to another, but collective, from a group of nerve fibers to a group of ganglion corpuscles" (Ramón y Cajal, 1901). Nowadays, the characterization of the functional glomerular map has led to a more thorough understanding of how the positional domain information translates to different odor responses such as innate or learned responses (reviewed in Mori and Sakano, 2011).

Below the glomeruli is located the EPL, similar to the molecular area of the cerebellum or the retina. This layer includes lateral dendrites of the mitral and tufted cells and apical dendritic processes of granular cells (**Figure 7A**). Cajal named tufted cells as that because of their robust peripheral dendrite branching into the olfactory glomeruli. They are divided into external, middle and deep, dependent on the location of their soma. Cajal also described the presence of axonal collaterals from mitral cells in this molecular layer.

The next stratum is the MCL, composed by mitral cells. Mitral cell bodies, as described by Cajal, form a regular single row and owe their name to their appearance (**Figure 7A**). They are the principal output cells of the OB and, in most mammals, are characterized by a single apical dendrite through the EPL that branches into an apical tuft within the glomerulus (**Figure 7B**). Mitral cells are one type of the projection neurons (**Figure 7C**), whose entire development terminates at postnatal stages (Blanchart et al., 2006). Within the glomeruli, mitral cells interact and receive inputs through synaptic contacts with periglomerular and granule cells. Mitral cells are the bridge connecting directly the periphery with higher integrative structures (Ramón y Cajal, 1904; reviewed in Gire et al., 2013). Their inputs come from OSNs and external tufted cells and send their outputs to various cortical structures (Hayar et al., 2004; Gire et al., 2012).

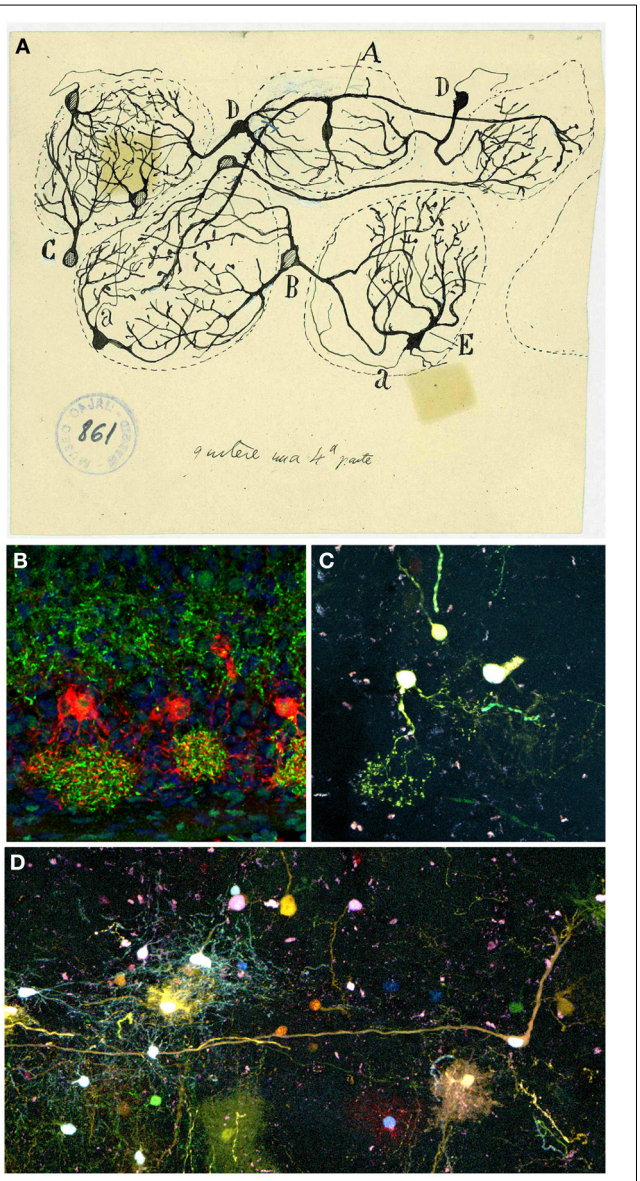

FIGURE 6 | Periglomerular cells. (A) Original drawing by Cajal's disciple Blanes, showing periglomerular cells of a cat glomerular layer (Blanes, 1898). Cell branching into three glomeruli *(A)*. Biglomerular cells *(B–D)*. Monoglomerular cells *(C–E)*. *(a)* Axon. Cajal Legacy (Instituto Cajal, CSIC, Madrid, Spain). **(B)** Periglomerular cells immunolabeled for tyrosine hydroxylase (TH, red) protein and Dab1 protein (green) on mouse olfactory bulb sections at P3. Panel was taken by Eduardo Martin-López. **(C,D)** Adult periglomerular cells contributing to one **(C)** or more glomeruli **(D)** labeled after E13 *in utero* electroporation of different plasmids with the *UbC-Star Track method* (Figueres-Oñate and López-Mascaraque, 2013).

Cajal also described the centrifugal feedback that mitral cells receive from cortical structures (Ramón y Cajal, 1901), and recent studies provided a functional explanation to these projections (for review see Gire et al., 2013).

Below the MCL layer, the IPL is populated by most axon collaterals of tufted cells (**Figure 8A**), while the GcL contains many interneurons like the granule cells and the short-axon cells. The granule cells are small spiny ovoid cells with an apical process extending radially into the EPL and short secondary dendrites confined to the GcL (**Figures 8B–E**). Golgi reported that these cells showed no evidence of axons Golgi (1875) and Blanes (1898) stated that they were not glia as Kölliker claimed (Kölliker, 1891). Additionally, Cajal described different types of short-axon cells within the GcL: Golgi cells, Cajal cells, and Blanes cells. Blanes cells are interneurons with a significant electrophysiological role, as they provide inhibitory inputs onto granule cells and appear to be excited by mitral cells, which could be a novel mechanism for encoding short-term olfactory information (Pressler and Strowbridge, 2006). The different subpopulations of these interneurons were classified by Cajal on morphological and

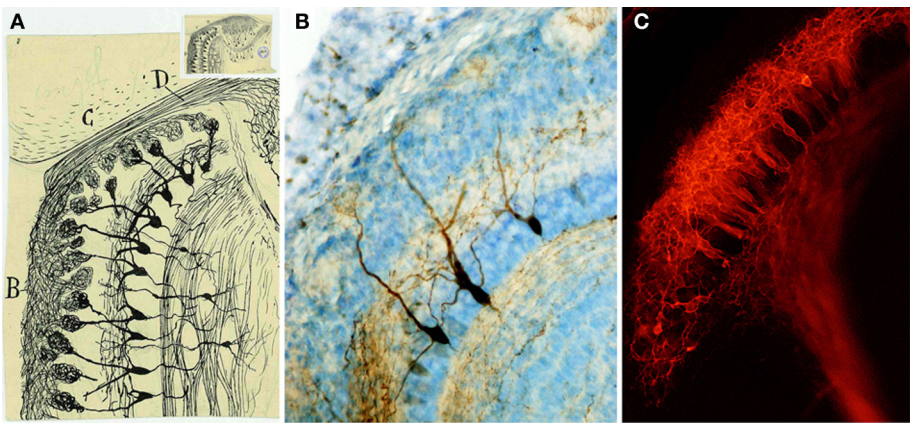

FIGURE 7 | Mitral cells. (A) Magnified detail of the original Cajal figure (upper inset). Horizontal mouse olfactory bulb section at 20-days-old (Ramón y Cajal, 1901). Olfactory bulb *(B)*, frontal cortex *(C)*, Olfactory nerve *(D)*. Cajal Legacy (Instituto Cajal, CSIC, Madrid, Spain). **(B)** Mitral cells labeled after BDA injection into the lateral olfactory tract at P5 (Blanchart et al., 2006). **(C)** Retrograde labeling of mitral cells after DiI injection into the lateral olfactory tract at E17 (Blanchart et al., 2006).

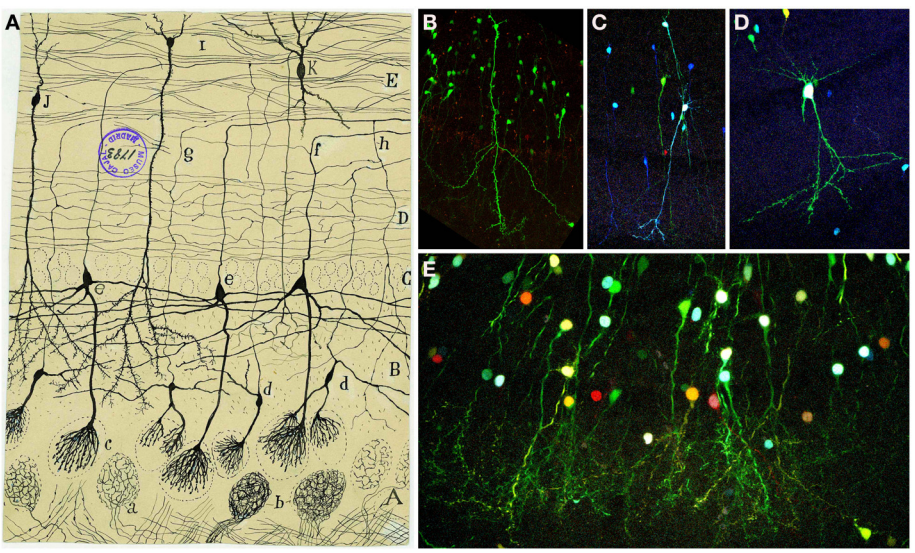

FIGURE 8 | Granular cells. (A) Original Cajal drawing of an olfactory bulb section from a few days cat brain (Ramón y Cajal, 1901). *G*lomerular layer *(A)*, outer plexiform layer *(B)*, mitral cell layer *(C)*, inner plexiform layer *(D)*, grains layer and white matter *(E)*. *(a)* Terminal arborization of an olfactory fiber; *(b)* glomerulus with several endings; *(c)* mitral plume; *(d)* tufted cells. Cajal Legacy (Instituto Cajal, CSIC, Madrid, Spain). **(B–E)** Granule cells in the olfactory bulb of young adult mice (P20) labeled after E12-14 *in utero* electroporation of different plasmids with the *UbC-Star Track* method. (Figueres-Oñate and López-Mascaraque, 2013). **(B,C)** Granule cells with similar to (i,j) in Cajal's drawing. **(D)** Tufted cell. **(E)** Granular cells branching their processes in the glomeruli.

spatial basis. Since his work, the diversity of GcL cells has also been based on molecular and physiological features (Price and Powell, 1970; Schneider and Macrides, 1978; López-Mascaraque et al., 1986; Crespo et al., 2001; Kosaka and Kosaka, 2005).

In summary, Cajal plotted the dynamic scheme of the OB, pointing out the need to give a special significance to the protoplasmic processes of mitral and tufted cells, which penetrate into the glomerulus and are in intimate contact with the olfactory fibrils. The olfactory fibers never depart from the glomerular territory and neither axons of central origin enter in the glomeruli, which is against Golgi's assertion. Cajal was also a pioneer in the description of different axonal projections of tufted and mitral cells to the OC (Ramón y Cajal, 1904).Indeed anatomical and physiological differences suggests that mitral and tufted cells may serve different functions and possibly contribute to different aspects of the olfactory code including perception of odorants (Nagayama et al., 2004; Shepherd et al., 2004). Although mitral and tufted cells innervate different cortical targets, the circuitry and projection sites of the tufted cells are not yet well-understood and are still one of the main focus of research in the field (for review see Mori and Sakano, 2011). Besides, the two main inhibitory interneuron types described by Cajal in the OB have a significant role in the olfactory processing: periglomerular cells mediate lateral inhibition at the level of the glomeruli (Aungst et al., 2003), while granule cells mediate dendrodendritic inhibition onto the lateral collaterals of mitral cells (Schoppa et al., 1998). These synapses formed between lateral dendrites of mitral cells and granule cells was suggested to be inconsistent with Cajal's Law of Dynamic Polarization (for extensive reviews see Shepherd et al., 2007; Sassoè-Pognetto, 2011).

NEUROGLIA IN THE OLFACTORY BULB

Cajal and his colleagues played an important role in describing glial cells. They initiated an active discussion regarding where to encompass those, at that time, unknown cells into the functional map of the brain. In different species, Cajal identified these cells, closely related to the cell bodies of neurons, as neuroglia. Then, he could not draw any definitive conclusion about the physiological role of neuroglial cells, but he presupposed an insulating role: protection to prevent contact between nerve fibers (Ramón y Cajal, 1896). This insulating theory of the neuroglia was originally developed by Cajal's brother, Pedro, and it was always supported by Cajal: "By rational conjecture, we have defended in several manuscripts the thesis, initially suggested by my brother, that both the epithelial and neuroglial cells have a role insulating the fibers and nervous cells, preventing contacts between close but dynamically independent elements" (Ramón y Cajal, 1897).

Focusing on the OB, Golgi briefly described the glia in this structure, but one the most important descriptions was done by Cajal's disciple, De Castro (1920). De Castro made a careful comparison between the neuroglia of human OB to other higher mammals by using the Cajal-improved sublimated-gold technique and the reduced silver impregnation method (**Figures 9A,D**). With these staining methods, Fernando de Castro showed the neuroglial distribution in the OB as well as the abundance and importance of the vascular glial end-feet in different brain areas. He also suggested that neuroglial cells may release neuroactive substances and directly participate in neural transmission (De Castro, 1951). In addition, he noticed how astrocytes were closely related to blood vessels through their end-feet, raising questions about their specific function: "What role does the neuroglia play in the vascular foot? Would it be entrusted with any function or would it be just a mere support organ? Difficult in every respect is the solution to the problem" (De Castro, 1920). Recently, the processes of protoplasmic astrocytes arranged around blood vessels (**Figures 9B,C**) were labeled after *in utero* electroporation of the Star Track plasmid mix (García-Marqués and López-Mascaraque, 2013) into the lateral ventricles. After embryonic electroporation of the OB progenitors with *UbC-Star Track* method (Figueres-Oñate and López-Mascaraque, 2013), clones of glial cells surrounding several glomeruli are located in adult olfactory bulbs (**Figure 9E**).

While the glomerular structure and neuronal connectivity has been extensively described, both the role and connectivity of neuroglia in the OB have yet to be characterized. Within the OB, astrocytes do not just play an insulating or supporting role, but they are also an active part of the sensory integration in the olfactory glomeruli, interacting with their neuronal counterparts, in a glomerulus-specific manner (Roux et al., 2011). Although the olfactory astroglia was defined as a syncytium, the advent of molecular and genetic techniques changed the experimental approaches to determine the progeny of single cells, shedding light to a further network specialization (Houades et al., 2008). A promising approach is the *in vivo* clonal analysis, Star Track, based on the combinatorial expression of different gene reporters (García-Marqués and López-Mascaraque, 2013; García-Marqués et al., 2014) that makes possible to trace the progeny of targeted GFAP progenitors (**Figures 9D,E**). Besides the classification based on morphology and location of glial cells, we show the presence homogeneous glial clones which indicates the existence of separate progenitors for each glial population (García-Marqués and López-Mascaraque, 2013).

Regardless from the glial elements mentioned above, Cajal also described the presence of myelin fibers within the OB using the Weigert-Pal staining technique (**Figure 10A**). "The medullated fibers are relatively abundant around the glomeruli and even within them. The periglomerular fibers are generally very thin and correspond with cylinder-axis of the inferior tufted cells [...]. The intraglomerular fibers have a more difficult interpretation. [...] In general, it can be assumed that said fibers [...] end within the same glomerular area. [...] As is well known, the olfactory fibers and the grains expansions lack myelin" (Ramón y Cajal, 1890b). Cajal and his disciples also observed what they called "*third element*" or "*adendritic cells*" (De Castro, 1920), known today as microglia. The use of molecular markers, selectively expressed by these cells in the OB, revealed the distinct glia subtypes (**Figure 10B**).

OLFACTORY CORTEX

The olfactory cortex is a phylogenetically old cortical structure. It is formed by all brain regions receiving direct axonal input from mitral and some tufted cells (Allison, 1954; Price, 1973), making the olfactory system the only sensory modality without thalamic relays. Among several areas, the OC includes the

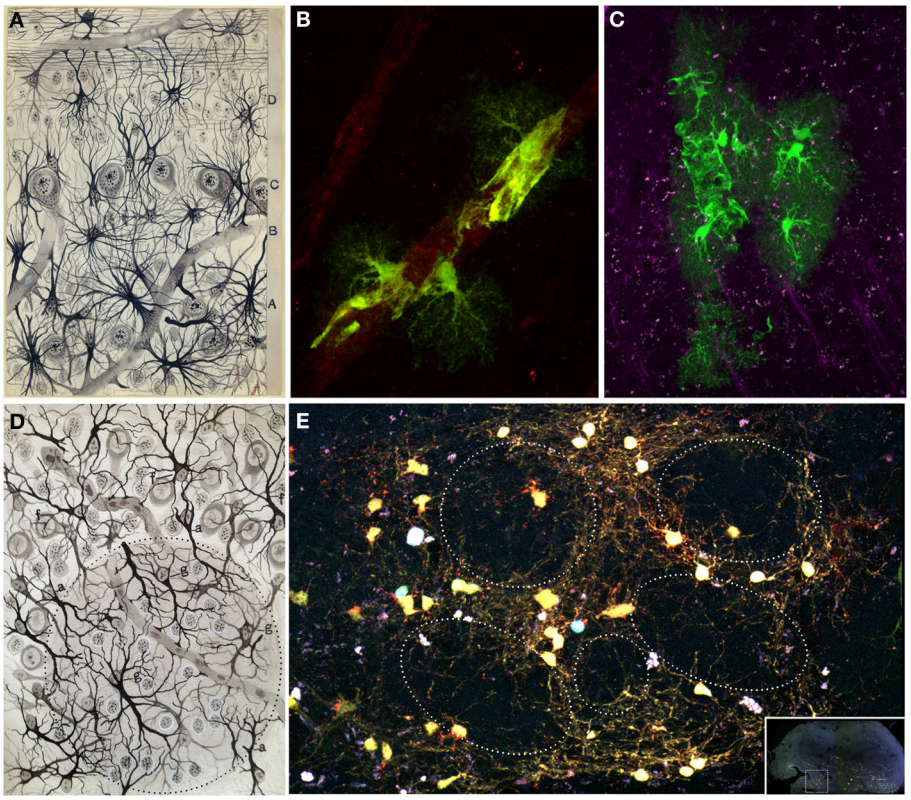

FIGURE 9 | Glial cells. (A) Original Fernando de Castro drawing of human olfactory bulb stained with Cajal's gold chloride sublimate method. Superficial substratum of the molecular layer with numerous cephalopodic cells *(A)*, deep substratum *(B)*, mitral cell layer *(C)*, grains layer *(D)* (De Castro, 1920). **(B,C)** Processes of protoplasmic astrocytes arranged around a blood vessel labeled after *in utero* electroporation of the *Star Track* plasmid mix (García-Marqués and López-Mascaraque, 2013) into the lateral ventricles. These perivascular end feet are represented in the olfactory bulb **(A)** by Fernando de Castro. Panel **(B)** modified from Martín-López et al. (2013) and panel **(C)** were taken by Eduardo Martin-López. **(D)** Fernando de Castro drawing illustrating the glomerular layer of the adult dog stained by the Cajal's gold chloride sublimate method. *(g)* Intraglomerular fibrous elements; *(a)* radioglomerular corpuscles; *(f)* fibrous cells located superficial to the molecular zone, displaying most of their extensions oriented toward deepest layers. Note numerous nuclei located in this region, corresponding to Cajal's adendritic glia (De Castro, 1920). **(E)** Clone of glial cells surrounding several glomeruli in an adult (7 months) olfactory bulb labeled after E13 *in utero* electroporation of different plasmids with the *UbC-Star Track* method (Figueres-Oñate and López-Mascaraque, 2013).

anterior olfactory nucleus, the entorhinal cortex, the piriform cortex (primary OC), *tenia tecta*, cortical amygdaloid nucleus and the olfactory tubercle. Axons from the OB projecting to the OC constitute the lateral olfactory tract (LOT), located on the outer and lower side of the olfactory pedicle named by Calleja and Cajal as the "*external root*" (Calleja, 1893; Ramón y Cajal, 1901).

Despite its heterogeneity throughout the rostro-caudal axis, the OC displays a three-layer organization: layer 1 is subdivided in layers 1a and 1b; layer 2 contains semilunar cells and a large number of pyramidal-like cells and layer 3 is formed by different pyramidal cells (Valverde, 1965). Cajal and his disciple Calleja (1893) distinguished five layers in the OC: fibrillar layer or outer root layer, molecular or plexiform layer, layer of small and large pyramids, layer of polymorphs corpuscles and white matter (**Figure 11A**).

The fibrillar layer (layer 1a) is formed by LOT fibers while the molecular or plexiform layer (layer 1b) receives associational fibers from deeper cells and includes collaterals of the olfactory fibers, tufts of pyramidal cells and dendrites of deeper horizontal cells. The layer of small and large pyramids (layer 2) appears like a "flexible and undulating belt quite well demarcated from the bordering areas" (Ramón y Cajal, 1901). It contains cells with different morphologies, including semilunar cells (superficial part) and a large number of pyramidal-like cells (in deeper regions). While the semilunar cells usually lack descendent axonal projections, deeper cells display axonal processes penetrating into the white matter. As Cajal postulated, "the configuration of the neurons from said layer is highly variable, being able to discover, even in the deepest planes, multiple elements whose shape is triangular, stellated or fusiform, though they never lack a radial dendrite directed to the second layer" (Ramón y Cajal, 1901). At the deepest level, the polymorph cells layer (layer 3) includes the most voluminous cells with descending axonal collaterals that penetrate into the white matter. Recently, the development of novel tools for the clonal analysis of the brain neural lineages, the *UbC-Star Track* method (Figueres-Oñate and López-Mascaraque, 2013), evidenced the large variety of morphologies within the OC (**Figures 11B–F**). Finally, the white matter is formed by the

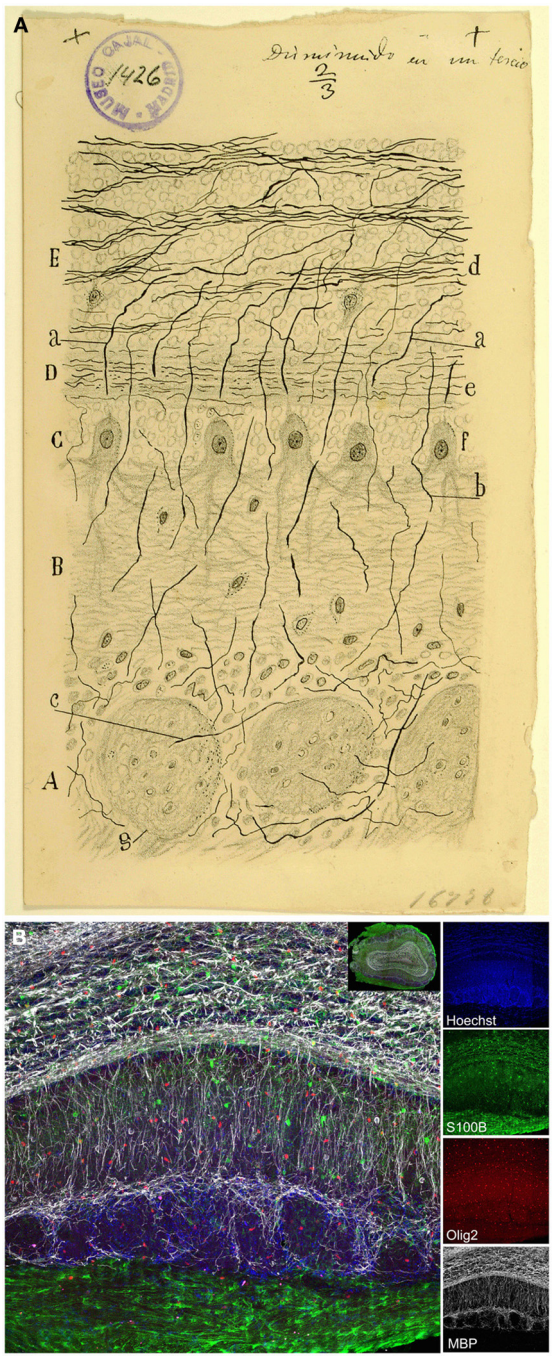

FIGURE 10 | Myelin. (A) Original drawing from Cajal of an olfactory bulb section of a month-old rat. Weigert-Pal method (Ramón y Cajal, 1890b). Layer of the glomeruli *(A)*, lower molecular layer *(B)*, mitral layer *(C)*, higher molecular layer *(D)*, coating the grains *(E)*. *(a)* myelin fiber corresponding to the cylinder-axis of mitral cells; *(b)* cylinder-axis of mitral cells; *(c)* core fiber from within the glomeruli; *(d)* bundles of fibers in the layer of grains; *(e)* thin horizontal strands from the higher molecular area; *(f) mitral* cells; *(g)* glomerulus; Cajal Legacy (Instituto Cajal, CSIC, Madrid, Spain). **(B)** Immunohistochemistry with different glial markers: Olig2 (oligodendrocyte progenitors, red), S100β (astrocytes, green), and myelin binding protein (MBP, gray). Nuclei labeled with Hoechst (blue). Inset shows overall view of the olfactory bulb (coronal section) labeled with the markers explained above.

concurrence of axonal projections from overlying layers, forming a labyrinthic and irregular plexus, that complicates the definition of their routes: "In summary, the fibers or second order conductors coming from the bulbar and frontal cortex, underlying the external root, follow two routes: ones, the majority, go backwards deeply to reach the corpus striatum incorporating to the corona radiata; others travel toward the inside and backwards and enter the anterior commissure. Being unable to sufficiently follow those conductors, we ignore if any of them reach Ammon's horn" (Ramón y Cajal, 1901).

This anatomical organization may underlie the fact that mitral and tufted cells project to the OC through different pathways and toward different targets suggesting the possibility that they carry different odor information (reviewed in Mori and Sakano, 2011). The diverse cortical projections of a single mitral cell, the broad distribution of mitral cells axons and the overlapping of their information at their target neurons provide the basis for a diversification and combinatorial integration of the olfactory information processing (Ghosh et al., 2011). Recent work using anatomical and physiological techniques demonstrated that individual neurons in the piriform cortex receive convergent input from mitral/tufted cells connected to multiple glomeruli located all over the OB (Apicella et al., 2010; Davison and Ehlers, 2011; Miyamichi et al., 2011). The precise scheme of the olfactory pathway displayed by Cajal (**Figure 1A**) opened the door to the anatomical basis of olfactory processing (Gire et al., 2013). OSNs expressing the same odorant receptor converge in one glomeruli of each hemisphere (Mombaerts et al., 1996; Mombaerts, 2006). This spatial pattern, termed *odotopic map* is just applied for the first olfactory station (OSN to OB). However, although much is known about how odors are represented at the level of OB, the nature of odor representations in this cortex and the integration of the odor activity of output OB neurons into higher brain regions, essential for the cortical odor representations, are still debated (for review see Bekkers and Suzuki, 2013).

OLFACTORY SYSTEM PERSPECTIVES

> "The functional specialization of the brain imposes to the neurons two main gaps: inability to proliferate and irreversibility of the intra-protoplasmatic differentiation. It is because this reason that, once development is over, the growth and regeneration of axons and dendrites are irrevocably dried up. In the adult brains the nervous pathways are fixed, finished, immutable. Everything may die nothing is regenerated itself. It belongs to the science of the future to change, if possible, this cruel decree" (Ramón y Cajal, 1913).

Unlike other brain structures, the OB is not a simple relay nucleus, but a center for information processing and storage. Cajal missed one of the most important characteristics of the OB, the cell turnover: "Nature has given us a limited amount of brain cells. Here is a capital, large or small, that nobody can increase as the neuron is unable to multiply" (Ramón y Cajal, 1931). However, adult neurogenesis is among the most important brain discoveries opening new debate about the function and integration of these cells into the system. The adult mice brain retains a proliferative area, the subventricular zone (SVZ), which maintains proliferative functions through live. Astrocyte-like cells (B cells)

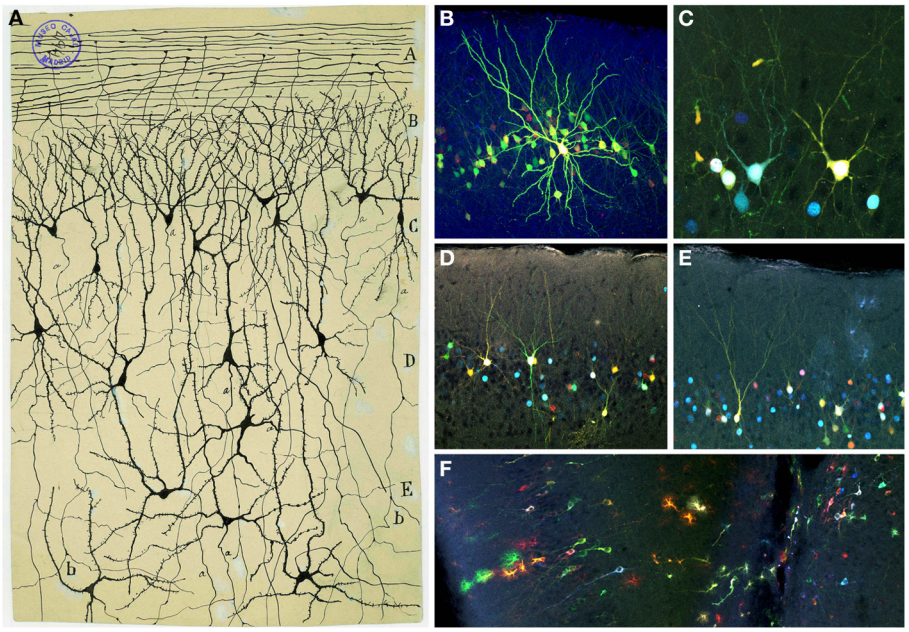

FIGURE 11 | Olfactory cortex. (A) Original Cajal drawing showing the olfactory cortex layers (Ramón y Cajal, 1901). *O*lfactory fibers layer *(A)*; plexiform layer *(B)*; layer of polymorphic superficial cells *(C)*; layer of the pyramids *(D);* deep polymorphous cells *(D). (b)* Bifurcation of axons. Cajal Legacy (Instituto Cajal, CSIC, Madrid, Spain). **(B–F)** Different cell morphologies in the adult mouse olfactory cortex labeled after E12 *in utero* electroporation of different plasmids with the *UbC-Star Track method* (Figueres-Oñate and López-Mascaraque, 2013). Note the presence of cells with either arachnoid morphologies, similar to those in **(B)**, and crescent-shaped cells similar to **(C)**. **(D,E)**. View of different morphological neuronal types. **(F)** Several neurons along with different glial clones.

divide to produce neuroblasts via intermediate progenitors. These neuroblasts migrate along the rostral migratory stream to the OB, where they differentiate and migrate to their final positions in the granular or periglomerular layers (Kriegstein and Alvarez-Buylla, 2009). Strikingly, a spatial patterning within the SVZ indicates that interneuron subtypes depend on their generation area (Merkle et al., 2007). Moreover, the temporal differences in the production of interneurons are related to their subtype specification and functional integration in the system (Batista-Brito et al., 2008).

To conclude, Cajal opened up an essential work to our current understanding of this system. These classical studies provided the basis for anatomical, physiological, and molecular studies. Now, more than a century later, the use of state-of-the-art approaches such as cell type specific optogenetic manipulations, *in utero* electroporation, *in vivo* genetic fate mapping and cell ablation, electrophysiological and live-cell imaging techniques, patch-clamp recordings and two-photon microscopy *in vivo* and in brain slice preparations can help understanding how odor information is represented and processed by the olfactory system.

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The Cajal school and the physiological role of astrocytes: a way of thinking

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Cajal is widely recognized by the scientific community for his important contributions to our knowledge of the neuronal organization of the nervous system. His studies on neuroglial cells are less recognized, yet they are no less relevant to our current understanding of the cellular bases of brain structure. Two pioneering studies published a century ago –"Something about the physiological significance of neuroglia" (Ramón y Cajal, 1897) and "A contribution to the understanding of neuroglia in the human brain" (Ramón y Cajal, 1913)—focused on glial cells and their role in brain physiology. Novel findings obtained using state-of-the-art and sophisticated technologies largely confirm many of the groundbreaking hypotheses proposed by Cajal related to the structural-functional properties of neuroglia. Here we propose to the reader a journey guided by the ideas of Cajal through the recent findings on the functional significance of astrocytes, the most abundant neuroglial cell type in the nervous system. Astrocyte–neuron interaction, which represents an emerging field in current neuroscience with important implications for our understanding of the cellular processes underlying brain function, has its roots in many of the original concepts proposed by Cajal.

Keywords: astrocytes, neuron-glia communication, Cajal, tripartite synapses, gliotransmission

One hundred years ago Cajal published two studies centered on glial cells: "Algo sobre la significación functional de la neuroglia" (Something about the physiological significance of neuroglia) in 1897 and "Contribución al conocimiento de la neuroglia del cerebro humano" (A contribution to the understanding of neuroglia of the human brain) in 1913, which proposed pioneering concepts regarding the relevance of glial cells in brain function that in many instances have been confirmed by recent evidence obtained using novel and sophisticated techniques. In this article, we propose to the reader a voyage starting from Cajal's original ideas to the most recent evidence revealing the functional significance of the neuroglia. While we will see how he opened new venues for the understanding of neuroglia and how his ideas have been largely confirmed by later studies, he must not be considered a scientific visionary; rather, he had an unparalleled capacity to extract general and dynamic physiological conclusions from observations of static images (**Figure 1**).

¿"Qué significación funcional debemos otorgar a la neuroglía? Desgraciadamente, en el estado actual de la ciencia no es posible contestar a la importante pregunta más que mediante conjeturas más o menos racionales. En presencia de este problema, el fisiólogo se halla, por falta de métodos, totalmente desarmado"

What functional significance can be attributed to the neuroglia? Unfortunately, the present state of science does not allow to answer this important question but through more or less rational conjectures. When facing this problem, the physiologist is totally disarmed for lack of methods (Ramón y Cajal, 1899).

This sentence reflects the incipient state of the technology at the end of the nineteenth century, which, however, did not prevent him from proposing ideas and hypotheses that were not fully misguided. At that time, one of the prevailing ideas concerning the function of glial cells postulated that they served to provide structural consistency to the nervous system in areas not occupied by neurons. In contrast, Cajal disagreed with this simple function ascribed to the neuroglia: ***¿Qué van a sostener corpúsculos pequeñísimos, aislados, flexibles, delicadísimos, mucho más delicados y pequeños que las células nerviosas mismas?*** (*What could hold these tiny, isolated, flexible, very delicate cells, much more delicate and smaller than the nerve cells?*) (Ramón y Cajal, 1895). In contrast, he proposed the *insulation theory*, that is, that astrocytes would serve as cellular insulators that separated the activity of neighboring neurons. While this hypothesis was not confirmed by subsequent studies, it is noteworthy that it was probably the first time that a direct involvement of astrocytes in neuronal function was proposed. Indeed, Cajal indicated "***No estimamos las hipótesis que acabamos de exponer como teorías exentas de reproche. Pero no por esto las hipótesis racionales, que tienen su punto de partida en algunos hechos conocidos, dejan de ser legítimas y hasta fecundas. Una hipótesis científica representa una dirección nueva, un camino que se traza a la observación y a la experimentación, el cual, si no conduce inmediatamente a la verdad, suscita siempre investigaciones y críticas que nos aproximan a ella***" (*We do not consider this hypothesis exempt from reproach. But rational hypotheses based on some actual facts are legitimate and even fruitful. A scientific hypothesis represents a new direction, a path traced for observation and experimentation, which, if it does not immediately lead to the truth, always raises*

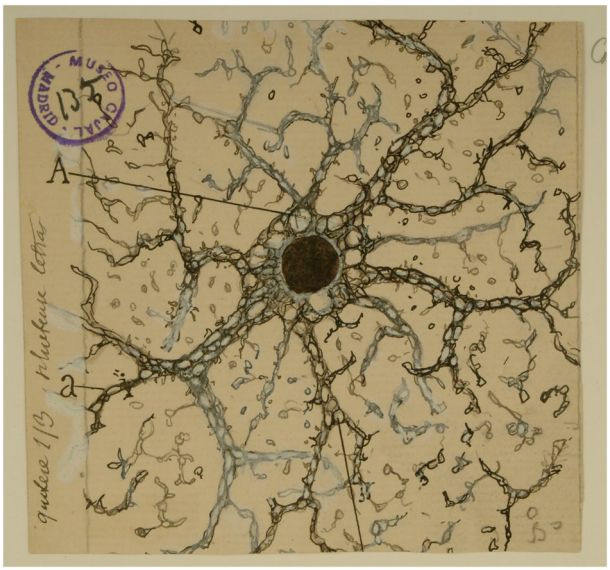

FIGURE 1 | Cajal's drawing showing a "neuroglia" of the pyramidal layer and stratum radiatum of the Ammon horn from adult man autopsied 3 h after death. (A) Indicates the large vacuoles of the soma; (a and b), the gaps of the expansions intended for the gliosomas. Reproduced from an original drawing, with permission of the Instituto Cajal.

investigations and criticisms that bring us closer to it) (Ramón y Cajal, 1895).

In the 1980s, cellular biology and neuroscience underwent a technological revolution led by the development and use of novel tools such as the patch-clamp technique, fluorescence imaging, and confocal and multiphoton microscopy, which allowed the detailed visualization of structural properties and physiological processes of cells. Using these novel techniques, the physiologist was no longer unarmed but rather endowed with an arsenal of potent tools to investigate the function of astrocytes. Until then, astrocytes were considered to simply provide trophic and structural support for neurons. This *Nutrition Theory* originally proposed by Golgi (1903) has been consistently confirmed, and astrocytes are recognized as fundamental cells providing the necessary metabolic and nutritional support for the proper development and function of neurons (for a review see, e.g., Bélanger et al., 2011). However, this theory limited the function of astrocytes to a passive role without direct involvement in information processing in the brain. Nevertheless, recent evidence indicates astrocytic glycogen breakdown and lactate release is essential for the maintenance of long-term synaptic strength, suggesting that metabolic support from astrocytes is required for long-term memory formation (Suzuki et al., 2011).

The idea that astrocytes play passive roles in brain function probably derived from the fact that electricity is the main biophysical substrate underlying brain physiology. While neurons use electrical events to convey information, astrocytes are not electrically excitable cells. Furthermore, neurons were recognized to be directly in contact with the external world, receiving information from the sensory organs and transmitting information to endocrine organs and muscles. In contrast, astrocytes are confined to the central nervous system without direct physical communication with the environment. However, the use of fluorescence microscopy and calcium-sensitive fluorescent dyes in the decade of 1990s revealed that astrocytes display cellular excitability based not on electrical events but on variations in intracellular calcium concentration (Cornell-Bell et al., 1990; Charles et al., 1991; Perea and Araque, 2006; Perea et al., 2009; Zorec et al., 2012), which serve as cellular signals with important consequences for the physiology of the nervous system. Indeed, transient variations in cytosolic calcium in astrocytes occur spontaneously, but more importantly, they can also be evoked by synaptic activity and sensory stimuli, indicating that astrocytes sense neuronal activity and synaptic transmission (Wang et al., 2006; Perea et al., 2009; Takata et al., 2011; Navarrete et al., 2012; Araque et al., 2014). Indeed, astrocytes express several G protein-coupled neurotransmitter receptors, which upon stimulation activate phospholipase C leading to inositol triphosphate (IP_3) production and calcium mobilization from internal stores. This astrocyte calcium signal can be elicited by a wide variety of neurotransmitters released from synaptic terminals, such as glutamate, gamma-aminobutyric acid (GABA), norepinephrine, dopamine, acetylcholine, serotonin, adenosine triphosphate (ATP) and nitric oxide (for reviews see Perea et al., 2009; Araque et al., 2014). Endocannabinoids released from postsynaptic neurons can also signal to astrocytes (Navarrete and Araque, 2008, 2010; Min and Nevian, 2012).

Consequently, astrocytes are now recognized to receive signals from neurons, actively responding to neuronal and synaptic activity with cytosolic calcium elevations evoked by neurotransmitters.

While synaptic activity is the input signal detected by astrocytes, what is the output of the astrocytic activity and what are its functional consequences?

"La neuroglía de la substancia gris vendría a constituir una vasta glándula endocrina intercalada entre las neuronas y plexos nerviosos, destinada quizás a elaborar hormonas asociadas a la actividad cerebral" *(The gray matter neuroglia would constitute a vast endocrine gland intertwined with neurons and nerve plexus, intended perhaps to produce hormones associated with the brain activity)* (Ramón y Cajal, 1913).

Accumulating evidence obtained in the last 15 years has confirmed this original idea expressed by Cajal. Indeed, astrocytes can release neuroactive substances, called gliotransmitters, which include glutamate, GABA, ATP/adenosine, or D-serine (for reviews see Volterra and Meldolesi, 2005; Perea et al., 2009; Araque et al., 2014). These gliotransmitters may activate receptors in neurons, exerting different and complex effects depending on the neuronal receptor subtypes activated and their pre- or postsynaptic localization, that result in the regulation of the neuronal excitability and synaptic transmission and plasticity (Volterra and Meldolesi, 2005; Perea and Araque, 2010; Araque et al., 2014). Moreover, astrocyte signaling can regulate neural network function (Porto-Pazos et al., 2011), as recently described in the cortex where astrocytes regulate UP states (Poskanzer and Yuste, 2011; for a review see Araque and Navarrete, 2010) (**Figure 2**).

Evidence demonstrating the calcium-based astrocytic excitability elicited by synaptic activity and the

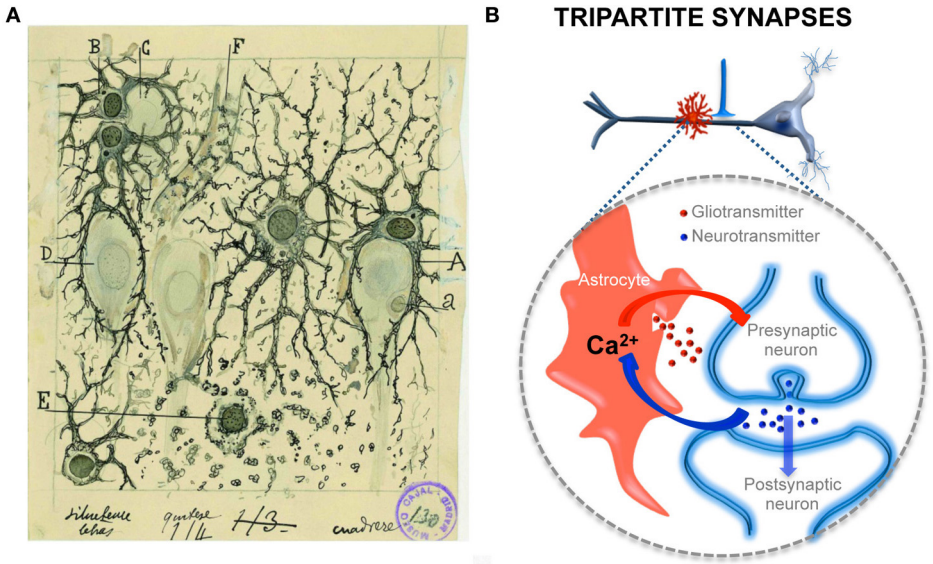

FIGURE 2 | Structural and functional relationships of neurons and astrocytes and tripartite synapses. (A) Cajal's drawing showing "neuroglia" of the pyramidal layer and stratum radiatum of the Ammon horn (from adult man autopsied 3 h after death). Original labels: A, large astrocyte embracing a pyramidal neuron; B, twin astrocytes forming a nest around a cell, C, while one of them sends two branches forming another nest, D; E, cell with signs of "autolysis"; F, capillary vessel. Sublimated gold chloride method. Reproduced from an original drawing, with permission of the Instituto Cajal. **(B)** Scheme of one axon establishing a synapse on an apical dendrite of a prototypical pyramidal neuron and an astrocyte located close to it (in red). The large dashed circle illustrate an enlarged schematic view of the tripartite synapse, where the pre- and postsynaptic neuronal elements (in blue) are surrounded by astrocytic processes (in red). It also depicts the transfer of information between neuronal synaptic elements and astrocytic processes. Astrocytes respond with Ca^{2+} elevations to neurotransmitters (blue dots) released during synaptic activity and, in turn, control neuronal excitability and synaptic transmission through the Ca^{2+} -dependent release of gliotransmitters (red dots).

calcium-dependent release of gliotransmitters that control synaptic transmission and plasticity has led to the establishment of a new concept in synaptic physiology, the Tripartite Synapse, in which astrocytes are integral elements of the synapses and actively exchange information with the neuronal elements (Araque et al., 1999; Halassa et al., 2009; Perea et al., 2009). This concept implies that the astrocytes directly play active roles in the transfer and storage of information in the brain and that the coordinated action of both neurons and astrocytes are involved in brain function.

Therefore, recent experimental findings regarding astrocyte physiology are in agreement with the original ideas expressed by Cajal based on observations of purely morphological data and on acute interpretation of those observations. Indeed, as he noted in 1897, "***Ciertos focos grises, ricos en plexos de expansiones dendríticas y de arborizaciones nerviosas, contienen muchas fibrillas de neuroglía y, al revés, ciertos focos pobres en dichos apéndices, son asimismo escasos en corpúsculos de Deiters o neuróglicos [astrocitos]***" and "***La neuroglía abunda donde las conexiones intercelulares son numerosas y complicadas, y no por el hecho de existir contactos, sino con la mira de reglarlos y dirigirlos de manera que cada expansión protoplásmica solo se ponga en relación íntima con un grupo especial de ramificaciones nerviosas terminales***" *(Certain gray nuclei enriched with plexus of dendritic expansions and nerve arborizations contain many neuroglia fibrils and, conversely, certain nuclei containing few of these appendices are also scarce in Deiters or neuroglia corpuscles [astrocytes]* and *the neuroglia is abundant where intercellular connections are numerous and complicated, not due to the existence of contacts, but rather to regulate and control them, in such a manner that each protoplasmic expansion is in an intimate relationship with only a particular group of nerve terminal branches)* (Ramón y Cajal, 1897).

Besides the intimate contact of astrocytes with synapses, they are also in close contact with blood vessels and capillaries. Cajal also proposed the physiological importance of astrocytes in the regulation of brain microcirculation.

"Todo astrocito de la substancia blanca o gris está provisto de un aparato chupador o pedículo perivascular. El aparato chupador constituye no sólo una disposición constante de los astrocitos de la substancia blanca, sino uno de los factores neuróglicos más importantes de los centros. Semejante generalidad, junto con el hecho de que en los animales de pequeña talla (conejo, cobaya, etc.), y en los en curso de evolución (perro y gato de pocos días), el órgano chupador constituye la más espesa, y a veces la única expansión perceptible y bien coloreable del astrocito denotan que el susodicho apéndice debe desempeñar cometido fisiológico de primer orden."

(Every astrocyte of the white or gray matter is provided with a sucking apparatus or perivascular pedicle [end foot]. The end foot is not only a constant characteristic of astrocytes in the white matter, but one of the most important neuroglial factors in the centers. Such generality, along with the fact that in small animals (rabbit, guinea pig, etc.) and in developing animals (few day-old cats and dogs) the

end foot is the thickest, and sometimes the only perceptible and well-stained expansion of the astrocyte, indicates that such an appendage must play a first-order physiological role) (Ramón y Cajal, 1913).

"El objeto de tales elementos es suscitar, por contracción de los referidos apéndices, dilataciones locales de los vasos, y, por ende, congestiones fisiológicas ligadas a la mayor o menor intensidad de los procesos psíquicos."

(The purpose of these elements is to provoke, by contraction of such appendages, local dilation of the vessels, and thus physiological congestions linked to the intensity of the mental processes) (Ramón y Cajal, 1895).

Astrocytes are currently recognized as key elements involved in the regulation of brain capillary blood flow during functional hyperemia, that is, the local increase in blood flow produced during neuronal activity that allows the local delivery of oxygen and nutrients in functionally active brain regions with greater energetic requirements. Indeed, recent findings have shown that regional increases in astrocyte calcium levels, produced by neurotransmitter release during neuronal and synaptic activity, stimulate the release of gliotransmitters and vaso-active compounds that regulate localized dilation or constriction of brain capillaries (for recent reviews see Iadecola and Nedergaard, 2007; Carmignoto and Gomez-Gonzalo, 2010; Petzold and Murthy, 2011; Newman, 2013) (**Figure 3**), providing compelling evidence for Cajal's idea proposed more than a hundred years ago.

"La corteza cerebral humana discrepa de la ·de los animales, no sólo por la cantidad enorme de células de tipo glandular que contiene, sino por la pequeñez de éstas y la riqueza del plexo gliomatoso intersticial." *(The human brain cortex differs from that of other animals not only in the huge amount of glandular cells [astrocytes] that contains, but in their smallness and the wealth of the interstitial glial plexus)* (Ramón y Cajal, 1913).

This initial observation made by Cajal has also been confirmed by recent evidence showing that human neocortical astrocytes are larger and extend more primary processes than those of non-primate mammals (Oberheim et al., 2006, 2009; Matyash and Kettenmann, 2010). Moreover, some special anatomically defined subclasses of astrocytes are specifically present in the human neocortex (Oberheim et al., 2009). Based on this evidence, it has been proposed that astrocytic complexity has permitted the increased functional competence of the adult human brain (Oberheim et al., 2006, 2009; Navarrete et al., 2013). In addition and in agreement with Cajal's idea, it is noteworthy that the ratio between glial cells vs. neurons increases along the phylogenetic scale, e.g., from around 0.1 in nematodes to around 10 in primates (Sherwood et al., 2006; Herculano-Houzel, 2012; Lewitus et al., 2012). Although an exhaustive quantification is still lacking, the high number of glial cells in mammals with superior brain functions may be indicative that astrocytes may provide a greater degree of complexity and computational capacity to the brain. Likewise, it is noteworthy that during human evolution, the brain size increased around 300% with respect to its primitive ancestors, whereas the number of neurons only increased by 125% (De Felipe, 2011). Therefore, the major difference in brain volume between humans and primates is not only due to a higher development of the neuronal neuropil but also to a higher number and complexity of astrocytes. Perhaps what makes us human is in part due to astrocytes.

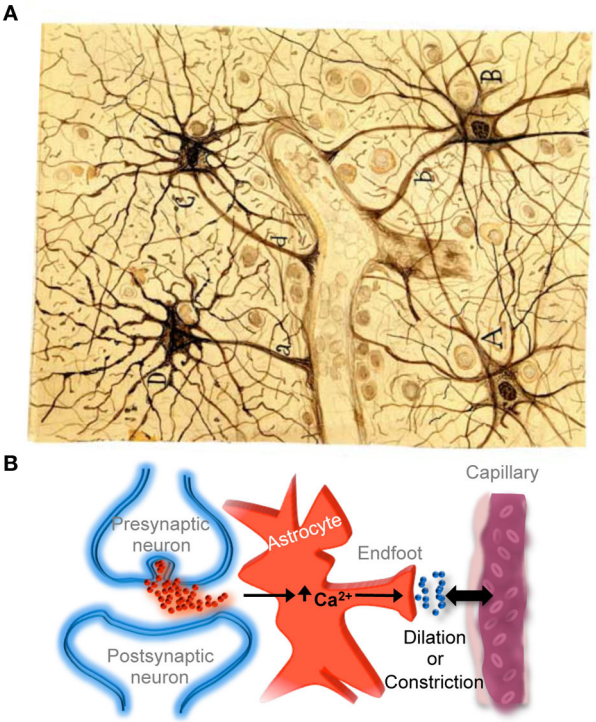

FIGURE 3 | Astrocyte-mediated control of neurovascular coupling. (A) Cajal's drawing of fibrous astrocytes of human cerebral cortex surrounding a blood vessel. The original slide was impregnated by the sublimated gold chloride method. Reproduced from an original drawing, with permission of the Instituto Cajal. **(B)** Scheme illustrating that neuronal synaptic activity can signal astrocytes to regulate cerebral microcirculation. It should be noted that much of the neurovascular coupling is achieved through effects on smooth muscle cells that are present in arterioles but not in capillaries.

In conclusion, the naïve notion that the function of glial cells in general, and astrocytes in particular, was merely to provide trophic and structural support, with no relevant contribution to brain function, has been overcome by recent findings obtained using sophisticated experimental techniques. These findings demonstrate that astrocytes are active cellular players involved in the processing, transfer, and storage of information by the nervous system (**Figure 2**). In many cases, these findings have confirmed experimentally many of the original ideas proposed by Cajal regarding the physiological significance of neuroglia.

"El prejuicio de que las fibrillas neuróglicas son a las células nerviosas lo que los haces colágenos del tejido conectivo a los corpúsculos musculares o glandulares, es decir, una trama pasiva de mero relleno y de sostén (y cuando más, una ganga destinada a embeberse en jugos nutritivos), constituye sin duda el principal obstáculo que el observador necesita remover para formarse un concepto racional de la actividad de los corpúsculos neuróglicos." *(The prejudice that the relation between neuroglial fibers and neuronal cells is similar to the relation between connective tissue and muscle or gland cells, that is, a passive for merely filling and support*

(and in the best case, a gangue for taking nutritive juices), constitutes the main obstacle that the researcher needs to remove to get a rational concept about the activity of the neuroglia) (Ramón y Cajal, 1899).

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REVIEW
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From the Cajal alumni Achúcarro and Río-Hortega to the rediscovery of never-resting microglia

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Under the guidance of Ramón y Cajal, a plethora of students flourished and began to apply his silver impregnation methods to study brain cells other than neurons: the neuroglia. In the first decades of the twentieth century, Nicolás Achúcarro was one of the first researchers to visualize the brain cells with phagocytic capacity that we know today as microglia. Later, his pupil Pío del Río-Hortega developed modifications of Achúcarro's methods and was able to specifically observe the fine morphological intricacies of microglia. These findings contradicted Cajal's own views on cells that he thought belonged to the same class as oligodendroglia (the so called "third element" of the nervous system), leading to a long-standing discussion. It was only in 1924 that Río-Hortega's observations prevailed worldwide, thus recognizing microglia as a unique cell type. This late landing in the Neuroscience arena still has repercussions in the twenty first century, as microglia remain one of the least understood cell populations of the healthy brain. For decades, microglia in normal, physiological conditions in the adult brain were considered to be merely "resting," and their contribution as "activated" cells to the neuroinflammatory response in pathological conditions mostly detrimental. It was not until microglia were imaged in real time in the intact brain using two-photon *in vivo* imaging that the extreme motility of their fine processes was revealed. These findings led to a conceptual revolution in the field: "resting" microglia are constantly surveying the brain parenchyma in normal physiological conditions. Today, following Cajal's school of thought, structural and functional investigations of microglial morphology, dynamics, and relationships with neurons and other glial cells are experiencing a renaissance and we stand at the brink of discovering new roles for these unique immune cells in the healthy brain, an essential step to understand their causal relationship to diseases.

Keywords: microglia, discovery, Cajal, Achúcarro, Río-Hortega, imaging, neuroanatomy, phagocytosis

The Discovery of Microglia

To us current investigators of microglia it is difficult to appreciate the 100-year research that has led us to where we are today. This road was plagued with obstacles, from the development of novel methods to visualize cells, to the difficulties in comparing results from different labs, which

hindered the reach of a consensus nomenclature for the different cell types that constitute the neuroglia of the central nervous system (CNS). Neuroglia was first described by Virchow in 1846 as an adhesive substance connecting neurons, but it took 75 more years to realize that the neuroglia is composed of cells belonging to three major types: astrocytes, oligodendrocytes, and microglia (Garcia-Marin et al., 2007). One particularly critical point in this discovery was the systematic testing of different methods of fixation and impregnation of the brain tissue to allow the selective and complete staining of the different types of neuroglial cell populations. Most of the impregnation methods relied on the use of silver ions combined with other metals to bind to different (unknown) proteins, many of which are still being used by neuropathologists. Among these, labeling methods were eventually found that allowed the complete visualization and identification of microglia in 1919. In this process, two Spanish researchers were instrumental: Nicolás Achúcarro and Pío del Río-Hortega, both alumni of the Santiago Ramón y Cajal School. In spite of their limited microscopy techniques, these researchers were thorough anatomists that from the simple observation of fixed tissue were able to infer important postulates about the nature (ectodermic vs. mesodermic origin), structural plasticity (motility), and function (phagocytosis of neuronal debris) of microglia that are still valid today (Kettenmann et al., 2011).

In the early 1900s, Cajal was the undisputable world leader in functional neuroanatomy. His critical contributions to our understanding of the CNS granted him, along with the prominent Italian neuroanatomist Camilo Golgi, a 1906 Nobel prize in Medicine. Cajal's Laboratory of Biological Research was located in Madrid, Spain, where he trained many generations of neuroanatomists in the development of new methods, systematic observation of the brain tissue, and detailed drawing to provide functional hypotheses about the roles played by the different types of brain cells. Cajal is best known for his seminal observations of neurons and their connectivity using the Golgi staining, leading to propose in 1888 the "neuron doctrine" (as opposed to the "reticular theory"), but by the end of the century he became very interested in neuroglia and started to develop novel methods to visualize them. In 1897 he published his first paper entirely devoted to neuroglia, where he speculated that a main role of glial cells was to preserve neuronal circuits and avoid inappropriate contacts (Ramón y Cajal, 1897; Garcia-Segura, 2002; Navarrete and Araque, 2014). At that time, the term "astrocyte" had already been introduced by Michael von Lenhossék (Garcia-Marin et al., 2007) and used to describe both protoplasmic and fibrous star-shaped cells. It was also clear that astrocytes were not the only type of neuroglial cell in the brain. In fact, a myriad of cells had already been described by different investigators each using their own methods of staining, but considering the lack of documentation available today, it still remains unclear whether they were actually discussing about the same cell types (**Table 1**).

To the Cajal School belongs the Basque-born psychiatrist Nicolás Achúcarro, both a colleague and an alumnus of Cajal (Vitoria Ortiz, 1977). After graduating as a medical doctor at the University of Madrid, Spain in 1904, Achúcarro traveled to Munich, Germany, to work in Alois Alzheimer's lab as a scientist and psychiatrist. There he became interested in neuroglia, and particularly in the rod cells *(Stäbchenzellen*), a cell type that Franz Nissl had discovered in 1898 while observing human autopsy cases of mentally ill patients with paralysis (Vitoria Ortiz, 1977; Rezaie and Hanisch, 2014). Achúcarro was able to visualize these cells in the brains of rabbits infected with rabies or damaged by focal or inflammatory injury using a Scharlach Red (specific for fatty tissue) and hematoxylin (which stains nuclei) staining. In particular he observed cells whose shape was adapted to that of neurons, localized around the "necrotic foci" (*sic*) in the pyramidal cell layer of the hippocampus. These cells were full of fatty degeneration products (that were called "protagonoid substances"), likely resulting from the degeneration of nervous structures. Since these "granuloadipose" cells were concentrated around the lesion area, he hypothesized that their role was to phagocytose the damaged neurons. Further, he thought that their elongated, rod shape was related to their active movement between the degenerating neurons (Achúcarro, 1909; de Castro, 1977).

After leading the Laboratory of Pathological Anatomy of the Federal Psychiatric Hospital in Washington DC, USA, Achúcarro returned to Madrid to join Cajal's laboratory. In 1910 he set up his own "Histology Laboratory" at the Students' Residency, which was part of the Free Teaching Institution, a precursor of the Spanish National Research Council. From Cajal he acquired the latest staining techniques and developed his own tannin and ammoniacal silver nitrate method (Achúcarro, 1911). With his staining, Achúcarro was able to clearly differentiate phagocytic, granuloadipose, and non-fibrous rod cells, from stellate cells with vascular end-feet and neuroglial fibrilles, which respectively correspond to our current understanding of microglia and astrocytes (Achúcarro, 1913) (**Figure 1**). Achúcarro strongly supported the idea that neuroglial cells must exert other functions than merely supporting neurons, an idea that had been widely accepted since Virchow. It was also clear to him that glial cell dysfunction could itself produce brain diseases, even without any primary damage to neurons (Achúcarro, 1913). This idea of glial cells primarily causing "gliopathies," as opposed to secondarily reacting to neuronal damage in "neuropathies," is re-emerging in today's research (Verkhratsky et al., 2012).

Another strong matter of discussion at the time concerned the origin of the granuloadipose cells. Achúcarro had initially observed what seemed to be an intermediate form between the granuloadipose cells and astrocytes, leading him to conclude that granuloadipose cells were derived from neuroglia, and thus had an ectodermic origin, supporting the thesis of many researchers including Nissl, who later reconsidered this theory (Achúcarro, 1913). Later, he observed what seemed to be granuloadipose cells migrating into the brain parenchyma from blood vessels, as Alzheimer had stated before, and inferred that some of these cells could simply be circulating monocytes (de Castro, 1977). However, there was no method available at that time to discriminate between these two alternative hypotheses. In fact, the neuroectodermic origin of astrocytes and the bone-marrow origin of circulating monocytes were found relatively early, but the unique origin of microglia as cells derived from the embryonic yolk sac had remained unknown (Alliot et al., 1999) and was only

TABLE 1 | The first steps of glia research.

Cell type	Year	Author	Term	Origin	Function	First location
Glia	1856	Virchow	Neuroglia	Ectodermal	Connection with neurons	Brain tissue
Astrocyte	1891	Van Lenhossék	Astrocyte	Ectodermal	Structural support	Brain tissue
Microglia and oligodendroglia	1913	Cajal	Third element	Mesodermal		Brain tissue
Microglia	1841	Gluge	Inflammatory corpuscles	Mesodermal	Phagocytosis	Damaged brain
Microglia	1856	Virchow	Foam cells	Mesodermal	Phagocytosis	Atherosclerosis plaques
Microglia	1899	Nissl	Rod cells	Mesodermal	Phagocytosis	Cerebral palsy
Microglia	1908	Achúcarro	Granuloadipose cells	Ectodermal/ Mesodermal	Phagocytosis	Brain of rabbits with rabies
Microglia	1910	Merzbacher	Scavenger cells	Mesodermal	Phagocytosis	Demyelinating CNS disorder
Microglia	1919	Del Río-Hortega	Microglia (mesoglia)	Mesodermal	Phagocytosis	Brain tissue
Oligodendroglia	1899	Robertson	Mesoglia	Mesodermal	Phagocytosis	Human and canine brain
Oligodendroglia	1921	Del Río-Hortega	Oligodendroglia (interfascicular glia)	Ectodermal	Support, isolation, nutrition	Brain tissue

Table of the different terms used by the listed authors to designate glial cells, and their opinion on their presumed origin and function. Thorough accounts on the history of their discoveries can be found elsewhere (Rezaie and Hanisch, 2014). After Virchow coined the term "neuroglia" in the mid-nineteenth century to describe a substance that connected neurons, different researchers observed what seemed to be different types of glial cells in a variety of pathological conditions. Astrocytes (protoplasmic and fibrous) were readily identified by Van Lenhossék, but the rest of the neuroglial cell types received different names under different researchers and it was unclear whether they were in fact the same cell types. Ramón y Cajal grouped them under the term "third element," but it was Río-Hortega who finally settled the issue and unambiguously discriminated between oligodendrocytes and microglia.

recently confirmed using fate mapping strategies (Schulz et al., 2012; Ginhoux et al., 2013; Kierdorf et al., 2013).

The work of Achúcarro on granuloadipose cells sparked the interest of Cajal on these cells, although they both acknowledged that the tannin and ammoniacal silver nitrate method used by Achúcarro was producing a partial and inconsistent labeling (de Castro, 1977; Vitoria Ortiz, 1977). This led Cajal to recall his former observations when he solely used formol as a fixative, to obtain a more selective staining of neuroglial processes, and to ultimately develop his formol uranium nitrate and sublimated gold chloride method (Ramón y Cajal, 1913; de Castro, 1977; Vitoria Ortiz, 1977), which soon became the standard to visualize fibrous and protoplasmic neuroglia (i.e., astrocytes) (Garcia-Marin et al., 2007). Nonetheless, this method barely stained the other type(s) of glial cell(s), and in 1913, Cajal introduced the controversial term "third element" to describe what he thought was the remaining class of glial cells: dwarf, adendritic, and apolar cells of the white matter, with perineuronal and perivascular location, and a mesodermal origin (Ramón y Cajal, 1913; Garcia-Marin et al., 2007).

These studies could not be pursued as Achúcarro became gravely ill in 1915 and passed away at a young age in 1918 from a self-diagnosed Hodgkin's lymphoma. He was deeply missed by his colleagues and Cajal, who wrote a wholehearted obituary (Ramón y Cajal, 1977). Nonetheless, his laboratory remained active and a student of his, Pío del Río-Hortega, was designated by Cajal to take the lead (Cano Diaz, 1985). Río-Hortega graduated in Medicine at the University of Valladolid, Spain in 1905. After unsuccessfully trying to join Cajal's lab for unclear reasons, Río-Hortega worked as a clinician and completed his doctorate courses at the University of Madrid, to finally obtain his PhD title from the University of Valladolid for his work on brain tumors in 1908. In 1913 he returned to Madrid and was admitted to Achúcarro's lab, with whom he developed a close master-student relationship (Prados y Such, 1977; Cano Diaz, 1985). After some brief stays in London and Berlin, the First World War forced Río-Hortega to return to Achúcarro's lab in 1915, which was now located just next to Cajal's Laboratory of Biological Research (Prados y Such, 1977; Cano Diaz, 1985). Río-Hortega professed a great admiration for Achúcarro, whom he considered an ingenuous and stimulating, benevolent and generous mentor (Prados y Such, 1977).

Notwithstanding his great scientific contributions and large body of publications, Río-Hortega entered in strife with some members of the Cajal School and Cajal himself (Cano Diaz, 1985; Rezaie and Hanisch, 2014). It is unclear whether behind this conflict were purely scientific reasons or more personal problems (Cano Diaz, 1985). The fact is that Río-Hortega's findings contradicted Cajal's view of the "third element" and that he left Cajal's lab in 1919 to establish his own lab back at the Student's Residency in 1920 (Cano Diaz, 1985; Rezaie and Hanisch, 2014). Río-Hortega was particularly involved with the search for simple, specific, and replicable methods to stain the nervous tissue, because he acknowledged, like Cajal before him, that novel findings required novel techniques (Cano Diaz, 1985). He acquired the methods of Cajal and Achúcarro, of Golgi and others and developed many variations, among them his famous silver carbonate method (a modification of Achúcarro's ammoniacal silver method) which provided an exceptional visualization of glial cells (Río-Hortega, 1916, 1917). With this method, Río Hortega was able to obtain smaller micelles that impregnated the finest details of the brain tissue (Rezaie and Hanisch, 2014) and revealed in great depth the morphology of what he classified as two independent cell types: microglia and interstitial cells (which he later

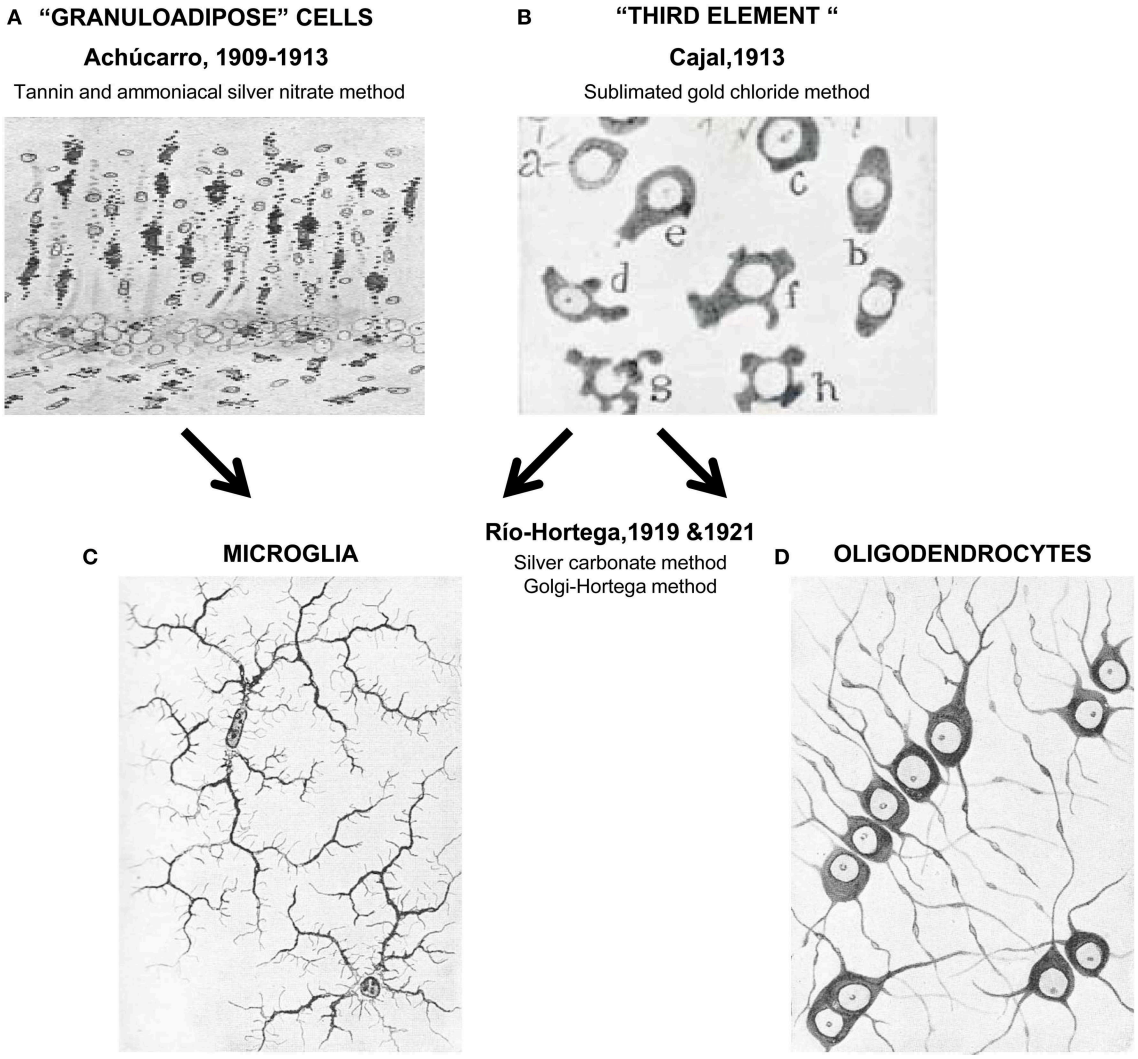

FIGURE 1 | Unraveling the nature of the "third element." In the early years of the twentieth century Achúcarro developed a novel method of staining (tannin and amoniacal silver nitrate) that allowed him to visualize what were at the time called "granuloadipose cells" that is, a group of cells that seemed to phagocytose degradation materials in the brain of rabid rabbits **(A)**. Stimulated by the interest of Achúcarro in glia, Cajal developed his famous sublimated gold chloride method that allowed him to beautifully visualize astrocytes but left the remaining neuroglial cells poorly stained (a–h, in **B**). To describe these apolar, dwarf cells he introduced the term "third element" of the nervous system. Río-Hortega continued the work of Achucarro and in 1919 developed his silver carbonate method, finally enabling him to resolve microglia **(C)** from what he initially called interfascicular cells and later oligodendrocytes (using his Golgi-Hortega method; **D**). Achúcarro's images are reprinted with permission from Achúcarro (1909). Cajal's image is reprinted with permission from Garcia-Marin et al. (2007). Río-Hortega's images are reprinted with permission from Río-Hortega (1919c).

named oligodendrocytes) (Río-Hortega, 1919a,b,c). Río-Hortega also realized that these two cell types were in fact what Cajal had called "adendritic" cells and that the master had not been able to discriminate between them because of an incomplete visualization of their processes using the sublimated gold chloride method of staining.

To Río-Hortega, it was clear that microglia have a small, dark nucleus, no signs of centrosome, a Golgi apparatus, some gliosomes (granules), and a profuse tree of processes: *"[microglia] are characterized by their small, dark nucleus enveloped by scant protoplasm and its long, tortuous, ramified expansions adorned with lateral spines"* (Río-Hortega, 1919b). His drawings of these cells stained with the silver carbonate method shockingly resemble our current labeling of microglia using modern immunostaining and transgenic labeling strategies (**Figure 1**), revealing the strengths of his fixation and staining techniques, as much as the exceptional quality of his artistic talents. In addition, Río-Hortega was a skillful writer who provided a clear articulation of systematically collected evidences and arguments in support of his conclusions. His technical prowess led him to discover

that microglia are regularly distributed throughout the brain, showing a higher density in gray than in white matter areas (Río-Hortega, 1919c). He also proposed that, in contrast to astrocytes, microglia are highly migratory and structurally dynamic, because their shape adapts to the contours of the other nervous structures (Río-Hortega, 1919b), as Achúcarro had observed before him (Achúcarro, 1909). In his own words: "*The plasticity of their protoplasm when they insinuate through the interstices that separate the fibers as they follow their cross directions, when they go parallel to the cells and wrap them in their processes, and when they stretch in places where the nerve structures follow the same direction, and develop their expansions in the same way as neuron processes form their plexuses, and so on, show very clearly that their shape is mutable and conditional; that their protoplasm is capable of plasticity; and that they have, in short, a quality inherent to the migrant corpuscles, among which, in all probability, microglia must be included."* (Río-Hortega, 1919b).

But he believed that it was during pathological conditions that microglia showed their true nature: "[…] *the nomadic nature of this [microglia] is best revealed during the destructive processes of the nervous tissue, when the apparent rest that it enjoys during normality turns into migratory and phagocytic activity*" (Río-Hortega, 1919b). Following the time course of a stab wound in the newborn cat, he proposed that microglia transformed into the granuloadipose cells of Achúcarro, becoming hypertrophic or ameboid and full of lipidic phagocytic inclusions (Río-Hortega, 1919c). Río-Hortega described a full progressive-regressive (*sic*) morphological cycle of microglia, from the ramified, sedentary (*sic*) forms found in normal physiological conditions to the amoeboid, phagocytic forms observed during injury, which to him resembled the morphology they display during development: "[…] *the forms acquired by microglia while passing, in pathological cases, from the normal or sedentary form to the globular form, are an accurate reflection of those which are successively acquired over the course of normal development. Our latest research confirms that in their evolution microglia go from the rounded to the stellar shape with branches, while in their involution they tend to regain the original form*" (Río-Hortega, 1919c). This analysis of microglial function based on their morphology has permeated even to our days, and has for many years been interpreted as a cascade of stereotypical activation from the ramified, resting form to the "activated," hypertrophic cell, culminating into the phagocytic amoeboid state (Graeber, 2010; Streit et al., 2010; Tremblay et al., 2011). As we will discuss later, even though all those morphological stages can be observed in particular contexts of health or disease, the cascade of microglial "activation" in itself is now viewed as an oversimplification of microglial functional repertoire.

Río-Hortega's experiments also settled the problem of the granuloadipose cells nature that had been concerning researchers since Nissl back in 1898, clearly establishing them as microglia for once. Nonetheless, the origin of microglia still remained undetermined as discussed above. To Cajal, the "third element" was composed solely of cells of mesodermic origin. Río-Hortega, however, argued that the problem dwelt in the confusion between different cell types (i.e., microglia and oligodendrocytes), thus leading to contradictory observations (Río-Hortega, 1919b). He stated that oligodendrocytes had an ectodermic origin, and were part of the neuroglia. As astrocytes, oligodendrocytes adopted a permanent shape and location once development was completed. They had a round or polyhedral, epithelial-like cell body; few, long and poorly ramified processes; and were preferentially located in the white matter, aligned with the nerve tracts (Río-Hortega, 1919b). He proposed that oligodendrocytes were homologous to the Schwann cells of the peripheral nervous system and formed membrane expansions around the myelin wraps, which at the time were thought to be of axonal origin, although to him the analogy between oligodendrocytes and Schwann cells rather evidenced a glial origin of myelin (Río-Hortega, 1922). These ideas were initially contested by alumni of Cajal such as Lorente de No and de Castro (1923). De Castro later changed his mind and fully acknowledged the relevance of Río-Hortega's discoveries (de Castro, 1977). In contrast to oligodendrocytes, Río-Hortega claimed that microglia were clearly of mesodermic origin because of their late appearance in the brain parenchyma during development, often in close association with blood vessels. Microglia also showed strong analogies of shape, staining properties, nuclear structure, and phagocytic capacity with circulating monocytes (Río-Hortega, 1919a). Considering these properties as strong evidence for an origin outside of the brain, he believed that microglia truly represented a "third element" (Cano Diaz, 1985; Gill and Binder, 2007). In fact, he profusely referred to microglia as "mesoglia" (Río-Hortega, 1919b).

Río-Hortega eventually published his findings in Cajal's journal (Rio-Hortega, 1920) but Cajal answered in the same issue (Ramón y Cajal, 1920) that "mesoglia" had already been described back in 1900 by a Scottish researcher, William Ford Robertson (Rezaie and Hanisch, 2014). Robertson had used a platinum method to visualize these "mesoglia," which he described to be of mesodermal origin, not related to nerve fibers, and phagocytic due to their lipidic inclusions (Iglesias-Rozas, 2013). However, Robertson's paper did not provide any images and it seems that his staining was particularly difficult to reproduce (Iglesias-Rozas, 2013; Rezaie and Hanisch, 2014). Cajal acknowledged that his "third element" was in fact composed of three different cell types whose morphology, structure and function were likely different: dwarf satellites, the interfascicular cells of Río-Hortega, and the microglia or Robertson/del Río cells (Ramón y Cajal, 1920). To Cajal, the priority in the discovery of microglia was Robertson's, although he conceded that he had not been able to read Robertson's original work but rather citations by other authors (Ramón y Cajal, 1920). The conundrum was solved by Wilder Penfield, an American neurosurgeon who visited Rio-Hortega's lab to learn his techniques and apply them to his epilepsy research (Gill and Binder, 2007; Rezaie and Hanisch, 2014). Penfield was able to compare Robertson and Río-Hortega's preparations. His observations suggested that Robertson's "mesoglia" were in fact Río-Hortega's oligodendrocytes, which decisively prompted to abandon the term "mesoglia" altogether (Gill and Binder, 2007; Rezaie and Hanisch, 2014). Although Robertson was indeed the first researcher to visualize oligodendrocytes, he was notoriously wrong in his claims about their origin and function. It was Penfield indeed who in 1924 finally settled down the dispute and established that

the three main types of glial cells in the brain are astrocytes, oligodendrocytes, and microglia, giving the full credit to Río-Hortega for his discovery of microglia and oligodendrocytes (Gill and Binder, 2007). The initial reluctance of Cajal and his alumni to accept Río-Hortega's findings tarnished his reputation in Spain, despite his worldwide recognition. Nonetheless, the long-expected reconciliation with Cajal finally took place in 1928, when the old master was 77 years old (Cano Diaz, 1985).

In the meantime, Río-Hortega continued to study microglia on his own, in his small laboratory at the Student's Residency. He was particularly interested in their phagocytic function, which he described thoroughly (Río-Hortega, 1919c). In particular, he found that microglia contained large amounts of granularities, some of which were enclosed in vacuoles, as early as they appeared in the nervous parenchyma during development. He hypothesized that "*before young neurons and embryonic neuroglia acquire their definitive shape and location, [may] occur the fragmentation of some delicate appendices or phenomena of disintegration and de-assimilation that require the intervention of microglial macrophages to gather and transform the resulting debris*" (Río-Hortega, 1919c). Further, in foci of experimental necrosis (*sic*) microglia appeared as voracious macrophages, filled by adipose granules, as well as entire erythrocytes and leukocytes. The need for an increased phagocytosis, argued Río-Hortega, fueled microglia to proliferate during pathological conditions, an interesting observation that deserves to be tested experimentally. Indeed, after migration and phagocytosis, proliferation was to Río-Hortega the ultimate response of microglia to injury. Microglial proliferation, reasoned Río-Hortega, explained why the damaged tissue was soon filled with microglia in the absence of infiltration from circulating monocytes (Río-Hortega, 1919c). Even today, the infiltration of these cells into the brain, and their differential contribution to brain disorders compared with resident microglia remains an open area of study (Gomez-Nicola and Perry, 2014; Prinz and Priller, 2014; Prinz et al., 2014).

Later, in 1928, Río-Hortega was appointed head of biological research at the Institute of Cancer in Madrid. From there he continued to work on microglia and oligodendrocytes, among other topics comprising the morphology and function of intracellular organelles; the classification and nomenclature of brain tumors; and the histology of the pineal gland, the hematopoietic organs (spleen), the digestive and urinary systems, etc. (Cano Diaz, 1985). Due to his leftish political views, and in spite of having a worldwide recognition (he was twice nominated for the Nobel Prize), he had to leave Spain during the Civil War (1936–1939). He spent some time at the Hôpital de la Pitié, Paris and the University of Oxford, UK, where he was awarded an honoris causa doctorate. He then exiled to Argentina in 1940 to settle a new lab and continue his research until he died of a self-diagnosed genital cancer in 1945 (Castellano and Gonzales, 1995; Palmero and Del Río-Hortega, 2002). His legacy continued with Isaac Costero, a student in his lab who learned tissue culture techniques at the Paul Ehrlich Institute in Frankfurt, Germany and implemented the first culture of microglia from the human brain, which he imaged in time-lapse to confirm the motility and phagocytic capacity of microglia (Rezaie and Hanisch, 2014); and with Wilder Penfield, who became a famous neurosurgeon specialized in the treatment of epilepsy, notorious for his functional mapping of the brain, and a pioneer in the study of glial scars (Gill and Binder, 2007).

The Renaissance of Functional Neuroanatomy

The principles of functional neuroanatomy established by the Cajal school of thought are now more than ever in vogue in microglial biology: a detailed and systematic morphological analysis of the various cell types and their interactions one with another, that leads to biologically relevant functional hypotheses which can then be directly tested using tools from modern molecular biology combined with state-of-the-art imaging. To this renaissance illustrated in **Figure 2**, three imaging techniques have been critical in the last decade: transmission electron microscopy (TEM) to obtain high resolution images; confocal microscopy to scan large areas of tissue; and two-photon microscopy to observe microglia in real time in the living brain. In particular, three postulates derived from the original observations of Achúcarro and Río-Hortega, on which the second part of our review focuses, still shape our current research on microglia: (1) their unique origin, (2) their morphological plasticity, and (3) their extreme capacity for phagocytosis [for a systematic review of different aspects of the history of microglial research refer to (Rezaie and Hanisch, 2014)].

A major revolution in the field came from studies showing that, unlike most tissue resident macrophages, microglia are not constantly replaced by bone-marrow derived monocytes from the blood, but in fact are seeded during early embryonic development from the infiltration of yolk sac derived precursors (Ginhoux et al., 2010; Schulz et al., 2012; Kierdorf et al., 2013). The ectodermal vs. mesodermal origin of microglia was a contended topic at the times of Achúcarro, Río-Hortega, and Cajal, and still continued to be highly debated until the 1990s, when it finally became clear that microglia belong to the myeloid lineage (Ginhoux et al., 2013). This idea settled the field for over two decades, during which mature microglia were mostly considered as macrophages orchestrating the brain inflammatory response to pathological insults, even though these cells were also shown to have a remarkable down-regulated inflammatory phenotype in the healthy brain, and to be extremely stable and long-lived in comparison with other macrophage populations (Lawson et al., 1992). In fact, it was not until their precursors from the yolk sac were traced using transgenic fate mapping strategies (Ginhoux et al., 2010; Schulz et al., 2012; Kierdorf et al., 2013) that the community realized their unique properties and functions, particularly in normal physiological conditions. Their repertoire of physiological roles discovered so far in the developing and mature CNS comprises the regulation of neural progenitors survival, blood vessel growth, developmental cell death, axonal sprouting, and neuronal firing, synaptic activity and plasticity, among others, in addition to neuronal circuit remodeling through the phagocytosis of newborn cells and synaptic elements as discussed below (Eyo and Dailey, 2013; Bilimoria and Stevens, 2014; Nayak et al., 2014).

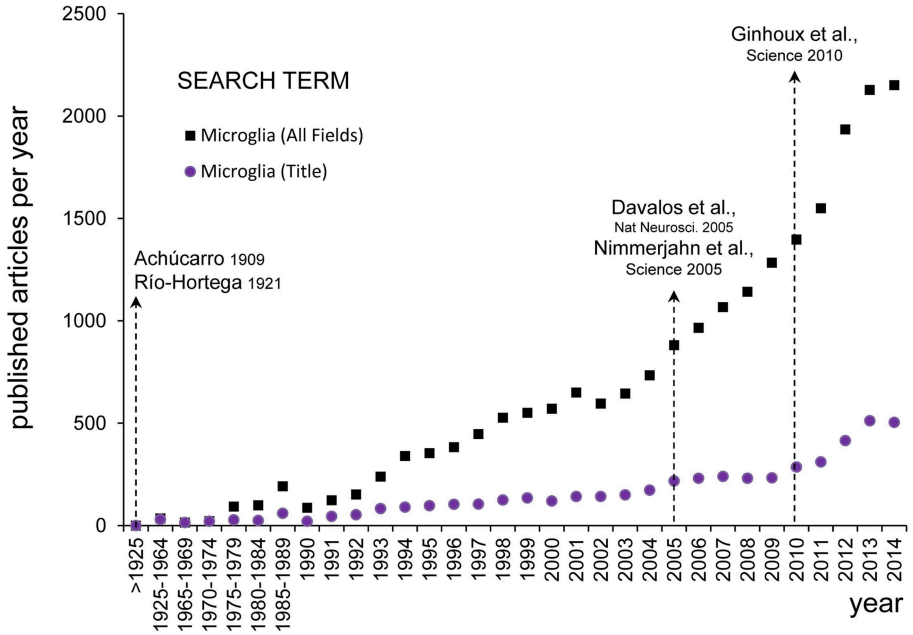

FIGURE 2 | Evolution of microglial research. The numbers of published papers per year about microglia was assessed on PubMed Central (www.ncbi.nlm.nih.gov) using the search term "microglia" (All Fields) and setting the Date of Publication from January 1st to December 31st from 1900 until 2014 (black squares). Since the earliest descriptions of Achúcarro and Río-Hortega at the beginning of the twentieth century, there were barely any papers discussing microglia published before 1990 (456 papers in total), from when there was a steady increase until the end of the Century (356 papers per year). It was not until the early 2000s that there was an exponential growth of microglial research (1180 papers per year in 2000–2014). The inflexion point seems to be located around 2005, after the publication of the seminal papers by Davalos and Nimmerjahn on the extraordinary motility of microglia in the adult brain in physiological conditions (Davalos et al., 2005; Nayak et al., 2014). To detect more specifically papers focused on microglia, we performed another search looking for the term "microglia" in the title (Title Field) (purple dots). Over the period 1990–2014, the percentage of papers with "microglia" in their title over the total number of papers including "microglia" in any field was maintained at a fairly constant rate of 24 ± 0.9% (mean ± SEM). When searching for papers with "microglia" in their title a second inflexion point is also evident around 2010 (155 papers per year in 2000–2004, 231 papers per year in 2005–2009, and 406 papers per year in 2010–2014), coincident with the publication of the influential paper by Ginhoux on the unique origin of microglia from the embryonic yolk sac (Ginhoux et al., 2010). This figure does not intend to be a systematic analysis of the most influential papers on microglia research, but to evidence that the rediscovery of the findings by Achucarro and Río-Hortega shapes our current research in microglial biology.

Another breakthrough occurred in the last decade when microglial dynamics were examined for the first time in the intact brain of living animals, through the skull of transgenic mice where they were fluorescently labeled, using two-photon microscopy (Davalos et al., 2005; Nimmerjahn et al., 2005). These important observations confirmed Achúcarro and Río-Hortega's hypothesis that microglia are extremely dynamic (Achúcarro, 1909; Río-Hortega, 1919b). The degree of their structural plasticity surpassed all expectations: microglial processes were found to survey their surrounding environment on a time scale of minutes, in contrast to neurons and other types of neuroglial cells which show no comparable structural remodeling *in vivo* (Majewska and Sur, 2003; Eom et al., 2011; Hughes et al., 2013; Lamantia et al., 2014). With these observations, microglia have emerged as the most dynamic cell type of the mature CNS. Their dynamism is far from being random, but microglia continuously respond to neuronal activity and behavioral experience in the healthy brain (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009; Tremblay et al., 2010). From the recent literature, an important distinction between microglial motility (of processes) and mobility (migration of cell body) has to be made (Eyo and Dailey, 2013). In particular, microglia are extremely motile in the mature healthy brain, even though they migrate very little (Tremblay et al., 2010; Hefendehl et al., 2014). During normal aging and neurodegenerative conditions, including Alzheimer's disease and amyotrophic lateral sclerosis, however, microglia acquire the ability to migrate, confirming Río-Hortega's idea that it is during pathological conditions that microglia would show their dynamic behavior at best (Río-Hortega, 1919b). In these conditions, their surveillance of the environment (motility) is concomitantly reduced (Damani et al., 2011; Dibaj et al., 2011; Krabbe et al., 2013; Hefendehl et al., 2014), possibly because they become involved with many other tasks that are required for the inflammatory response.

Microglial motility is not fully understood but has emerged as a major property underlying their immune surveillance function during normal physiological conditions. It has been estimated that microglia could scan the whole brain parenchyma every few hours (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009; Tremblay et al., 2010). Among the structures contacted by microglia in the healthy brain stand out synaptic elements, particularly pre-synaptic axon terminals and post-synaptic dendritic

spines. Microglial contacts with synaptic elements have been observed *in vivo* with two-photon microscopy, showing durations varying between 5 and 30 min, as well as in fixed tissue using TEM (Wake et al., 2009; Tremblay et al., 2010; Sogn et al., 2013). Importantly, these contacts are widespread, because almost all (∼94%) microglial processes directly juxtapose synaptic elements, axon terminals, dendritic spines, perisynaptic astrocytic processes, and synaptic clefts in decreasing order of frequency, in adolescent mouse cerebral cortex (Tremblay et al., 2010). Serial section TEM with 3D reconstruction further revealed that a single microglial process can make multiples contacts with synaptic elements, at multiple synapses simultaneously, sometimes with morphological specializations in the form of finger-like protrusions wrapping around dendritic spines and axon terminals (Tremblay et al., 2010). Clathrin-coated vesicles, which are responsible for the endocytosis of membrane-bound receptors and their ligands (Le Roy and Wrana, 2005), were also observed inside of microglial processes and synaptic elements, specifically at their sites of contact (Tremblay et al., 2010), suggesting reciprocal exchange of molecular signals (which remain to be identified) via clathrin-mediated endocytosis. These two types of specializations indicate that never-resting microglia interact functionally with excitatory synapses (Tremblay and Majewska, 2011). While the function of these contacts remains to be determined, it is now clear that in normal physiological conditions microglia both sense and react to neuronal activity (Miyamoto et al., 2013; Wake et al., 2013; Tremblay et al., 2014).

The last seminal observation of Achúcarro and Río-Hortega concerned microglial capacity for phagocytosis. Microglia are indeed the brain professional phagocytes, and they engulf cellular debris in larger amounts and more rapidly than other brain cells with phagocytic capacity, such as astrocytes (Parnaik et al., 2000; Magnus et al., 2002). A longstanding view over the last century is that microglia need to be activated in order to become efficient phagocytes, and that phagocytic microglia should necessarily adopt an ameboid morphology (Sierra et al., 2013). However, recent findings show that ramified, surveillant microglia are true phagocytes that engulf cells undergoing apoptosis, the major form of cell death, through terminal or *en passant* branches in the adult healthy brain (Sierra et al., 2010). Using as a model the hippocampal neurogenic cascade, where newborn neurons undergo apoptosis throughout adulthood, and a combination of TEM, confocal microscopy, and unbiased stereology methods of quantification it was shown that apoptosis and microglial phagocytosis are tightly coupled, and that phagocytosis of apoptotic cells is fully executed under 90 min on average (Sierra et al., 2010). Two-photon *in vivo* imaging and TEM also revealed that microglia make use of their potential as phagocytes in the healthy brain to eliminate axon terminals, dendritic spines, and possibly entire excitatory synapses during post-natal development, adulthood and aging. In particular, microglial processes harboring phagocytic structures were encountered *in vivo* (Nimmerjahn et al., 2005; Tremblay et al., 2010). At the ultrastructural level, phagocytic inclusions with distinctive features of axon terminals (synaptic vesicles) and dendritic spines (post-synaptic densities) were frequently observed inside of microglial cell bodies and processes, and their prevalence was found to be regulated by neuronal activity and behavioral experience (Tremblay et al., 2010, 2012; Paolicelli et al., 2011; Schafer et al., 2012). This unique capacity of microglia to engulf and remove cellular debris is undisputable, but many open questions remain: Do microglia actively select which cells or neurites/spines/synapses require to be removed? What are the downstream effects of phagocytosis on microglia, synapses, and the surrounding brain parenchyma? What are the functional consequences on neuronal plasticity, learning and memory? Is microglial phagocytosis in the diseased brain equally fast and efficient, and how does it contribute to disease pathogenesis?

The Future of Microglia

In these 100 years of microglial history, functional neuroanatomy based on different microscopy techniques has played a central role in enabling us to study these cells in their normal physiological state, thus providing invaluable insights into their possible implication in the pathogenesis of various neurodevelopmental and neurodegenerative diseases where changes in their morphology, dynamics, and gene expression pattern have been described. A recurrent problem across the years has been the development of selective staining tools to specifically visualize microglia and manipulate their gene expression, as required to investigate their causal relationship to the pathogenesis of diseases. To date, and to the best of our knowledge, all "microglia-specific" transgenic mouse lines expressing fluorescent reporters such as the green fluorescent protein (GFP) are not microglia-specific, as they also label the small proportion of brain macrophages located in the meninges and perivascular spaces of the brain (Davalos and Fuhrmann, 2014), as well as the peripheral, circulating monocytes which could contribute to replenishing the microglial population under certain (Gomez Perdiguero et al., 2015) pathological conditions (Ginhoux et al., 2013). Furthermore, the most commonly used reporter mouse line for live imaging and inducible expression based on the Cre recombinase system is a knock-in where the constructs replace the endogenous fractalkine receptor (CX3CR1) locus (Goldmann et al., 2013; Wolf et al., 2013; Yona et al., 2013). Although the heterozygous CX3CR1$^{GFP/+}$ mice have been extensively used for imaging, they show functional deficits in synaptic plasticity, learning, and memory compared to wild-type mice (Maggi et al., 2011; Rogers et al., 2011) and thus may not be the best model to study the roles of microglia in the healthy brain.

In recent years, fate mapping strategies have enabled to specifically visualize cells of the microglial lineage (Ginhoux et al., 2010; Schulz et al., 2012; Kierdorf et al., 2013). Last year, gene profiling and quantitative mass spectrometry analysis also allowed to identify for the first time a molecular "signature" of microglia in the healthy brain [which was subsequently shown to be altered in neurodegenerative diseases (Butovsky et al., 2014a)], leading to the development of selective antibodies to visualize particular phenotypes of microglia vs. monocyte-derived macrophages (Butovsky et al., 2014b). Novel microglial-specific fluorescent probes such as CDr10a and CDr10b may also be useful for live imaging (and isolation), although their specificity remains to be tested (Leong et al., 2014). In the near future, these advancements

should lead to the development of improved strategies for both the visualization and conditional manipulation of gene expression in microglial cells of various phenotypes, leading to an explosion of discoveries regarding their distinctive roles across brain development, function, plasticity, and disease.

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Pío del Río Hortega and the discovery of the oligodendrocytes

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Pío del Río Hortega (1882–1945) discovered microglia and oligodendrocytes (OLGs), and after Ramón y Cajal, was the most prominent figure of the Spanish school of neurology. He began his scientific career with Nicolás Achúcarro from whom he learned the use of metallic impregnation techniques suitable to study non-neuronal cells. Later on, he joined Cajal's laboratory. and Subsequently, he created his own group, where he continued to develop other innovative modifications of silver staining methods that revolutionized the study of glial cells a century ago. He was also interested in neuropathology and became a leading authority on Central Nervous System (CNS) tumors. In parallel to this clinical activity, del Río Hortega rendered the first systematic description of a major polymorphism present in a subtype of macroglial cells that he named as oligodendroglia and later OLGs. He established their ectodermal origin and suggested that they built the myelin sheath of CNS axons, just as Schwann cells did in the periphery. Notably, he also suggested the trophic role of OLGs for neuronal functionality, an idea that has been substantiated in the last few years. Del Río Hortega became internationally recognized and established an important neurohistological school with outstanding pupils from Spain and abroad, which nearly disappeared after his exile due to the Spanish civil war. Yet, the difficulty of metal impregnation methods and their variability in results, delayed for some decades the confirmation of his great insights into oligodendrocyte biology until the development of electron microscopy and immunohistochemistry. This review aims at summarizing the pioneer and essential contributions of del Río Hortega to the current knowledge of oligodendrocyte structure and function, and to provide a hint of the scientific personality of this extraordinary and insufficiently recognized man.

Keywords: Del Río Hortega, myelin sheath, oligodendroglia, oligodendrocyte precursor cell (OPC), Ramón y Cajal

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Biographical Sketch of Del Río Hortega

Pío del Río Hortega (1882–1945) was, with the exception of Ramón y Cajal, the most prominent figure of the Spanish school of neurology (Andres-Barquin, 2002; Pasik and Pasik, 2004; De Carlos and Pedraza, 2014). He revolutionized the study of neuroglia by developing and improving metallic impregnation techniques that he applied to the study of the group of non-astrocytic cells. These cells were poorly stained with the methods available at that time, and were known after Ramón y Cajal as the "third element" of Central Nervous System (CNS), neurons and astrocytes being the "first and second element", respectively (Ramón y Cajal, 1913a). With the staining tools he developed, Del Río Hortega was able

to identify two kinds of cells and to unveil their origin: microglia, the true "third element" due to its mesodermic origin; and oligodendroglia, included with astrocytes as second element due to their shared ectodermal origin (Del Río Hortega, 1918, 1920, 1924).

Pío del Río Hortega studied Medicine (1899–1905) and even as a student, he committed himself to follow a career in research which was focused on neurohistology and pathology all his professional life (exhaustively reviewed in Cano-Díaz, 1985; Del Río Hortega, 1986; López-Piñero, 1990). With some delay but with an enormous capacity for sustained hard work, he began his postdoctoral training in 1911, in Nicolás Achúcarro's laboratory in Madrid (Spain), the year after. Achúcarro was Del Río Hortega's true mentor and inculcated in him a deep interest in neuroglia before he worked in several European laboratories for short periods. After finally returning to Spain in 1914, he had the opportunity to share scientific interests with Ramón y Cajal, to whom he always felt great admiration, since Cajal's and Achúcarro's laboratories were located in the same building though each did independent research. Following closely in Achúcarro footsteps, and stimulated by Cajal's third element, Del Río Hortega, now working full time in the laboratory, began to search for more stable variations of Cajal's and Achúcarro's metallic impregnation methods to study this cell class (reviewed in Castellano-López and González-de Mingo, 1995). In that fertile scientific environment, Del Río Hortega made numerous adjustments to the staining procedures which accounted for more than one hundred variations by the end of his career.

After developing modifications of Achúcarro's ammoniacal silver method (Del Río Hortega, 1916), Del Río Hortega challenged the accuracy of Ramón y Cajal's concept about the third element of CNS which grouped non neuronal (first element) and non-astrocytic (second element) cells (Ramón y Cajal, 1913b, 1916; García-Marín et al., 2007). Later, he described his silver carbonate staining technique which was the methodological key to identify two distinct elements: the microglia, the "true third element", and what he called initially "interfascicular cells" and later oligodendroglia (Del Río Hortega, 1918, 1920, 1921). Ramón y Cajal and others were not convinced particularly regarding the existence of oligodendroglia (reviewed in Pasik and Pasik, 2004). Perhaps, this skepticism delayed the immediate acceptance of these cells, and contributed to a misunderstanding between the two scientists which ended up with the dismissal of Del Río Hortega from Cajal's laboratory in 1920 and his move to a new one, promoted in some ways by Ramón y Cajal himself. He was aware of Del Río Hortega talent as researcher and although they never worked again together, their relationship improved later on.

Once in his own laboratory, Del Río Hortega continued frantically with his investigations and created an important school with outstanding pupils from Spain and abroad. Among them was Penfield, who greatly supported and replicated the results of Del Río Hortega, and thus, contributed to the international recognition of Del Río Hortega's discovery of oligodendroglia (Penfield, 1924; Gill and Binder, 2007). This intense activity was favored by the commitment of the Spanish Government of that time to guarantee high standards in science, an atmosphere that helped Del Río Hortega to develop a well equipped Laboratory of Histology and Pathology, as he named it (Andres-Barquin, 2002; De Carlos and Pedraza, 2014). At the same time, Del Río Hortega himself was an active advocate of science both within and outside of the academic circles. Del Río Hortega became internationally recognized for his contributions to the understanding of glia in the healthy nervous system and also in disease, mainly in cerebral tumors. Unfortunately, the Spanish civil war (1936–1939) forced him into exile which interrupted the development of his school, though he strived to keep it alive in the midst of difficulties while working abroad in Oxford and Buenos Aires (reviewed in Cano-Díaz, 1985; López-Piñero, 1990).

Silver Carbonate Staining Method of Del Río Hortega

All along his career, del Río Hortega had a great interest in improving metallic impregnation techniques to advance the characterization of neural cells (reviewed in Castellano-López and González-de Mingo, 1995; Pasik and Pasik, 2004). He developed new modifications to the Achúcarro's ammoniacal silver staining (Del Río Hortega, 1916), applied Cajal's formol uranium nitrate and gold chloride sublimate methods (Ramón y Cajal, 1913b, 1916), as well as the Golgi's method. This array of techniques gave him and those who used them, an almost complete picture of the morphology of the protoplasmic and fibrous astrocytes (FAs), cells known as the second element of the CNS, neurons being the first element. However, these methods did not stain the remaining cell types of the CNS which were termed by Ramón y Cajal as the third element which in his own words was composed solely of "corpuscles without processes" grouping adendritic, apolar dwarf cells that were present in white matter, perineuronally and as perivascular satellites (Ramón y Cajal, 1913a, 1916; García-Marín et al., 2007).

The identification of these cells was possible when Del Río Hortega described a method of using silver carbonate to stain glial cells (Del Río Hortega, 1918) with precise timing of the formalin-ammonium bromide fixative introduced by Ramón y Cajal (1913b). Del Río Hortega never explained how (i.e., a mistake, an intuition, or a test) he happened to introduce lithium carbonate with silver nitrate to precipitate it as silver carbonate (Del Río Hortega, 1918), but it could be said that in the best Cajalian tradition, he doggedly tried modification after modification of methods to selectively stain cell types. His discovery provided Del Río Hortega with a new tool to transform morphological and physiological concepts of the CNS. For the first time, this method clearly distinguished two cells types with distinct cytoplasmic expansions in the previously so-called third element group, which Del Río Hortega termed microglia and oligodendroglia (Del Río Hortega, 1920, 1921). He focused his research efforts on microglia and found its mesodermal origin (the true third element), its surveillance function and phagocytic capacity in pathology in a remarkably precise fashion, which was soon accepted by the scientific

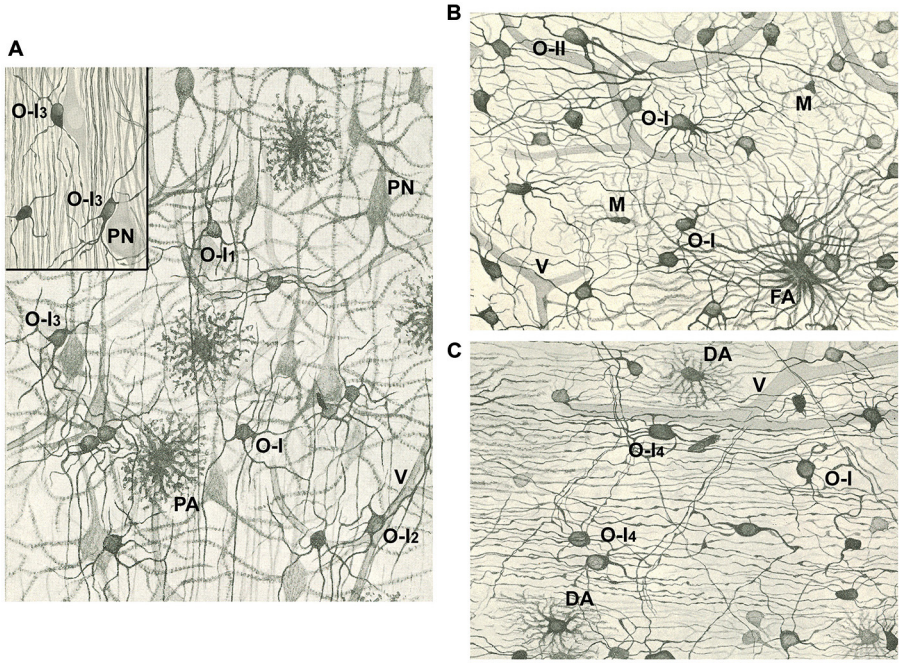

FIGURE 1 | Drawings of the cerebral cortex (A) and white matter (B,C) after staining with the Golgi-Hortega method or the silver carbonate procedure by Hortega (inset in A). (A) Notice pyramidal neurons (PN), protoplasmic astrocytes (PA), vessels (V), and type I oligodendrocytes (OLGs; O-I) with variable number of processes, many of them divided in "Y" or "T". Some OLGs have processes mainly oriented in the direction of projecting axons ($O\text{-}I_1$), while others have a perivascular ($O\text{-}I_2$) or perineuronal ($O\text{-}I_3$; see inset) localization. **(B)** Note a fibrous astrocyte (FA), some OLGs of the first type (O-I) and one of the second type (O-II), as well as microglia cells (M). **(C)** See type I OLGs similar to those in **(A, B)** (O-I) or with long processes that follow axons ($O\text{-}I_4$), and two dwarf astrocytes (DA). Vessels (V) are also drawn in **(B,C)**. Magnification in Figures **(A–C)** is similar. Modified from Del Río Hortega (1928).

community. However, there was still much debate on the existence of oligodendroglia as a distinct CNS cell type, particularly by Ramón y Cajal and others (reviewed in Pasik and Pasik, 2004). It was not until 1924 when the confirmation of oligodendroglia as a variety of neuroglia of ectodermic origin (part of second element as astroglia was) was broadly accepted (Del Río Hortega, 1924; Penfield, 1924; Gill and Binder, 2007).

Contribution of Del Río Hortega to Understanding Oligodendroglia

Del Río Hortega rendered the first systematic description of oligodendrocytes (OLGs) in an article published in 1928 (Del Río Hortega, 1928; **Figures 1**, **2**). Nevertheless, the complete story of his discovery had already begun when he described microglia (Del Río Hortega, 1920; Castellano-López and González-de Mingo, 1995; Pasik and Pasik, 2004) as the third element, mentioning the existence of a new cell type of neuroglia, the interfascicular glia, made up by cells showing very fine processes and arranged in groups among axonal tracts. Surely this distinction was only made possible using the new silver carbonate impregnation method developed by him (Del Río Hortega, 1918). In 1921, he named these cells as oligodendroglia or glia with very few processes (Del Río-Hortega, 1921), because they were present not only in white matter but diffusely distributed in all regions of the CNS and commonly grouped next to neurons in gray matter. He was aware that as many other histochemical techniques involving metallic silver impregnations, his silver carbonate method had very specific requirements, which did not, however, guarantee reproducible results in every preparation. Despite the results were very variable in terms of staining, he predicted the relationship of oligodendroglia with myelination, its implication in neuronal trophism, and its ectodermal origin. In fact, one year later (Del Río Hortega, 1922) proposed that these cells were functionally similar to Schwann cells in the CNS and responsible for myelination. However, the demonstration of oligodendroglia as cells that produce and maintain the myelin sheaths that insulate CNS axons had to wait for the introduction of electron microscopy in the 1960s (reviewed in Verkhratsky and Butt, 2007; Butt, 2013). This temporal gap, together with difficulties in oligodendroglia staining until the introduction of immunohistochemical techniques, and that the seminal articles by Del Río Hortega were published in Spanish, made his discovery of oligodendroglia not recognized internationally, as his discovery of microglia was, and restricted to scientists, who were histologists (Castellano-López and González-de Mingo, 1995; Pasik and Pasik, 2004).

Del Río Hortega published a thorough review of his discoveries about morphology and functionality of oligodendroglia in 1928 (Del Río Hortega, 1928). By this time he had introduced a new metal impregnation protocol

based on the Golgi method, known as Golgi-Hortega technique, which provided detailed information on the morphology of these cells, which he renamed as OLGs. He noted three kinds of OLGs according to their neighboring relationship: interfascicullar (alignment of closely apposed cells in rows along axonal tracts); perineuronal (juxtaposing neuronal soma) and perivascular (abutting blood vessels but lacking contacts; **Figures 1**, **2**). He was astonished with the complexity of oligodendrocytic morphology which he profusely illustrated with drawings and photomicrographs in a review (Del Río Hortega, 1928). Accordingly, he tried to classify OLGs according to their soma size and shape, number and characteristics (orientation) of cellular processes, their distribution within CNS, manner of interaction with axons and size of the axons with which they were associated. As a consequence of this analysis, he grouped OLGs into four subtypes (I to IV), while recognizing the absence of clear boundaries among them.

Type I OLGs or Robertson's OLGs, are named so because this type was probably the only one observed by Robertson (Robertson, 1899), have small rounded cell body (15–20 μm diameter) and a high number (from 5 to 20 or more) of very fine processes emerging in multiple directions and towards axons that are usually thinly myelinated. They are present in gray (nearly all perineuronal OLGs are of the first type) and white matter (frequently arranged in interfascicular series; **Figures 1**, **2**).

Type II OLGs or Cajal's OLGs, named as a tribute to him, are only present in white matter. They are polygonal or cuboidal in shape (20–40 μm) with fewer and thicker processes than type I OLGs, which are directed to axons and attached to them longitudinally (**Figure 1B**).

Type III OLGs or Paladino's OLGs because Paladino, although associated with many misinterpretations, had intuited that myelin had a neuroglial origin (Paladino, 1892). Theseare also less abundant than types I and II. They are present in white matter with thick myelinated fibers (as brain stem and spinal cord) and are distinguished by one to four processes emanating from a bulky cell body and directed toward axons (**Figure 2**).

Type IV OLGs or Schwannoid OLGs, due to their similarity in appearance, are very elongated cells with flattened somata, and found adhered and extended mono or bipolarly to medium or large thickness axons in white matter of brainstem and spinal cord (**Figure 2B**).

This classification was not made for purely descriptive purposes. In fact, he also made a synthesis about the morphological and physiological knowledge of OLGs creating the concept of neurogliona (Del Río Hortega, 1942), by suggesting that OLGs have a close association with neurons and attributing to them hypothetically mechanical, trophic and myelinogenic functions. Although many observations along his scientific career supported the formation of myelin by OLGs, either directly or by supplying axons with needed materials, he was cautious enough not to consider them as definitive. This conclusion could be regarded as an example reflecting his high standards of scientific reasoning and intuition (Cano-Díaz, 1985; López-Piñero, 1990; Castellano-López and González-de Mingo, 1995; Andres-Barquin, 2002; Pasik and Pasik, 2004; Gill and Binder, 2007).

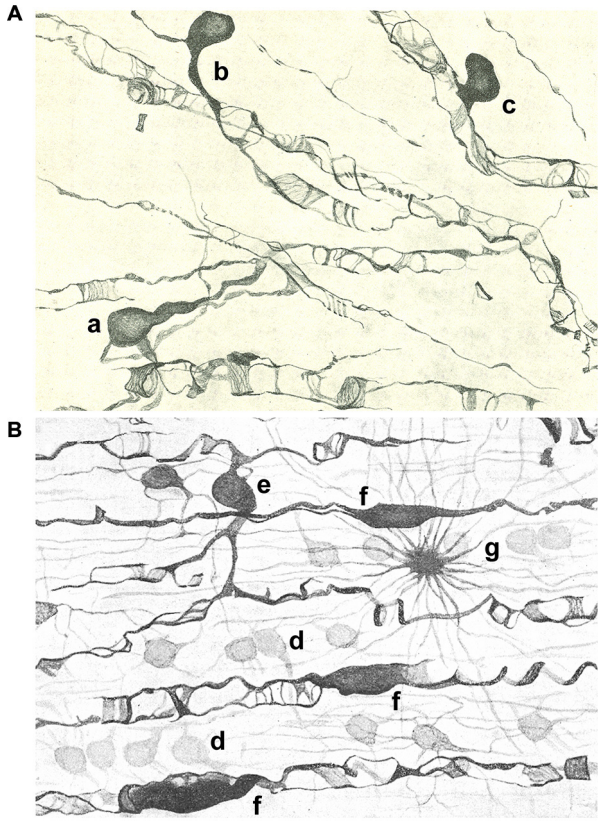

FIGURE 2 | Drawings of the subcortical (A) and spinal cord (B) white matter after staining with the Golgi-Hortega method. (A) Display of oligodendrocytes of the third type with different kind of processes around axons are represented: one has two clear and long processes (a), while others are endowed with a single process divided into an acute angle for two axons (b) or in T that ensheathes a nerve fiber (c). **(B)** Illustration of oligodendrocytes of the first (d); third [e; similar to a in panel **(A)**] and fourth type (f) as well as a fibrous astrocyte (g). Modified from Del Río Hortega (1928).

Scientific Legacy of Del Río Hortega on Oligodendrocyte Knowledge

The oligodendrocyte phenotypic diversity proposed by Del Río Hortega was initially neglected, but it has been later on confirmed by electron microscopy, intracellular dye injection, immunohistochemistry and more recently with genetic tools (Butt, 2013). This could be due to the fact that his studies were made mainly in gyrencephalic brains while current consensus about OLGs has been mainly obtained from lysencephalic ones. Indeed, Del Río Hortega's contribution to the field has been often overlooked and reference to his pioneer ideas are not included in recent relevant papers (for example: Nishiyama et al., 2014; Dumas et al., 2015; Zeisel et al., 2015). This oblivion is unfair since we learned from his discoveries that OLG phenotypes are related to the number of axons myelinated per OLG and the diameters of fibers they myelinate. As a result of that finding,

we now classify OLGs in two distinct phenotypes defined by the caliber of the axon they myelinate, i.e., below and above of 2–4 μm of diameter which correspond to Del Río Hortega's types I/II and III/IV (Verkhratsky and Butt, 2007; Butt, 2013). In addition, although he did not specifically mention it, he did suggest that there was a direct relationship between the axon caliber and the internodal length (i.e., the length between two nodes of Ranvier, the unmyelinated axonal gap where action potentials are generated), as well as with the width of the myelin sheath.

As of today, it is not clear how OLG polymorphism impacts the thickness and width of the myelin sheath and the functioning of the myelinated axons. In addition, recent evidence about axonal metabolic support provided by OLGs (Morrison et al., 2013; Saab et al., 2013) could be related to the concept of neurogliona suggested by Del Río Hortega (1942). It is outstanding that, as with Ramón y Cajal, he related morphology to function usingmicroscopy and neurohistological preparations impregnated with innovative and specific staining methods exclusively. This reveals an enormous capacity for hard work, deep observational abilities and exceptional artistic skills.

Current data show a population of adult oligodendrocyte progenitor cells, called NG2-glia or polydendrocytes, which provide a pool of slowly proliferating cells that generate OLGs throughout life (Nishiyama, 2013; Nishiyama et al., 2014). Del Río Hortega already observed this cell population in white matter (Del Río Hortega, 1928). He described it as a cell type with ambiguous character sharing with OLGs the size and shape of soma, but differing from them by the number and characteristics of its expansions: very numerous, not very long, dichotomized at acute angles several times with a semiprotoplasmic appearance similar to that of astrocytes which display a crown-like shape though its diameter is much smaller. He named them as dwarf astrocytes (DA) and although he did not propose a particular biological significance for those cells, they could possibly correspond to polydendrocytes, whose morphological descriptions are very similar (See **Figure 1C**). We now know that OLGs are not the only fate of polydendrocytes, particularly during development since they can differentiate into astrocytes (Nishiyama, 2013; Nishiyama et al., 2014).

Another exciting OLG type described by Del Río Hortega was the perineuronal one whose soma lie apposed to neuronal soma (Del Río Hortega, 1928). They are non-myelinating cells and although their role is not clear, they could provide neurotrophic and metabolic support for neurons as he suggested, an idea that others extended to pathology showing that they could produce myelin in response to demyelination (Nishiyama et al., 2014).

Del Río Hortega observations and interpretations have been instrumental to contemporary neurobiology. He anticipated concepts that were dormant during decades, due in part to the neurocentric view of the CNS, and of the view that astrocytes are the relevant glial cells in the understanding of physiology of CNS and its pathology. More recently, the interest in OLGs has had a renaissance with the increasing attention to translational research on demyelinating diseases, and ultimately, provide justice to the pioneer contributions to our knowledge of oligodendroglia made possible by Del Río Hortega. It would be difficult to imagine a coherent story of OLGs without recognizing his contributions.

Molecular Epilog

Historically, OLGs have been classified using location and morphology, as started by Del Río Hortega (1928), in combination afterwards with molecular markers (reviewed in Butt, 2013). Although the majority of OLGs in any one category tend to look alike (see **Figures 1**, **2**), very recently the analysis of the RNAs expressed in these brain cells (Zeisel et al., 2015) has revealed the possibility of classifying OLGs into a half-dozen classes according to progressive changes in previously known and novel gene expression markers along OLG differentiation. The harmonization of morphological and genetic criteria to classify OLGs remains to be done, and reveals the complexity of oligodendroglia. All in all, this open question reveals that the knowledge of the OLG network organization, pioneered by Del Río Hortega almost a century ago, is still an open question which needs further exploration.

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REVIEW
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Pío del Río-Hortega: A Visionary in the Pathology of Central Nervous System Tumors

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The last 140 years have seen considerable advances in knowledge of central nervous system tumors. However, the main tumor types had already been described during the early years of the twentieth century. The studies of Dr. Pío del Río Hortega have been ones of the most exhaustive histology and cytology-based studies of nervous system tumors. Río Hortega's work was performed using silver staining methods, which require a high level of practical skill and were therefore difficult to standardize. His technical aptitude and interest in nervous system tumors played a key role in the establishment of his classification, which was based on cell lineage and embryonic development. Río Hortega's approach was controversial when he proposed it. Current classifications are not only based on cell type and embryonic lineage, as well as on clinical characteristics, anatomical site, and age.

Keywords: histogenesis, gliomas, Rio Hortega, brain tumors, classification

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INTRODUCTION

I am satisfied to be able to summarize the contribution of Pío del Río Hortega to the field of neuropathology, in particular, tumors of the central nervous system. As a pathologist, I am keenly aware of the classifications of central nervous system tumors and can now provide a context for many of the contributions of Dr. Pío del Río Hortega.

As stated in other sections of this monograph, Río de Hortega was an illustrious character, both at the human level and at the scientific level. A self-taught man with an extraordinary knowledge of laboratory techniques, he was a leading scientific figure in the cities where he worked. The combination of expertise, dedication, and an undeniable persistence and capacity for work, together with considerable talent, led him to make key discoveries in the history of neuroscience, including microglia and oligodendroglia (as described in other chapters of this Special research Topic; see Pérez-Cerdá et al., 2015; Tremblay et al., 2015).

His techniques, especially the silver carbonate staining method, enabled him to work on tumors of the central nervous system and develop his highly practical classification (Polak, 1947; Llombart Rodríguez, 1965; Obrador, 1965).

HISTORICAL CONTEXT

The first descriptions of brain tumors date from the period of Virchow, who described gliomas arising from neuroglial cells. Virchow made a distinction between myxoglioma, gliosarcoma, glioma durum, and glioma hemorrhagicum, which are composed of glial cells that sometimes contained fibers. Virchow's pioneering comparison of neoplastic cells with normal brain tissue

laid down the scientific foundations for all subsequent classifications of tumors of the central nervous system. The main studies at the end of the nineteenth century and beginning of the twentieth century were performed by Simon (1874), who described spider cell glioma, and Tooth and Conheim, who reported that tumors arose from embryonic remnants.

Between 1900 and 1950, the various classifications of central nervous system tumors led to decades of confusion over terminology. The more notable studies of the period were by Ribbert (1910, 1918) who speculated about the histogenesis and etiology of glioma, particularly in his paper on spongioblastoma and glioma ("Über das Spongioblastoma und das Gliom" [On spongioblastoma and glioma]). Some authors feel that this study had a negative effect on the classifications of glioma that were produced during the following 20 years. In his study, which was based on theoretical deductions, Ribbert concluded that differentiated glial areas can never return to a lower grade of differentiation and that gliomas, glioblastomas, and spongioblastomas would therefore necessarily have to be explained by the presence of embryonic remnants whose growth had stopped at various stages of differentiation. According to this hypothesis, Ribbert believed that tumors stemmed from embryonic stages and not from changes occurring in the most differentiated cells. Nevertheless, Ribbert paved the way for the cytological study of tumors and for more specific studies based on impregnation methods, of which Río Hortega was a major proponent. The histogenetic and embryological approach adopted by Ribbert was modified by the cellular approach espoused mainly by Río Hortega. The contributions of the French school (Lhermitte and Dumas, 1916; Cornil, 1924; Roussy and Oberling, 1932) around the 1920 made it possible to distinguish between fibrillary astrocytoma, four subgroups of non-fibrillary glioma (round, spindle-shaped, polymorphic, and ameboid cells), glioblastoma, and spongioblastoma. The classification included ependymomas with choroid plexus tumors, which were separate from the other gliomas. The histogenetic approach was maintained in the studies by Globus and Strauss (1925) and in those of Bailey and Cushing (1926), where a distinction is made between various histogenetic cell types in glioma. The authors recognize the considerable internal heterogeneity of these tumors, to the extent that their classification placed considerable emphasis on the predominant cell type. The same authors performed an exhaustive study of brain tumors based on morphologic characteristics and on correlations with the patient's prognosis after surgery (Bailey, 1924; Bailey and Bucy, 1929). Their classification developed from the concept that tumor cells could arise from a medullary epithelium parent cell, which could differentiate into other glial, neural, or choroid cells. These cells could then differentiate even further. In theory, tumors could develop at each of these phases of differentiation. This period saw the first description of oligodendromas and cerebellar medulloblastomas. Although these tumors had been described as sarcomas or neuroblastomas by other authors, Bailey and Cushing (1925) have the merit of separating them from neuroblastoma based on their gross appearance, origin, form of growth, and spread along the spinal cord.

The 1926 classification of Bailey and Cushing is similar to the present one. It distinguished between tumors of the central nervous parenchyma, as follows:

(1) Astrocytoma (grades I–IV), pilocytic astrocytoma, glioblastoma multiforme, oligodendroglioma, ependymoma, choroid plexus papilloma, pinealoma, colloid cyst, and medulloblastoma.
(2) Meningeal tumors: meningioma, malignant meningioma, meningeal sarcoma, and meningiomatosis.
(3) Tumors of the cranial pairs: neurinoma.
(4) Tumors of the pituitary gland: adenoma, invasive adenoma, carcinoma, and craniopharyngioma.
(5) Vascular tumors: hemangioblastoma.
(6) Embryonal tumors: dermoid cysts and teratomas.

This classification had an enormous impact on neuroscience and neurosurgery, although it was criticized by several authors, mainly Scherer, who stressed the lack of correlation between clinical and pathological aspects in several tumors and the fact that a very high number of tumors (up to 30%) could not be classified following the authors' criteria. In parallel, authors such as Cushing focused on clinical classifications that described tumors with a favorable prognosis, for example cerebellar astrocytoma, which they stressed was different from cerebral astrocytoma, despite the histogenetic similarity between the two. Using data from studies of the child brain, the authors described non-recurring cystic tumors that were well-defined in terms of growth stage and whose classification was highly relevant at the time. This distinction was not based merely on histogenetic and cytologic principles, but on clinical, histopathologic, and clinical data. Important as well the contributions of Penfield (1931).

The publications and lectures of Río Hortega during the 1930s played a major role in promoting histogenetic classification, largely thanks to very accurate silver staining techniques. Most authors from this period and thereafter felt that his classification contained the most exhaustive collection of images until then. Río Hortega's classification was not based on clinical findings or anatomical site, but on morphological and histogenetic data.

TUMORS OF THE CENTRAL NERVOUS SYSTEM: THE CONTRIBUTION OF RÍO HORTEGA

During the initial stage of his training, Río Hortega analyzed brain tumors in four studies. One of these was his doctoral thesis ("Causas y Anatomía Patológica de los Tumores de Encéfalo" [Causes and Histopathology of Brain Tumors]), which he defended under the direction of his tutor, Leopoldo López García, between the years 1911 and 1912.

Río Hortega wrote papers on the histopathology of carcinomas and of the nervous system in patients with brain tumors (1911a), the pathophysiology of brain tumors (1911b), and abnormalities of nerve tissue and general symptoms of brain tumors (Río-Hortega, 1911a,b, 1912, 1914a,b,c; Río-Hortega and y Costero, 1928; Río-Hortega and y Álvarez Cascos, 1930).

During the following phase of his training, Río Hortega performed a study of subcutaneous giant cell glioma, the results of which were published in 1926 (Río-Hortega, 1926). The third phase (1928–1936) saw the appearance of his most important contributions to the field of neuro-oncology. In 1930, he published a series of monographs analyzing the cytologic and histogenetic characteristics of specific tumor groups, beginning with a detailed consideration of meningeal exotheliomas, which he discussed and included in the differential diagnosis of what was then known as Cushing meningioma. He described variations of meningioma, such as xanthomatous tumors and fascicular tumors, which have been reported sporadically by other authors. The examination of these histopathologic forms led him to propose three major patterns: (a) a predominantly syncytial pattern; (b) a pattern based on fibrillary differentiation of cytoplasm that tended to arrange itself in plaques and bundles; and (c) a pattern involving more epithelioid and lobulated morphological differentiation. Similarly, he described the formation of acervuli and psammoma bodies in meningioma, the pineal gland, and the colloid plexus (Río-Hortega, 1930c).

The year 1932 saw the publication of the major study "La estructura y sistematización de los gliomas y paragliomas" [Structure and systematization of gliomas and paragliomas], which, with more than 260 pages and 200 images, was the fruit of the techniques that Río Hortega had been developing using mainly silver carbonate staining (Río-Hortega, 1932, 1933a,b).

He performed the study using his in-depth knowledge of the histology of the glia and brain and had to seek the help of neurosurgeons and other pathologists to compile a sufficiently large series of brain tumor samples for study and classification. The French neurosurgeon Clovis Vincent was of inestimable help during this period.

The results, which were based on neuroembryological data, pointed to four potential evolutionary pathways of the primitive medullary epithelium (neuroblasts, glioblasts, pineoblasts, and choroideoblasts). Given the considerably heterogeneous nature of brain tumors, Río Hortega thought that it was important to classify them into histologic types with common embryological findings. Therefore, he tried to define two large groups of tumors, with emphasis on histological and embryological lineage. The first group comprised gliomas and the second paragliomas, which included all those tumors formed by immature or mature elements of the nervous system and tumors arising from choroidal folds and the pineal gland.

His systematic typing of the gliomas according to the degree of maturity of the cell components or the degree of differentiation enabled him to define the following entities (Río-Hortega and y Jiménez de Asúa, 1921; Río-Hortega, 1930a,b, 1932, 1933a,b; Pineda et al., 1962; Diaz, 1985) (**Figure 1**):

(1) Embryonal glioblastoma or spongioblastoma.
(2) Heteromorphic glioblastoma.
(3) Isomorphic glioblastoma (**Figures 2**, **3**).
(4) Astroblastoma.
(5) Astrocytoma (**Figure 4**).
(6) Oligodendroglioma, with a distinction between oligodendrocytomas and oligodendroblastomas (**Figure 5**).
(7) Glioepitheliomas, which included ependymal tumors (ependymocytoma and ependymoblastoma).

The classification of paragliomas included neuroma from neuroblasts (neuroblastoma), neurocytoma, pineal tumors (pinealcytoma, pineoblastoma), and choroid plexus tumors, which he termed chorioepitheliomas (**Figure 6**).

At the International Cancer Congress on the Scientific and Social Fight Against Cancer held in Madrid in 1933, he provided a more extensive summary of his classification in a lecture based on 287 pages of text (315 pages including the bibliography) and 248 images.

The classification, which to a certain extent complemented those proposed by Roussy and Overling and especially that proposed by Bailey and Cushing, differed in major areas, some of which are worthy of mention. Río Hortega's classification was based on the cytologic and embryologic characteristics of tumor cells, irrespective of their location, and therefore included tumors such as cerebellar medulloblastoma alongside tumors with a blastic lineage from within the brain. This distinction between medulloblastomas and other neuroblastic or primitive tumors was controversial at the time and continues to be so today.

In his lecture, Río Hortega stressed the need for international harmonization of the nomenclature applied to tumors of the nervous system, as also proposed by Roussy and Overling. The groups he suggested in the lecture were as follows:

(1) Tumors arising from choroidal folds, the pineal valve, and homologous evaginations of the diencephalon that develop in the embryo.
(2) Tumors arising in the parenchyma of the brain and spinal cord and visual system, which is a prolongation of the brain.
(3) Tumors arising in the sympathetic nervous system, but not all those that develop from sympathogonia.
(4) Tumors arising in the nerve roots and in the peripheral nerves from interstitial cells or parenchymatous cells, depending on the interpretation of neoplastic elements (**Figure 7**).
(5) Tumors arising in the meninges owing to proliferation of cells or to new vascular formations (**Figure 8**).
(6) Tumors arising from hyperplasia in the parenchyma of the pituitary gland and from dislocated epidermal germ cells and invaginations of the Rathke pouch.

The fourth stage of Río Hortega's life (1936–1945), which was spent in exile, saw the publication of several papers on the nervous system (see below) (Río-Hortega, 1940a,b,c, 1941a,b, 1942, 1943).

In 1940, after his stay in Oxford, he performed a study on tumors of the optic nerve. During the same year, once he had settled in Argentina, he published a study on neuroblastomas, in which he concluded that there were no nervous system tumors with bipotential cells that were able to progress to neuroblasts or glioblasts and that most of the so-called medulloblastomas should be termed neuroblastomas, which is the term that corresponds to their lineage. His approach was considered scientific in terms of its embryological interpretation.

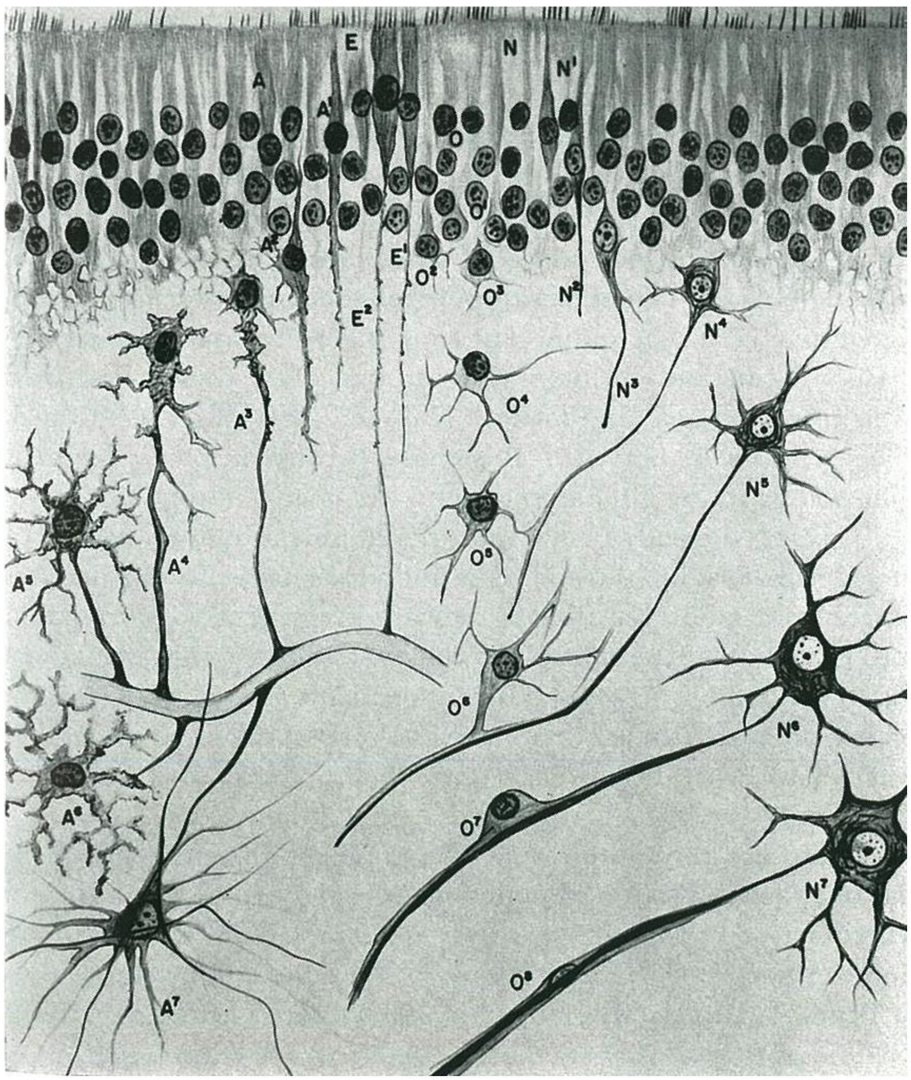

FIGURE 1 | Morphological evolution of the cells that are derived from the neural epithelium in the central nervous system (taken from Río-Hortega, 1933a).

This interpretation remains controversial today. The embryological approach did not take histopathological characteristics into account. In addition, the neuroblastoma group included tumors that developed from other precursors, namely, medulloblasts, which develop in the molecular layer of the cerebellum.

His paper entitled "Del glioepitelioma al glioblastoma isomorfo" [From glioepithelioma to isomorphic glioblastoma], which was published in 1941, discussed and criticized the use of the term ependymoma—suggested by Bailey and Cushing—for tumors associated with the ependymal wall.

In 1943, Río Hortega (Río-Hortega et al., 1943) performed a cytological study of neurofibromas (also known as lemmocytomas), in which he described the histologic characteristics of the tumors and the elements that characterize them. He made an in-depth examination of the constitution of these tumors, discussed the identification of the main elements of Schwann cells and the embryonic origin thereof, and examined the specific differentiation of multiple neurofibromas and neurinomas (solitary schwannomas). His findings remain in force today.

He added new pathological information after previous papers (Cushing, 1917; Kernohan et al., 1931; Kernohan and Ody, 1932; Scherer, 1933).

In 1944, Río Hortega reported the results of an extensive study on oligodendrogliomas, which he classed as a gliomatous ectodermal variety characterized by small cells with a spherical nucleus (Río-Hortega, 1944a,b). His description of the nucleus as "very round" continues to be of use today in the diagnosis of oligodendrogliomas. Similarly, he observed that oligodendrocytes tend to be arranged in dense or diffuse patterns and never in perivascular patterns. Río Hortega established three

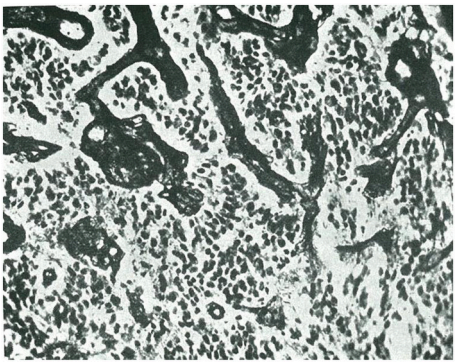

FIGURE 2 | Isomorphic glioblastoma, as termed by Rio-Hortega (taken from Río-Hortega, 1933a).

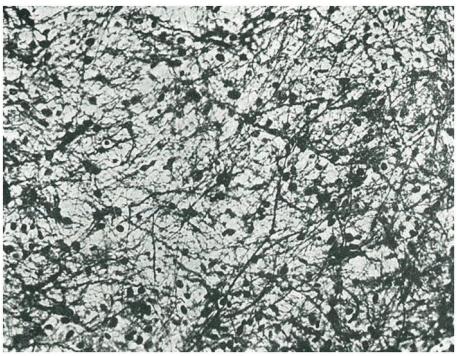

FIGURE 4 | Example of fibrous astrocytoma (taken from Río-Hortega, 1933a).

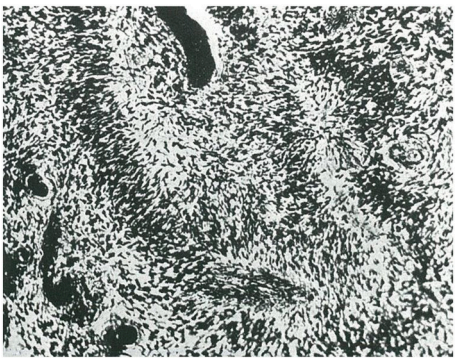

FIGURE 3 | Another example of isomorphic glioblastoma with wave-like arrangement of glioblasts (taken from Río-Hortega, 1933a).

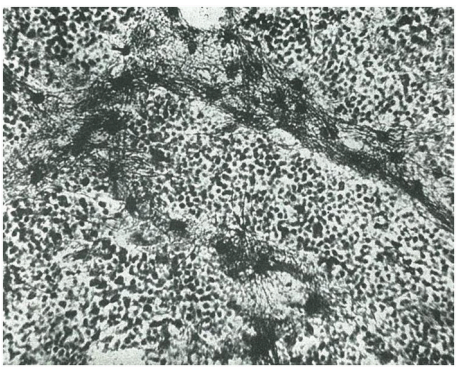

FIGURE 5 | Examples of oligodendrocytoma (taken from Río-Hortega, 1933a).

cytological types of oligodendroglioma: (a) those whose cells have a spherical nucleus surrounded by a characteristic light halo and wrapped in a small layer of protoplasm that projects a varying number of fine and long appendages; (b) a more infrequent type of oligodendroglioma, which is formed by large neoplastic oligodendrocytes; and (c) a type that includes tumors with a non-uniform structure. As Río Hortega pointed out, the neoplastic oligodendrocyte evolves morphologically to the extent that it takes on the characteristics of an astrocytoma.

The year 1944 is also notable for Río Hortega's cytological study of tumors of the optic chiasm and nerve. The tumors described at this level that can be classed as gliomas, which were similar to brain tumors, with a moderately expansive or infiltrative character. The several cell types that can be identified for tumors of the optic nerve and chiasm include the following: (1) cells with small, round nuclei; (2) cells with bipolar, spindle-shaped, and long nuclei; (3) cells with a tripolar cytoplasm and thick prolongations; (4) cells with multipolar cytoplasm and fibroid and undulating prolongations; (5) cells with multipolar cytoplasm that invade the vasculature. Río Hortega reached the conclusion, albeit indefinite, that there are two basic neoplastic types in the formations he studied: one characterized by long elements (Schwann oligodendrocytes) and another defined by multipolar elements that give it the appearance of astrocytes (Ortiz de Picon, 1983).

CLASSIFICATION OF CENTRAL NERVOUS SYSTEM TUMORS AFTER RÍO HORTEGA

Current classifications of nervous system tumors are mixed, based on cytological and histogenetic criteria, as well as on histopathological variants that are of clinical and prognostic importance.

The main studies published after Río Hortega include that of Kernohan and Sayre (1952), Miller et al. (1952) who began to grade gliomas by establishing a correlation between microscopy findings, degree of malignancy, and prognosis.

In 1965, Zülch stressed the importance of other factors, such as patient survival, and included the concept of clinical malignancy (Zülch, 1965). Finally, the first classification of the World Health Organization was published in 1979 (Zülch, 1979) and classified tumors as follows:

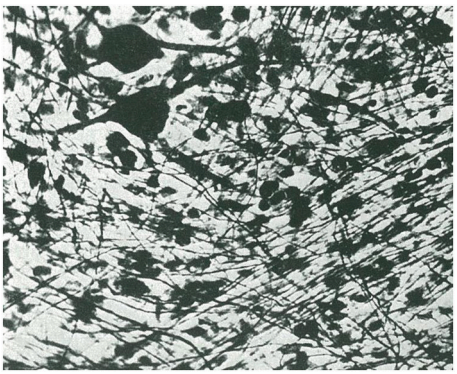

FIGURE 6 | Neurocytoma. Note the gangliocytic cells and a plexus of unmyelinated fibers (taken from Río-Hortega, 1933a).

FIGURE 7 | Neurinoma. Grouping of the nuclei in rows or palisades (taken from Río-Hortega, 1933a).

(1) Tumors of neuroepithelial tissue, including astrocytoma, glioblastoma multiforme, oligodendroglioma, ependymoma, pinealcytoma, medulloblastoma, gangliocytoma, ganglioglioma, and neuroblastoma.
(2) Meningeal tumors, such as meningioma and meningeal sarcoma.
(3) Tumors of nerve sheath cells, such as neurinoma and neurofibroma.
(4) Primary cerebral lymphoma.
(5) Tumors arising in blood vessels, such as hemangioblastoma.
(6) Germ cell tumors, such as germinoma and teratoma.
(7) Metastatic tumors.
(8) Malformative tumors and tumor-like lesions, such as craniopharyngioma, epidermoid cyst, dermoid cyst, and colloid cyst of the third ventricle.
(9) Local extensions from regional tumors, such as glomus jugulare tumor and chordoma.
(10) Tumors of the anterior pituitary, such as pituitary adenoma.
(11) Unclassified tumors.

This classification serves as the basis for the subsequent editions of the World Health Organization classification until the year 2007 and the subgroups that are currently being incorporated. The most notable new additions are as follows:

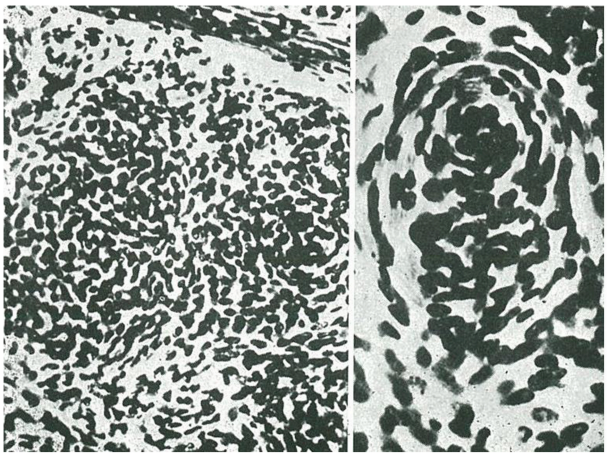

FIGURE 8 | Meningeal exothelioma. The disordered cells are forming clusters and concentric layers or acervuli (taken from Río-Hortega, 1933a).

(1) Variants of grade 1 astrocytomas, such as fibrillary, protoplasmic, and gemistocytic astrocytoma.
(2) Pilocytic astrocytoma as an independent entity.
(3) Subependymal giant cell astrocytoma.
(4) Astroblastoma.
(5) Anaplastic (malignant) astrocytoma.

A distinction is also made between oligodendroglial tumors and oligoastrocytic tumors [oligoastrocytomas and anaplastic (malignant) oligodendrogliomas].

Within the ependymal tumors and colloid plexus tumors, it is important to distinguish between variants of ependymomas, such as myxopapillary ependymoma, papillary ependymoma, subependymoma, and anaplastic ependymoma. At the level of the colloid plexus, we must distinguish between colloid plexus papilloma and colloid plexus carcinoma.

The neuronal tumors include variants such as gangliocytoma, ganglioglioma, ganglioneuroblastoma, gangliocytoma, anaplastic (malignant) ganglioglioma, and neuroblastoma.

Among the poorly differentiated and embryonal tumors it is important to identify glioblastoma (with its two subvariants, glioblastoma with a sarcomatous component and giant cell glioblastoma), medulloblastoma, medulloepithelioma, primitive polar spongioblastoma, and gliomatosis cerebri.

The classification covers tumors of the meningeal and related tissues, such as meningioma, with at least 11 morphological variants depending on the predominance of the Schwann, angiomatous, and papillary component. Similarly, the anaplastic (malignant) variant of meningioma is a distinct entity.

The classification still includes vascular tumors (e.g., hemangioblastoma and a malignant variant known as monstrocellular carcinoma), primary malignant lymphoma, and several variants of germ cell tumors. The previously cited group of malformative tumors and tumor-like lesions is extended to include enterogenic cysts, lipoma, hypothalamic neuronal

hamartoma, nasal glial heterotopia (nasal glioma), as well as various vascular malformations (capillary telangiectasia, arteriovenous malformations, and Sturge-Weber disease).

The 2007 classification continues to include new entities, mainly anatomical-clinical conditions where it is very important to distinguish between gliomas with a high and low degrees of malignancy based on cytological criteria. The new types of low-grade glioma described include angiocentric gliomas, which are variants of glioneuronal tumors (e.g., rosette-forming or papillary tumors), and cytological variants of tumors of the anterior pituitary (e.g., pituicytoma and spindle cell oncocytoma). We can also distinguish between pilocytic tumors and their pilomyxoid variants, which have a poorer clinical prognosis (Louis et al., 2007).

In the coming years, it will be necessary to add the molecular abnormalities underlying the transformation and malignancy of these tumors. Our knowledge is expected to increase thanks to amplification of genes such as EGFR in glioblastoma, loss of alleles on chromosomes 1p and 19p in oligodendroglioma, and mutations in genes such as in p53 and IDH1 in low-grade astrocytoma that progresses to malignant astrocytoma. Intra- and inter-tumoral heterogeneity could be understood as resulting from cancer stem cells and the accumulation of various molecular abnormalities.

As has occurred with other types of tumor, especially lymphoma, whose classifications have for decades been based merely on morphological or clinical criteria, a joint approach to classification is probably the most suitable for clinical practice. Cytological abnormalities, location, and histopathological characteristics could facilitate a more in-depth study of the various types of tumor. It is important to remember that the major objective of any classification is that the information it provides be of use in clinical practice. Only thus can the patient receive the best and most personalized treatment possible.

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The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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The Cajal School in the Peripheral Nervous System: The Transcendent Contributions of Fernando de Castro on the Microscopic Structure of Sensory and Autonomic Motor Ganglia

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The fine structure of the autonomic nervous system was largely unknown at the beginning of the second decade of the 20th century. Although relatively anatomists and histologists had studied the subject, even the assays by the great Russian histologist Alexander Dogiel and the Spanish Nobel Prize laureate, Santiago Ramón y Cajal, were incomplete. In a time which witnessed fundamental discoveries by Langley, Loewi and Dale on the physiology of the autonomic nervous system, both reputed researchers entrusted one of their outstanding disciples to the challenge to further investigate autonomic structures: the Russian B.I. Lawrentjew and the Spanish Fernando de Castro developed new technical approaches with spectacular results. In the mid of the 1920's, both young neuroscientists were worldwide recognized as the top experts in the field. In the present work we describe the main discoveries by Fernando de Castro in those years regarding the structure of sympathetic and sensory ganglia, the organization of the synaptic contacts in these ganglia, and the nature of their innervation, later materialized in their respective chapters, personally invited by the editor, in Wilder Penfield's famous textbook on Neurology and the Nervous System. Most of these discoveries remain fully alive today.

Keywords: Nobel Prize, history of neuroscience, Santiago Ramón y Cajal, spanish neurohistological school, superior cervical ganglion, development, synapse, chemoreceptors

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INTRODUCTION

Today it is common knowledge that in Vertebrates an autonomic (or vegetative) nervous system governs the visceral components of the body and controls the internal environment in close integration with the somatic nervous system. The autonomic nervous system has sensory and motor ganglia, and the latter can belong to the sympathetic or parasympathetic subdivisions. In addition there are two related but semi-independent systems in the heart and the enteric system (Standring, 2008). At the beginning of the 20th century, although knowledge of the general microscopic structure of the nervous system was accumulating fast due to, among others, the capital contributions by Santiago

Ramón y Cajal (1852–1934), several neural structures remained poorly understood. Amongst these were the relatively small groups of neural cells forming the sensory and autonomic ganglia, all of them external to the mechanical protection offered by the skull and vertebrae lining the vertebral canal. The concept of the autonomic nervous system had been proposed by the British neurophysiologist John Langley (1852–1925):

"I propose the term "autonomic nervous system" for the sympathetic system and the allied nervous system of the cranial and sacral nerves and for the local nervous system of the gut" (Langley, 1898).

With this concept, Langley modified previous descriptions by Christian Bell ("vegetative nervous system") and François-Xavier Bichat ("ganglionic nervous system"). Langley reserved Winslow's "sympathetic nervous system" to those ganglia positioned closely to the thoracic and lumbar spinal cord, and he coined that of "parasympathetic" for the cranial and sacral ganglia involved in the visceral innervation. This was maybe the first global conclusion after experimental work started around 1889 when blocking the of peripheral ganglia with nicotine had made it possible to distinguish between preganglionic fibers projecting to ganglionic cells and other fibers surpassing ganglia to innervate organs (Langley and Dickenson, 1889). Langley proposed that it is a single sympathetic cell that connects the CNS and the final effector organ. He considered each ganglion as a switching station and classified efferent nerves as "preganglionic" or "postganglionic". In a series of research articles during the 1890s, Langley and his then young pupil Charles S. Sherrington (1857–1952) established the concept of an "innervation field" by describing the distribution of sympathetic fiber terminals in the skin (for a summary, see Todman, 2008). In 1921, the German physio-biochemist Otto Loewi (1873–1961) published his famous experiment on the beating hearts of frogs (with and without vagus nerve, respectively), which allowed him to propose that a chemical substance ("Vagusstoff"), liberated by nerve terminals, controls the frequency of heart contractions (Loewi, 1921). This "Vagussoff' was later identified as acetylcholine by Henry Hallett Dale (1875–1968), who identified also a related molecule in (ortho)sympathetic neurons: adrenaline. Thus, acetylcholine and adrenaline were the first neurotransmitters identified (Dale and Richards, 1927; Dale and Dudley, 1929). It was in the parasympathetic system where the chemical component of synaptic transmission was first recognized (although we know today that there are cases of electrical synapses). For these important discoveries Loewi and Dale received the Nobel Prize in Physiology or Medicine in 1936.

In spite of all these capital developments in the understanding of the physiology of the peripheral nervous system the study of the fine morphology of the associated anatomical structures and their interconnectivity remained technically and logistically very challenging. Many histologists had tried to resolve the morphological details, among them Alexandre Dogiel, Santiago Ramón y Cajal, Michael von Lenhossèk, Gheorghe Marinescu, Jean Nageotte, Károly Schaeffer, Max Bielschowsky, and Giusseppe Levi, for merely citing the most relevant ones (**Figures 1A–C**; good reviews on prior works can be found in, respectively, de Castro, 1932a, 1951). Dogiel (1852–1922) was the first identifying different types of neuron in somatosensory, sympathetic and parasympathetic ganglia (Dogiel, 1899). Studying the enteric ganglia with different histological methods (Ehrlich and Golgi methods, respectively), Dogiel described particular cells with short dendrites later named after him. Cajal discovered the stellate cells with long dendrites (Ramón y Cajal, 1899; **Figure 1D**; "colossal dendrites" in his own words). This is a good example of the complementarity of the different studies in clear (and sane) scientific competitiveness. It is therefore not surprising that the interpretations of all these pioneers differed almost as much as their nationalities or as the animal species studied. In its apogee at that time, the debate between supporters of "neuronism" (i.e., Cajal) and "reticularism" (**Figures 1D,E**) was even bitter in this particular field because researchers like Kölliker and Dogiel (both cannot generally be considered as reticularists) thought that the interstitial Cajal cells in the gut were fibroblastic while Cajal proposed their neural origin (for a review on this specific topic, see Szentágothai, 1975; García-López et al., 2009).

Although the contributions by Giuseppe Levi (1872–1965) on the sensory ganglia were really remarkable (Levi, 1908), important debates took place on the intraganglionic axon collaterals and on the nature of the "atypical cells" [cells with fenestrated forms, tangled, or with cell processes in balls (terminology of those days)]. This was undoubtedly why Ramón y Cajal entrusted his young pupil Fernando de Castro (1896–1967) to work on the microscopic structure of the sensory ganglia that, with time, would crystallize in a brilliant PhD thesis:

"Hey, guy, here are some badly-known details, for example the interpretation of typical and atypical cells found in the sensory ganglia. We really do not know beyond what is described under experimental conditions. In the human we do not know what exactly the atypical forms are, especially in normal conditions and in young humans" (Gómez-Santos, 1968).

In other words, are the atypical forms of cells observed in the sensory ganglia a fruit of pathological processes affecting ganglia or can they be observed in normal conditions, too?

DE CASTRO'S FIRST STEPS IN SCIENCE: STRUCTURE OF THE HUMAN SENSORY GANGLIA

With this commission received from the Maestro (Ramón y Cajal), de Castro started to accumulate material from autopsies. He systematically collected Gasser's (Vth cranial nerve; somatic sensory) and vagus plexiform ganglia (Xth cranial nerve; autonomic sensory), in order to systematize the findings by his predecessors Cajal and Bielschowsky who had obtained them with neurofibrillary silver impregnations. "Normal" material was obtained from premature human fetuses to young adults (40–45 years-old) died accidentally. Ganglia in pathological cases included specimen obtained post mortem from patients suffering from a large diversity of diseases, ranging from infectious

FIGURE 1 | A tribute to some pioneers of the study of the autonomic nervous system. (A) Photographic self-portrait of Santiago Ramón y Cajal; his profile is outlined by a chalk sketch of a human brain. **(B)** Portrait of the recognized Russian histologist Alexander Dogiel. **(C)** The Russian neuroscientist Lawrentjew looking at the microscope. **(D)** A detailed drawing from a sympathetic neuron by Santiago Ramón y Cajal, as a good example of the neuronist interpretation of the fine structure of the nervous system. **(E)** Dogiel's interpretation of neurons from the Auerbach plexus (published in: Dogiel, 1899), a good example of reticularist vision of the organization nervous system **(A–D)** are part of Archive Fernando de Castro.

diseases (syphilis, tetanus, tuberculosis, rabies, Kala-azar, etc.) to metastatic cancers, intoxications and alcoholism, osteomalacia, diabetes, hyperthyroidism, amyotrophic lateral sclerosis and traumata. He applied the silver methods of Cajal, Achúcarro and Río-Hortega. De Castro confirmed that monopole neurons are the most abundant cell type, up to 70% of the total cells in the normal sensory ganglia, slightly more than calculated by Cajal and Marinesco and three times more than the number obtained by Levi (de Castro, 1922). This type of cell was even more abundant in pathological conditions, especially in the prenatal samples. Both Levi and de Castro confirmed that the largest of these cells occurred in cervical and lumbar ganglia and within a single ganglion, at its poles (de Castro, 1922). It is remarkable that although Levi and Terni had previously described a relationship between the size of the ganglionic neurons and the volume of peripheral tissue innervated by its axon (Terni, 1914), de Castro briefly cited this observation without intellectual additions (de Castro, 1922) and ignored it in his next chapter on the subject (de Castro, 1932a), maybe due to the fact that these observations had been made in reptiles. A bulk of posterior data in the same sense as those described by the Italian pioneers, including many observations in mammals, resulted in what is known as the "neurotrophic theory" (for a compilation of this, see Purves, 1988). De Castro's observations confirmed prior descriptions from Cajal, Dogiel and others with respect to the presence of bipolar cells in normal ganglia but he reported that neurons with intraganglionic branches (Dogiel's type VIII) were only present in pathological circumstances, in open contradiction with Dogiel (Dogiel, 1908; de Castro, 1922). While Ramón y Cajal was the first to describe satellite cells in somatic sensory ganglia, Dogiel hypothesized that they had mesenchymal origins, but it was de Castro with an elegant combination of different histological staining techniques who clearly demonstrated their ectodermal nature and their function as "neuro-neuroglial symbiosis" (de Castro, 1922). De Castro also confirmed previous observations of Cajal and Levi (1908) and as a result, suggested that Dogiel's type V, VI and VII should be considered as variations of the same cell type (fenestrated cells), present in both normal and pathological conditions (Dogiel, 1908; de Castro, 1922). In all these specific questions, the state of the art remained almost unaltered for at least 10 years (de Castro, 1932a).

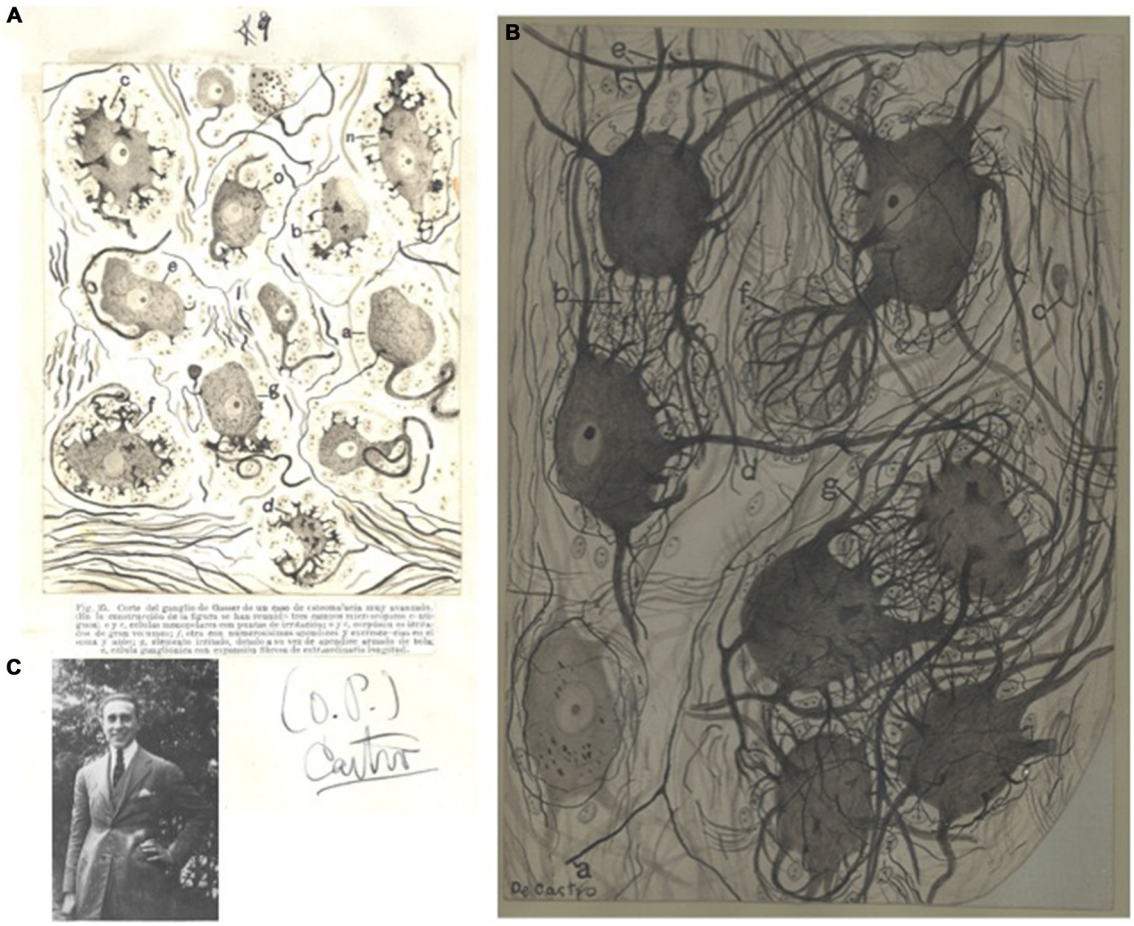

FIGURE 2 | First works of Fernando de Castro in the structure of the peripheral nervous system. (A) Image from the original PhD thesis of Fernando de Castro, with his hand-drawn illustrating some pathological forms of neurons from the Gasser's ganglion from a patient of osteomalacia. The typewritten figure legend (in Spanish) for the defense of the thesis is conserved for the reader, as well as the signature from de Castro at that time. This figure was published in de Castro (1922). **(B)** de Castro's original hand-sketch of a portion of a sympathetic lumbar ganglion in normal condition (human, 38-year old) originally stained with the Cajal's method, and illustrating preganglionic (*a*) and intraganglionnic endings (*d*) over dendritic bushes, accesory dendrites forming bushes (*b,g*), a protoplasmic process forming collaterals (*c*) and a pericellular dendritic nest (*f*). This schema was published in de Castro (1923c, 1933). **(C)** Image of a young Fernando de Castro (1922) at his family house in Cercedilla, in the mountains close to Madrid **(A–C)** are part of Archive Fernando de Castro.

In the study of the ganglia obtained from cases with pathological conditions, (de Castro, 1922) appeared involved in a curious debate at that time. Some experts in the field (Dogiel, Michailow, Cajal—initially) assumed that the ball-ended processes arising from ganglia after nerve transection had a trophic function. De Castro leaned towards the alternative view that these balls grew to repair nerves after their destruction (Marinesco, de Castro, Cajal—in a second stage). This alternative view was strongly supported by the *in vitro* regenerative studies performed mainly by Marinesco and Minea (Marinesco and Minea, 1912, 1914). The current perspective on the subject is a concept of molecular differentiation between both trophic and tropic cues in growing and re-growing of axons (Tessier-Lavigne and Goodman, 1996; de Castro, 2003). At the beginning of the 1930s, following the initial descriptions of nerve regeneration by Langley (Langley, 1898, 1900), de Castro undertook complex experiments including reinnervation and crossed anastomosis between autonomic motor fibers and sensory ganglia. He as well studied the behavior *in vitro* of explants from ganglia (de Castro, 1930, 1933, 1934, 1937), but these studies were mostly focused on subjects outside the scope of the current review.

In his research on experimental re-innervation and regeneration, de Castro produced several of his most memorable histological preparations and drawings reflecting the diversity of cells and the complex relationships between neurons (**Figure 2**; de Castro, 1921, 1922). The publication of these intense and meticulous studies had important consequences. Some of them are easily tangible. For instance, de Castro's PhD thesis, named "Estudio de los ganglios sensitivos del hombre en estado normal y patológico. Formas celulares típicas y atípicas" defended at the Medical School of the Universidad de Madrid (Spain) in 1922 (**Figure 2A**), obtained the highest possible qualification ("Sobresaliente") and was 1 year later awarded by

the Real Academia Nacional de Medicina with the Rodríguez Abaytúa Prize. But the ultimate award for de Castro's scientific career was the definitive and full scientific and technical recognition by the Maestro, Santiago Ramón y Cajal. This recognition did not weaken: it would last until the death of Cajal in 1934 and would determine several of the milestones in the scientific trajectory and human life of Fernando de Castro.

THE SECOND CONQUEST: THE FINE STRUCTURE OF AUTONOMIC GANGLIA

Undoubtedly impelled by the success of his research on the histology of the human somatic sensory ganglia, as well as by the evident lack of studies with neurofibrillary methods at that time, Fernando de Castro re-assumed a research line which he briefly explored in the very first years of his scientific career (de Castro, 1916): a serious study of the histology of autonomic ganglia. This research line can be considered as completed with the publication of a monograph and a series of shorter articles which includes maybe the most important comparative study between mammalian species including primates and humans to that date (de Castro, 1923a,b, 1926, 1927), although important morpho-functional observations derive from works mainly devoted to other subjects than sympathetic ganglia (for details about the latter three studies, see below de Castro, 1932a,b, 1937, 1942; de Castro and Herreros, 1945; **Figure 3**). Together with research by other colleagues (Van Gehuchten, Lenhossek, Retzius, Köelliker, Cajal, Mihailov, etc.), de Castro's contribution during 25 years of work in this field affirmed that the preganglionic connections wrap in spirals onto ganglionic cells to form the pericellular nests described by Ehrlich in the frog (although the number of these nests are largely lower in mammals; Ehrlich, 1888; de Castro, 1923a,b, 1932b). De Castro also affirmed that these dendritic nests, far from being accidental arrangements, are receptive sites for specific synaptic contacts from preganglionic fibers (de Castro, 1923b). Indeed, the fibers climbing along the dendrites, forming what they called the "receptive plaques", were pointed by Cajal and de Castro as maybe the most frequent form of intercellular connection in the sympathetic ganglia (de Castro, 1923a,b, 1933, 1951). It was almost a decade after these first descriptions by de Castro that synapses were suggested to be present at the terminal boutons of the preganglionic fibers (de Castro, 1930, 1933; Lawrentjew, 1931, 1934a,b; Kolossow and Sabussow, 1932; Fedorow and Matwejewa, 1935; Bullón Ramirez, 1945; Bullón-Ramirez, 1947). These morphological descriptions contributed to the notion that three main types of neuron can be distinguished in the sympathetic motor ganglia (big, medium size and small neurons, big and small neurons each approximately 25% of the population, and medium size 50%). In each ganglion cells of these three types are intermingled and distributed in an apparent arbitrary way (de Castro, 1932b, 1937, 1950), and each type of preganglionic fiber contacts exclusively one type of ganglionic cell (Billingsley and Ranson, 1918; de Castro, 1923a, 1932a,b, 1937), which coincides with electrophysiological recordings showing four different potential waves in sympathetic ganglia (Bishop and Heinbecker, 1932; Eccles, 1935a,b,c). In this sense, de Castro's observations on the nature of the axons of Dogiel's Type II cells confirmed that they project either to other neurons within the same ganglia or in other neighbor ganglia, while they never end in the enteric mucosa. These observations confirmed previous reports (Dogiel, 1899; Ramón y Cajal, 1905; Billingsley and Ranson, 1918; de Castro, 1923b). Thus, the sensory nature of these fibers, as proposed originally by Dogiel, could be discarded. Posterior denervation studies demonstrated that the number of intraganglionic synapses is significantly larger than that of terminal boutons (de Castro and Herreros, 1945). In the latter article the positioning of the synaptic boutons close to astrocytes suggests the presence of what has been described at the turn of the 21st century as "tripartite synapses" (Araque et al., 1999; Perea et al., 2009). Developmental evidence drove de Castro to suggest that the apparent disorder and arbitrary distribution of ganglionic cells derive from germinative centers or spheres disseminated within the ganglia (de Castro, 1923a, 1932a,b). Although they appear in these studies as modest details, de Castro's mind caught details here that remain uncontested and are still very important for our current perception of the structure and functioning of the nervous system. For example, he clearly stated that the ganglia are literally invaded by mesenchymal structures that lie interposed between the ganglionic neuronal components (somata, dendrites, axons). There always appeared to be a tiny glial mantle around neuronal components, forming a kind of "neuronal atmosphere", for instance protecting axons once they loose their myelin sheaths (de Castro, 1937; de Castro and Herreros, 1945; **Figure 3**). In the ganglia the Schwann cells behave as the oligodendrocytes in the CNS, but de Castro also suggested that expansions emanated by Schwann cells form the intermediate portions of synapses, i.e., thin lamina interposed between the preganglionic fibers and the ganglionic neurons (de Castro, 1937; del Río-Hortega and Prado, 1941; de Castro, 1942; del Río-Hortega and Prado, 1942). It should be quoted here that de Castro, together with B.I. Lawrentjew, was among the first scientists specifically studying regeneration of synaptic contacts in the sympathetic system (Lawrentjew, 1925, 1934a,b; de Castro, 1930). In this series of scientific articles, de Castro showed in detail the cytoarchitecture of sympathetic and parasympathetic autonomic motor ganglia in humans, in other primates and in several large mammals. As a result of this research, de Castro was in 1924 awarded with the Martínez y Molina Prize (again from the Spanish Real Academia Nacional de Medicina). At that time the exhaustive and expert works of Fernando de Castro in the field of the histology of somatic sensory and autonomic ganglia had gained international recognition. The most clear example of this came by hand of the famous American neurosurgeon and neuropathologist Wilder S. Penfield (1891–1976), founder of the prestigious Montreal Neurological Institute (Canada): penfield invited de Castro to write two chapters for the first edition of his celebrated treaty "Penfield Cytology and Cellular Pathology of the Nervous System" (de Castro, 1932a,b). Penfield himself juicily described his "Quixotian adventure" (in his own words): his trip from the Presbyterian Hospital in New York, USA to 1924's Madrid to work in the laboratory of Pío del Río-Hortega. In particular he describes his visit to Cajal's laboratory on

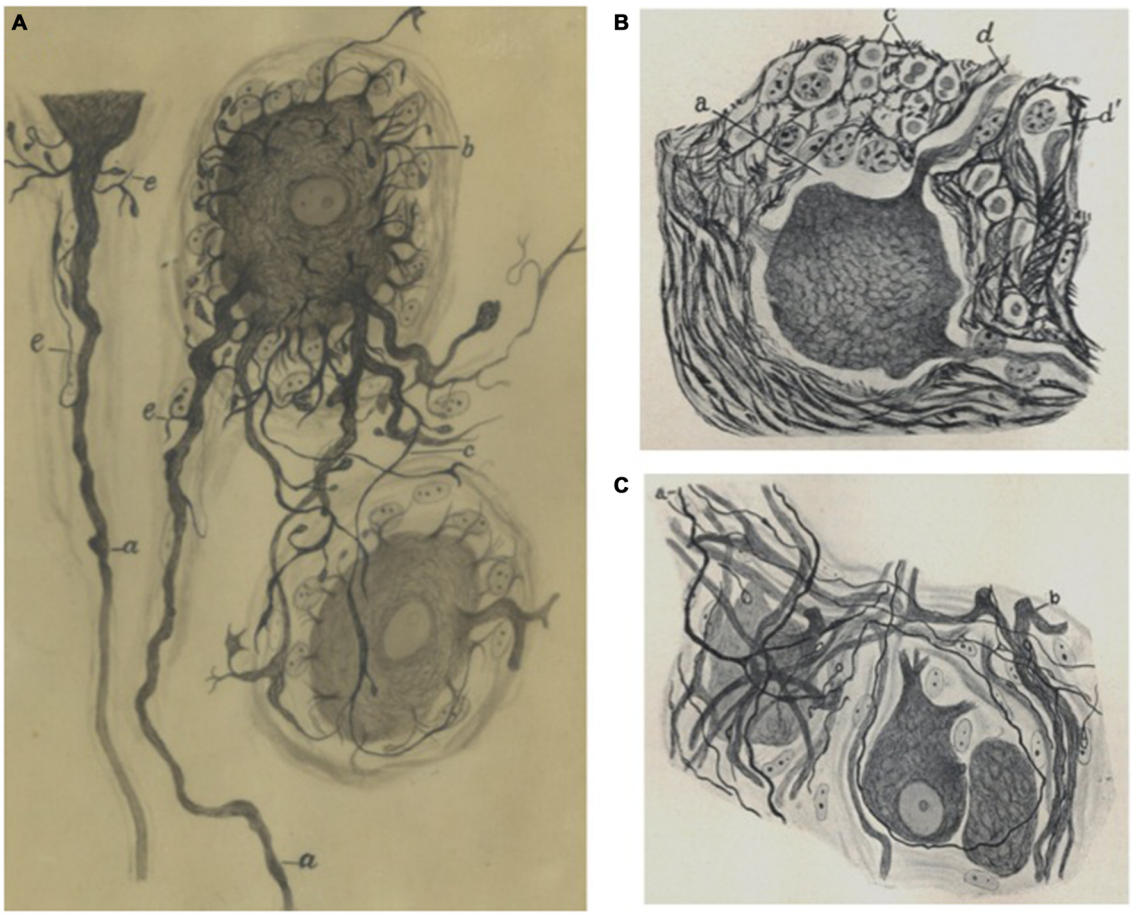

FIGURE 3 | Sympathetic neurons by Fernando de Castro. (A) de Castro's hand-made schematic illustration of sympathetic neurons stained with the Cajal's method, showing short long (*a* –the axón arises from this dendrite at a distance from the soma, *c*), short dendrites (*b*). This image was published in de Castro (1933). **(B)** Partial view of a sympathetic ganglion (normal condition) of an adult cow (de Castro, 1937). **(C)** Portion of a sympathetic ganglion with regenerated preganglionic fibers (*a*) after a vagus-sympathetic crossed anastomosis (de Castro, 1937; **A)** is part of Archive Fernando de Castro.

May 11th, to meet Cajal, Fernando de Castro and Domingo Sánchez:

"Cajal looked at his watch and I looked at Asúa. But at that moment, a young fellow, Fernando de Castro, came in. Cajal seemed to brighten up and said that de Castro was master of his (Cajal's) gold method for neuroglia and suggested that I could work sometimes at a table where de Castro would teach me.

Cajal left us then and I did stay on to talk with de Castro. Dr. Sánchez insisted that I should examine with his microscope the complicated structure of an insect's brain, explaining that the brain of an ant or a bee was just as vast in its complexity as the brain of man or any other mammal. I marveled at what he showed me and at the beautiful sections of mammalian sympathetic nerve cells on de Castro's desk." (Penfield, 1977).

Because, indeed, Fernando de Castro was personally entrusted by Ramón y Cajal to direct the technical training and the research of all the fellows and researchers who arrived between 1924 and 1932 from the entire world to learn and work at the Cajal Institute, like, among many others, Deszö Miskolczy (1894–1978; considered as the father of Neuroscience in Hungary), Howard Florey (1898–1968; awarded with the Nobel Prize in Physiology or Medicine in 1945), André Dewulf (Belgium), or Clemente Estable (1894–1976; Uruguay). A number of visitors became significant friends of Fernando de Castro (**Figures 4B,C**). Penfield did not formally work at Cajal's laboratory, partly due to the so vaunted distancing between the Maestro and his disciple, as Penfield himself writes:

"There was no doubt that, as I had chosen Hortega, I should continue behind him. Unfortunately, there was no extra time to work with de Castro. Hortega had spread off. The most recent discoveries came from him and his research was still far from completed. But I worked on, day by day, sitting at the desk beside Don Pío del Río-Hortega" (Penfield, 1977).

One of the most characteristic aspects of de Castro's research is the exhaustive study of synaptic connectivity established within autonomic motor ganglia. This could be

FIGURE 4 | Some proofs of the long-term friendship developed by Fernando de Castro with other disciples and visitors of the laboratory of Santiago Ramón y Cajal. (A) Manuscript letter from Rafael Lorente de Nó to Fernando de Castro describing the excellent formation and situation of de Castro in the neuroscientific panorama of the mid 1920's. **(B)** Original charcoal portrait of Fernando de Castro by Ferenc Miskolzy (made in 1926, in Madrid), Hungarian painter and brother of the founder of modern Hungarian Neurology, Deszo Miskolzy, disciple and translator of Cajal's books and close friend of de Castro for years. The painter came to Spain because he wanted to visit exiled last Austro-Hungarian empress, Zita, exiled in Spain. Following recommendations of his brother Deszo, he visited Fernando de Castro at Madrid, who showed him his scientific drawings and his deep interest and knowledge in Art. The painter gifted this charbon portrait to the Spanish neuroscientist as a proof of the close frienship of both Miskolzy brothers and Fernando de Castro. **(C)** Madrid (Spain), December 1958, from the left to the right, Florencio Bustinza (1902–1982; born at Liverpool, pharmacologist and profesor of Biology at Madrid), Sir Howard Florey (Nobel Prize in Physiology or Medicine 1945) and Fernando de Castro. Bustinza was personal friend of Sir Alexander Fleming and Sir Howard Florey since 1948, and Fernando de Castro kept frienship with the latter since his time at Cajal's laboratory to learn histological technique during the mid 1920s, **(A–C)** are part of Archive Fernando de Castro.

considered as his first interest in the synapse. This interest represents a continuum along the remaining of de Castro's career.

A DRASTIC CHANGE OF DIRECTION WITH CONSEQUENCES FOR DE CASTRO'S SCIENTIFIC CAREER

After 1925, Fernando de Castro combined his work on autonomic ganglia with the study on the innervation of the aorto-carotid region. This work fundamentally changed the field (for specific reviews in this subject, see de Castro, 2009; González et al., 2014—included in this current Special Research Topic; **Figure 4A**). In the attempts to prove his hypothesis that neurons located in the carotid bodies act as sensory chemoreceptors that detect changes in the chemical composition of circulating blood, Fernando de Castro took an alembicated experimental way that should pass through… the orthosympathetic ganglia! Lesions of the superior cervical ganglion were instrumental to study degeneration and regeneration of the intra-ganglionic synapses in the carotid bodies (de Castro, 1930), as an easier and indispensable step to attack regeneration of "his" aorto-carotid fibers in the future. The 1929 article represents a prelude of a full reorientation in de Castro's research during the decade starting in 1930: in agreement with Cajal's advice, Fernando de Castro had begun studying neural tissue *in vitro* and regeneration of the nervous system in collaboration with the un-discussed leader of the field at that time, Giusseppe Levi (1872–1965). De Castro spent several periods in Levi's laboratory. These postdoctoral fellowships had the potential to be very successful for Fernando de Castro. However, political events (Giuseppe Levi was imprisoned by the fascist Mussolini government after a problem between Levi's son and the police) and unexpected health problems of the young Spanish neurohistologist (for details in this novelistic episode,

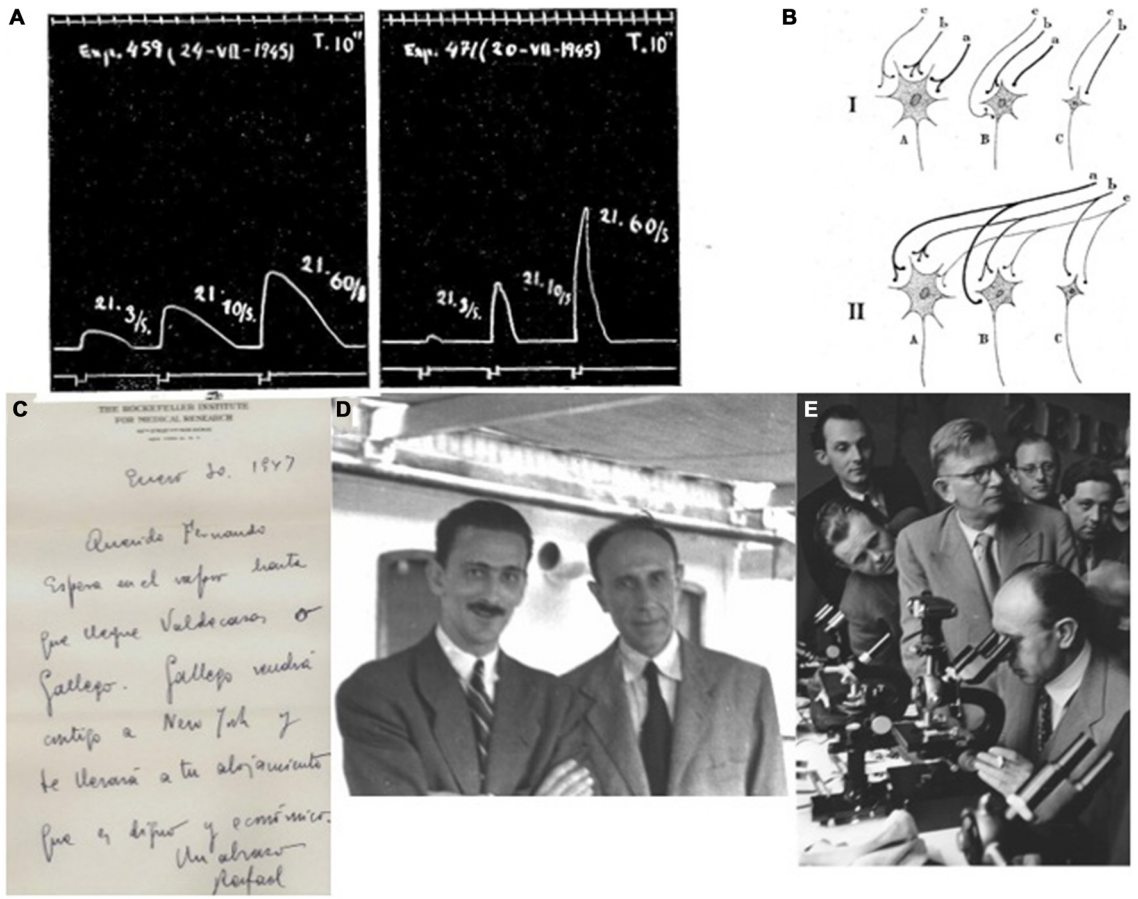

FIGURE 5 | Post-war de Castro on the autonomic nervous system and their synaptic structure. (A) Electromiographic recordings of the nictitant membrane of the adult cat where the sympathetic superior cervical ganglion has been innervated by rami from the VI-c and VII-c nerves (de Castro and Herreros, 1945). This 10 s-recording shows that the intensity of the contraction correlates with the frequency of the tetanic stimulation. **(B)** Schematic representation of the preganglionic convergence of fibers (*a*, *b*, *c*) onto ganglionic cell types (*A*, *B*, *C*). The thickness of the fibers is representative of their thickness *in vivo*. In this it is assumed that ganglion cells can trigger when activated by two boutons simultaneously, or in the slow fibers (*c*) when a sinchronic impulse via a-b facilitates it (de Castro and Herreros, 1945). **(C)** Original letter from Rafael Lorente de Nó to Fernando de Castro (dated at the Rockefeller Institute, New York on January 30th, 1947): *"Dear Fernando, Wait at the shipboat till the arrival of Valdecasas or Gallego. Gallego will come with you to New York and will bring you to your accomodation, that is worthy and economic. Hugs, Rafael"* (translated by the autor of this work from Spanish). **(D)** Antonio Gallego (1915–1992) and Fernando de Castro on board of the *Motomar* steamship, in their way back to from New York to Spain (1947) after their respective first scientific experience at the USA. **(E)** Fernando de Castro (at the microscope), invited speaker to expose the cytoarchitecture of the autonomic nervous system. Became one of the main characters in the final offical defeat of reticularists at the 34 Tagung Deutschen Gesselchaft fü Pathologie (Wiesbaden, Germany; 1950). His friend, the German histologist established in Chile, Emil Herzog (on foot, with glasses, just behind Fernando de Castro) acts as de Castro's master of ceremony at that time, **(A–E)** are part of Archive Fernando de Castro.

see de Castro, 2009; Santarén and Sánchez-Ron, 2009) seriously limited the planned research. The direct and indirect results of the experiments undertaken in the Italian collaboration became incorporated in work published in the immediate years after de Castro had returned to Madrid (de Castro, 1934, 1937).

In this period, de Castro's colleague and friend, Rafael Lorente de Nó (1902–1990; together with de Castro, the last and youngest direct disciples of Cajal) strongly advised Ramon y Cajal that de Castro should never abandon the study of the aorto-carotid innervation (de Castro, 1972, 2009; Santarén, 2014). While Lorente's advice was correct, the decision by de Castro and Cajal to continue the aorto-carotid innervation research line may be considered in the light of world history to have had a negative impact on to de Castro's scientific career. Civil war erupted in Spain in 1936 and fighting reached Madrid towards the end of that year. Fernando de Castro, being in charge of protecting the equipment and collections of the Cajal Institute, became fully occupied in protecting the Institute from literal disappearance during the almost 3 years (1936–1939) that the Spanish Civil War ravaged Madrid (de Castro, 2009; De Carlos and Pedraza, 2014; González et al., 2014). In the mean time the Belgian physio-pharmacologist Corneille Heymans (1892–1968) took advantage of the opportunity and won the race to functionally demonstrate the origin in the carotid body of the chemical reflexes. Heymans consequently

was awarded in 1938 with the Nobel Prize in Physiology or Medicine.

POST-WAR STUDIES ON THE SYNAPTIC ORGANIZATION OF THE SYMPATHETIC GANGLIA

Times changed and Fernando de Castro decided to attack one of his scientific dreams, postponed for years due to the Spanish Civil War (1936–1939) and the Second World War (1939–1945). The study of structure and function of synapses had significantly progressed in these years already, and de Castro assumed that the study of synapses in the autonomic nervous system would really be profiting since the structure of the ganglia is simpler than that of the CNS and his particular knowledge on the fine structure of the sympathetic ganglia would undoubtedly be of great help in this new research (**Figures 5A,B**). As soon as the political circumstances permitted, de Castro contacted his old friend Rafael Lorente de Nó who had emigrated to the USA in 1931 to fulfill a fellowship at the Rockefeller University in New York. Fernando de Castro's travel request to the USA was accepted and granted by the Junta de Relaciones Culturales (Spain), de Castro arrived in New York at the beginning of 1947, to work with Herbert S. Gasser (Nobel Prize in Medicine or Physiology on 1944, shared with Joseph Erlanger) and Lorente de Nó and to learn the basics of electrophysiology and electrophysiological recordings (**Figures 5C,D**).

In these years, de Castro insisted on the fact that is the protoplasmic glia that is interposed between the pre- and the postsynaptic elements (de Castro, 1942, 1951). De Castro showed that pericellular nests are the way through which presynaptic fibers contact postsynaptic neurons, although these nests appear to be larger and more frequent in Amphibians and Reptiles than in Mammals. According to de Castro there are more synapses than morphologically identifiable terminal boutons in sympathetic ganglia, as he demonstrated by sectioning preganglionic fibers (de Castro and Herreros, 1945). This drove him to propose that preganglionic fibers form in these autonomic ganglia a kind of diffuse connection beyond the terminal boutons (de Castro, 1951).

CONCLUDING REMARKS

The work by Fernando de Castro to get his PhD degree at the beginning of the 1920's decade produced capital ammunition to destroy the reticularist conception of the organization of somatic sensory and autonomic nervous ganglia (**Figure 5E**). De Castro described the delicate morphological details of the ganglionic cells and the distribution of the synaptic connections in such a meticulous and convincing way that it revolved the field. De Castro's work in this field fully granted him the technical and intellectual recognition by his tutor, Santiago Ramón y Cajal, and it prepared him for the study of the innervation of blood vessels, particularly those in the carotid region, to identify the controversial nature of this innervation triggering the cardio-respiratory reflexes. He was the first to identify arterial chemoreceptors in the carotid bodies. After the forced break due to both the Spanish Civil War and the Second World War, Fernando de Castro continued working on sympathetic ganglia to study synapses and synaptogenesis. For the rest of his scientific career till his death in 1967, both the arterial chemoreceptors and the autonomic and somatic sensory ganglia remained his principal research lines. His histological descriptions remain fully recognized and actual today.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Fernando de Castro and the discovery of the arterial chemoreceptors

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When de Castro entered the carotid body (CB) field, the organ was considered to be a small autonomic ganglion, a gland, a glomus or glomerulus, or a paraganglion. In his 1928 paper, de Castro concluded: *"In sum, the Glomus caroticum is innervated by centripetal fibers, whose trophic centers are located in the sensory ganglia of the glossopharyngeal, and not by centrifugal [efferent] or secretomotor fibers as is the case for glands; these are precisely the facts which lead to suppose that the Glomus caroticum is a sensory organ."* A few pages down, de Castro wrote: *"The Glomus represents an organ with multiple receptors furnished with specialized receptor cells like those of other sensory organs [taste buds?]...As a plausible hypothesis we propose that the Glomus caroticum represents a sensory organ, at present the only one in its kind, dedicated to capture certain qualitative variations in the composition of blood, a function that, possibly by a reflex mechanism would have an effect on the functional activity of other organs... Therefore, the sensory fiber would not be directly stimulated by blood, but via the intermediation of the epithelial cells of the organ, which, as their structure suggests, possess a secretory function which would participate in the stimulation of the centripetal fibers."* In our article we will recreate the experiments that allowed Fernando de Castro to reach this first conclusion. Also, we will scrutinize the natural endowments and the scientific knowledge that drove de Castro to make the triple hypotheses: the CB as chemoreceptor (variations in blood composition), as a secondary sensory receptor which functioning involves a chemical synapse, and as a center, origin of systemic reflexes. After a brief account of the systemic reflex effects resulting from the CB stimulation, we will complete our article with a general view of the cellular-molecular mechanisms currently thought to be involved in the functioning of this arterial chemoreceptor.

Keywords: Fernando de Castro, carotid body, arterial chemoreceptorss, sensory physiology, ion channels, transduction cascade

INTRODUCTION: THE CAROTID BODY UNTIL FERNANDO DE CASTRO[1]

Hardowicus Wilhelmus Ludovicus Taube made his dissertation to obtain the greatest honors in Ars Medica the thirty first of January of 1743. The title of the dissertation was "De vera nervi intercostali origine" (The true origin of intercostal nerves), and in chapter 17 it reads "..., qui retro Carotides, ad ipsum interna and externa fecenditis angulum Ganglion minutum efficient, cujus ramuli, quantum video, in tunicis hujus arteriae desinunt," what in a free translation would mean: "... behind the carotid arteries, where the internal and the external carotid arteries form their angle, Ganglion minutum forms, whose small twigs, as far as I can see end in the arterial walls." Thus, ganglion minutum is the first name given to the carotid body (CB), which represented a small nervous structure located in the back of the carotid artery bifurcation. A few years later, Albrecht von Haller in his "Elementa physiologiae corporis humani" referred to the CB with the name of ganglion exiguum, as part of a plexus formed by branches of the superior cervical ganglion. Johann E. Neubauer, who provided the first drawing of the CB, referred to it as ganglion parvum; he depicted the CB as a small rectangular structure located in the angle of the carotid artery bifurcation but without any physical contact with the arteries themselves. Carolus S. Andersch, the discoverer of the petrosal ganglion which is the inferior or main ganglion of the glossopharyngeal nerve, named the CB, gangliolum intercaroticum, indicating in the name two important traits: its small size and its location. We want to explicitly state that even if Andersch discovered the inferior main sensory ganglion of the glossopharyngeal nerve (the superior, accessory or superior glossopharyngeal ganglion is named after his discoverer

[1]The historical account we are providing here has been made after a careful reading of Adams book. Adams W. E. The comparative morphology of the CB and carotid simus. Charles C Thomas, Publisher: Springfield, Illinois, USA, 1958.

Johann Ehrenritter, the Ehrenritter ganglion), he did not describe any participation of this cranial nerve (the IX cranial pair) in the innervation of the CB.

In 1833–1834 August Franz Joseph Karl Mayer, the creator of the term histology, rediscovered the CB in man and several other species and named it ganglion intercaroticum. Besides describing it as a constant organ with a well defined relationship to the angle of the common carotid artery bifurcation through a small ligament, which in fact is a pad of fibroelastic tissue through which its arterial supply reaches the organ. In addition to its reddish color, its compactness and its size, like a grain of rice in man, the most important trait of the carotid he discovered was that, in addition of its connection with the superior cervical ganglion by one or more branches, the CB receives innervation from the glossopharyngeal through a fine branch that, running down parallel to the external carotid artery, ends up branching in the ganglion intercatoricum. We want to emphasize here to the fact that, in spite of the discovery of the innervation by the glossopharyngeal in the first third of the 19th century, it took almost a century to recognize the sensory nature of the innervation (de Castro, 1928).

Hubert von Luschka in 1862 found that the purported ganglion intercaroticum was not a ganglion, but a gland that he named glandula intercarotica. He provided many details about the size and anatomical variations in humans; he described a structure of the CB typical of a gland with glandular tubes and a close association with sympathetics, like the adrenal gland. Unfortunately, Luschka considered sympathetic innervation as the only nervous supply to the CB. He also described dispersed ganglion cells or small microganglia in his glandula intercarotica; Fernandode Castro described these ganglion cells in great detail in his 1926 article. It should be mentioned that Luschka was a very prestigious anatomist with multiple structures described and named after him (for a comprehensive list see Tubbs et al., 2011) and therefore his belief that the CB was a gland rooted deeply in the literature of that time. According to Noble et al. (1997) Luschka was the first to describe a CB tumor. A completely different point of view was proposed by Julius Arnold, son of Friedrich Arnold, the teacher of Luschka and his predecessor as anatomy professor in Tübingen. In his 1865 article, Julius Arnold presented the CB as formed by a complex net of small arteries, capillaries and venules that created a small round or oval shaped ball; it was not glandular tubes but rather convoluted vessels that gave this appearance. He named the CBs glomeruli arteriosi intercarotici, and the terminology has reached our days when we speak of glomic tissue or glomoid to refer to each of the nests of glomus cells (chemoreceptor cells) present in the CB along with the network of small vessels or capillaries surrounding each of them.

The glandular nature of the CB gained support from embryological studies, as Luschka himself and his disciples/followers proposed that it derived from the pharyngeal endoderm. Adams (1958) points out that Luschka's proposal was based on an elementary mistake, as the CB was mistaken for a parathyroid gland. Arnold's glomerular conception also received support from embryology as when in 1887, Kastchenko proposed that the CB was of mesodermal origin as it derives from a thickening of the adventitia in the vicinity of the origin of the internal carotid artery, close to the nodose ganglion. We do not want to proceed any further with this history before clarifying the embryological origin of the CB. At the beginning of 1970s, Le Douarin and coworkers developed a technique to create viable chimeras in avian embryos: grafts of the neural rhombencephalic primordium from 6 to 10-somite quail embryos were implanted in the homologous region of chick embryos of the same age, and after appropriate time the CBs of the host were histologically and immunohistochemically examined. Since quail cells are characterized by having interphase nuclear chromatin condensed in large masses while chicken cells have homogeneously distributed, uncondensed chromatin, it is possible to distinguish cells from each species; additionally, quail cells are dopamine-rich while chicken cells contain serotonin, making it possible to distinguish, on the basis of the spectrum of formaldehyde-induced fluorescence, the cells of quail origin which form part of the chicken CB. This type of studies allowed the French authors to demonstrate that, in fact, the parenchymatous cells, at least in the avian CB, derived from the neural crest, being neither endodermical nor mesodermical, but neuroectodermical (Le Douarin et al., 1972; Pearse et al., 1973).

It was the presence of biogenic amines in the CB cells that prompted Le Douarin to include them in APUD (amine content and/or amine precursor uptake and decarboxylation) series of endocrine polypeptide cells. The neuroectodermal origin of CB cells in mammals has also been demonstrated with different techniques, including genetic markers (e.g., Kameda, 2009). It is worth noting that neural crest cells migrate during embryogenesis according to their position along the embryo axis to give origin to many structures including most of the peripheral nervous system: the sympathetic and parasympathetic enteric ganglia, satellite glial cells in ganglia, dorsal root ganglia, Schwann cells, adrenal medullary chromaffin cells, and the CBs (see Hempleman and Warburton, 2013).

In retrospective, what we know today of the embryological origin of the CB (and adrenal medulla), provides a clear example of sound judgement, of the capacity of discernment of the scientists in the last part of the 19th and the early 20th century, when the technical support for their observations were rather limited. Thus, Kohn in 1900 recognized in his studies the observations made by Kastchenko on the thickening of the adventitia in the vicinity of the origin of the internal carotid artery, but added that this has nothing to do with the origin of the typical cells of the CB which arrive there along the sympathetic plexus growing in the carotid bifurcation. It would appear that Kohn asked himself how a structure should be named that is clearly neither a ganglion, nor a gland or a glomus. This answer was that since the CB, like adrenal medulla, is one of the organs connected to the sympathetic, it should be named paraganglion intercaroticum. This origin of today's concept of paraganglion, as a group of cells, that not being neurons, are derived from the neural crest and are located in the vicinity of sympathetic or parasympathetic ganglia, was born in the past century. The first quarter of the 20th century was largely occupied by the discussion of the true nature of the CB as a paraganglion. The point was that the adrenal medulla, the best example of paraganglion, exhibited a very positive chromaffin reaction, i.e., a marked capacity to form yellow–brown precipitates when sections

Table 1 | Catecholamine content in the CB of several mammalian species.

Animal species	Dopamine (DA)	Norepinephrine (NE)	DA/NE
Cat	726 ± 114	697 ± 94	1.05
Rabbit	750 ± 78	143 ± 15	5.25
Rat	210 ± 24	45 ± 7	4.6
Mouse	225 ± 20	25 ± 3	9.0
Calf	598 ± 121	29 ± 4	20.6
Man	1.32 ± 0.4	1.63 ± 0.4	0.8[a]
	40 (at birth)–10 (young adult)[b]	–	–

Data are expressed as nmole/g fresh tissue and have been obtained in our laboratory except for the data in humans. The data in humans are quite variable due to the fact that they have been obtained in necropsies with variable times elapsing from dead.
[a] *From Perrin et al. (1984).*
[b] *From Lack et al. (1985).*

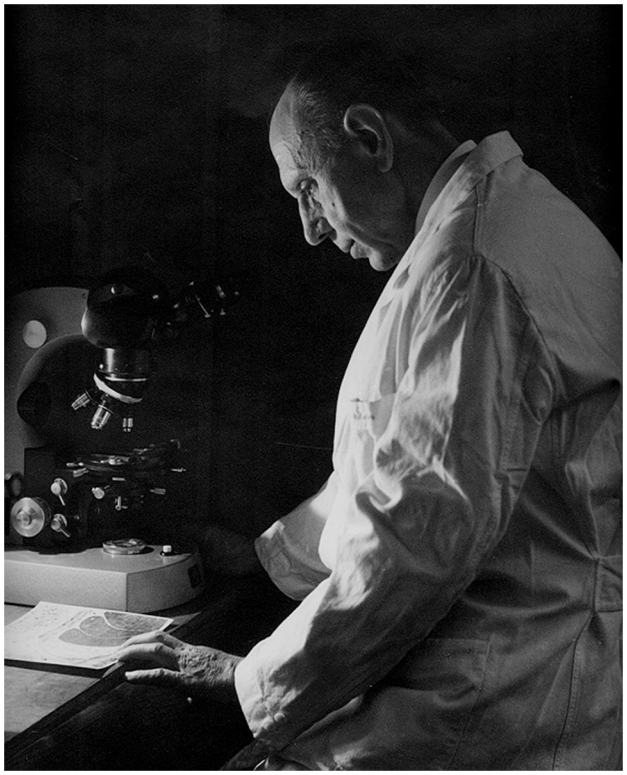

FIGURE 1 | Fernando de Castro (1896–1967). The picture was taken in 1967 (Courtesy of Fernando-Guillermo de Castro from the Archive Fernando de Castro).

of the adrenal medulla were exposed to chromate and dichromate salts due to the adrenomedullary cells content in epinephrine and norepinephrine, but the CB showed, at best, a weak chromaffin reaction. Recognition of this fact, lead to the classification of paraganglia as chromaffin paraganglia (or sympathetic, as in the adrenal medulla) and non-chromaffin paraganglia (or parasympathetic, as in the CB). Thus, since according to Verna (1997) only those cells immunopositive to norepinephrine or its synthesizing enzyme, dopamine beta hydroxylase, exhibit chromaffin reaction, it follows that the classification of the CB as a chromaffin or non-chromaffin paraganglion could be species-related, according to the content of norepinephrine (see **Table 1**) and to the subjective appreciation of chromaffinity or pseudo-chromaffinity (is yellowship, chromaffin?). In establishing comparisons with the chromaffinity of the adrenal gland it should also be taken into account that the concentration of catecholamine in the adrenal gland is much higher than in the CB. It should be noted that some authors in the initial third of the 20th century also described the CB as a mixed paraganglia, or containing both chromaffin and non-chromaffin cells.

THE 1926 ARTICLE OF FERNANDO DE CASTRO

In the Introduction to his 1926 article, de Castro (**Figure 1**) regretted the scarcity of studies devoted to the study of structure, innervation and function of the CB in contrast to the great profusion of embryological works. He pointed out that after Kohn the CB was considered by most researchers as a paraganglion, which like the organ of Zuckerkland or aortic paraganglion (located in the abdominal aorta in the vicinity of the origin of the inferior mesenteric artery or by the bifurcation of the aorta), the cardiac paraganglion or paraganglion of Wiesel (located in the fatty tissue around the left coronary artery among the branches of the cardiac plexus that supply the left auricle), and possibly also the coccygeal glomus gland of Luschka (located at the tip of coccyx) would represent supplementary adrenomedullary glands. de Castro also wrote that chromaffinity being the most conspicuous property of these paraganglia there was not unanimous agreement on the ability of the CB cells to reduce chromic salts. Nevertheless, there was general agreement on the spherical or ovoid form of the CB cells, their distribution contiguous to capillaries, and the richness of vessels and nerves of the organ. de Castro concluded the Introduction stating that little was known about the structure of the cells themselves, the nature and origin of the nerve fibers of the organ, and the way the nerve fibers end on the cells; these aspects were the targets of his 1926 study.

As to the methods of the paper (section I in the paper) we wish to underline a few details. (1) de Castro used in this study young and adult mice, rats, rabbits, cats, and dogs as well as mice and cat embryos at several developmental stages, mature human fetuses and human tissue from adult humans accidentally killed. (2) In the study of nerves de Castro used as staining procedure Cajal's reduced silver nitrate method. In small animals and embryos, in order to prevent possible destruction or disturbances of the anatomical relationships, he fixed the entire head with several fixatives that included nitric acid as decalcifier, and serially sectioned the entire piece: this procedure allowed him to follow the nerves from their origin and to precisely define their anatomical relationships. (3) To study the cellular structure he used a new set of fixatives and staining procedures. (4) To experimentally study the innervation of the CB and the carotid region in general, he used cat and dog preparations in which the cervical sympathetic

chain had been resected and/or the glossopharyngeal had been sectioned distally to the petrosal ganglion.

With this methodological armamentarium de Castro in 1926 made a series of salient findings that we shall try to faithfully summarize. From section II of the paper, devoted to the general organization of the innervation of the CB, we highlight the following aspects: de Castro wrote that in almost all studied species, the CB is surrounded by nerves which as a whole form a periglandular plexus (notice that de Castro follows the main trend in naming the CB as a gland). In mice the disposition of the fibers is much simpler and it is not feasible to talk of a truel periglandular plexus. Similarly, in humans, where the CB commonly is formed by several independent lobules (also common in calves; Sanz-Alfayate et al., 2001) dispersed in the conjunctive and fat tissue around the carotid sinus, there is not a true periglandular plexus. The fibers surrounding the CB, whether or not forming a true periglandular plexus, have three origins: (a) the greatest part comes from the superior cervical sympathetic ganglion and most of them are unmyelinated (i.e., postganglionic fibers) although there are some fibers of sympathetic origin which are middle sized myelinated (i.e., preganglionic that would terminate in sympathetic neurons located in the periphery of the CB or directly on chemoreceptor cells; see Verna, 1997); (b) the second contingent in quantitative importance comes from the glossopharyngeal via its intercarotid branch or *nerve intercarotidien* (the carotid sinus nerve, CSN), and is formed by myelinated fibers of middle size, although there are some myelinated fibers and some unmyelinated fibers; (c) finally, the smallest contingent is represented by filaments of fibers escaping from the pharyngeal branch of the vagus nerve. This organization of the periglandular plexus would explain two facts frequently disputed in recent literature: (1) for any given species, the levels of NE in the CB would vary from laboratory to laboratory according to their thoroughness on the dissection of the organ, i.e, depending on the elimination or not of the sympathetic fibers; (2) the effect of sympathectomy on the NE levels of the CB would vary from species to species according to their richness in NE-containing chemoreceptor cells (in the rat and rabbit, and probably in the mice, with few NE cells, sympathectomy would cause a great decrease in NE levels, while in the cat, the effect would be very modest; Gonzalez et al., 1997).

The interstitial plexus is formed almost exclusively by fibers of the CSN which penetrate the CB at different points, most commonly at superior or cephalic pole, and distribute and divide in the connective tissue that separates the clusters of parenchymatous cells; if the fibers are myelinated, it is commonly observed that, on dividing, they loose their myelin. The sympathetic fibers of the periglandular plexus do not penetrate the CB of the cat, dog, and man, but in mice, two or three thick sympathetic nerves penetrate and traverse the CB and, therefore, contribute to form the interstitial plexus. The fibers of the interstitial plexus, either isolated or in groups of up to eight or more, escape to form the periglomerular plexus which surrounds every cluster of parenchymatous cells. The periglomerular plexus forms on many occasions a true basket or nest around the cell clusters. Fibers of this plexus are both myelinated and unmyelinated and divide profusely and, upon dividing, myelinated fibers almost always loose the myelin sheath. From the

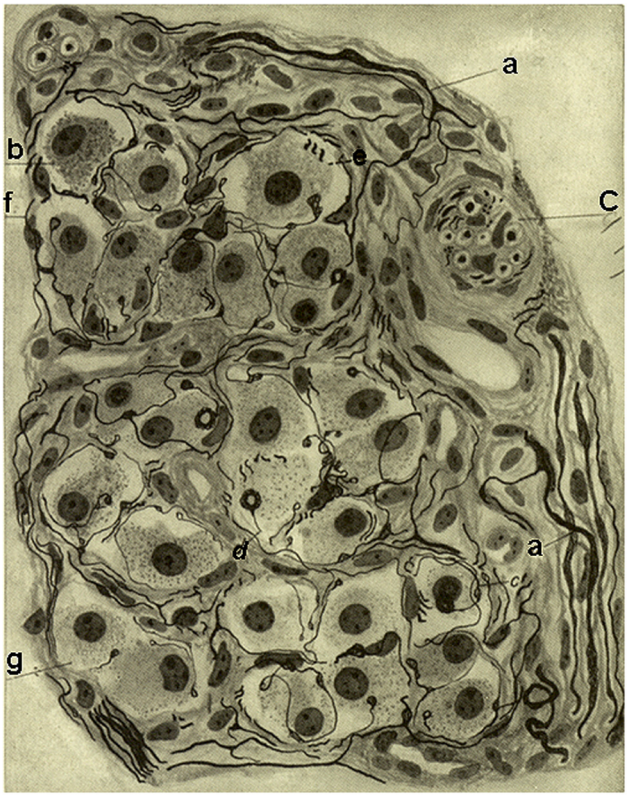

FIGURE 2 | Segment of a glomerulus of the intercarotid gland (the CB) of a young man. c, nerve with myelinated and unmyelinated fibers; a, division of myelinated fibers; b, glandular(chemoreceptor) cell; e, glandular cell with a nerve ending in mallet; g, another cell with vacuolated cytoplasm; f, fiber with varicosities; d, nerve ending forming a thick ring. Stained by the Cajal's reduced silver nitrate method (in de Castro, 1926; courtesy of Fernando-Guillermo de Castro from the Archive Fernando de Castro).

periglomerular plexus, fibers commonly thin unmyelinated, penetrate cell clusters or glomeruli and frequently run associated with capillaries in the fine trabeculae of connective tissue forming the intraglomerular plexus (**Figure 2**). It is common that in this plexus fibers become very thin giving the impression that they end, but careful examination with adequate impregnation shows that they continue and widen to form polymorphic endings on the surface of chemoreceptor cells, from truly fine endings to small buttons or disks up to big calyx-shaped endings that almost completely envelope the entire cells (see the splendid images obtained in the serial section analysis with the electron microscope performed by Nishi and Stensaas, 1974). In the cat, rabbit, and dog the intraglomerular plexus is clearly discernable, being even more evident and complex in the human CB. In mice, where the intraglomerular connective tissue is absent and the parenchymatous cells contact each other, the intraglomerular plexus is less evident, fibers traveling freely among chemoreceptor cells.

On reading section II of de Castro's paper, it strikes one that the author writes on no less than three or four occasions: the nerve endings do not penetrate the cell cytoplasm, but rather are always external to the cells and adapt to their surfaces. This

sentence and its reiteration might sound meaningless today. Yet, it should be recalled that Cajal (de Castro's mentor), the creator of the current theory of the nervous system organization known as the Neuron Doctrine (i.e., that the nervous system is made up of discrete individual cells, interrelated with one another by contact and not by continuity), had to defend his view against the proponents of the so-called reticularism (reticularist theory) which believed in the cytoplasmic continuity of nervous system cells, so that the cytoplasm of the neurons would form a continuous syncytium. The acrimonious attacks on the Neuron Doctrine are best exemplified by the Nobel Lecture given by Golgi in his sharing of the Nobel Prize with Cajal (see De Carlos and Borrell, 2007). Therefore, the statements of de Castro were meaningful in those years when the entity of neurons as independent cells was questioned.

Section III of the 1926 paper is devoted to the description of sympathetic (autonomic) neurons and microganglia in the CB and nervous plexuses just described. de Castro starts this section recognizing that Kohn has described these neurons/microganglia, and a few lines later states that the most important aspects of the biology of these neurons, i.e., the origin of their innervation and the destination of their axons, remain unknown. He asks himself, if these neurons innervate the parenchymatous cells of the gland (i.e., the chemoreceptor cells) or the CB blood vessels and other more distant structures? To answer these questions he used two main preparations: the perfused head of mice serially sectioned that allowed him to follow the trajectories of the fibers, and cat preparations which had been subjected to two different denervations 8–30 days before. The denervations consisted in either the removal of the cervical sympathetic chain or the section of the glossopharyngeal nerve cephalic to the origin of the CSN. His conclusions were: (1) Interspaced among the fibers of the periglandular and the interstial plexuses of the CB, at the origin of the CSN (particularly in the rat and mouse), and all along the length of the CSN there are autonomic neurons either isolated or in groups, forming true microganglia; as a whole these neurons are more frequent in the cat and dog than in the rat or mouse, and are very rare in man. (2) The preganglionic fibers of these neurons/microganglia have two origins, the brain stem (the medulla oblongata) and the spinal cord. Those fibers originating in the brain stem would belong to neurons located in the motor nuclei of the glossopharyngeal or vagus nerves, travel in the glossopharyngeal/CSN, and arborise on neurons located all along the CSN itself, in the periglandular plexus or inside the CB. The fibers originating from neurons located in the spinal cord come out via the superior cervical ganglion (forming part of the ganglio-glomerular nerves) and end up on neurons located in the periglandular plexus and in branches of the cervical sympathetics. The fibers with their origin in the brain stem degenerate on sectioning the glossopharyngeal and those originating in the spinal cord disappear after the removal of the sympathetic chain. (3) The axons from the autonomic neurons located in the CSN, on the surface, and inside the CB innervate the vessels of the CB and nearby structures; other contingents of fibers continue with sympathetic branches to innervate more distant structures.

We would not wish to close the description given by de Castro in 1926 of the autonomic neurons located in the glossopharyngeal and all along of the CSN without referring to the "efferent pathway." The efferent pathway is a functional concept to describe the fact that stimulation of the peripheral cut end of the CSN inhibited chemoreceptor discharge recorded from single or few fiber filaments split off from the main nerve trunk (Almaraz et al., 1997). The point was that the anatomical substrate, the origin of the fibers supporting the efferent inhibition was unknown and persisted after every imaginable denervation procedure. Recent experiments have demonstrated that the axons from the autonomic microganglia constitute the efferent pathway. In a set of experiments Fidone's laboratory re-described the autonomic neurons in the glossopharyngeal, in the origin of the CSN and along the CSN, and showed that these neurons are nitrergic which upon their activation release nitric oxide (and acetylcholine, implying that they are parasympathetic neurons). Nitric oxide and acetylcholine would cause vasodilation, with an additional supply of O_2 to the CB and thereby causing the efferent inhibition; a direct effect of NO on chemoreceptor cells would also contribute to the efferent inhibition (see Wang et al., 1995). It remained, however, to be known how these neurons are physiologically activated. The obvious assumption was that signals activating them would come via the preganglionic fibers located in the brainstem, and it surely occurs that way, but quite recently Colin Nurses's laboratory has made some brilliant experiments describing more direct forms of activation of these autonomic neurons. The Canadian authors confirmed the nitrergic-cholinergic nature of the autonomic neurons and showed that they are sensitive to hypoxia, so that hypoxia at the same time it augments the sensory activity provides a direct negative feed-back; they also showed that autonomic neurons express ATP receptors so that ATP released by chemoreceptor cells during hypoxic stimulation would activate the terminals of the autonomic neurons (or even the somas of neurons located inside the CB) promoting the genesis of nitric oxide which would be the ultimate effector. This last conclusion was obtained using a modification of the co-culture preparation developed in their laboratory. In this occasion they co-cultured clusters of chemoreceptor cells with autonomic neurons obtained from the glossopharyngeal-CSN and observed that application of ATP or hypoxia to the neurons caused a robust chemoreceptor cell hyperpolarization that was prevented by pre-incubation with NO scavengers and NOS inhibitors; additionally they showed that NO donors, but not ATP itself, was able to hyperpolarize chemoreceptor cells in clusters cultured without the autonomic neurons (see Campanucci et al., 2012).

Section IV of the paper is entitled: "The intercarotid gland (the CB) is not innervated by the sympathetics of the paravertebral sympathetic chain: anatomical and experimental data." Once again de Castro starts by referring to Kohn who proposed that the cells of the gland (the chemoreceptor cells) were innervated by the superior cervical sympathetic ganglion. In opposition to that, de Castro continues writing: in the cat there are many myelinated fibers (that could not be postganglionic sympathetic fibers), and what's more, nobody has shown that all unmyelinated fibers, or only a part of them, come from the sympathetic system of the spinal cord or from the brainstem autonomic system. As in section III he used several experimental approaches: serial sectioning of

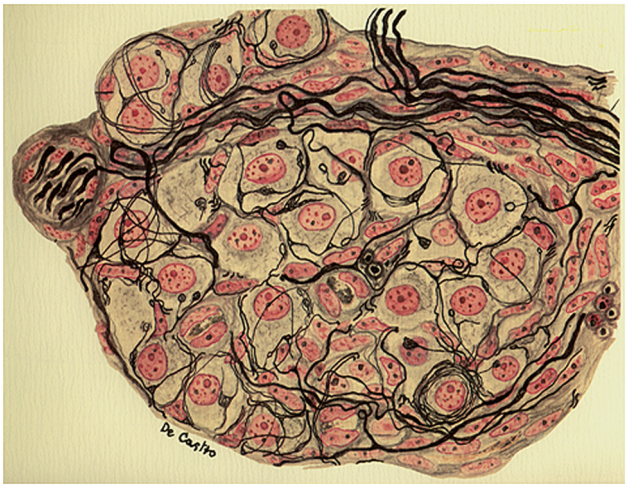

FIGURE 3 | Section from the intercarotid gland (the CB) of an adult cat 25 days after the surgical removal of the vertebral sympathetic chain. The image evidences fascicles of myelinated nerve fibers and parenchymatous cells normally innervated. The preparation was stained by the Cajal's reduced silver nitrate method and counterstained by the carmine method of Mayer (in de Castro, 1926, 1928, 1951; courtesy of Fernando-Guillermo de Castro from the Archive Fernando de Castro).

the head of the mouse and preparations of cat and dog whose cervical sympathetic chain had been removed 15–30 days in advance. Referring to the mouse, de Castro writes: silver nitrate impregnation evidences that the nerve fascicles that originate in the superior cervical ganglion (the ganglioglomerular nerves) either go around the CB or traverse it, but none of the fibers arborise to penetrate in the cell clusters. Even further, it becomes evident that the fibers that penetrate the cell clusters or glomeruli arriving from the cephalic (brain stem) autonomic system are thicker and stain more intensely than the sympathetic fibers. As shown in **Figure 3**, representing a trustworthy copy of the microscope field of an operated cat, the removal of the cervical sympathetic chain of both sides does not alter the organization of intraglomerular plexus, i.e., the number of myelinated and unmyelinated fibers that penetrate the cell clusters and innervate the glandular (chemoreceptor) cells are undistinguishable from those seen in control cats. This implies that the innervation of chemoreceptor cells comes via the glossopharyngeal and the CSN from its origin in the autonomic system of the medulla oblongata. Readers must appreciate the erroneous bias in de Castro's interpretation of his findings: since the CB purportedly was a gland, it must receive secretomotor motor innervation, and therefore the fibers which reach the glandular cells (chemoreceptor cells) via the CSN must have their origin in the brain stem (see below).

Section V of the paper is entitled: "Existence of a direct autonomic pathway for the intercarotid gland. Anatomical and experimental facts." de Castro begins this section stating that he had already communicated to the Spanish Society of Biology that the intercarotid gland, the CB, appears to be innervated exclusively by the glossopharyngeal nerve via the CSN; the innervation is direct, without the interposition of an autonomic peripheral ganglion, a fact that is contrary to the admitted rule for the innervation of other glands and tissues controlled by the brain-stem, thoracolumbar, and sacral autonomic nervous system. In the paragraphs that follow he emphasizes the discrepancies that existed in the literature of that time, regarding the source of innervation of the CB and opposes to the view recently put forward by Drüner (1925). This last author, based on macroscopic studies in humans, inappropriate to solve the difficult problem of the CB innervation in the opinion of de Castro, proposed that innervation of the CB was sensory or centripetal and that CB itself represented the sensory organ responsible for the carotid baroreflex recently described by Hering.

The preparations used by de Castro to describe the innervation of the CB are those used in previous sections of the study, the head of mice and rat serially sectioned and cat and dog preparations whose cervical sympathetic chain had been removed or their glossopharyngeal had been sectioned 1–4 weeks in advance. After a meticulous description of the observations made in mice and rat he concludes that an autonomic pathway exists in the glossopharyngeal nerve that provides almost the entire wealth of fibers that constitute the CSN; these are efferent (centrifugal) secretory fibers devoted to innervating the intercarotid gland and are unique in the sense that they innervate the gland without the interposition of ganglion cells. de Castro estimated that these direct secretory fibers represented about 2/3 of total CSN fibers, the remaining 1/3 being preganglionic fibers that synapse with the autonomic neurons and microganglia described above, with an additional contingent of sensory fibers that innervate blood vessels that would be described in the next section. Our findings in the rat and mouse, continues de Castro, have been confirmed in the cat and dog as the extirpation of the cervical sympathetic chain does not appreciably alter the innervation of the gland while the section of the glossopharyngeal at its exit from the skull causes complete degeneration of the CSN, almost total disappearance of the interstitial and periglomerular plexus and complete loss of the intraglomerular plexus and its endings; occasionally after the section of the glossopharyngeal it is possible to see some myelinated axons which would be sensory fibers coming from the pharyngeal branch of the vagus nerve, from glossopharyngeal neurons displaced from their usual location (in the petrosal ganglion) or autonomic axons of the neurons located along the glossopharyngeal-CSN nerves, but in no case do they end on the glandular cells.

The description given by de Castro in this section is precise and it agrees with the descriptions given by modern morphologists (e.g., McDonald, 1981; Verna, 1997): nearly all the fibers ending on the glandular cells of the intercarotid gland (i.e., on the chemoreceptor cells of the CB) reach the organ via the CSN. Yet, de Castro was wrong in two aspects: in attributing the fibers an origin in the brain stem and in assigning them a secreto-motor function. Needless to say that de Castro corrected these two errors in his 1928 study, as we will see below. What lead de Castro to these erroneous conclusions? Obviously de Castro was short in his experimental observations. He performed sections on the glossopharyngeal at its exit from the skull, and this means distal to the glossopharyngeal sensory ganglion (petrosal ganglion), but he did not perform glossopharyngeal sections cephalic to the petrosal ganglion. In the cat (and also in the rat), the petrosal ganglion is located deep in the base of the skull, dorsomedial to the tympanic

bulla really non-accessible and incapable of being seen from a ventral approach. That means that what indeed emerges from the base of the skull is the glossopharyngeal distal to the ganglion. As a consequence de Castro's sections certainly caused the degeneration of fibers having their somas in the brain stem, but in addition all sensory neurons having their somas in the petrosal ganglion also degenerated. However, although de Castro was strictly faithful describing his observations, it is our opinion that he was biased in the interpretation by the current view that the CB was a gland, and obviously glandular cells must have an efferent secreto-motor innervation. In spite of the suggestion of Drüner, de Castro in 1926 had not yet considered that the CB could be a sensory organ, and certainly not the sensory element in the Hering's reflex (see below), and therefore he did not search for a sensory innervation.

Section VI of the paper is devoted to the sensory innervation of the intraglandular vessels and to the innervation of the internal carotid artery. In the introduction to this section de Castro states that he is going to refer to the carotid sinus reflex described by Hering: mechanical stimulation by application of pressure of the region where the common carotid artery divides causes a diminution of the heart rate frequency and a vascular dilatation and, as a consequence, a fall in blood pressure. Already Hering had verified that the reflex remains intact after the removal of the superior cervical ganglion, but it disappears on sectioning the first branch of the glossopharyngeal (the CSN); additionally, Hering had also shown that the electrical stimulation of the central stump of the sectioned CSN mimicked exactly the mechanical stimulation of the carotid sinus region. Hering named this depressor nerve, "s*inusnerv,*" a name that de Castro considered inadequate (he preferred the name, nerve *intercarotiden*) because such a nerve would contain in addition to the sensory or centripetal fibers innervating the carotid sinus region, sensory fibers innervating the intraglomic vessels and the fibers innervating the CB itself which he thought at that time were autonomic centrifugal in nature.

Working in mice and rat de Castro describes that some (or most) of the sensory fibers mediating the depressor reflex (baroreceptor fibers), which arrive to the initial portion of the internal carotid artery via the CSN, come from the pharyngeal branch of the vagus nerve or even from the nodose ganglion itself. de Castro also described more complicated paths for the baroreceptor fibers of the vagus in their route toward the sinus region and assumes a comparable situation in the cat and dog. de Castro was surprised by the richness of innervation of this area, an aspect that nobody had noted before. The baroreceptor fibers mediating the depressor reflex are myelinated and upon reaching the origin of the internal carotid artery, which is a very densely innervated area, divide in the adventitia of the artery forming multi-shaped terminals in all species. In some cases, the fibers give rise to a few branches which form meniscus-shaped terminals of different sizes; on other occasions the fibers follow an arborisation pattern with progressively thinner branches which form small blebs in their trajectory or their end acquiring a varicose-like aspect. In all cases the terminals rest on the external elastic membrane of the artery. However, in a study using serial semithin and thin sections Böck and Gorgas (1976) confirmed the observations made by de Castro regarding the superficial disposition of the baroreceptor endings in mice, finding additionally that in the guinea pig the terminals enter the media and approach the innermost layers near the intima.

de Castro also performed denervation experiments in the cat and the dog. He found that removal of the superior cervical ganglion did not alter the innervation of the carotid sinus-origin of the internal carotid artery, and therefore gave histological support to the above mentioned physiological experiments performed by Hering. However, and contrary to the findings reported by Hering, de Castro found that sectioning of the glossopharyngeal early on its exit from the brain resulted in degeneration of the distal stump of the glossopharyngeal as well as degeneration of the CSN, but still the innervation of the internal carotid artery in its origin remained intact, implying that in large animals, as is the case in mice and rat, most of the depressor fibers would have their origin in the vagus nerve. This last conclusion was erroneous as de Castro himself recognized in later studies: most if not all baroreceptor fibers responsible for the carotid sinus baroreflex are in fact of glossopharyngeal origin having their soma in the petrosal ganglion (de Castro, 1940, 1951; see also Belmonte and Gallego, 1983).

In this section de Castro also described a comparable innervation of the small intraglomic arteries and arterioles: myelinated fibers of glossopharyngeal origin penetrate the CB and from the interstitial plexus arrive to the innervated vessels. The fibers divide in the adventitia and form a nearly complete ring around the vessel to terminate forming varicose arborizations or meniscus, with the extension of the terminal arborisation being proportional to the diameter of the vessel; yet, the complexity of the terminals is smaller than that of their counterparts in the carotid sinus or the origin of the internal carotid artery. Although the sensory terminals most commonly are located in the adventitia most densely in branching points of the arteries, occasionally they reach the muscular layer or even approach the endothelium. de Castro underlines that these sensory fibers should not be confused with sympathetic fibers which terminate in the muscular layer. Regarding the function of this sensory innervation of the intraglomic vessels, de Castro considers that it would be different from the general baroreceptor (depressor) fibers present in the carotid sinus/origin of the internal carotid artery which support Hering's reflex. He envisions the innervation of the intraglomic vessels as forming a reflex loop capable of regulating the functional activity of the CB (**Figure 4**). However, in his 1928 and 1951 studies de Castro concludes that the sensory system located in the intraglomic vessels also exerts an influence on the regularization of the arterial pressure, albeit smaller than that played by the carotid sinus innervation (see below under 1928 article).

Section VII of the article is devoted to the study of the chromaffin reaction of the CB. As we have mentioned above the chromaffinity of the CB was a much debated issue in the initial third of the 20 century, the discussion being linked to the concept of paraganglion, the embryological origin and the putative function of the CB itself. de Castro found that in the CB of man and cat fixed with formol-dichromate there are not cells exhibiting the typical brown chromaffin reaction (Henle's reaction) as are seen in the suprarenal glands of the same individuals used as controls; at most in the CB cells there is a faint yellow and freely spread

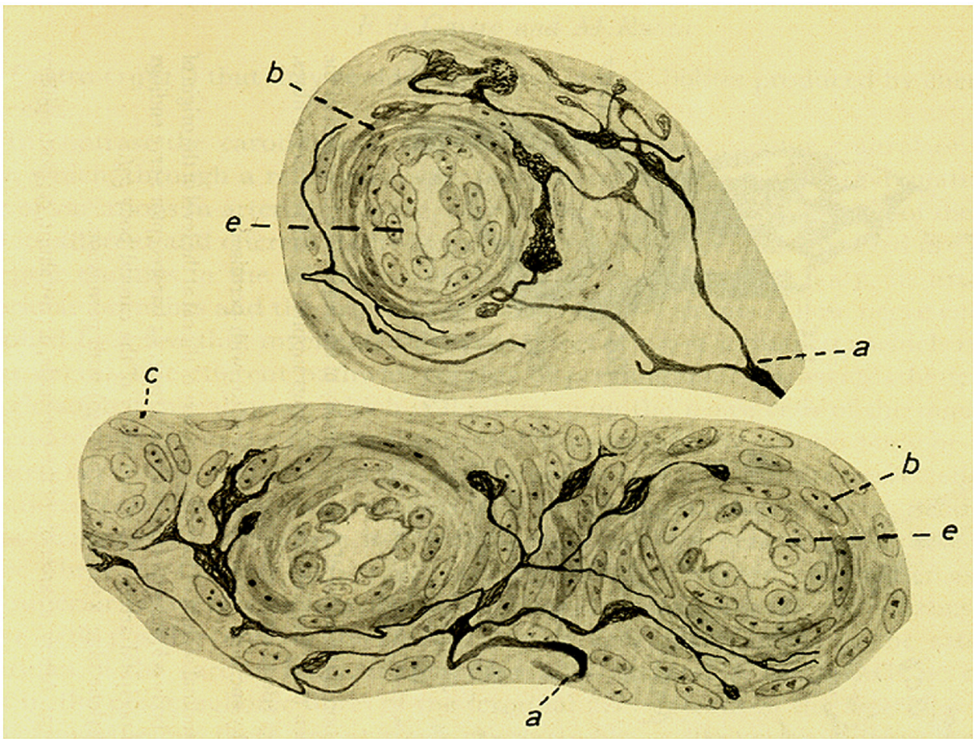

FIGURE 4 | Intra carotid body arterioles (e) receiving sensory innervation from two myelinated fibers (a) which arborise to terminate preferentially in the adventitia although some endings reach the muscular layer (b). (c) Smooth muscle cells. Branches form varicosities and terminate up forming small meniscus, buttons or hammer-like endings. Note that in the lower figure the fiber and its terminals are located in the angle formed by the branching of one artery. The preparation was stained by the Cajal's reduced silver nitrate method (in de Castro, 1926; courtesy of Fernando-Guillermo de Castro from the Archive Fernando de Castro).

(non-granular) stain of the cytoplasm of the cells. In addition, the cells of the sections of the suprarenal glands also fixed with formol-dichromate when later stained with Giemsa stain acquire a granular aspect with a very striking greenish color, thought to be in agreement with other authors that grains would contain adrenaline, the secretory product of the cells. Contrary to that, the cytoplasm of CB cells after the same fixation and staining procedures acquires a pink tint. Since in addition CB cells stained in orange-red with Sudan III, de Castro concluded that the yellow staining of the cells did not represent a true, even if weak, chromaffin reaction, but rather a manifestation of the richness in lipoid material of these cells. Finally, CB cells did not exhibit the typical Vulpian reaction (development of a green color when a weak solution of ferric chloride is applied to the cells) seen in the cells of the suprarenal gland. At this point we want to refer to the study of Lever et al. (1959) which, along with additional observations, represents the full publication of their initial observation of the presence in chemoreceptor cells of osmiophile granular bodies (or dense-core granules or chromaffin vesicles) made in 1957 (Lever and Boyd, 1957). After using a significant number of histochemical tests they conclude that data strongly suggest the presence within the cytoplasm of chemoreceptor cells of a phenolic amine or some closely related chemical, because the relative order of staining intensities obtained with these tests was strikingly similar in the CB and in the adrenomedullary cells. The authors, also state that the intensity of staining was much less in the CB than in the adrenal gland, estimating in at least a factor of ten the difference in the intensity of the reactions between both tissues. Interestingly enough, the levels of catecholamine per unit weight in the adrenal gland are over one order of magnitude greater than in the CB. Agreeing with de Castro's descriptions, Lever et al. (1959) also refer to the richness in lipoid like material and the presence of vacuoles in the cytoplasm of chemoreceptor cells.

de Castro also performed denervation and physiological experiments aiming to solve the issue of the chromaffinity of CB cells. He observed that denervation of adrenal glands in cats caused an augmentation of the chromaffinity in the denervated vs. the contralateral side with intact innervation. In agreement with other authors, he also observed that in normally innervated adrenal glands repeated injections of insulin (so as to produce a hypoglycaemic shock) caused a marked diminution, almost a depletion, of the brown grains (green after Giemsa staining) in the cytoplasm of adrenal gland cells. Since denervation of the glands prevented the action of insulin, de Castro concluded that the discharge of adrenalin was controlled by the central nervous system, and neither insulin nor the resulting hypoglycaemias have a direct effect on adrenomedullary cells. Contrary to the situation with the adrenal gland, the denervation of the CB did not cause any improvement in the chromaffin reaction, leading de Castro to conclude that there is no histochemical basis to consider the CB as

a chromaffin organ capable of generating adrenaline. As pointed out by Adams is his monograph (Adams, 1958) it was precisely this study of de Castro that lead Kohn's disciples to the coining of the concept of non-chromaffin or parasympathetic paraganglion. Ironically, when this concept began to be used in 1934, de Castro had already shown that the CB was a sensory organ rather than a supplementary gland. It should be noted, however, that the term parasympathetic paraganglia continues to be in use particularly by clinicians and pathologists to refer to tumoral masses of neural origin lacking an adrenergic trait, associated primarily to the vagus nerve, and usually located in the base of the skull, in the neck or in the mediastinum (Welander et al., 2011).

Section number VIII is devoted to the structure of the CB cells with a particular emphasis on mitochondria, Golgi apparatus (Golgi reticle) and lipoid material. The cells, which are disposed forming clusters, are frequently round or ovoid and on occasion they have processes that appear to anastomose. Part of the surface of the cells faces blood capillaries, this part of the cells being named the vascular or metabolic pole. The cell cytoplasm has a fine granular appearance and presents vacuoles either isolated or forming groups. They only have one nucleus although exceptionally they might have two. The nucleus is not centered, being usually displaced to the pole opposed to the capillaries, and the chromatin is usually diffuse although smaller cells have more condensed chromatin.

According to de Castro, in the cells of the CB nobody had studied before the chondriome or chondrioma, i.e., the mitochondrial content of the cells taken as a whole. We consider that the morphological description of mitochondria and additional organelles given by de Castro in those days has a limited value for today's readers, as evidently ultrastructural analysis during the second half of the past century with the electron microscope has allowed the acquisition of very neat and precise images (see for example Verna, 1979). Nonetheless, there are certain observations which demonstrate the great capacity of discernment of de Castro. For example, during the description of mitochondria he states that circular mitochondria with a clear or empty center are not seen in the cells of the experimental animals, being occasionally noticeable in human cells, particularly if a long time has elapsed between death and the autopsy of the individuals. In other words, de Castro clearly noticed that late or poor fixation of tissues resulted in a loss of mitochondrial matrix. Another fact that strikes one on reading this section of the paper is the profusion of fixative and staining procedures used by de Castro to optimize the observations of particular organelles. Finally, also notable is the preoccupation transmitted by de Castro regarding the purported secretion of chemoreceptor cells. The notion behind this appeared to be: if indeed the CB is a gland, where is the secretion product formed? He stated not to have obtained experimental support to attribute either to mitochondria or to the Golgi apparatus the origin of the product of secretion. The pioneer work of Lever and Boyd (1957) demonstrated that chemoreceptor posses dense core granules and, as they suggested and we know today, they contain catecholamine. Dense core granules of chemoreceptor cells represent synaptic vesicles which secrete their content in response to the physiological stimulation of the CB not to the blood stream but rather to the synaptic spaces created by the cells and the sensory nerve endings innervating them (Iturriaga et al., 2009).

In the last section of the article de Castro makes some general conclusions that deserve to be mentioned. (1) Presently, we lack specific facts providing clues about the functional significance of the so-called intercarotid gland (the CB). (2) The CB is not a vital organ necessary for survival or for the correct development of the entire organism, as is the case for the thyroid gland. (3) On the other hand it cannot be admitted that the CB is a rudimentary or involutional organ as it persists for the entire life span of individuals without signs of degeneration or atrophy. (4) Rather, the complex innervation of the CB cells as well as the great vascularization of the organ and the profuse innervation of the CB vessels favors the notion of a very important role, perhaps assisting the depressor nerves of the carotid sinus in the regularization of arterial blood pressure through the secretion of a hypotensive substance. In the 1928 article, Fernando de Castro set the key bases that lead to satisfactory answers to these queries.

THE 1928 ARTICLE OF FERNANDO DE CASTRO

There are two central themes in the planning or design of this study, which, by its own right, has originated a new area of physiology, namely the area of arterial chemoreception. In the first part of the study, de Castro develops the idea of the carotid sinus baroreceptor reflex as described by Hering and describes experiments on the structure and innervation of the carotid sinus area in several mammalian species giving full support to the notion that the origin of the reflex is the carotid sinus and its profuse innervation. The second part of the paper describes new denervations of the CB by cutting the rootlets of the glossopharyngeal at their exit from the brain stem, representing therefore a denervation of the CB cephalic to petrosal ganglion; this denervation procedure would leave intact the sensory innervation of the organ and cause degeneration of those fibers-endings having their somas in the brainstem. It is worth noting that in the title of 1926 study, de Castro refers to the intercarotid gland while in the 1928 he refers to the glomus caroticum.

As in the 1926 study, in this new work there is also a section on material and methods in which he states that to study the region of the common carotid artery bifurcation he has used material obtained from young men, monkeys, and calves as well as common laboratory animals from dogs to mice. He underlines the promptness in fixation of the tissues commonly in pyridine and the preferential use of Cajal's reduced silver nitrate method to stain the fibers and their terminals. As an alternative fixative, de Castro provides the formula for his two-step fixation procedure in choral hydrate and somniphene solutions, and as alternative staining procedures he used the Bielschowsky's silver and the Ehrlich-Dogiel methylene blue methods, emphasizing the need for careful handling of the last method to avoid the appearance of artifacts (formation of bubbles in the nerve fibers and terminals which may acquire the aspect of a string of beads).

Under the heading of macroscopic and microscopic anatomy of the carotid sinus, de Castro starts describing that in humans in the division of the common carotid artery, or more precisely in the origin of internal carotid artery, there is a constant and normal

dilation or enlargement, the carotid sinus. Its external appearance is variable which has leaded some authors to classify them by their form. In laboratory animals, the sinus is very prominent. For example, in the cat whose internal carotid artery is very thin, the sinus has a large cone-shaped form with the base in the common carotid artery and with the internal carotid artery starting at the apex of the cone. As previously recognized by other authors, de Castro confirms the particular structure of the artery wall at the sinus level: it becomes thinner than the proximal and distal zones, this thinning being due to the absence of the media or muscular layer with an elastic layer and an adventitia well preserved (elastic segment). Since in the cow the internal carotid artery is absent (the common carotid artery gives origin to a thick eternal carotid artery, a glossofacial artery and the occipital artery), de Castro wanted to determine if in the area of the common carotid artery branching there was anything like the elastic segment of other mammals. He observed that indeed the occipital artery, which is responsible for the blood supply of the encephalon, presented dilation in its origin with a structure like that seen in the sinus-origin of the internal carotid artery of other mammals. In the guinea pig, which also lacks an internal carotid artery, it is also the occipital artery which is enlarged in its origin and presents the elastic segment (see Böck and Gorgas, 1976). However, in this same article the German authors observed that in the wall of the mouse carotid sinus a corresponding "elastic segment" is not evident. Similarly in the rat, according to Yates and Chen (1980), the vascular wall of the baroreceptor field in the internal carotid artery exhibits neither a marked dilation to form a carotid sinus nor histological differences in the intima and media compared to other parts of the carotid artery

The topography and density of the innervation present minimal variations, other than those derived form the anatomical localization of the elastic segment in cows. There is a band or belt around the elastic segment where the sernsory terminals concentrate, although the limits of the disposition of the endings are not always precise, with zones in the upper and lower ends in which the innervation is less dense. In the cow, the zones of maximal density of innervation are the internal zones of the occipital artery at the angle formed by the occipital artery and the external carotid artery. There is later in the paper an ample, meticulous, and beautiful description of the morphology of the sensory fibers and terminals in the carotid sinus of men and other mammals that we will present in a very condensed manner. The innervating fibers, usually myelinated of different diameters, have their origin in the sensory ganglia of the vagus or glossopharyngeal (note that in the 1926 paper he sustained that they were vagal fibers) and from the periglandular plexus reach the sinus by its superior pole. In the carotid sinus of men, de Castro distinguishes at least two types of branching pattern for the fibers on arriving to the adventitia: (1) diffuse arborisations which are originated from small or middle sized myelinated fibers and follow a rather regular division pattern giving branches of first, second, third and even larger orders; in their trajectories they might have small varicosities and in the terminals capricious forms (leaves, rackets, mallets,. . .). (2) circumscribed ramifications which are originated from medium to thick sized myelinated fibers and follow an arborisation pattern like grapevines or bushes; branches are spiny and zigzagging,

FIGURE 5 | Sensory ending in a tangential section in the carotid sinus of a man. (A) Thick myelinated axon that arborises in a rather beautiful pattern with different details in the terminals. (f) A branch of the main fiber. (g) Nerve terminal in racket. The preparation was stained by the Cajal's reduced silver nitrate method (in de Castro, 1928; courtesy of Fernando-Guillermo de Castro from the Archive Fernando de Castro).

and the terminals draw small triangular structures (**Figure 5**). In the rest of the mammals studied, the same two types of branching patterns are distinguishable having an overall complexity that parallels the size of animals. de Castro shows a clear parallelism between the large sizes of the architecture of the sensory fibers their arborisations and terminals in the occipital artery of the cow vs. the much simpler pattern of branching of the sinus of the mouse.

Finally, de Castro devotes a small section to describing the relationships between the disposition of the sensory branches and the organization of the adventitia of the carotid sinus area: Knowledge of this relationship, he states, would aid understanding the mechanism of activation of the sensory fibers when changes in blood pressure occur. As a general rule de Castro observes that the fascicles of collagen fibers in the adventitia have a parallel disposition following the longitudinal axis of the sinus region and the branches of the fibers are disposed among those fascicles. On occasion this regular pattern is lost due to the existence of oblique fascicles of conjunctive tissue, being in these areas where the zigzagging branches of the sensory fibers are housed. de Castro reasoned that natural stimulation of the sensory apparatuses would occur by

their compression between the collagen fascicles when the wall of the arteries becomes distended by the arterial pressure. In this manner the sensory innervation of the carotid sinus area would be transmitting constantly the level of the arterial blood pressure to brain stem centers and, as the experiments of Hering have demonstrated, an increase in the pressure of the area would trigger the sinusreflex.

The next section in the 1928 article is devoted to the sensory innervation of the intraglomic vessels. de Castro expanded the description of the sensory innervation of intraglomic vessels given in 1926. The fibers and their terminals would be distributed in small arteries and arterioles and even in precapillaries where the sinusoids of the CB are initiated. de Castro restates de simplicity of the innervation and the general branching pattern of the nerve fibers in comparison to those present in carotid sinus and provides some magnificent images obtained in longitudinal views of intraglomic vessels (**Figure 6**).

Once again de Castro calls attention to the sympathetic innervation of the intraglomic arterial vessels that would come mostly from intraglomic or periglomic sympathetic neurons receiving their preganglionic innervation via the ganglioglomerular nerves. From a functional point of view he declines his previous 1926 beliefs: that innervation of the intraglomic vessels would represent the afferent arm of a reflex which via the sympathetic innervation would promote profuse secretion of the purported carotid gland and that the sensory innervation of the CB vessels would not have a complementary role to that of the carotid sinus. In this 1928 study de Castro denervated the carotid sinus region by careful application of phenol solutions that would destroy the carotid sinus-internal carotid artery nerve endings, and this denervation preserved, albeit attenuated, the typical systemic depressor response when the pressure in the cannulated common carotid artery was augmented; he concluded that the sensory innervation of the intraglomic vessels is functionally complementary to the innervation of the carotid sinus.

In spite of these observations, the existence of a reflex loop initiated in the sensory innervation of intraglomic arteries and arterioles and closing at the sympathetic innervation of the same vessels cannot be dismissed. Obviously this reflex would not control the secretion of the carotid gland, because the CB is not a gland, but it could control the chemosensory activity in the CSN. This reflex loop would represent an efferent control of the CB function, in addition to the above described efferent pathway. Although we do not yet know the circumstances in which this sympathetic based efferent control would work there are some well established facts in that regard. For example, Eyzaguirre and Lewin (1961) demonstrated that stimulation of either the preganglionic sympathetic trunk or the ganglio-glomerular nerves produced an increase in the chemosensory activity (non-baroreceptor sensory activity recorded in the CSN) that was attributed to a decrease in CB blood flow (and therefore to a decrease in CB O_2 supply) because sympathetic stimulation lacked effects in the isolated superfused preparation. The observation that stimulation of the sympathetic supply to the CB augments chemosensory activity has been ratified by many laboratories, but the doubt remains: under which circumstances are the sensory nerve endings of intraglomic vessels activated to initiate the reflex loop? The truth is that we do not know. Lahiri's laboratory (Lahiri et al., 1986) showed that the responses of the ganglioglomerular nerves were associated with the respiratory responses to hypoxia and hypercapnia, so it could be speculated that the changes in blood flow that occur in the CB during its physiological stimulation contributes, via the sensory innervation of intraglomic vessels to set the sympathetic tone directed to the CB vessels and thereby to fine tuning the chemosensory activity in the organ.

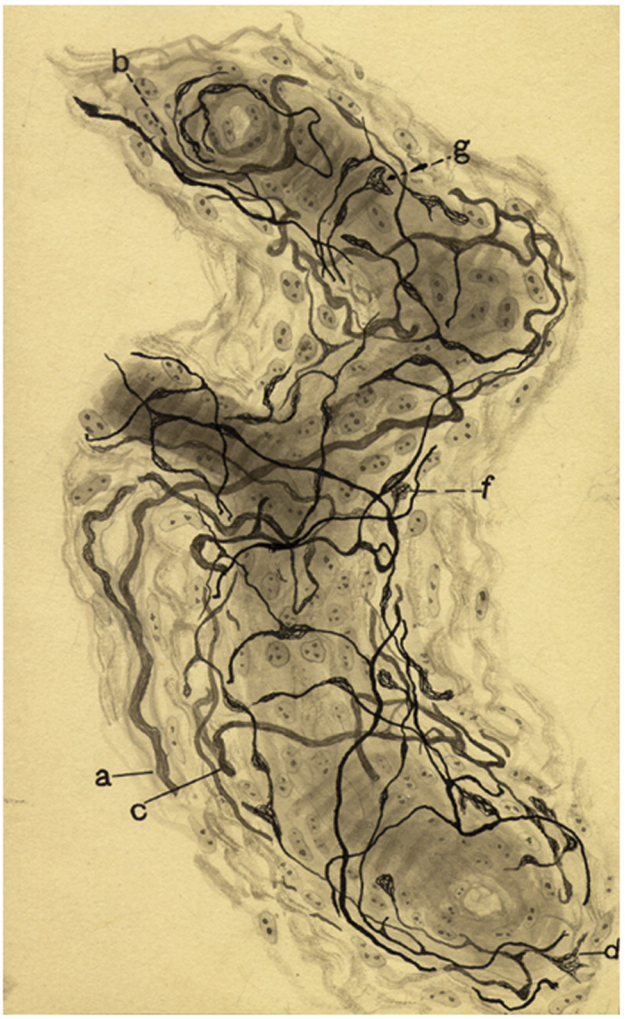

FIGURE 6 | Sensoy nervous plexus in an artery of small caliber from a dog of two months. (a,b,c) Myelinated axons. (d) Varicose nerve terminal, (g,f) Terminal meniscus. The preparation was stained by the Cajal's reduced silver nitrate method (in de Castro, 1928; courtesy of Fernando-Guillermo de Castro from the Archive Fernando de Castro).

The next section of the article is entitled New facts on the innervation of glomus caroticum (the CB) and its likely function. de Castro splendidly summarizes his previous findings along with many of the experimental approaches he employed to discover the function of the CB. He states that the CB is not the origin of the sinus reflex, the CB does not meet the criteria to be considered a paraganglion, in sum, as he had stated at the beginning of this 1928 paper: "up to now I have being unable of finding a function for

the CB." Aiming for a definitive answer, de Castro performed two physiological and anatomical experiments. In the first experiment performed in cats he approached the phossopharyngeal through the bulla tympanica and sectioned the nerve placing a thread of silk in the distal stump to handle it adequately; he later sectioned the peripheral branch of the glossopharyngeal going to the tongue thereby obtaining an easy-to-handle preparation with the intact CSN and a small, much thicker segment of the glossopharyngeal. When he stimulated this CSN preparation while recording the arterial blood pressure in the femoral artery no changes were observed, but if he stimulated the intact CSN without the prior proximal sectioning of the glossopharyngeal he obtained the Hering' s reflex in all its clarity. He concluded that the stimulation of the functional nerve of the CB had not provided any insight as to the physiological significance of organ. The second experiment performed in 17 cats and three dogs was more decisive. The experiments consisted in the intracranial sectioning of the roots of the glossopharyngeal (9th) and vagus (10th) and occasionally also of the accessory (11th) nerves. 5–12 days after the surgery the glossopharyngeal and the vagus in their extracranial segments as well as the area of the division of the common carotid artery were collected for histological analysis. It was found that in glossopharyngeal there were a very small number of degenerated fibers with an even smaller number in the CSN, so that the appearance of the CSN was essentially normal. The CB cells exhibit an intact innervation after the intracranial section of the glossopharyngeal, there being no differences, other than the expected vascular congestion, between the operated and the contralateral control CB. These findings obliged de Castro to conclude that the trophic centers (the neuronal somas) of the axons innervating the CB cells are located in the sensory ganglia of the glossopharyngeal, i.e., the petrosal ganglion, and not in the brain stem as he had concluded in his previous study. To assure that the surgical procedures and therefore the conclusions were correct, de Castro stimulated electrically the glossopharyngeal and the CSN of the operated side and no response was obtained, while on stimulating the contralateral side he obtained the typical depressor response.

It is in the conclusion of this section where de Castro demonstrates unequivocally the sensory nature of the CB and where he creates the concept of chemoreception. de Castro wrote: "In sum, the glomus caroticum is innervated by centripetal fibers, whose trophic centers are located in the sensory ganglia of the glossopharyngeal nerve, and not by centrifugal secretory fibers as is the case in the glands. The CB is a special sensory organ, whose function was not an enigma. We hypothesized that the CB is a sensory organ devoted to perceive some qualitative modifications of the blood, and not a system devoted to detect the variations of the blood pressure; this last function residing in the carotid sinus and also in part in the arteries and arterioles of the CB itself."

In the following section of the paper that he entitles General considerations and discussion, de Castro devotes great efforts to refute the notion of the CB as the origin of the Hering's reflex, because it was a notion that was gaining adepts in spite of the really conclusive experiments of Hering and de Castro himself. He also refuted the notion of the CB as a (sympathetic) paraganglion because of the dubious (negative) nature of the chromaffin reaction and to the fact that some authors found that the CB extracts caused hypotension while those of the adrenal gland caused an elevation of blood pressure. He also refuted his previous notions of the CB as a special gland non-classifiable among the paraganglion group. Finally, he refused to accept an involutional or rudimentary functional significance to the CB. However, he maintained the notion of a possible cooperative significance of the innervation of the CB vessels in the final setting of the Hering reflex.

Later de Castro reiterates that the intracranial section of he glossopharyngeal has made evident that the innervation of the CB cells is sensory and therefore the CB as a whole represents a sensory organ, the only one presently known, responsible for detecting changes in the qualitative composition of the blood, and possibly, using a reflex pathway might affect the functional activity of other organs. Certainly de Castro did not know the nature of stimulus triggering the reflex nor the targets of the reflex itself, but he clearly outlined the CB chemoreflex. de Castro conceives the parenchymatous elements of the CB (i.e., the chemoreceptor cells) as having two poles, one vascular and other nervous. Through the vascular pole the cells are in a close relationship with the sinusoidal capillaries that surround the cell clusters to taste the blood composition. By the nervous pole the cells are innervated by polymorphic nerve endings. The nerve endings or nerve fibers would not be stimulated directly by the blood, but rather by the mediation of the parenchymatous cells whose products of secretion would activate the stimulation of the centripetal fibers (see also de Castro, 1951).

Certainly, de Castro in these two articles (1926 and 1928) here summarized has built a frame, a circuit through which neural orders must circulate to contribute to maintain the internal equilibrium of the organisms, the adjusting to physiological processes and a potential mechanism of defense in disease. It remained to be established how the circuit is put into motion, how it is activated, how it works, where it impinges to contribute to homeostatic equilibrium. These aspects of the CB physiology started to be developed almost immediately in the laboratory of Corneille Heymans, being the merit of the Belgian authors the discovery of the qualitative changes in the composition of the blood that the chemoreceptor cells detected (see the title of the paper) as well as the description of lungs as the main target of the chemoreflex (Heymans et al., 1930). To fully appreciate the significance of de Castro's work we copy as a foot note a fragment of the Award Ceremony Speech given by Prof. G. Liljestrand in 1938 when Corneille Heymans received the Nobel Prize in Physiology and Medicine[2].

PRESENT STATUS AND PERSPECTIVES IN CB MECHANISMS AND POTENTIAL PATHOPHYSIOLOGICAL SIGNIFICANCE

The systemic targets of the carotid chemoreflex had largely been described by the middle of 1980s (Fitzgerald and Lahiri, 1986).

[2]http://www.nobelprize.org/nobel_prizes/medicine/laureates/1938/press.html …The glomus …has been considered …a sort of endocrine gland similar to the medulla of the suprarenal glands. de Castro (1928) demonstrated that the anatomy of the glomus could no be compared to that of the suprarenal medulla. de Castro suggested that the glomus was an organ whose function was to react to variations in the composition of the blood ... an internal gustatory organ with special "chemo-receptors." Heymans et al. (1930) undertook to find out whether these supposed chemo-receptors were responsible for the respiratory reflexes produced by modifications in the composition of the blood.

Physiologically, the CB plays a homeostatic and adaptive role. Chemoreceptor cells are naturally stimulated by hypoxia and hypercapnia and the secretion of chemoreceptor cells, the release of their neurotransmitters, activate the sensory nerve endings of the CSN which via the glossopharyngeal nerve carry the activity to the brain stem; the information incoming from the CB is integrated at this level to reflexly generate proportional ventilatory and cardiocirculatory responses aiming to normalize blood gases and to minimize the deleterious effects of their alterations. Aside from the respiratory and cardiocirculatory systems the reflex initiated at the CB has many more targets, being capable of initiating, whether directly or indirectly, a large array of systemic responses that help to cope with the original unbalances that stimulated the CB (Fitzgerald and Shirahata, 1997). However, in recent years a new concept is emerging on assigning to the CB a great significance in pathological processes. It is thought that processes such as the origin and development of hypertension particularly when associated to the obstructive sleep apnea syndrome, insulin resistance and type II diabetes, heart failure and obesity share increased sympathetic activity as a pathophysiological mechanism; this sympathetic hypertony being originated in part by afferent signals emerging from the CB. In turn, these new concepts are creating the notion that the inhibition of the CB activity, the CB denervation or even the surgical removal of he CB might have a favorable impact on the morbidity and mortality of those processes (Paton et al., 2013; Ribeiro et al., 2013).

One of the most fundamental questions in CB physiology was, and to some extent continues to be, the mechanisms involved in the O_2 sensing in chemoreceptor cells. Literature up to the middle 1980s was full of imaginative hypotheses with few supportive experimental data (Fidone and Gonzalez, 1986). Our laboratory has been collecting experimental observations indicating that hypoxia must depolarize chemoreceptor cells, because it promoted the release of dopamine from the cells by a process that was Ca^{2+}-dependent and sensitive to antagonists of voltage operated Ca^{2+} channels. In a search for mechanisms capable of producing the depolarization required to activate the voltage-operated Ca^{2+} channels we found that rabbit carotid chemoreceptor cells expressed K^+ channels that were selectively inhibited by acute hypoxia (Lopez-Barneo et al., 1988). This finding originated the formulation of the so-called membrane hypothesis for hypoxic chemotransduction that we have formulated as shown in the **Figure 7**. Although the expression of O_2-sensitive K^+ channels was confirmed in all studied species by different laboratories, and therefore the membrane hypothesis gained wide support, there are many fundamental facts that remain unanswered. For example, how hypoxia couples to K^+ channels to alter their kinetic properties? Since there is a marked inter-species diversity O_2-sensitive channels it would appear that chemoreceptor cells expresses a unique O_2 sensor capable of interacting with the different channels. If the existence of a sensor is accepted, how does it interact with the channels? Are the beta-like regulatory subunits required? (see Pérez-García et al., 1999).

As Conde and Peers (2013) discuss, the discovery of those very basic mechanisms go beyond the pure academic interest of expanding knowledge. Thus, if as we have described in preceding paragraphs the hyperactivity of the CB is involved in the pathogenesis of several clinically relevant processes, the adequate manipulation of the basic machinery involved in chemoreceptor cell activation might represent the most rational approach for their treatment. The membrane model for chemoreception, as depicted in **Figure 7**, was formulated over twenty years ago (González et al., 1992), and consequently there have been significant additions to it, being worth noting the proposal of several mechanisms acting as primary oxygen-sensors and/or elements coupling the sensor to K^+ channels (Gonzalez et al., 2010). Second messengers capable of modulating the flow of information in the transduction cascade have also being described with the cAMP-EPAC system having a particular relevance as a positive regulator of the hypoxia triggered responses (Rocher et al., 2009). Finally, among others, Prabhakar's laboratory has generated a significant number of studies showing that hydrogen sulfide, which is produced in chemoreceptor cells, is capable of stimulating the CB by a Ca^{2+} dependent mechanism, being proposed that maxi-K^+ channels are the potential targets-effectors of this gasotransmitter (Prabhakar and Peers, 2014).

Finally, another aspect of the general physiology of the CB that has received great attention but it is not satisfactorily resolved, is, using de Castros's words, the product of secretion of chemoreceptor cells that stimulates the chemosensory nerve endings,

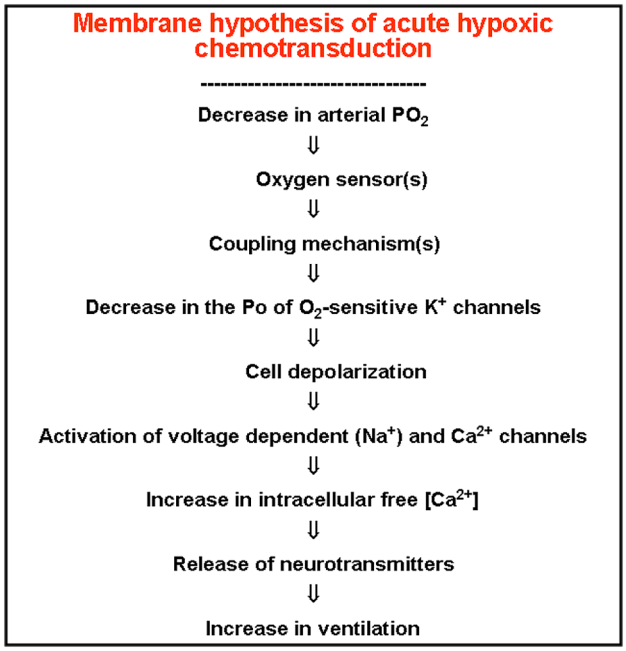

FIGURE 7 | The membrane model of acute hypoxic transduction proposes the cascade of events depicted in the figure. On lowering PO_2, an O_2^- sensor, hypothetically located in the plasma membrane, would experiment a conformational (and reversible) change, which transmitted to oxygen-regulated K^+ channels would cause modifications in their kinetic properties, resulting in a decrease in their opening probability. The ensuing depolarization activates voltage dependent Ca^{2+} channels and entry of Ca^{2+} in the cells triggering the exocytotic release of neurotransmitters, increase in CSN action potentials and in ventilation (redrawn from González et al., 1992).

i.e., the nature of the neurotransmitter(s) responsible for setting the activity in the CSN. Historically, acetylcholine was the initially proposed and most firm candidate to represent the key "conveyor" of information from chemoreceptor cells to the chemosensory terminals which at some early point in the nerve fiber would generate conducted action potentials driving the information to the brainstem. The cholinergic hypothesis was based on experimental observations obtained in the cat, and was rejected because while acetylcholine and nicotinic agonists were powerful chemostimulants and classical nicotinic antagonists blocked these responses, they were ineffective to eliminate the chemosensory activity elicited by natural stimuli. The progressive discovery of neurotransmitter systems in the central nervous system, including the neuropeptides, and the appearance of the concepts of co-storage and co-release of neurotransmitter substances invaded the CB field as chemoreceptor cells appeared to express every single neurotransmitter system. In the sensory synapse established between chemoreceptor cells and the CSN terminals, as is the case in every synapse studied, the existence of postsynaptic, presynaptic and extrasynaptic receptors for the putative neurotransmittes was recognized. This complex neurochemical arrangement, added to the intricate anatomical organization of the CB, has made it very difficult to interpret the classical pharmacological experiments and to demonstrate the criterion of identity of action of any candidate neurotransmitter. Difficulties have been amplified by the existence of genuine differences among species, both in the concentrations of one or another neurotransmitter (e.g., dopamine vs. norepinephrine) and dominance of one or another receptor subtype (nicotinic vs. muscarinic). Many laboratories have their particular bias in the study of one or another neurotransmitter system, and therefore articles giving a wide coverage to neurotransmission in the CB are not abundant. Among those articles, our 1994 comprehensive review (Gonzalez et al., 1994) has a broad section which compiles what could be considered classical findings on every neurotransmitter system. In our review we discuss at length the great controversy existing in the understanding of the function of dopamine in the CB. Yet there are three facts that we consider firmly established: (1) Although in most species dopamine is considered an inhibitory neurotransmitter-neuromodulator, in the rabbit, species in which we have carried out most of our experiments, dopamine appears to fulfill the criteria to be considered an excitatory neurotransmitter at the synapse between chemoreceptor cells and chemosensory nerve terminals (see the article by Iturriaga et al., 2009); (2) in every species studied there is a reasonable parallelism between the intensity of natural stimulation and the amount of dopamine released by chemoreceptor cells, making the measurement of the release of dopamine a very valuable index of the activity of chemoreceptor cells, both in intact organs or in isolated cells, and (3) the dopaminergic (catecholaminergic) trait, i.e., the immunohistochemical positiveness to tyrosine hydroxylase, is probably the most universally accepted criterion to identify chemoreceptor cells, both in organ sections or in tissue culture. In more recent times the interest for the study of neurotransmitters in the CB has not weakened. We want to mention the recent study by Conde et al. (2012) showing that in the rat ATP and adenosine are key neurotransmitters involved in hypoxic CB chemotransduction, with a more relevant contribution of adenosine during mild hypoxia, and a more prominent role for ATP in high-intensity hypoxia. However, there is no doubt that Colin Nurse's laboratory has been leading the study of neurotransmission in recent years (Nurse, 2010). This laboratory using rat as their experimental species developed the aforementioned simplified co-culture preparation in which chemoreceptor cells or cell clusters were plated together with neurons dissociated from the petrosal ganglion which in a lapse of time of less than one week formed functional synapses. Under these conditions it was possible to record in the petrosal neurons spontaneous postsynaptic-like potentials and burst of action potential when the preparation was stimulated by a hypoxic pulse. In this preparation it was possible to study both in the postsynaptic element (the petrosal neuron) and in the presynaptic element (the chemoreceptor cells) the effects of agonists and antagonists of putative neurotransmitters. The conclusions attained with this preparation are that ATP and acetylcholine released by chemoreceptor cells and via $P2X_{2/3}$ and nicotinic receptors would act as excitatory neurotransmitters in the postsynaptic element of the chemoreceptor cell-sensory terminal synapse. GABA would also be released by chemoreceptor cells and via $GABA_A$ receptors also present in the sensory nerve endings would be an inhibitory neurotransmitter. Serotonin and adenosine would act on autoreceptors in chemoreceptor cells as positive modulators while GABA, dopamine and histamine would be negative modulators of chemoreceptor cell activity. The undeniable merits of Nurse's experiments might have a potential handicap, namely that in the co-culture conditions some neurotransmitters and their receptors might change their expression. For example, while the findings of Nurse's laboratory unequivocally would indicate that acetylcholine is being release form chemoreceptor cells, Gauda's laboratory (Gauda et al., 2004) working with rat pups from 2 to 28 days of postnatal age showed that tyrosine hydroxylase positive chemoreceptor cells of freshly obtained CB tissue do not express the most reliable cholinergic markers, choline acetyltransferase and vesicular ACh transporter studied by semiquantitative in situ hybridization histochemistry and immunohistochemistry.

In conclusion, the working program outlined by de Castro on his discovery of arterial chemoreceptors still represents the path to follow: we must deepen our understanding of the molecular mechanisms used by chemoreceptor cells to "taste" hypoxia and hypercapnia, we must devise new preparations, probably based in imaging techniques, that allow faithful study of the communication between chemoreceptor cells and the sensory nerve endings, and finally, we must attempt to understand how the blood flow of the CB is regulated and how it affects the functioning of the arterial chemoreceptor. The fulfillment of this program would certainly allow the understanding of the CB malfunctions and the correction of CB-linked pathological processes.

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Some predictions of Rafael Lorente de Nó 80 years later

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Rafael Lorente de Nó, the youngest of Santiago Ramón y Cajal disciples, was one of the last Century's more influential researches in neuroscience. This assay highlights two fundamental contributions of Rafael Lorente de Nó to neurobiology: the intrinsic organization of the mammalian cerebral cortex and the basic physiology of the neuron processes.

Keywords: Lorente de Nó, cerebral cortex, summation coding, Ammon's horn, hippocampus

BIOGRAPHICAL NOTE

Rafael Lorente de Nó stands out as one the most notable progeny that Santiago Ramón y Cajal bequeathed Neuroscience. A gifted, precocious intellectual, Lorente de Nó was born in Zaragoza, Spain, in the Kingdom of Aragón, on the 8th of April, 1902, and died in Tucson Arizona, on the 2nd of April, 1990. Initially guided by Pedro Ramón y Cajal, a Gynecologist and Professor of Histology at Zaragoza, Spain, Lorente de Nó learned from him the routine histological techniques and, notably, silver impregnations introduced earlier by Camilo Golgi himself. After this early exposure, Lorente de Nó traveled in 1920 to Madrid, where the bold 18-year-old is introduced to Santiago Ramón y Cajal. Following a memorable interview (Larriva-Sahd, 2002), Don Santiago, was perceptive enough to accept "paisano Lorente" in his laboratory as his youngest disciple. Soon afterwards Lorente de Nó embarks in his first studies on spinal cord regeneration (Lorente de Nó, 1921) and cerebral cortex (Lorente de Nó, 1922), obtaining the degree of Medical Doctor at the enviable age of 21. Among the most important studies Lorente de Nó carried out under the direct supervision of the Master are those relating to spinal cord regeneration, brain-stem nuclei and, especially, to the mouse cerebral isocortex, which resulted into 15 published works (see Larriva-Sahd, 2002). His experiments on the VIIIth cranial nerve earned him the attention of Nobel Laureate Robert Bárány, who was at that time in Spain visiting Cajal's lab (De Carlos and Pedraza, 2014). His experimental work on the vestibular system preceded his contribution to elucidating the structure of brainstem nuclei underlying cranial nerve reflexes. In 1924 Lorente de Nó traveled to Uppsala, and later to Berlin, where he worked with Robert Bárány, and with Oskar and Cecile Vogt, respectively. His most important studies on the brainstem nuclei and reflexes resulted from his interaction with Professor Bárány and were published in five different languages (i.e., French, Swedish, Russian, German, and English). In 1931 Lorente de Nó sailed to America and become head of the Neuroanatomical Laboratory at the Central Institute for the Deaf in St. Louis Missouri. In 1935 he was appointed lecturer at the Washington University, and the following year he moved to the Rockefeller Institute until his formal retirement in 1970. Lorente de Nó's most notable contributions to understand the physiology of nerve fibers and the microscopic organization of the cerebral cortex were performed during this period, although he later acknowledged that a substantial part of both ideas and results (**Figures 1–3**) of this work were conceived when he was still living in the Old Word (Larriva-Sahd, 2002). In the early 70's Lorente de Nó was invited by Dr. Victor Goodhill to join the University of California in Los Angeles, as a visiting Professor. From this late period resulted a long series of papers on the physiology of nerves and his detailed account on the VIII cranial nerve (Lorente de Nó, 1981). In 1981, he moved to Tucson Arizona where he died in 1990.

Lorente de Nó was member of the American Physiological Society and the American Association of Anatomists, the National Academy of Sciences, and the American Academy of Arts and Science. In addition to honorary degrees by the University of Uppsala, Clark University, and Rockefeller University, Lorente de Nó received the Karl Spencer Lushly Award, and the Award of Merit in 1986, and held an Honorary Membership at the UCLA-Brain Research Institute in 1972 (Woolsey, 2001).

It is debatable which of Lorente de Nó numerous contributions are the most relevant, even within the confines of a single publication; perhaps his most celebrated papers pertain to two fundamental areas of current neuroscience: his contributions to the modular organization of the mammalian cerebral cortex and to the basic physiology of the neurons. Since both topics

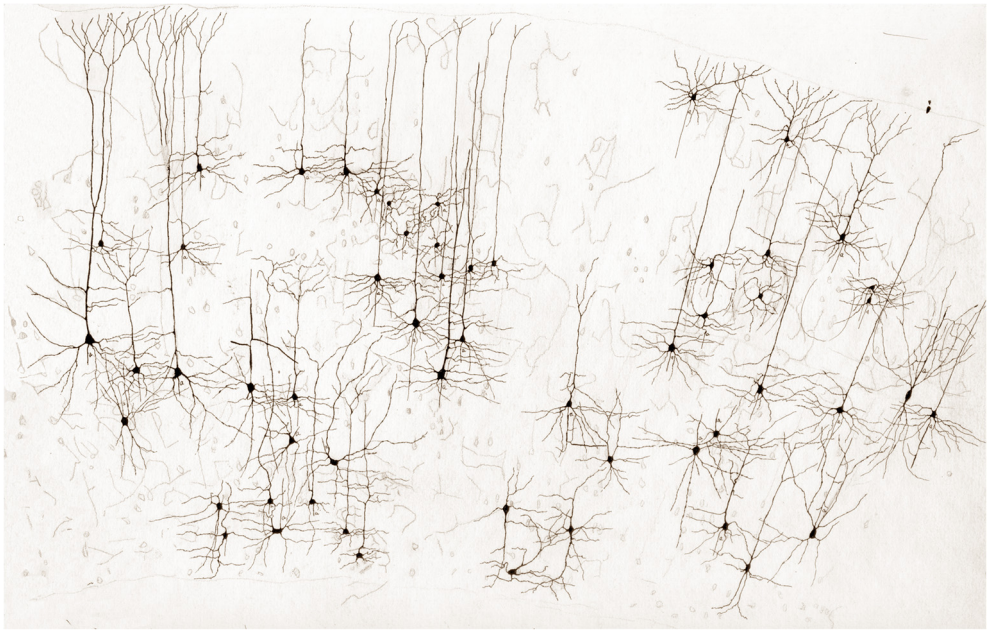

FIGURE 1 | Survey, unpublished drawing from the rat cerebral cortex by Lorente de Nó. Golgi-Cox technique.

were primarily presented by Lorente de Nó in his papers of the organization of the cerebral cortex, they are used here to provide a conceptual frame. Before his demise, Lorente de Nó left to this author a number of his original drawings; some dealing with different aspects of neocortical organization are presented here (**Figures 1–3**).

COLUMNAR ORGANIZATION OF THE MAMMALIAN CEREBRAL CORTEX (FIGURES 1–4)

Every researcher dealing with functional or developmental aspects of the mammalian cerebral cortex has a somewhat different idea on what a cortical column might be. In fact, rather than being a well-defined concept, the term cortical "column" is often a context-defined notion (Valverde, 1986; Mountcastle, 2003; Horton and Adams, 2005; Rockland, 2010; Merchant et al., 2012). While this issue is beyond the scope of the present brief assay, the term cortical "module," or "elementary unit" was coined by Lorente de Nó to refer to a vertical neural quantum having distinct extrinsic afferences and interneurons converging onto single or a group of pyramidal cells (vide infra); the axon of the latter is both the output and recurring element of the unit. Clearly, at the time Lorente de Nó defined the existence of cortical modules (**Figure 4A** and insert), every element structuring them was previously identified (see Valverde, 1986). Thus, incoming fibers to the cerebral cortex were elegantly featured by Ramón y Cajal (1891) [also termed Ramón fibers by Kölliker (Ramón'sche Fasern)] (see Lorente de Nó, 1922) (**Figure 3C**); likewise, the existence of interneurons (**Figures 2A,C**, **3B**) was defined earlier by C. Golgi and fully corroborated afterwards. The same applied to cortical pyramidal cells (see Jones, 1984) (**Figures 1**, **2B**, **3B**). Both cortical distribution and interaction between short-axon neurons and pyramidal cells were also noted by Ramón y Cajal himself. Furthermore, the notion of the central nervous system composed of linear series of neurons as understood by Sherrington and Ramón y Cajal prevailed until the 1930's (see Ramón y Cajal, 1904). While Lorente de Nó added an unprecedented number of novel cell-types and interactions among them, the basic ingredients that he utilized to develop his tenets on cortical organization were previously known. In a very straight forward way, Lorente de Nó conceived that geniality "is to utilize facts that have been previously neglected or assumed to be irrelevant for a given phenomenon" (Larriva-Sahd, 2002), and he did. Thus, he gathered (**Figure 4A**) previously characterized elements to advance two cardinal points, these are, their synaptic interactions–as reveled by the Golgi technique (see Wang et al., 2004)—and their potential function. Although it has been assumed (see Mountcastle, 2003) that the concept of cortical "module" was presented first in the memorable chapter released in 1949 (Lorente de Nó), this happened quite a while earlier. Time matters, especially because as earlier as 1933 he *anticipated* the existence of physiologically distinct elemental units nearly 20 years before that intracellular recordings came to our technological armamentarium. In fact, the essence of the concept created by Lorente de Nó imparts to structural elements a physiological implication: it is, therefore, binomial. Structurally, each vertical "elemental unit," or "cylinder" is composed of afferent fibers, short-axon neurons and pyramidal cell(s) endeavor to produce a common physiological response. Indeed, what he wrote casts no doubt: "but no matter what kind of cylinders we choose, -all are equally well justified and everyone will have a real physiological existence at determined moments of cortical function" (Lorente de Nó, 1933). This furnished a tangible working hypothesis that, with the advent of electrophysiological studies, was successfully tested: recording electrodes coursing tangentially in the primary

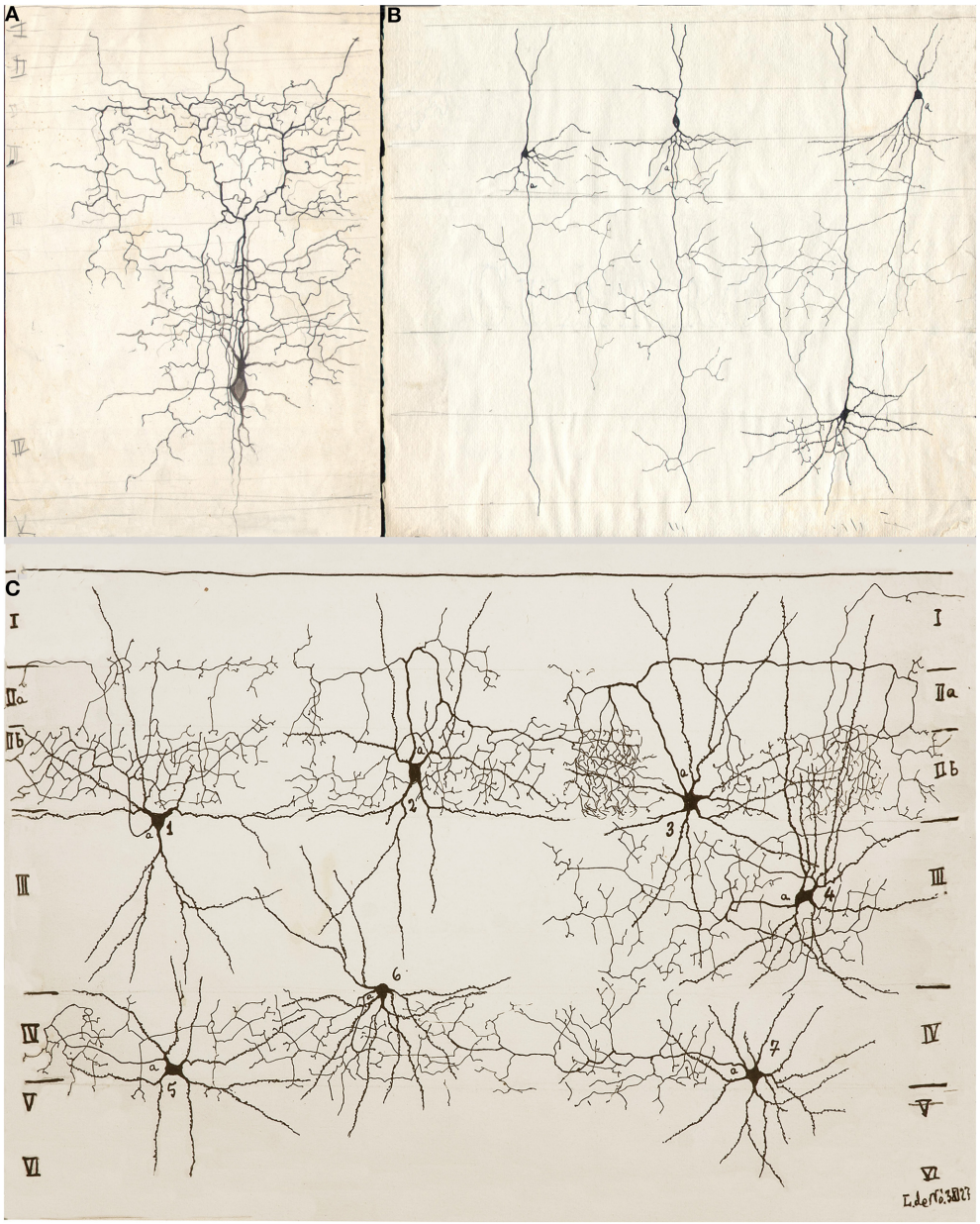

FIGURE 2 | Drawings from Lorente de Nó performed between 1922 and 1927 in the Ramón y Cajal laboratory. (A) Short-axon neuron with an ascending axon that distributes primarily in layer III (III). **(B)** Three pyramidal cells with numerous axon collaterals to layer IV and an interneuron (upper right), whose descending axon (a) resolves in layer III. **(C)** Seven examples of shot-axon neurons (see Lorente de Nó, 1949). To note is that while the axon of each cell ramifies profusely, it remains confined to the homonymous layer. Newborn mice, rapid Golgi technique.

somatosensory (Mountcastle and Powell, 1959) and visual (Hubel and Wiesel, 1959) cortices proved that, the cerebral cortex is, in fact, composed of distinct vertical functional modules.

As previously stated, at the time Lorente de Nó performed his studies on the brainstem and cerebral cortex, the prevailing notion was that the central nervous system was composed of linear series of neurons. Fortunately, Lorente de Nó had to perform physiological studies in experimental models with small mammals paralleling his cytological studies, which led him to challenge this early concept. In this context, scrutiny of his work on the entorhinal cortex (Lorente de Nó, 1933) adds another fundamental observation, that is, the interaction between superficial and deep pyramidal cell layers (**Figures 2A**, **4B**). While it had been recognized earlier that pyramidal cells in layer III (LIII) and deep layer V (LV) send projecting axons beyond the cortical confines, Lorente de Nó demonstrated that axon collaterals from pyramidal cells in either layer and those from pyramidal cells with ascending axons (**Figure 4B**, cells 6 and 7) converge on LIII interneurons and LIV pyramids themselves. This frequent axonal association and that from the short-axon neurons

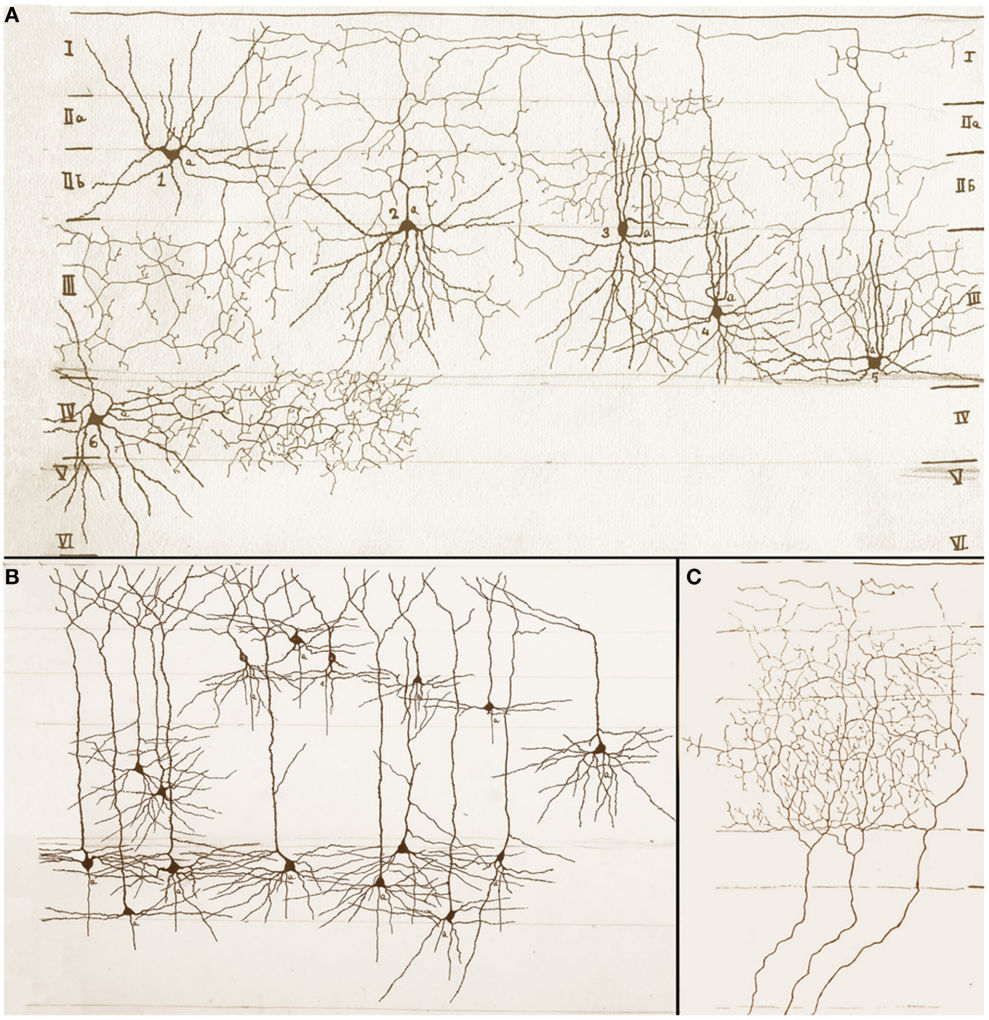

FIGURE 3 | Assorted drawings of the mouse cerebral cortex by Rafael Lorente de Nó. (A) Examples of short-axon neurons that include ascending (cells 2, 4 and 5), descending (cell 1), and horizontal (cells 3 and 6) axons. Roman numbers at either side of the drawing designate cortical layers, which are bounded by soft pencil. **(B)** Examples of superficial and deep pyramidal cells; axons have been omitted. **(C)** Talamo-cortical fibers distributing throughout layers I to III (bounded with soft pencil).

in LIII back to the adjacent pyramids (**Figure 4B**), led Lorente de Nó to suggest: "The activity of the cortex does not end with the first discharge of the pyramids, the afferent volley starts the autogenous activity of the cortex, that may be brought of [*sic*] in the following way (**Figure 4B**). The pyramids with recurrent axis cylinder (**Figure 4B** cells 6 and 7) and the deep pyramids (**Figure 4B**, cells 4 and 5) on the on hand, and the superficial pyramids (**Figure 4B**, cells 1, 2, and 3) on the other, constitute a closed chain of neurons, so that impulses may travel around, provided the refractory period of each cell is somewhat shorter than the time necessary for impulses to travel through the chain. For the chain superficial pyramids and pyramids with recurrent axis cylinder (alone), this condition can hardly be accomplished, but if one or several neurons with short axis cylinder (**Figure 4B**, cell 8) are intercalated the time relations within the chain will allow such autogenous activity." As one might expect from Lorente de Nó postulation, voltage-sensitive optical visualization in slices from the cerebral cortex, two clusters of cellular activation would be detected following electrical stimulation of thalamic afferences. In fact, such stimuli (Laaris et al., 2000) revealed two foci that appear to correspond to supra- and infra-granular pyramids. Further, administration of the NMDA receptor antagonist to the medium attenuate significantly that response.

Regarding the concept of the central nervous system organized as longitudinal successions of neurons, Lorente de Nó (1933a) wrote what could be a closing statement: "The conception of the reflex arc as a unidirectional chain of neurons has neither anatomic nor functional basis. Histologic studies with Golgi's method show the universality of the existence of plural parallel connections and of recurrent, reciprocal connections. Study of vestibulo-ocular reflexes by isotonic recording of the eye muscles in the rabbit after various experimental lesions of the reflex centers leads to a physiologic interpretation in terms of closed "self-reexciting" chains of neurons. Nystagmus is an alternating

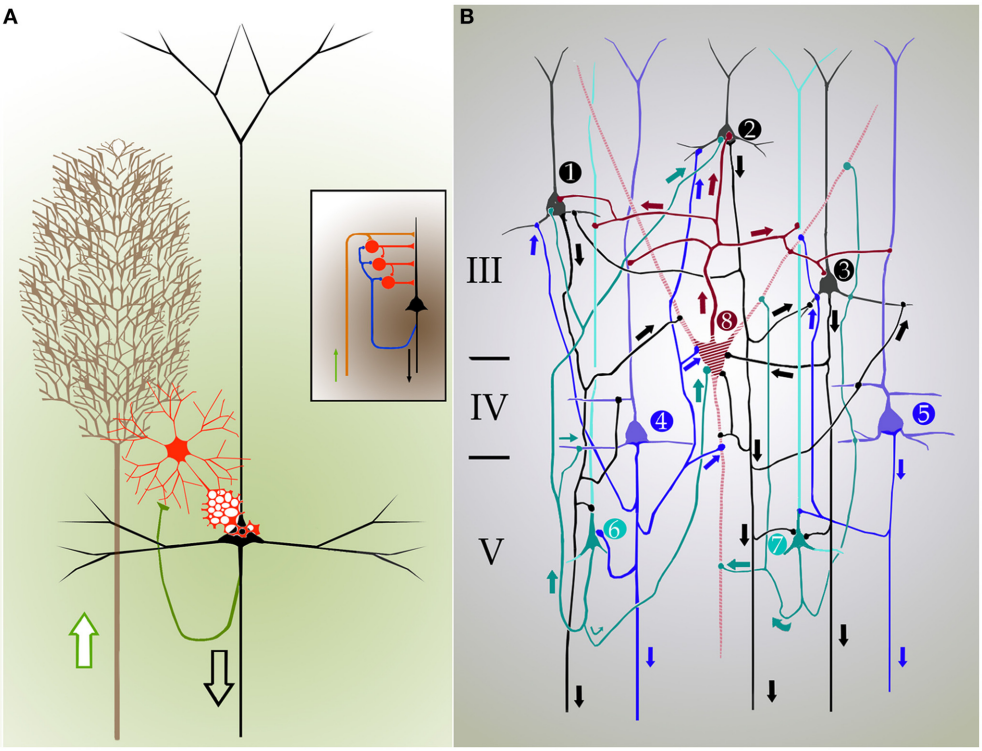

FIGURE 4 | Cartoons depicting the inner organization and synaptic interactions in the mammalian isocortex. Arrows designate directionality of nerve impulses. **(A)** Cylindrical unit composed of afferent fibers (brown), short-axon (red), and pyramidal cells. Axon impulses arrive via talamo-cortical fibers (brown), and target short-axon neurons (red), and pyramids (black) that send efferent axons. A set of collaterals from the pyramidal cell axon (green) ascend back to the interneuronal pool and adjacent homologs. Inset. Synaptic interactions between individual components of the cortical elemental unit that represent the structural basis for cortical reflexes. **(B)** Diagram showing a closed, self-exciting chain in the entorhinalis cortex. 1, 2, 3–pyramids (black) of layer III; 4, 5–deep pyramids (blue); 6, 7–pyramids with recurrent axis cylinder of layer V (V). 8–cell with short axis cylinder of layer III (III). The discharge of pyramidal cells 1, 2, 3 and that of the small pyramids with recurrent axis cylinder (6,7) have raised in cell 8 a certain amount of central excitatory state (c.e.s.), and when the deep pyramids (4,5) discharge cell 8 reaches threshold c.e.s. The impulses of cell 8 excites again cells 1, 2, 3, that had already being facilitated by recurrent collaterals, and the cyclic process start again. The discharge of such closed chains will constitute the autogenous activity of the cortex; the frequency of impulses is dependent on the number of active links within the chain. **(A,B)** modified from Lorente de Nó (1933 and 1949).

reflex in which the peripheral labyrinthine stimulation sets into activity a machinery which gives rise to nystagmus in the same way that the spinal cord sets up the stretch reflex as a response to a skin stimulus. In the vestibular system there is no "localization" of reflexes in anatomic nuclei; the whole system is a functional unit; reflex reactions may be produced as long as the reflex arc is closed through one of the multiple pathways; on the other hand, lesions in any part of the vestibular system modify all the reflexes"(Lorente de Nó, 1933a). In précis, Lorente de Nó's identification of clusters of neurons linked by the sort of interaction just described, together with the inner circuitry of the tuberculum acousticum (Lorente de Nó, 1981) represent biological substrata to what latter on was known as a "feed-back loop."

NEURON AS A TIME-SPACE DECODING DEVICE

In addition to the enduring contribution made by Lorente de Nó to the knowledge of inner structure and circuitry of the hippocampal formation (see Andersen et al., 2007), both the scholarly revision of previous work and the presentation of results he afforded, render this work an indispensable ingredient to both the novice and to the settled researcher. Possibly the best known contributions contained in this work are the plan of parceling of the Ammon's horn (AH) resulting in its current nomenclature (**Figure 5**) and the distribution of distinct afferences to the pyramidal cell (**Figure 6**). From Lorente de Nó's writing prior to this latter study it is clear that he was concerned with the proportions, location, and interactions of neurons. While Ramón y Cajal and Camilo Golgi performed their drawings from information kept in mind after observing the specimen, Lorente de Nó developed a variant of the Germanic approach. Researchers, particularly Oskar Vogt and his disciples, who were concerned with the proportions and dimensions of every layer or nuclei, utilized either camera lucida drawings or even photographic reproductions from large-format negatives. Lorente de Nó designed a hybrid system consisting of a projecting prism placed directly at the eye piece of the microscope. This straightforward procedure allow him to focus the microscopic image directly onto the working-table; then, the image was accurately copied (**Figures 1–3**); "every drawing should be a

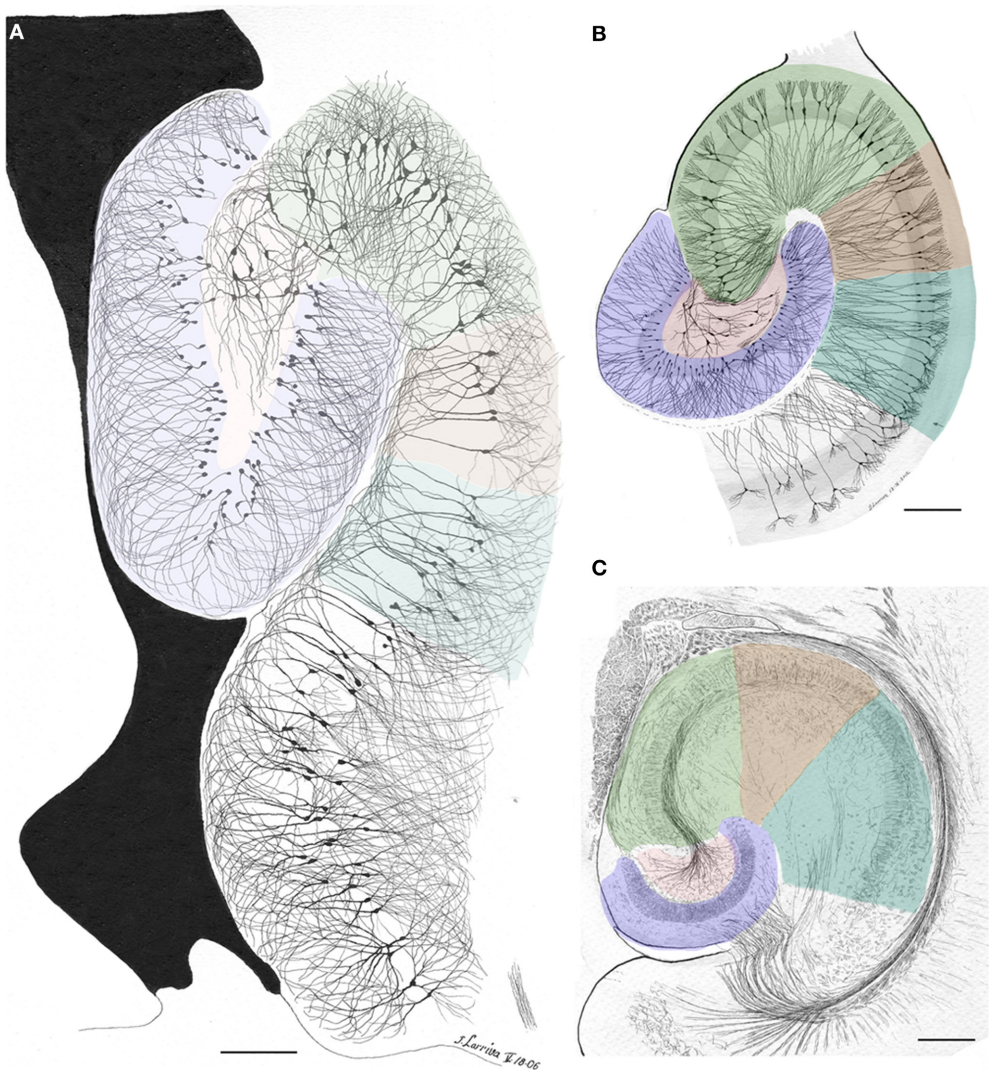

FIGURE 5 | Survey camera lucida drawings by the present author depicting the Ammon's horn sectors in accord with Lorente de Nó parceling (colored). (A) Sagittal section through the dorsal hippocampus. Golgi-Cox technique. **(B)** Horizontal section through the ventral hippocampus of an adult rat. The actual width of the pyramidal cell layer as seen in Nissl-staining is shaded in gray. Golgi-Cox-Nissl technique. **(C)** Section obtained at a site comparable to that shown in "B" after the reduced-silver technique. Calibration bars = 200 μm.

replica of a neuron," he said (Larriva-Sahd, 2002). Following this method Lorente de Nó was able both to obtain a huge number of neuron samples and to detect subtle differences among pyramidal cells as a function of position from the entorhinal cortex to the dentate gyrus (**Figures 5A,B**). Moreover, he noted that, in addition to the well-known contribution of basket-cells to perisomatic axonal fibrils (Ramón y Cajal, 1904), afferences from each extrinsic [i.e., incoming tract(s)] (**Figure 5C**), and intrinsic (i.e., short-axon neurons) source distribute and terminate in different domains of the pyramidal cell (**Figure 6**). This cornerstone observation followed by his own interpretation shaped one of the fundamental concepts of current neurobiology. "…it has been possible to establish that the synapses of different kinds are not mixed but rather grouped in special regions of the cell; some on the body, some on the origin of the dendrites, some on the end, etc., although, of course the fields of distribution of the synapses of different kinds often overlap"(**Figure 6**). "But even without knowing what the multiplicity of connections really signify, one can conclude from the simple fact of its existence that the dendrites may not conduct in the same way as the axon, or in other words, that the central synapse cannot be compared with the neuro-muscular junction." "The only possibility for cell Py1 (**Figure 6**) "using" all the impulses seems to be, first, that each synapse sets only a subliminal (chemical or other) change able to summation and second, that the conduction through the synapses is not follow by a refractory period. The subliminal changes are summated first in the dendrites and then in the surrounding of the axon takes place. When the change reaches the threshold value, an explosive discharge through the axon takes place axon. The axon–as well as any other nerve fiber–enters in

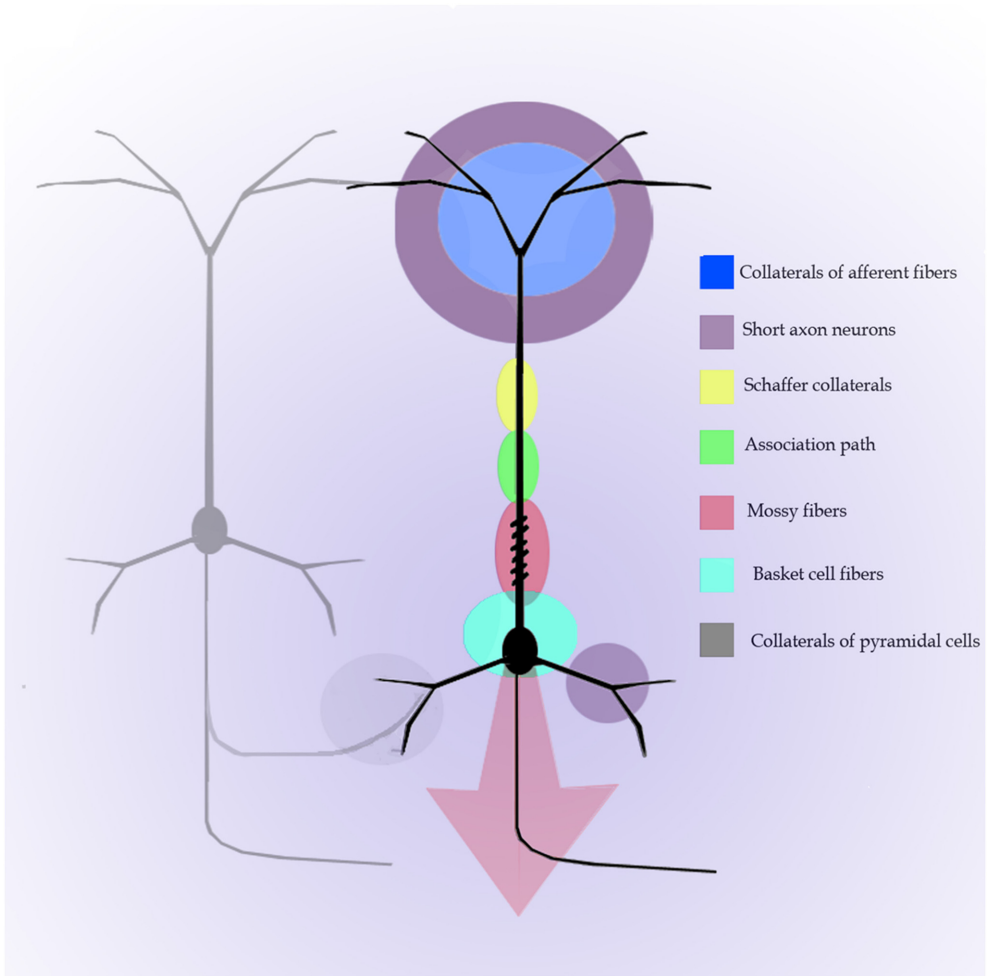

FIGURE 6 | Diagram of the distribution of afferences into distinct parts of the pyramidal cell of the Ammon's horn. Modified and colored from Figure 34 in Lorente de Nó (1934).

a refractory state, but the cell body and dendrites do not do so, they continue receiving and adding subliminal changes until the threshold value is reached again and the axon has recovered." In dimensioning implications of Lorente de Nó observations and tenets in this regard Swanson (1993) wrote: "This prediction is remarkably close to our modern view of central neuron physiology which awaited the impetus of the microelectrode (Brock et al., 1952). Nevertheless, as early as 1934, Lorente de Nó stated that the grater question in neurophysiology was how dendritic changes are summated, even though informed opinion at the time was skeptical of subthreshold summation in central neurons." The recent implications of the distribution of afferents in the AH pyramidal cell are clearly presented by current authoritative authors (Somogyi and Klausberger, 2005), who wrote about the hippocampus: "These results suggest roles for specific interneuron types in structuring the activity of pyramidal cells via their respective target domains, and accurately timing and synchronizing pyramidal cell discharge, rather than providing generalized inhibition. Finally, interneurons belonging to different classes may fire preferentially at distinct time points during a given oscillation. As different interneurons innervate different domains of the pyramidal cells, the different domains will receive GABAergic input differentiated in time. Such a dynamic, spatio-temporal GABAergic control, which evolves distinct patterns during different brain states, is ideally suited to regulating the input integration of individual pyramidal cells contributing to the formation of cell assemblies and representations in the hippocampus and, probably, throughout the brain."

CONCLUDING REMARK

It is obvious that Lorente de No's contributions, his observations, interpretations, and models of neurobiological events are widely utilized in contemporary neurobiology, often without citing his original works. To underscore their relevance, one could imagine what our understanding of neurobiology be without them. Granting that it is not reasonable to say that his explanations would not have been attained by anyone else, it would be difficult to imagine a coherent story about a neuron without sub-threshold excitability, a decoding nerve cell without

temporo-spatial summation, or a cortical column without a neuronal substratum.

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The influence of James and Darwin on Cajal and his research into the neuron theory and evolution of the nervous system

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In this article we discuss the influence of William James and Charles Darwin on the thoughts of Santiago Ramón y Cajal concerning the structure, plasticity, and evolution of the nervous system at the cellular level. Here we develop Cajal's notion that neuronal theory is a necessary condition to explain the plasticity of neural connections. Although the roots of the term "plasticity" in reference to neuroscience are not completely clear, Cajal was an important figure in the propagation and popularization of its use. It is true that he carried out a large *number* of studies throughout his career in favor of the neuronal theory, but perhaps one of the most interesting aspects of his studies was his innovative capacity to interpret structure as being the result of evolutionary mechanisms, i.e., natural selection. This capacity would ultimately lead Cajal to the conclusion that, in relation to the histology of the nervous system, such selection occurs in the establishment of connections between cells. The present article is divided into five sections: (1) Learning and general notions of organic plasticity in the 19th century; (2) The idea of "mental" plasticity proposed by James; (3) Neuronal theory and "structural" plasticity: general considerations; (4) Evolutionary factors of the nervous system in Cajal's work; and (5) Final considerations.

Keywords: neural plasticity, evolution of the nervous system, reticular theory, neuron theory, history of neuroscience

INTRODUCTION

The origins of modern neuroscience was highly influenced by the revolutionary biological thinking that arose in the second half of the 19th century with the theory of Charles Darwin (1809–1882) concerning the evolution of the species through natural selection. An important example is the strong influence of the theory of evolution on the embryologists (Coleman, 1983). In this early period, explanations were based on the phylogenetic evolution of organisms. The relationships between the development of the organism (ontogenesis) and of the species (phylogenesis) resulted in the theory of recapitulation, which proposed that in developing from embryo to adult, animals go through stages resembling or representing successive stages in the evolution of their remote ancestors. This idea influenced naturalistic thinking (Gould, 2010):

> "*Naturalists had at the time an infinite belief in the actual existence of a straight parallel and even may be an identity between the developments of a full-grown chick from a chicken egg, for instance, and the entire species of chicks from a more primitive bird.*"
>
> (Coleman, 1983, p. 85)

The overall way of thinking of academic circles in Europe in the second half of the 19th century was influenced by the theories of evolution (Baumer, 1977), with the reach of this influence extending beyond the boundaries of biology and similar sciences.

As a result, given the general importance placed on evolution by the academic community at the time, it follows that the study of the nervous system would be approached from an evolutionary standpoint. This paper aims to examine, at the cellular level, the notion of evolution of the nervous system presented by Santiago Ramón y Cajal (1852–1934). The notion that neuronal theory is a necessary condition to explain the plasticity of neural connections will also be explored. It is important to highlight that the studies in the field of neuroscience converged increasingly in the twentieth century to the point that the notion of the plastic nervous system was generally accepted as a critical mechanism for learning and memory (Barlow, 1972, 1995; Azmitia, 2002, 2007; Fields and Stevens-Graham, 2002; DeFelipe, 2006).

The sheer number of studies in favor of the neuronal theory that Cajal carried out over the course of his career was impressive. However, perhaps one of the most interesting aspects of his studies was his innovative capacity to interpret the structure as being the result of evolutionary mechanisms, i.e., natural selection and his proposal that, ultimately, in relation to the histology of the nervous system, such selection might occur in the establishment of connections between cells.

The present article has been divided into five sections: (1) Learning and general notions of organic plasticity in the 19th century; (2) The idea of "mental" plasticity proposed by William James (1842–1910); (3) Neuronal theory and "structural" plasticity: general considerations; (4) Evolutionary factors of the nervous system in Cajal's work; (5) Final considerations.

LEARNING AND GENERAL NOTIONS OF ORGANIC PLASTICITY IN THE 19TH CENTURY

In the world of neuroscience the notion of learning encompasses a wide range of events involving both biological factors and those related to the interaction between the organism and its environment at both, a biological dimension and the role of interaction between the organism and its environment. Learning is closely associated with memory mechanisms and implies plastic changes in the brain at different levels (genes, molecules, synapses, etc.), which lead to structural and functional reorganization of neural networks.

Here, we will not attempt to perform an exhaustive review of the concept of learning, but rather our intention is to briefly outline this area in order to proceed with a historical and conceptual analysis of the notion of plasticity in biological terms, since learning and plasticity are closely related.

The concept of plasticity as an indicator of changes in the organism, at both the anatomic and behavioral level, was extensively discussed within the area of psychology in the late 19th century and early 20th centuries. Analysis of the relationships between the biological and the psychological traits of the organism was present in scientific debates around this time period (Galton, 1880; Ebbinghaus, 1885; Calkins, 1896; Thorndike, 1898; Small, 1901; Yerkes, 1901; Pavlov, 1904; Köhler, 1917).

It should be noted that the experimental approach for the study of the psychology of learning attracted considerable interest from psychologists at the time. It was then that experiments with labyrinths were first used to investigate the cognitive and emotional skills of animals (Small, 1901; Yerkes, 1901). The theory guiding most of these studies assumed that our mind operates by association (the association theory) of elements subject to experimental treatment due to the relation between stimulus and response.

In a landmark study, the sociologist and politician Leonard T. Hobhouse (1864–1929), opposed the notion of learning as a mere process of fixing connections and associations between stimulus and response (Hobhouse, 1901). Such new notion became widespread thanks to a study by Wolfgang Köhler (1887–1967) using chimpanzees (Köhler, 1917). Köhler concluded that chimpanzees can learn by associating stimuli and not only through the stimulus-response relation.

It was James (1879), first drew attention to the idea that there were anatomical changes associated with plasticity although objective results were not provided to corroborate this idea. In the next section, we will address James' notion of plasticity and then discuss how Cajal presents this notion in relation to the connections between the cells of the nervous system, which in our opinion is a broader notion with a better set of experimental results compared to other contemporary notions.

THE IDEA OF PLASTICITY PROPOSED BY WILLIAM JAMES

William James is considered a pioneer in transforming psychology into an independent science (Kinouchi, 2009). It took 12 years to write the well-known reference book for beginners in psychology: *The Principles of Psychology* (James, 1890). For the purposes of our analysis, we have examined two chapters in detail, namely chapter IV – *Habit* – and chapter V – *The automaton theory*. Both chapters were published before the book. Chapter IV was originally published in February 1887 in *Popular Science Monthly* under the title *The laws of habit* and chapter V was originally published in 1879 in *Mind* # 4 (pp. 1–22). However, in the book *The Principles of Psychology* (James, 1890), these chapters were revised and updated based on the debate established. Thus, we will refer to the book instead of the original chapters.

In the first chapter of the book *The Principles of Psychology*, James (1890) establishes as the central theme what he considers to be the subject matter of psychology, as per the quote below.

> *"(. . .) the fact that the brain is the one immediate bodily condition of the mental operations is indeed so universally admitted nowadays that I need spend no more time in illustrating it, but will simply postulate it and pass on. The whole remainder of the book will be more or less of a proof that the postulate was correct."*
>
> (James, 1945, p. 16)

According to James, there was a hierarchical division of the nervous system in such a way that the lower centers respond to sensory stimulus, while the cerebral hemispheres are responsible for perception and conscious actions. Perceptions involve the grouping of sensations while conscious considerations are expectations of sensations to be felt based on previous experience of the sensations felt. In this explanatory model, memory has a central role. James indicates that our memory is located in the cerebral hemispheres. He also affirms that cerebral functions represent the crucial differences in terms of the variety of responses seen between an animal that has no cerebral hemispheres and another animal with cerebral hemispheres. While the latter would respond to absent objects the former would only respond to present stimuli.

In the section where he writes about training the cerebral hemispheres, James justifies his idea that reflexes may be influenced by both physical and psychic factors:

> *"I hope that the reader will take no umbrage at my so mixing the physical and mental, and talking of reflex acts and hemispheres and reminiscences in the same breath, as if they were homogeneous quantities and factors of one causal chain. I have done so deliberately; for although I admit that from the radically physical point of view it is easy to conceive of the chain of events amongst the cells and fibers as complete in itself, and that whilst so conceiving it one need make no mention of ideas, I yet suspect that point of view of being an unreal abstraction. Reflexes in centers may take place even where accompanying feelings or ideas guide them."*
>
> (James, 1945, p. 33)

The question underlying such an argument is how hemispheric processes that correspond to what James called recollection of the "spirit" (mental processes) can be organized. In answering this question, it is important to consider, firstly, the process that occurs in the brain when it is stimulated. For instance, the visual perception of an object will be reproduced giving an idea of the same object when internally stimulated by other brain processes. Another clue is that when processes are stimulated jointly or immediately after one another, a stimulus arising from any of the given processes tends to stimulate the other processes in a sequential order. James named this second hypothesis the law of association. The third hypothesis states that any stimulus spreading to the lower centers tends to also spread to the higher centers (cerebral cortex) and produce a general idea. The result of these three

hypotheses is that all ideas tend to ultimately produce or restrain a motor response that would otherwise be produced.

We notice that James refers to a problem that was the focus of academic debate in the second half of the 19th century, namely expanding physiological explanations to account for thoughts and ideas. James' line of thinking does not align him with either side of the debate. He found in Darwinian Theory a way to transcend these two sides between physiological determinism and social determinism (Kinouchi, 2006).

A crucial moment for this discussion took place in the last three decades of the 19th century. The 1870s were the beginning of a period that basically rejected the phrenological view that discrete cortical areas represented individual functions. An alternative to this idea was that although the cerebral hemispheres were associated with certain functions, they were not acting in isolation, but rather in conjunction with the entire organism. Thus, this idea of continuity or cooperation between the parts was not incompatible with the existence of reflex, such as motor reflexes associated with the spinal cord. This scenario led researchers to question whether it was the cerebral cortex alone that creates states of consciousness. With this idea of cooperation in mind, perhaps it would not be unreasonable to suggest that there are also levels of consciousness in the lower centers? This and other questions arose in studies carried out during this period

Another important concept is the notion of habit. James distinguished inherent or instinctive habits from variable or acquired habits – with this second category of human beings' habit being acquired by education or learning.

James begins his considerations on the changes that occur in organisms by analyzing the physical world. The particles in the physical world do not change due to their nature. However, mass that is made up of matter can have changes. Such changes occur in a compound structure. James wrote:

> *"(. . .) Plasticity, then, in the wide sense of the word, means the possession of a structure weak enough to yield to an influence, but strong enough not to yield all at once. Each relatively stable phase of equilibrium in such a structure is marked by what we may call a new set of habits. Organic matter, especially nervous tissue, seems endowed with a very extraordinary degree of plasticity of this sort; so that we may without hesitation lay down as our first proposition the following, that the phenomena of habit in living beings are due to the plasticity of the organic materials of which their bodies are composed."*
>
> (James, 1945, p. 106)

Therefore, the starting point for the notion of plasticity explored by James was a certain property of the physical world, with organic matter being the ultimate product. James was optimistic about the likelihood of future explanations accounting for changes to the most intimate part of organic matter.

The early ideas on the process of forming a habit can be better understood in two essays from the 1870s, one by Léon Dumont (1837–1877) and the other by Albert Lemoine (1824–1874; Dumont, 1876; Lemoine, 1876). James appears to have been influenced by Dumont's essay on the physical characteristics of habit formation.

James also interpreted the adaptive properties of consciousness as being a plastic mechanism. In the chapter on The Automaton-Theory, James presents a widespread theory in the second half of the 19th century, in which the notion of the "reflex" in the nervous system (a behavior that is mediated via the reflex arc) was extrapolated to conscious acts to explain conscious behavior. Based on this concept, attributing a function to consciousness in mechanical terms is not necessary, since this theory explains the causal relations between stimulus and motor response without the necessity for an external agent whose nature differs from that of the organic elements involved.

According to Kinouchi (2006), James does not fully agree with this physiological view alone. The idea of consciousness in terms of evolution has a unique role in James' line of thinking. Indeed, evolution would to an extent explain the role of consciousness. What would be the possible deficiencies of the nervous system in animals whose consciousness appear to be more developed? In James' view, the key characteristic that indicates such possible deficiencies is instability.

This brief explanation of the idea of plasticity from James' perspective leads to some key points. Firstly, plasticity occurs in the "matter" of the nervous system. Nevertheless, James does not provide experimental results to corroborate this idea. Another key point refers to the adaptive role that consciousness has by providing organisms with the capacity to change in response to the environment. In the next section, we will analyze how Cajal introduced the concept of plasticity at the level of connections between the cells of the nervous system.

NEURONAL THEORY: GENERAL CONSIDERATIONS

In the 19th century there was a widespread idea that nerve cells linked to each other through anastomosis (fusion), similar to pieces of a tubing system.

At that time, several authors supported the reticular theory proposing different types of networks, including Albrecht Von Kölliker (1817–1905), a major scholar in anatomy and embryology (Kölliker, 1868). However, it was Joseph von Gerlach (1820–1896), an enthusiast of Kölliker's ideas on the fusion of the nervous system, who really developed the reticular theory (Gerlach, 1872) through the use of a procedure of staining with ammoniated carmine and gold chloride that he introduced. He observed a very fine reticulum of nerve cell processes in the gray matter of the cerebral cortex, cerebellar cortex, and spinal cord and such observations led him to propose that the nerve impulse travels from cell to cell through fiber networks formed as meshes. Accordingly, nervous tissue would consist of a reticulum comprising a large number of pieces that were physically interconnected (Shepherd, 1991; Jones, 1994).

In the mid 1860s – a period in which the scientific community leaned toward the reticular theory – the ideas of Kölliker and Gerlach, who were experts in the subject, were readily accepted since there was no empirical evidence that ruled out this theory. This situation would start to change with a new method for staining tissue, the black reaction (*la reazione nera*), a method developed by Camillo Golgi (1843–1926) in 1873.

Thanks to the Golgi method, it was possible for the first time to observe neurons and glia in a histological preparation with all their parts (cell body, dendrites, and axon, in the case of neurons; cell body and processes in the case of glia (**Figure 1**; see below).

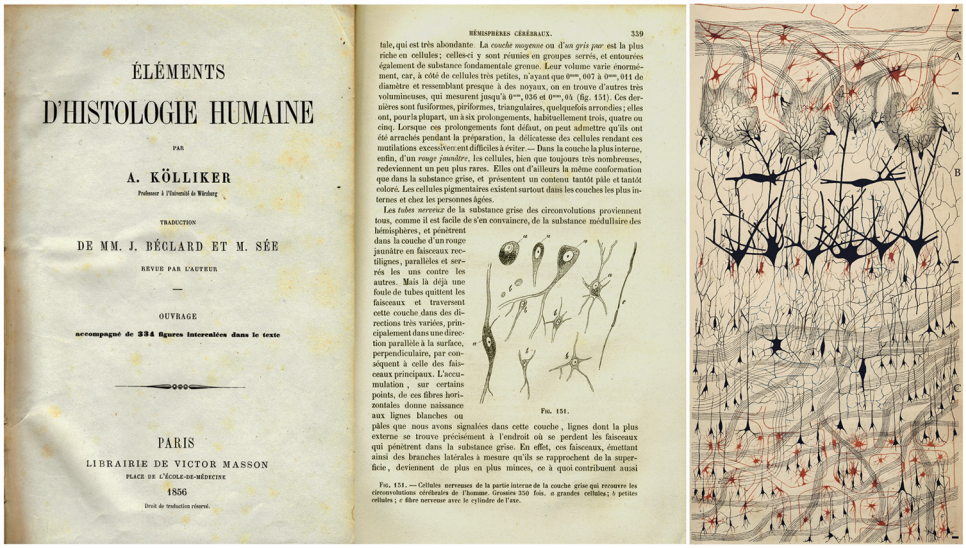

FIGURE 1 | Illustrations showing the evolution of the visualization of the structure of the nervous system. Left: drawing by Kölliker to show different cell types in the cerebral cortex (Published in 1856 in the French edition of Kölliker (1852), classic book Handbuch der Gewebelehre des Menschen). Right: the first illustration of a histological preparation by Golgi with his silver nitrate method ("reazione nera"; black reaction) to study the olfactory bulb of the dog (Golgi, 1875). Taken from DeFelipe (2010a).

Cajal used this revolutionary technique immediately after the psychiatrist and neurologist Luis Simarro (1851–1921) showed Cajal in 1887 a Golgi-impregnated preparation in his private laboratory. Cajal started to use the technique and went on to analyze practically the entire nervous system in several species. Cajal (1888) had published his first important article based on results obtained with this method in the avian cerebellum. In this study entitled *Estructura de los Centros Nerviosos de las Aves*, Cajal confirmed Golgi's conclusion that dendrites end freely but, unlike Golgi, Cajal added the decisive conclusion that this also applies to axons and their branches:

> "*We have carried out detailed studies to investigate the course and connections of the nerve fibres in the cerebral and cerebellar convolutions of the human, monkey, dog, etc. We have not been able to see an anastomosis between the ramifications of two different nervous prolongations, nor between the filaments emanating from the same expansion of Deiters [axons]. While the fibres are interlaced in a very complicated manner, engendering an intricate and dense plexus, they never form a net [. . .] it could be said that each [nerve cell] is an absolutely autonomous physiological canton [unit].*"
>
> (Santiago Ramón y Cajal, 1888)

Thus, by using this technique Cajal came to a conclusion that differs greatly from that defended by the followers of the reticular theory. He suggested that, instead of forming a continuous network, single nerve cells communicate with one another through a specific mechanism by contact or synapse, although the term "synapse" was coined later by Charles Sherrington (1857–1952) in 1897 to describe the hypothetical one-way contact between axon terminals and somata or dendrites (Foster and Sherrington, 1897).

Cajal proposed that neurons could be divided into three functionally distinct regions: a receptor apparatus (formed by the dendrites and soma), an emission apparatus (the axon) and a distribution apparatus (terminal axonal arborization). Thus, the new ideas about the connections between neurons led to novel theories concerning the relationship between neuronal circuits and brain function (DeFelipe, 2010b) and it was possible to trace the first circuit diagrams of the brain (**Figure 2**).

An important consequence of the Neuron Theory was the introduction of the concept of plasticity based on changes on the structure of the nervous system. Cajal had indeed used the term plasticity in the transactions of the International Medical Congress held in Rome in 1894 published in *La Veterinaria Española* (Cajal, 1894). Cajal explained his theory about cerebral gymnastics, clearly stating that the capacity to increase neuronal connections was a plastic mechanism in response to a continued stimulus.

> "*As opposed to the reticular theory, the theory of the free arborization of the cellular processes that are capable of developing seems not only the most likely, but also the most encouraging. A continuous pre-established net – like a lattice of telegraphic wires in which no new stations or new lines can be created – somehow rigid, immutable, incapable of being modified, goes against the concept that we all hold of the organ of thought that within certain limits, it is malleable and capable of being perfected by means of well-directed mental gymnastics, above all during its period of development. If we did not fear making excessive comparisons, we would defend our idea by saying that the cerebral cortex is similar to a garden filled with innumerable trees, the pyramidal cells, which can multiply their branches thanks to intelligent cultivation, sending their roots deeper and producing more exquisite flowers and fruits every day.*"

In this publication, Cajal applied the words "dynamism," "force of internal differentiation," "adaptations (of the neurons) to the conditions of the environment" and "plasticity," among others, to describe the potential of the brain to adapt to the environment. Cajal had been invited to deliver a plenary lecture at this congress

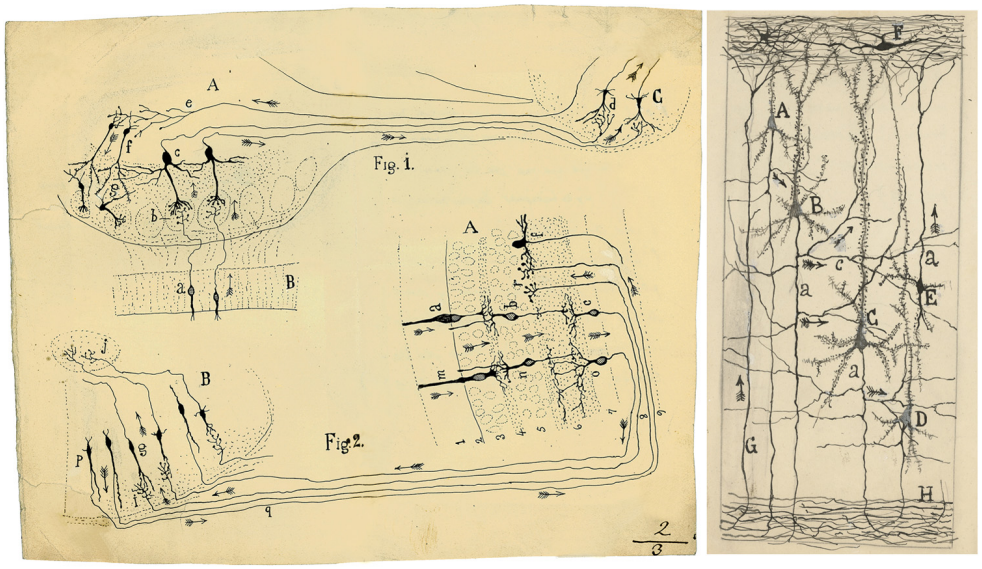

FIGURE 2 | (Left) Cajal's scheme showing the flow of current (arrows) in the visual and olfactory systems to support the Law of Dynamic Polarization. This drawing was reproduced in his article *Significación fisiológica de las expansiones protoplásmicas y nerviosas de las células de la substancia gris* (Rev. Ciencias Méd., 22: 673–679; 715–723, 1891). *Fig. 1.* Scheme of cellular connections in the olfactory mucosa, olfactory bulb, tractus, and olfactory lobe of the cerebrum. The arrows indicate the direction of the currents. *A*, olfactory bulb; *B*, mucosa; *C*, olfactory lobe. *a, b, c, d.* One-way or centripetal pathway through which sensory or olfactory excitation passes. *e, f, g,* Centrifugal pathway through which the [nervous] centres can act on the elements of the bulb, granules and nerve cells, whose protoplasmic processes penetrate the glomeruli. *Fig. 2.* Scheme of the visual excitation pathway through the retina, optic nerve and optic lobe of the birds. *A*, retina; *B*, optic lobe. *a, b, c,* represent a cone, a bipolar cell and a ganglion cell of the retina, respectively, the order through which visual excitation travels. *m, n, o,* parallel current emanating from the rod also involves bipolar and ganglion cells. *g*, cells of the optic lobe that receive the visual excitation and transfer it to *j*, the central ganglion. *p, q, r,* centrifugal currents that start in certain fusiform cells of the optic lobe and terminate in *r*, in the retina at the level of the spongioblasts; *f*, a spongioblast. (Right) Schematic drawing by Cajal to show synaptic connections and the possible flow of information through neural circuits in the cerebral cortex. Taken from *Neuronismo o reticularismo?* (Cajal, 1933). *A*, small pyramid; *B* and *C*, medium and giant pyramids respectively; a, axon; [*c*], nervous collaterals that appear to cross and touch the dendrites and the trunks [apical dendrites] of the pyramids; *H*, white matter; [*E*, Martinotti cell with ascending axon]; *F*, special cells of the first layer of cerebral cortex; *G*, fibre coming from the white matter. The arrows mark the supposed direction of the nervous current.

and, although he could not attend, it is likely that it was there that the term "plasticity" became popular (DeFelipe, 2006).

There is no doubt that some of Cajal's ideas regarding the influence of the environment, such as the influence of education in mental activities, had been proposed by a number of physicians, teachers and philosophers long before. Indeed, Sigmund Freud (1856–1939) used the word plasticity before Cajal, as did other neurologists and psychiatrists of the time, when referring to the "plasticity of psychic material," inferring that the brain or nervous system as a whole is "plastic." As discussed above, in *The Principles of Psychology* (James, 1890), the term "plasticity" referring to the nervous system appears in several passages, particularly to explain habits. James (1890) uses "plasticity" in a broad sense that does not necessarily imply a change in the external form of the structure, but may be "invisible and molecular, as when an iron rod becomes magnetic." Nevertheless, Cajal's contribution was crucial in trying to explain these facts from a structural or connectional point of view based on the Neuron Theory (DeFelipe, 2006).

EVOLUTIONARY ASPECTS OF THE NERVOUS SYSTEM IN CAJAL'S WORK

This topic is mainly based on the first five chapters of Cajal's classic book: *Textura del sistema nervioso del hombre y de los vertebrados* (Cajal, 1899–1904). Readers who are interested in going into further detail on this subject should consult Swanson (2007) and the references contained therein.

The idea of the nervous system being the central organism in the process of organizing and creating behaviors is the basis for most of the histological studies on the fine structure of the nervous system that approach this topic from an evolutionary perspective-properties such as sensation, thinking and willpower – when considered exclusively from an evolutionary point of view – are all a result of the evolution of the nervous system. Irritability was considered to be a fundamental property of the cell in the second half of the 19th century (e.g., Maestre de San Juan, 1885). With regards to this, the presence of flagella in certain locations in infusorians (nowdays named Cnidarians) led to a greater range of motor and sensory possibilities, which in turn translated into development beyond the level of the organism itself, that is, development at the evolutionary level (Cajal, 1899–1904).

The organization of sensory phenomena and the "division of work" will only occur, according to Cajal, in pluricellular organisms. Cajal frequently uses the expression "functional solidarity" to describe the functional specificity of organisms. He defines what we can call the origin of a proto-nervous system in coelenterates and mentions the work of zoologists Blanchart, Hertwit, Zoja, and

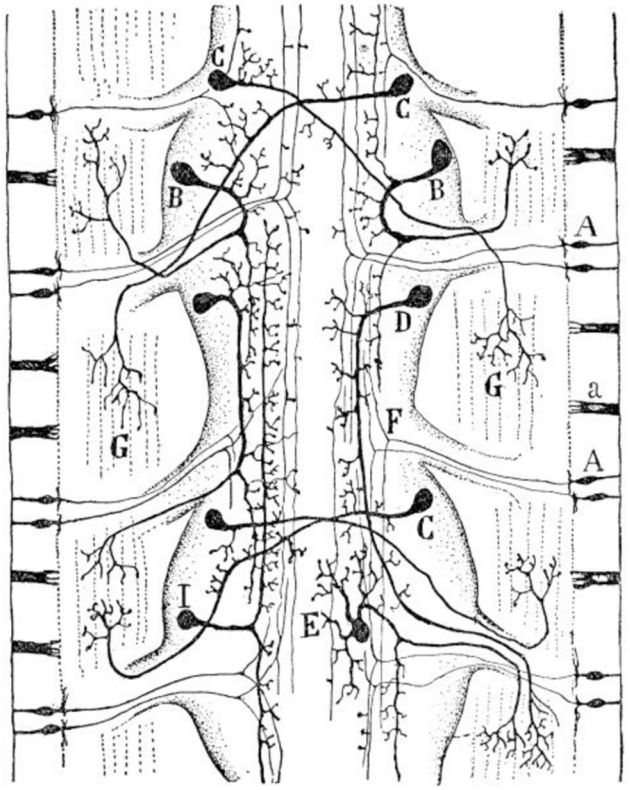

FIGURE 3 | Diagram of the sensory and motor nervous system of a worm (composite of two figures, one from Retzius and another by v. Lenhossék). (A) Sensory cells of the skin; **(B)** ipsilateral motor cells of central ganglia; **(C)** crossed motor cells; **(D)** ipsilateral longitudinal motor cells; **(E)** multipolar motor cell; **(G)** terminal ramifications of motor neurons in muscles; **(I)** interganglionar association cells. Taken from Cajal (1899–1904).

Wolff, who identified in polyps a "nervous system" comprising two distinct classes of neurons: motor neurons and sensory neurons.

A third class of nerve cells appear in worms (**Figure 3**); the association neuron (interneuron). Cajal believe that understanding these first agglomerates of nerve cells (proto-nervous system) was of utmost importance to understand the origin and differentiation of the nervous system. The following quotation by Cajal identifies the advantages that organisms gained after the differentiation of the association neuron:

> "*. . . sensory excitation can propagate not only to the motor cells of a particular ganglion but also to those residing in other ganglia;* in this way, the animal is *capable of reacting* after being stimulated at any point on the skin, *triggering most or perhaps the totality of the locomotor apparatus.*"
>
> (Cajal, 1899–1904, p. 04)

Cajal further describes the appearence of a fourth type of nerve cell, the psychomotor neuron, which is located in the cerebral ganglion or animal brain, from where it controls the others cells. Cajal believed that the information that reaches the skin activates the bipolar sensory cell and travels to the corresponding ganglion center, where the connection between the central arborization and the outgoing motor neurons facilitates the innervation of the muscle to be triggered. When considering animals that are more complex, Cajal affirms that a new element of connection – the association neuron – appears between the sensory neuron and the motor neuron.

The psychomotor cell found in the cerebroid ganglion of invertebrates and in the brain of vertebrates directs information from nervous centers (via voluntary "orders") and stimulates motor neurons. Cajal proposes a classification of the zoological scale in terms of the evolutionary order of appearance of cell types: (1) unicells and sponge-like structures: era of irritability; (2) celenterates: era of fundamental neurons; (3) lower invertebrates: era of association neurons; (4) vertebrates: era of the psychomotor neurons.

The above classification proposed by Cajal does not assume the existence of leaps among the mentioned groups of animals. In terms of evolution, the perfecting that each era imprints on the prior era directs the psychomotor neurons toward what Cajal called "functional solidarity" of the entire organism:

> "*The preponderance and directing excitatory or inhibitory action of the cerebroid ganglion is one of the most surprising phenomena provided by the evolution of the nervous system. [This phenomenon] leads to the emergence of memory, will and intelligence. Since there are no significant structural, morphological, chemical and evolutionary differences between neurons of the cerebroid ganglion and those populating the esophageal and abdominal ganglia, what is the reason for this hierarchical superiority reached by the encephalic ganglion?*"
>
> (Cajal, 1899–1904, p. 06)

The higher complexity of the psychomotor neuron is related to how information from the environment is processed. Cajal believes the main cause of this phenomenon is the existing dynamic relations established between the cerebroid ganglion and the outside world. Rather than receiving from the latter mere tactile and thermal stimulation – Like the abdominal ganglia, the cerebroid ganglion receives from sensory organs impressions which had already been organized, rather than mere tactile and thermal stimulation. These impressions include real images of the outside world that have fixed relations in time and space – the cerebroid ganglion receives from sensory organs impressions which have already been previously organized – real images of the outside world that have fixed relations in time and space.

In the preface of the book by Pedro López-Peláez, *Anatomía normal de la médula espinal humana* (Cajal, 1897), Cajal described how Corti's organ for hearing and cones and rods of the retina act as filters for complex patterns of waves received from the environment by selecting and organizing them in sound and image, respectively, and subsequently projecting them to the cerebral cortex, which transforms them into sensations, ideas and volitions. In the words of Cajal:

> "*The cerebrum of the vertebrates or the encephalic ganglion of invertebrates need not create images; they are given to them perfectly organized by the sense organs, with intensities proportional to the energy of the stimuli, which marvelous architecture constitutes the primordial cause of the superior mental activity of animals. In a word, the morphology and chemical composition of a cell, although very important for the type of psychic operation, do not exclusively determine the hierarchy of this operation, which chiefly depends on the quality of the excitation received from the outside world.*"
>
> (Cajal, 1899–1904, pp. 06–07)

Cajal agreed with the explanation given by Joseph Pierre Durand (1826–1900) and Auguste Henri Forel (1848–1931) on the conscious response from the spinal cord (Forel, 1896; Durand quoted in Cajal, 1899). If this relationship (stimuli generated from the environment) is unclear and diffused, i.e., if there is no precision in the relationship between extension and form, the raw material of sensation actually triggers motor impulses and conscious representations together with the basic response from the spinal cord. Cajal gives the example of tactile and thermal information that reaches the abdominal ganglion of invertebrates and the spinal cord of vertebrates.

The hypothesis of attributing consciousness to lower nervous centers, in particular to the spinal cord, was initially defended by Eduard Friedrich W. Pflüger (1829–1910) and subsequently expanded by Joseph Pierre Durand (1826–1900) and Auguste Henri Forel (1848–1931). This objective hypothesis tried to bridge the gap left by the differences between the cerebral ganglion and the sympathetic and spinal cord nervous centers. Cajal believed that if the optic nerve ended directly in the spinal cord, then the spinal cord would create not motor stimuli but visual images. Building upon this idea, Cajal mentions the principle of Pflüger which assumes that "the cause of an organic necessity is also the cause of satisfying this necessity."

The considerations presented so far in the evolutionary concept of major cell types of the nervous system in the zoological series aim to demonstrate the thesis defended by Cajal in which he affirms that the differentiation of cerebroid operations is subject to special sensory relationships. A widespread thesis on the matter was proposed by Theodor Meynert (1833–1892), (Cajal, 1899–1904). In Meynert's view, the functional diversity of nerve cells was related to the differences in their peripheral connections, and his intention was to explain why different functional regions of the cerebral cortex perform such different activities despite the fact that the structure of these regions appears to be identical.

The solution to this problem lies in the explanation of why parts of the epidermis that are metamerically associated with the cerebral ganglion become differentiated to form an eye. Herbert Spencer (1820–1903) explains that the appearance of sensory organs or structures results from combined operations between adaptation and natural selection (Spencer, 1871). This explanation influenced Cajal greatly, as did Spencer's line of thinking as a whole. However, Cajal believed that it is not easy to fully account for this problem via a progressively evolutionary approach. We note below the argument defended by Cajal:

> "*We must confess that, even applying the principle of natural selection, it is impossible to explain satisfactorily these marvelous apparatuses of relationship [with the environment] which are, as we have said, the probable efficient cause of the superior dynamic hierarchy of the cephaloid ganglion and of the directing role that it exerts over all other ganglion foci.*"
>
> (Cajal, 1899–1904, p. 08)

Cajal proceeds with his line of reasoning regarding the difficulty in satisfactorily explaining the leap from a sensory mechanism to a more developed level by considering the progression from one organism to another during evolution. The example given by Cajal is the panoramic vision of fish, reptiles and amphibians – animals in which the optic nerve fibers cross over completely. In higher mammals, vision is binocular and within a single field. The optic nerve in these animals crosses only partially, with part of it remaining uncrossed. Cajal refers to the fact that this partial arrangement can result in diplopia, causing imperfect vision when compared to lower vertebrates. Although highlighting this and other obstacles to the idea of progressive evolution, Cajal affirms in the French version of the *Textura* (Vol. 1, p. 10):

> "*This and other arguments do not lead us to reject the principle of selection. We have herein advanced this argument to show the need to accept that, concerning progressive evolution, there are factors that are as yet unknown.*"

Further improvement to the nervous system highlighted by Cajal was related to the significant levels of development achieved by sensory organs and that they are distinct in vertebrates, especially in mammals, as segments of various structures, such as the forebrain midbrain, intermediate brain and hindbrain. In relation to invertebrates, Cajal mentions the double ganglionic chain that they have, which blends into a single nerve cord.

In order to automatically control the background vegetative processes of the organism (such as digestion, circulation, secretion, etc.), Cajal states that there was the differentiation of a new ganglionic chain, the sympathetic ganglion, whose functions are partially independent from the cerebrospinal system.

Finally, a favorite subject of research for Cajal was the question of what is special about the neocortex of humans and how does it differ from that of other species? In the words of Cajal:

> "*At that time, the generally accepted idea that the differences between the brain of [non-human] mammals (cat, dog, monkey, etc.) and that of man are only quantitative, seemed to me unlikely and even a little offensive to human dignity . . . language, the capability of abstraction, the ability to create concepts and finally, the art of inventing ingenious instruments . . . do [these facets] not seem to indicate (even admitting fundamental structural correspondences with the animals) the existence of original resources, of something qualitatively new which justifies the psychological nobility of Homo sapiens? Microscope at the ready, I then launched with my usual ardor to conquer the supposed anatomical characteristic of the king of Creation, to reveal these enigmatic strictly human neurons upon which our zoological superiority is founded.*"
>
> (Cajal, 1917)

Thanks to the discovery of the Golgi method, it was possible to start the detailed study of the nervous system in order to compare the neuronal organization between different brain regions within a given species and between species. The idea was to determine whether it was possible to explain functional specialization through structural specialization:

> "*. . . for example, if an organizational detail is found exclusively in or is particularly exaggerated in the visual cortex, we will be justified in suspecting that it has something to do with [cerebral visual function]. Conversely, if an anatomical detail is repeated equally in all cortical regions, we will be justified in assuming that it is devoid of specific functional significance and instead is of more general [significance].*"
>
> (Cajal, 1899)

Thus, Cajal and other authors thought that it was essential to carry out comparative histological studies to see whether any structural peculiarities existed in the human cerebral cortex that might yield a key to specific human behaviors, a fundamental question in neuroscience which is still under debate (for reviews, see for example Rakic, 2009; DeFelipe, 2011; Sherwood et al., 2012; Kaas, 2013).

FINAL CONSIDERATIONS

The process of ganglionic centralization was essential in the evolution of the nervous system. The discussion about the learning processes – which in a way in itself suggests the existence of some plasticity – indicates that the concept of plasticity became commonplace in academic circles in the 19th century, especially in the second half of this century when it became particularly widespread.

William James was one of the first scholars to propose that the nervous system – the organ system chiefly responsible for the processes of consciousness – is subject to changes, i.e., that it has plasticity. James, however, did not attempt to explain his idea at the anatomical level.

Cajal, who enthusiastically supports this hypothesis, constructed an argument firmly grounded in the conclusions stemming from his experimental results in favor of the neuronal theory. Based on such results, he attributes to the nervous system – in terms of evolution – the property of changing itself in response to the relationship between the organism and its environment. We note that Cajal was not the first to use the term plasticity to refer to the nervous system, but he was without doubt the first to attribute plasticity to connections between nerve cells and to explain them in adaptive terms in the process of ganglionic centralization, including the morphological differentiation of cell types.

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Cajal and Pavlov: a comparison between two central neuroscientific schools of the twentieth century

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Santiago Ramón y Cajal (1852–1934) and Ivan Pavlov (1849–1936) share some important features in common: both of them made outstanding contributions to the study of the brain; the two of them came from peripheral countries which had remained in deep isolation from the principal centers of scientific progress in the nineteenth century (United Kingdom, France, and Germany); both of them achieved great recognition in their lifetime. Pavlov received the Nobel Prize in physiology or medicine in 1904; Cajal won it in 1906, together with the Italian physician Camillo Golgi (1843–1926). Moreover, Cajal and Pavlov established well-known neurological schools in their respective countries, some of whose disciples became prominent figures in the fields of anatomy and physiology.

Cajal's discovery of the individuality of nerve cells opened the way for some of the greatest neuroscientific developments of the twentieth century. He refined Golgi's silver staining technique and he offered a detailed description of the anatomy of the nervous system, culminating in his monumental *Textura del Sistema Nervioso del Hombre y de los Vertebrados* (1904–1906), perhaps "the most original work ever written in Neurology" (De Castro, 1981, pp. 31–32).

Initially interested in the study of digestion, Pavlov revolutionized the disciplines of psychology, and physiology by elucidating the nature of conditioned reflexes after a series of famous experiments with dogs. He and his disciples discovered the role of the brain in salivary and gastric secretion (Pavlov, 1902). His influence on our understanding of some forms of learning has been enormous (Todes, 2000, pp. 97–104),especially through key figures of the behaviorist school like John Watson (1878–1958).

Pavlov headed the department of physiology at the Imperial Institute for Experimental Medicine in St. Petersburg from 1890 until his death in 1936: around 45 years of leadership over numerous prominent disciples. Topics like the innervation of gastric glands, the physiology of pancreas and conditioned reflexes were fruitfully examined. His fame attracted relevant international professors and researchers, eager to work with the renowned Russian scientist. For example, in 1902 professors Konheim (Heidelberg University) and Chermak (University of Halle) carried their research under the direction of Pavlov. Russian disciples of Pavlov like Krasnogorsky and Nikiforovsky applied the study of conditioned reflexes to pharmacology. From 1891 to 1917, when the Russian Revolution took place, "more than 110 persons worked here during different periods of time under the direction of Pavlov" (Klimenko and Golikov, 2003, pp. 115).

The so-called "Spanish Neurological School," tragically affected by the 1936–1939 Civil War, was founded by Cajal. One of its principal precursors was Luis Simarro (1851–1921), and it had prominent names like Fernando de Castro (1896–1960), who made important contributions to the structure and function of sympathetic ganglia, the study of baroreceptors and the organization of synaptic complexes; Nicolás Achúcarro (1880–1918), known for his research on the macroglia and the architectonics of neuroglia in cerebral cortex; Pío del Río-Hortega (1882–1945), who discovered microglia and created an important histological method; and Jorge Francisco Tello (1880–1938), who studied the process of degeneracy and regeneration of nervous endings and contributed to our knowledge about the development of the nervous system (Gallego, 1983). The official founding date of this school was 1902, when an Institute for Biological Research was created so that Cajal, who had been recently awarded the Moscow Prize at the International Congress of Medicine that had taken place in Paris in 1900, could continue his work. Tello and Domingo Sánchez (a future leading researcher on the invertebrate nervous system) were among the first to participate in the new center and collaborate with Cajal. Achúcarro joint the Institute in 1911, and in the following years an amazing amount of neuroscientific achievements flourished (De Castro, 1981, pp. 57–61).

However, the working methodologies of both schools showed significant differences. The two schools achieved an almost unequaled degree of productivity in the first decades of the twentieth century, but whereas Pavlov's school preferred a more hierarchical organization, Cajal tended to grant more freedom to his students and collaborators (De Castro, 1981, pp. 51). According to Fernando de Castro, Cajal offered higher levels of independence and free intellectual movement to those who worked with him. In Pavlov's laboratory, every scientist seemed to fulfill

an "organic," and impersonal function, in which the footprint of the individual disciple became minimized. Pavlov exerted an almost complete control over the work of his collaborators. In the prolog to his book *The Work of the Digestive Glands* (1902), an English edition in which he summarized some of his principal discoveries, he remarks that the meaning of a certain experiment must be understood from the viewpoint of the "Laboratory," not of the individual researcher. De Castro names this methodology "direct" collaboration (De Castro, 1981, pp. 49), and he opposes it to the "indirect" way privileged by Cajal. In Cajal's school, the disciple could choose the research topic that was closer to his scientific interests. The master was regarded as an example to imitate and a source of advice rather than a true hierarchical authority to which he should report all his ideas, developments and findings.

The outstanding productivity of both schools indicates that the paths toward great scientific discoveries follow no general rule. Two schools guided by two different methodological principles managed to make some key contributions to our understanding of the nervous system. However, the greater freedom to pursue individual research that Cajal granted to his disciples may explain why the Spanish Neurological School managed to produce relevant figures in the field even when the activity of their master had declined or had disappeared.

The two schools also share a tragic destiny: the Civil War (1936–1939), and the diaspora of researchers to countries like Argentina and Mexico, meant the abrupt ending of the most glorious period of neuroscientific research in the history of Spain; after the 1917 Revolution, the international isolation suffered by many Russian scientists deprived Pavlov's school from its past splendor.

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REVIEW
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Santiago Ramón y Cajal and Ivan Petrovic Pavlov: their parallel scientific lives, schools and nobel prizes

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Santiago Ramón y Cajal was not only a great scientist but he was also a dedicated teacher who managed to create his own School in Spain. Cajal was active at the end of the XIX and the beginning of the XX century, a period in which Ivan Petrovich Pavlov, another great contemporary scientist, also established a strong School in Russia. While these two acclaimed scientists shared a similar vision on science, a view they also conveyed to their disciples, they applied quite distinct criteria in the way they dealt with their followers. Interestingly, despite the geographic and idiomatic barriers that had to be overcome, the paths of these two great figures of XX century science crossed at least three times. First when they competed for the City of Moscow Prize, second when they both attended the "Congreso Internacional de Medicina de Madrid" (Medicine International Congress in Madrid) in 1903 and finally, they competed on four consecutive occasions for the Nobel Prize in Physiology or Medicine. Here we discuss their scientific vision, their different attitudes in the interaction with disciples and the distinct circumstances in which their paths crossed.

Keywords: Cajal, Pavlov, nobel prize, madrid congress 1903, city of Moscow prize, history of neuroscience, teaching

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Introduction

Santiago Ramón y Cajal (1852–1934; **Figure 1**) and Ivan Petrovich Pavlov (1949–1936; **Figure 2**) are two important figures in science who were not only contemporary, but they also both had to overcome difficult conditions to carry out science in their respective countries. Nevertheless, both became prominent national and worldwide figures, gaining the highest recognition with the award of the Nobel Prize in Physiology or Medicine, and becoming models for their fellow scientists in Spain and Russia, respectively.

Cajal and Pavlov were born less than 3 years apart, they received the Nobel Prize in Physiology or Medicine within 2 years of one another, and they died within a 2 year interval. Although they had quite distinct personalities, their lives had some similarities, as an impulse to science in their homelands during the same period (**Table 1**). Their lives were both characterized by their tremendously disciplined willingness to work, a common vision of their work as scientists, a great concern for the future of science in their respective countries, and a strong commitment to young researchers and their disciples.

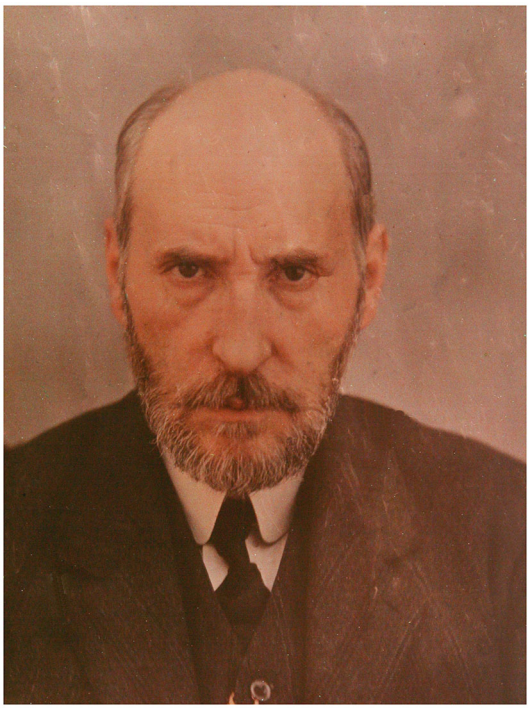


FIGURE 1 | Santiago Ramón y Cajal. Cajal in a photograph from approximately 1906 when he was awarded the Nobel Prize in Physiology or Medicine (image reproduced with permission from the Cajal Institute, Madrid).

FIGURE 2 | Ivan Petrovich Pavlov. Pavlov at his desk in the Imperial Military Medical Academy, St Petersburg in 1904, when he was awarded the Nobel Prize in Physiology or Medicine (image reproduced with permission from The Institute of Experimental Medicine of the Northwest Branch of the Russian Academy of Medical Sciences, St. Petersburg).

Cajal's and Pavlov's Vision of Science

Cajal's and Pavlov's vision of science was inherited from *positivist epistemology*, *scientific monism*, the *theory of evolution* and from *experimental methodology*. From this position, Cajal encouraged the adoption of science-based medicine in Spain, moving away from the *vitalism*, *metaphysics*, *dualism*, *and empiricism* that dominated much of the XIX century. It should be remembered that most medicine at the time was dominated by *vitalism* and *empiricism*. However, thanks to the new experimentalist approach it began to be considered that disease is not a defensive act of the "vital principle" but rather a consequence of a malaise of cells in the body, and that experimentation is the best way to overcome clinical empiricism.

Cajal and Pavlov rejected *vitalism* and *metaphysics* as a way of attaining knowledge, and they advocated the application of the scientific method. They conceived that all activities of the organism had physical basis. Against *dualism*, they defended the principle of *scientific monism*, which holds that mental activity could be reduced and explained by the physiology of the nervous system.

In agreement with *evolutionism*, Cajal and Pavlov based their ideas on the existence of a continuum in phylogenetic evolution that allowed them to perform studies on lower species and to some extent extrapolate the results to higher species difficult or impossible to investigate in the laboratory. Obviously, it was a true breakthrough for Cajal to be able to use techniques that stained the nervous cells in lower species and at early stages of the development. The nervous system of these animals is relatively simple, which implied that he could find principles applicable to the nervous system of different species, and even to humans.

Cajal and Pavlov considered that the prestige of a scientist depends on the original facts he produces. "Hypotheses come and go but the facts remain. Theories abandon us, whereas the facts defend us" (Ramón y Cajal, 1940). Cajal was always aware of the commitment that scientific work implies, writing: "... people insist little in a form of attention that could be called *brain polarization* or *chronic attention*, where all our powers are tirelessly focused on an object of study for months and even years" (Ramón y Cajal, 1940). Indeed, Cajal always advocated that overwork and excessive attention creates talent, as opposed to the widespread notion that it surges from thin air. Cajal did not regard himself as a genius but rather a stubborn workhorse of Spanish science.

Similarly, Pavlov in his letter to the Soviet youth, speaks about the quality he defined "passion for science": "The most important, I insist, is the intense and persistent concentration of the mind: the aptitude to think incessantly about a certain matter, to go to bed and to get up thinking about it constantly" (Frolov, 1937).

TABLE 1 | The lives of Cajal and Pavlov, their career milestones.

Year	Santiago Ramón y Cajal	Ivan Petrovich Pavlov
1849		Ivan Petrovich Pavlov, born on the 26th of September in Ryazan (Russia).
1852	Santiago Ramón y Cajal, born on the 1st of May in Petilla de Aragón (Spain).	
1875	Becomes Assistant in Anatomy at The School of Medicine in Zaragoza.	Becomes Doctor in Natural Sciences
1876	Obtains a permanent position as Assistant at the Hospital of Zaragoza.	Becomes Technician and Assistant at the Veterinary Institute of St. Petersburg.
1877	Becomes Doctor in Medicine.	Develops a new pancreatic fistula procedure.
1879	Obtains a permanent position as Director of the Anatomy Museums in Zaragoza	Graduates in Medicine.
1883	Becomes Full Professor at the University of Valencia.	He becomes Doctor in Medicine.
1884	He starts to publish in fascicles the *Manual de Histología*.	Is designated Assistant Professor at the Military Medical Academy of St. Petersburg.
1886		Is named Director of the Botkin laboratory.
1887	Cajal is introduced to the Golgi technique.	He works at the Botkin laboratory.
1888	Develops a *double impregnation procedure*. He postulates the autonomy of the neuron.	
1889	He attends the German Anatomical Society Congress. Kölliker supports him.	
1890	Cajal studies the embryonic development of the nervous system.	He becomes Full Professor at the Military Medical Academy of St. Petersburg.
1891	He presents for first time the law of dynamic polarization of neurons in a Congress held in Valencia.	Is designated Director of the Department of Physiology at the Imperial Institute of Experimental Medicine in St Petersburg.
1892	Is awarded a Full Professorship position at the Central University of Madrid.	
1895		Is designated Full Professor in Physiology at the Military Medical Academy of St. Petersburg.
1897	He begins the publication of his great treatise *Texture of the nervous system of man and the vertebrates* in fascicles.	Publishes his book *The work of digestive glands*.
1900	He was awarded the City of Moscow Prize.	
1903	Cajal participates in the XIV International Congress of Medicine in Madrid.	Pavlov participates in the XIV International Congress of Medicine in Madrid.
1904	Cajal finishes the book *Texture of the nervous system of man and the vertebrates*	Receives the Nobel Prize in Physiology or Medicine for his work on digestive physiology.
1905	The Science Academy of Berlin concedes the Helmholtz Gold Medal to Cajal.	The method of "artificial" conditional reflexes is introduced into his laboratory.
1906	Receives the Nobel Prize in Physiology or Medicine for his contributions to the knowledge of the nervous system.	
1911	*Histologie du système nerveux de l'Homme et des vertebrés* appears in French.	Pavlov begins extensive studies related to cortical inhibition.
1923		Publishes his book, *Twenty Years Experience in Objective Study of Higher Nervous Activity (Behaviour) of Animals*
1926	Inauguration of the Institute Cajal for Biological Research.	
1927		Publishes his book *Lectures on the Work of Large Hemispheres of the Brain*
1933	Publishes *Neuronism or reticularism*	
1934	Ramón y Cajal dies in Madrid at 22:45 on the 17th of October. He was 82 years old.	
1936		Pavlov dies in Saint Petersburg on the 27th of February. He was 86 years old.

Despite their similarities, as stated above, Pavlov's view on how a School should be managed contrasted considerably from that of Cajal (Blanco, 2014).

The Attitudes of Cajal and Pavlov Towards their Schools

Both Pavlov and Cajal can be considered as researchers who reached the category of maestro. Each of them achieved undisputed recognition in his area and, additionally, both developed new techniques. The technique developed by Pavlov was the preparation of the fistula from the parotid or submandibular gland that he used to study learning by conditioning. Cajal modified and improved the chromium silver technique developed by Golgi to stain nerve cells, and devised other novel staining methods (reduced silver nitrate, formalin-, uranium or sublimed gold). These techniques allowed him and his disciples to obtain new data and develop

novel theories, eventually forming a School of followers attracted by Cajal's reputation and the new techniques he had developed.

Two types of collaboration emerge within a School: direct and indirect (De Castro, 1952). Direct contributions are those that follow the lines identified by the maestro and are carried out under his supervision, without deviating from the pattern or main idea. In this case, the laboratory work will carry the hallmark of the school, which applies equally to all involved, and the laboratory and its work revolve around the maestro. By contrast, the indirect collaborator is much freer. He/she selects the topic to investigate irrespective of the desires of the maestro. In this case, the School's work will be distinguished by its originality and by the author's own personality. Here the laboratory's role varies and the investigator can follow different paths, parallel or not to those of the maestro.

Pavlov's school represents a clear example of direct cooperation between the disciples and the master. He was the head of the laboratory, he guided and planned the work of his many followers, intervening in their work if necessary, and he organized and selected the results of interest. Indeed, he evaluated all the individual experiments and he evaluated the inter-related approaches to understand the issues in question. Since Pavlov was the head and his disciples the hands, the basic ideas came from him and therefore, any intellectual property generated through the huge amount of data collected by his different disciples belonged to him.

Pavlov says in the preface to his book *The work of the digestive glands* (Pavlov, 1902) "I use the word "we" to indicate the entire laboratory. We always name the researcher when describing the experiments but the object of the experiment, its significance and its relationship to the series of experiments carried out is considered from the perspective of the laboratory, as opposed to any isolated opinion or the sole findings of the individual researcher. This way of working has a specific advantage for the reader, as it allows him to really ascertain how the findings were made, given that they are derived from a consistent line of study and shaped through harmonious experimentation" (De Castro, 1952).

Pavlov had 80 disciples during the first period of his scientific activity, which was focused on digestion (pre-1904). Over the years of his studies into higher nervous activity he mentored about 200 disciples, not counting foreign visiting students (Frolov, 1937). Among these, V. N. Massen, a gynecologist who established the initial aseptic and antiseptic procedures in the laboratory, has been highlighted as one of the most constant aides of Pavlov (Todes, 2002). N. I. Damaskin and E. A. Ganike (biochemists), and A. P. Sokolov (a histologist), also made important contributions. Ganike held a prominent place in the laboratory and he was considered as Pavlov's right hand man from 1894 until his death. He handled the finances and he oversaw various activities, and it was he who prepared the annual activity reports that were approved by Pavlov. Another of Pavlov's important disciples was Nikolai Kharitonov, his surgical assistant in whose absence Pavlov said he had lost his hands.

Pavlov's ideas spread internationally thanks to his disciples. For example, Gleb von Anrep, spread the word when working at the University of London and of Cambridge after 1920, and for 20 years when acting as the director of the Department of Physiology of the Egyptian University of Cairo. Boris P. Babkin, who introduced the ideas of Pavlov into England and Canada, was a member of the Canadian Royal Society. William N. Boldyreff immigrated to Japan in 1918 and in 1922 he went to the USA, where he became director of the Pavlovian Laboratory and Hospital in the state of Michigan until 1940. In Poland, Jerzy Konorsky developed Neuropsychology and William Gantt, who worked with Pavlov between 1925 and 1929, played an important role in developing the ideas of Pavlov in the USA (Klimenko and Golikov, 2003).

How Pavlov organized the laboratory resembled the production process in a factory. He was a strict manager who put his new disciples to the test while they were being trained, prior to offering them any research work. Ideally, an initial training was offered for the work that Pavlov had in mind in order to bring new members of the laboratory up to the level of "expert hands". The experiments they carried out and their results would have otherwise been considered useless. Once the best co-workers had been identified, he engaged them in research topics he considered most important. In the laboratory, Pavlov demanded timeliness, accuracy and quality of work. This research machinery or factory was essentially held together thanks to Pavlov's own personal qualities, his leadership, his energy and inspiration, as well as his exceptional organizational skills (Todes, 2000, 2002; **Figure 3**).

Conversely, Cajal's School was characterized by indirect collaborations in which intellectual freedom prevailed. Nevertheless, the characteristic features of Cajal's personality could always be detected in his disciples' work and their individual efforts helped consolidate his own research. This became especially relevant when their research objectives led them along paths distinct to those chosen by their maestro. Cajal never presented opposition to his followers expressing and developing their own independent ideas, that rather, he welcomed and solicited. Cajal did not want devotees of just a single book and followers of a single master. In his own words: "My aim is to offer support and to illuminate the way, fully respecting individual initiatives" (De Castro, 1952).

Cajal believed that an ambitious scientist should remain undisturbed during the training period because the available time was limited and had to be devoted to individual work. He was aware that for young scientists to be successful they must dedicate all their available energy to their work. As such, a young researcher should not establish a School, which would represent a distraction and absorb too much energy at a time when they had not gained sufficient experience (De Castro, 1952). For Cajal, teaching is a job for the wise and to maximize the benefits to be gained from them, efforts must be made to spread their ideas as widely as possible, guaranteeing the well being of the nation. The creation of a school is vital to the most successful researcher (De Castro, 1952).

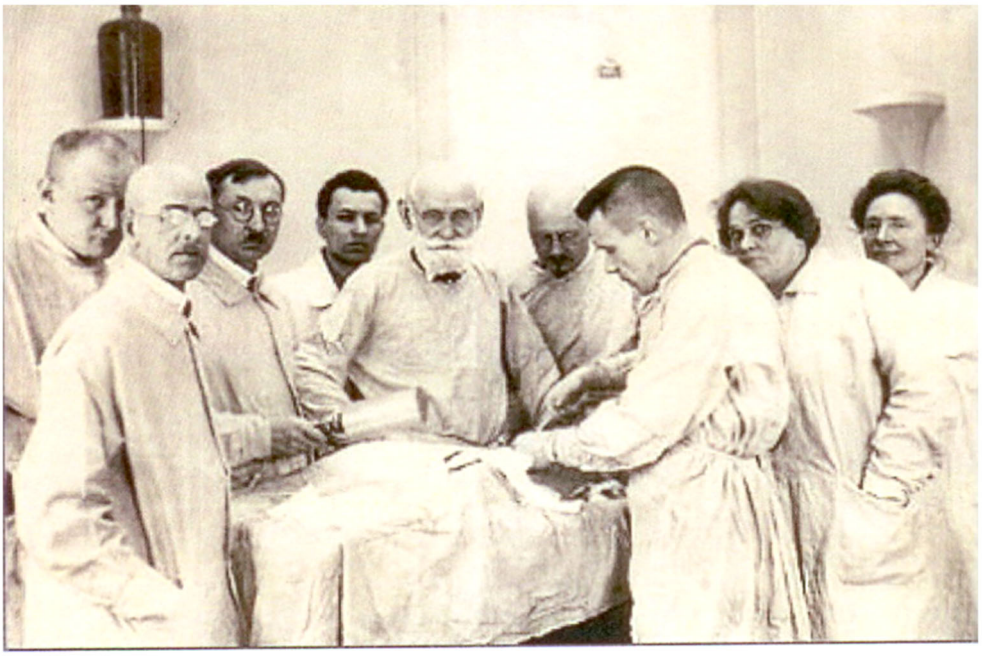


FIGURE 3 | Pavlov with three colleagues and disciples operating on a dog in the Physiology Department, Imperial Institute of Experimental Medicine, St Petersburg. Second on the left: G. von Anrep, operating: Alexander Speranskii (image reproduced with permission from The Institute of Experimental Medicine of the NorthWest Branch of the Russian Academy of Medical Sciences, St. Petersburg).

Cajal's School truly emerged in the early XX century when the Spanish state began to support him, providing him a well equipped laboratory and founding the "Laboratory for Biological Research". This progress followed the award of the Moscow Prize in 1900 at the International Congress of Medicine held in Paris. In previous years, Cajal had had young assistants who helped him with his laboratory work. For example, De Castro refers to Claudio Sala, Carlos Calleja, Isidoro Lavilla, Tomás Blanes, Federico Olóriz-Ortega, and Pedro Ramón y Cajal (Cajal's brother), although the authentic and first true disciples of the Master were Jorge Francisco Tello and Domingo Sánchez. The first original works coming from Cajal's disciples were produced by Tello and they first appeared in their laboratory journal. Nicolás Achúcarro, who trained with Luis Simarro (who first introduced Cajal to Golgi and silver nitrate methods) and Alzheimer, was a subsequent addition to this list, followed by Pío Del Río-Hortega, Gonzalo Rodríguez-Lafora, and José Maria Villaverde. The last of the direct disciples, who began working with Cajal between 1902 and 1916, were Rafael Lorente de Nó and Fernando De Castro, completing this first generation.

The generation mentioned above, trained in turn new disciples who gave rise to a second generation. Between 1916 and the outbreak of the Spanish Civil War, the School grew to the sum of 41 disciples, especially those working with Tello, De Castro and Del Rio-Hortega. Unfortunately, the onset of the Civil War led to a dispersion of the School, many of the disciples going into exile and the few who remained in the country living and worked in very difficult conditions (Aguirre, 2002).

Cajal's believed a teacher should guide his disciples, identifying adequate lines of research, steering them through the literature, and helping them acquire the necessary knowledge and skills (languages, artwork, writing, etc …). The teacher must gradually test the student's capacities, proposing accessible research topics derived from his/her basic interests. Once they have developed sufficient technical and speculative capacity, they will gradually face more stimulating research challenges (Ramón y Cajal, 1940).

To understand Cajal's vision, it is perhaps best to consider how he describes this in his own words: "When the noble investigator can work by himself, take care to imbue in him a pleasure for originality and allow him to suggest new ideas, even when they do not conform with the theories of the School. The true glory of the maestro is not to train his disciples to follow him but rather, to instill the wisdom that will make them capable of surpassing him. The truest ideal should be to create new unique spirits where possible, in order to drive the machine of progress. The generation of docile and indistinguishable followers indicates that the master has been more centered on himself than in furthering Science, and benefiting his country" (Ramón y Cajal, 1940, **Figure 4**).

Cajal, like Pavlov, embodied many of the qualities required for scientific success: an indefatigable capacity for work; a capacity to describe observations; a patience bordering on obstinacy to develop new methods; a dexterity and skill to replace expensive experimental set-ups with simple

FIGURE 4 | Cajal with some of his disciples in his laboratory. First and second on the left: G. Lafora and D. Sánchez, respectively, in the middle N. Achúcarro (Courtesy of the Cajal Institute, Madrid).

custom-made pieces of equipment; a continuity and indefatigable zeal to obtain facts; and above all, a flexibility to change or revise opinion and to correct errors. Cajal tried to convey these qualities to his disciples in a motivating environment, offering total freedom. Thus Pavlov and Cajal shared a similar vision of how science should be done, a view that they conveyed to their disciples (Ramón y Cajal, 1940; Asratian, 1949).

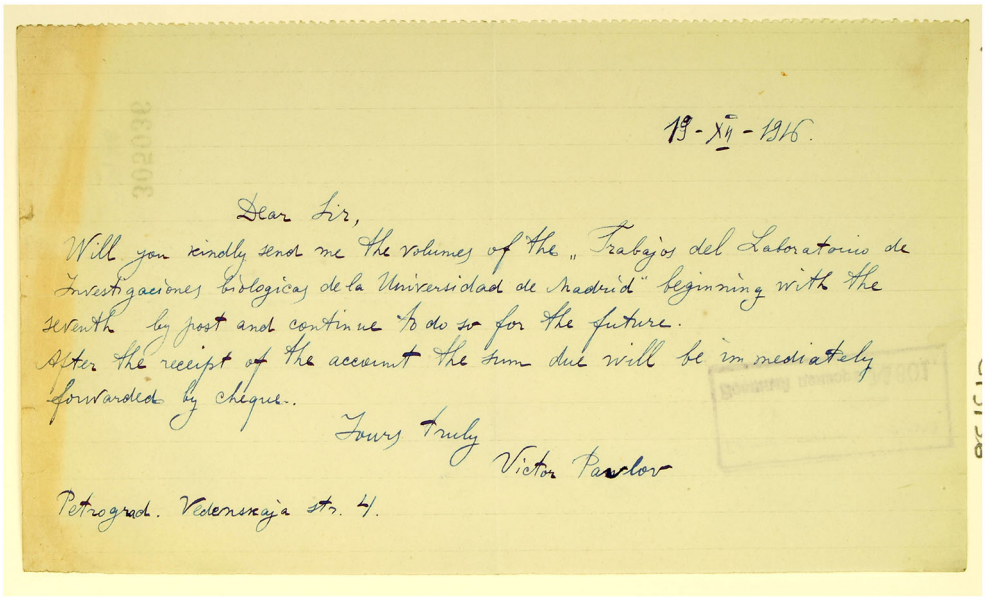
Did Cajal and Pavlov Ever Meet?

Given that we are considering two contemporary figures, one might expect that they would have maintained some contact, at least by correspondence. For example, Cushing (the father of modern neurosurgery) and Pavlov communicated by letter and they also met in person. Cushing also established indirect contact with Cajal through his disciple Penfield, who was also a disciple of Del Río-Hortega, a member of Cajal's school. Indeed, Penfield was the first American scientist who worked with Cajal's group (Aguirre, 2002; Zamora-Berridi et al., 2011).

There are no records of letters exchanged between these two scientists, neither at the Cajal Institute in Madrid (part of the CSIC—the Spanish National Research Council—and where the "Cajal's Legacy" is located) nor at St Petersburg branch of the Archive of the Russian Academy of Sciences (with holds Pavlov's papers). Only one letter addressed to Cajal from Victor Pavlov in 1916 has been found at the Cajal Institute, requesting copies of the journal edited by Cajal (**Figure 5**). Unfortunately, at present it is not possible to confirm whether or not this letter is really from Ivan Pavlov as it is not signed by him but rather by his son Victor Pavlov. Victor was a promising student but it was his daughter Vera who later joined Pavlov to perform research on conditioned reflexes (Todes, 2000). Therefore, it is uncertain whether the letter came from Ivan Pavlov's son or even from a completely different person. Indeed, one of Pavlov's biographers, Todes, who had direct access to various files while working in St. Petersburg, found no record of any written contact with Cajal. According to him, Pavlov must have known about Cajal's research but there are no records of discussions between them, possibly because of their different expertise and to language problems (the only language they had in common was German).

Todes also notes that the only mention of Cajal found in Pavlov's archives was a letter of December 1924 from Cushing to Pavlov. In this letter, Cushing tried to persuade Pavlov to write a book in English on conditional reflexes: "I only regret that we do not have access to your writings in English. I'm afraid we may have to learn Russian in the same way we have had to learn Spanish to follow the studies of Ramon y Cajal in Madrid" (Archive of the Russian Academy of Sciences, quoted in Zamora-Berridi et al., 2011).

Alternatively, contacts between Pavlov and Cajal could have been mediated through their disciples, although there is no


19-XI-1916.

Dear Sir,

Will you kindly send me the volumes of the „Trabajos del Laboratorio de Investigaciones Biologicas de la Universidad de Madrid" beginning with the seventh by post and continue to do so for the future.

After the receipt of the account the sum due will be immediately forwarded by chèque.

Yours truly

Victor Pawlow

Petrograd. Vedenskaja str. 4.

FIGURE 5 | Letter from the "Legado Cajal" from Victor Pavlov to Cajal in 1916 (Cajal Institute, CSIC, Madrid).

evidence for this. Therefore these two scientists might have met only once, at the international meeting in Madrid in 1903.

Congress in Madrid, 1903

Cajal was not only a speaker, but he was also a member of the Organizing Committee of the XIV International Congress of Medicine held in Madrid in 1903, where Pavlov also offered a lecture (Campos-Bueno, 2003; García-Albea Ristol and García-Albea Martín, 2010; Campos-Bueno and Martín-Araguz, 2012). The XIV International Congress of Medicine in April 1903 marked an important milestone for Psychology and Neuroscience, and for Science in general. As Campos-Bueno indicated, two discoveries that would mark a century in the study of the brain and behavior were unveiled before the public. "Both works considered the body as a whole and as such, both these methods had been developed *in vivo*, overcoming the limitations of classical Anatomy and Physiology. We refer to the works of Ivan Petrovich Pavlov and Santiago Ramon y Cajal" (Campos-Bueno, 2006).

Two of the four papers Cajal presented at the meeting are especially interesting: the first because it was presented just before Pavlov's and the second because it is transcendental to the defense of the neuron doctrine. Thus, it would appear highly likely that Cajal and Pavlov met on April 28th, 1903 (García-Albea Ristol and García-Albea Martín, 2010). Cajal's presentation was titled "*Plan of structure of the optic thalamus*" in which the mapping of the thalamus was thoroughly discussed for the first time, acting as a point of transit for the sensory pathways and other projections (Campos-Bueno and Martín-Araguz, 2012). Interestingly, after Cajal's presentation, Ivan Pavlov presented the world's initial discoveries on *Psychical Secretion*, later known as *Conditional Reflexes*. Since they presented their respective papers in the same auditorium, in the same event and on the same day, one preceding the other, and being two of the most distinguished scientific figures of the day, it is more than likely that they were formally introduced there. However, we have found no photographic or written record to confirm that such an encounter did in fact take place.

The talk delivered by Pavlov was titled "*Psychology and Experimental Psychopathology in animals*", in which he surprised the audience by presenting a series of new facts obtained through his research on the digestive glands (research that would lead him to obtain the Nobel Prize in the following year). Indeed, these studies laid the foundation for him to plan the following years of his research, from then on focusing on the nervous system and the animal's ability to learn in the midst of a changing environment (Pavlov, 1927, 1955). Thus, this paper contained the seed of future works and marked a turning point for Psychology, providing a scientific method to study learning and memory processes in higher mammals.

Cajal's lecture that followed was titled "*Critical considerations on the theory of A. Bethe on the structure and connections of nerve cells*". Cajal was already the leading figure of Spanish science at that time and he was engaged in a theoretical struggle with European reticularist scientists, given that he and other colleagues supported and defended the *neuron theory*. This hypothesis postulated that the nervous system was composed of independent, autonomous cells that communicated with other cells by contiguity rather than continuity, as argued by reticularists. As Cajal hinted (1923), the character of this speech was more controversial as its purpose was to stimulate a debate on the reticularist theories of Bethe, in order to propose, promote

and discuss the important issue of neuronal connections, and the fine structure of the nervous protoplasm. The importance of this paper is that it marked the beginning of a series of studies by Cajal, which would lead to a new method of histological staining and the confirmation of the neuron doctrine, as well as opening a line of research on degeneration and regeneration in the nervous system.

Simarro was present when Cajal delivered his speech and he also presented data obtained through his new technique to stain the neurofibrillary network (later modified by Cajal and that would ultimately become the reduced silver nitrate method), which demonstrated that their inner neurofibrils did not form part of an neuronal network as argued by the reticularists. An important feature of Simarro's method was that it allowed the cell to be studied as single entity. Interestingly, this staining was performed on a live animal before it was sacrificed, thus avoiding potential *post mortem* artifacts (Ramón y Cajal, 1923; Campos-Bueno, 2006; Frixione, 2009).

The studies of Cajal and Pavlov had some common ground. They both focused on the organism as a whole, overcoming the constraints of classical anatomy and, as previously indicated, they both developed innovative experimental techniques: Pavlov developed the permanent fistula, which allowed salivation to be measured in living, healthy dogs; Cajal developed new methods to stain neurons before the animals were sacrificed from the technique devised by Simarro. Both discoveries stressed the importance of cell contiguity as a functional feature of the brain. Finally, as a crucial element in their theories, they were both trying to find a basic unit for an objective investigation of mental activity (Campos-Bueno and Martín-Araguz, 2012). Pavlov presented reflexes as basic behavioral units thanks to his theory of conditional reflexes, while Cajal studied the nerve cell as the basic unit of the nervous system (Campos-Bueno, 2006).

The Moscow Prize

The Moscow Prize was the first time that Cajal and Pavlov were contenders for an academic award. Tsar Nicolas II established the award in August 1897, during the Russian convention of the XII International Congress of Medicine. The prize was to be awarded to the most original research submitted to subsequent medical congresses and its first recipient was presented with the award 3 years later in Paris. Pavlov attended the Paris meeting and was keen to be awarded this prize (Campos-Bueno and Martín-Araguz, 2012).

The Paris meeting was an important moment in the academic life of Pavlov and Cajal, since both competed for the Moscow Prize. However, they did not have the opportunity to meet there in person as Cajal could not attend the Paris congress in August 1900 due to health problems. The third scholar nominated to compete with Cajal and Pavlov was Metschnikoff. Cajal won the first edition of the award, obtaining 14 votes in favor as opposed to 6 obtained by Metschnikoff and 3 by Pavlov. At the same meeting, it was also decided that the following congress would be held in Madrid, in 1903 (Campos-Bueno and Martín-Araguz, 2012). The Spanish people rejoiced with this decision as, to some extent, it compensated for the recent military defeats, and as Cannon (1949) said: "Choosing Cajal was considered a kind of racial triumph".

The Moscow Prize was also very important for the academic life of Cajal, producing recognition by the Spanish government. He received the Great Cross of the Order of Isabel the Catholic, the Great Cross of Alfonso XII and he was appointed Counselor of Public Education. In the same year, 1900, he was also appointed Director of the "Alfonso XIII" National Institute of Hygiene (Cannon, 1949) and the government approved the establishment of a laboratory for Cajal, the "Laboratory of Biological Research" (De Carlos and Borrell, 2007).

Later, Cajal and Pavlov were to compete again, this time for the Nobel Prize in Physiology or Medicine. They were nominated together on 4 consecutive occasions (1901–1904), with Pavlov finally being awarded the Prize in 1904 and Cajal sharing it with Golgi in 1906.

Pavlov and the Nobel Prize

As mentioned above, Pavlov was nominated for the Nobel Prize in Physiology or Medicine for four consecutive years (1901–1904) and every time the Committee was faced with the question: "To what extent are the works from Pavlov's laboratory actually his own?" This question arose for good reason. At different lectures, Pavlov had openly acknowledged the collective efforts of the whole laboratory and he named several colleagues who had performed experiments on which his presentations were based. Therefore, the Committee did not know if Pavlov results were indeed original contributions or merely a compilation of the contributions of his colleagues. In the early XX century, the idea persisted that science was created by great minds and not by a machine or scientific apparatus as those in Pavlov's laboratory. The Nobel Committee finally decided that the work from Pavlov's laboratory was truly his merit even taking into account his scientific profile and his way of organizing the laboratory work (Todes, 2002). To assess the work of Pavlov, in 1901 the Nobel Committee carried out an evaluation entrusted to two eminent physiologists of the time, Johansson and Tigersted. They visited St. Petersburg on June 8th (1901) to witness Pavlov's experimental work directly. Pavlov prepared several dogs on which distinct experimental procedures had been performed and he briefly explained the most important results of the experiments to his visitors. The two physiologists were impressed by the work of Pavlov. From then until 1904, both became ardent supporters of the nomination of Pavlov for the Nobel Prize in Physiology or Medicine (Todes, 2000).

Despite this positive report, the Committee's doubts persisted, also because there were very few publications under Pavlov's name. The report did however serve for subsequent evaluations in the following years and his name remained in contention until 1904, when Pavlov obtained four votes to one, and in 1904 he was finally awarded the Nobel Prize in Physiology or Medicine (Todes, 2002).

Pavlov went to Stockholm to receive the first Nobel Prize awarded to a Russian scientist. At the age of 55,

Pavlov was at the height of his career and internationally recognized, receiving a financial incentive of 73,000 gold rubles (about U$ 36,000 at the time) that he invested in his laboratory and further research (Babkin, 1949; Fernández, 2006). Interestingly, Pavlov did not seem to give too much importance to such recognition and he certainly never referred to it during his life, not even in his short autobiography. However, it did represent an important recognition of his work and that of his colleagues, as well as for his country.

Pavlov delivered his Nobel lecture in Stockholm on December 12th, 1904. He started talking about the simple topic of bread and the fight for it, which has dominated many of the events of human life. He then described the fate of food and the process of digestion, and the results of his laboratory at St. Petersburg (the Institute of Experimental Medicine). He then stopped to ". . . express my deepest gratitude to all my colleagues", before he went on to describe the technical developments that had allowed them to surgically intervene in dogs, following the correct principles of anesthesia, asepsis and the proper maintenance of these facilities. He identified two key achievements: first, the digestive glands work differently depending on the nature of the food; second, this digestive process is orchestrated by the nervous system. His important discoveries provided knowledge on how nerves stimulate the gastric glands and pancreas, and how they are involved in digestive activity (Fernández, 2006).

Although Pavlov received the Nobel Prize for his research on the physiology of the digestive glands, at that time he was already interested in "psychic secretion" and what would be later known as "conditional reflexes". The end of his lecture was devoted to this new research topic and its importance, a psychological process that would be addressed in an essentially objective and experimental way.

The Nobel Prize brought money to Pavlov and his family, as well as worldwide fame. He was invited to join different scientific communities and became a member of the Russian Academy of Sciences in 1907. By then, he led three laboratories attracting many scientists from around the world (Todes, 2000). This was interrupted by World War I and later by the Bolshevik Revolution, although once in power, Lenin gave full support to Pavlov's work with his famous decree that allowed him to continue his research until the end of his life.

Cajal and the Nobel Prize

When Cajal received the Nobel Prize in 1906 he was at the peak of his international recognition: he had already received the Moscow Prize in 1900 and the Helmholtz Medal in 1905. The Helmholtz Medal was purely honorary, while the other two awards were associated with a significant financial compensation. As Cajal himself indicates, the Nobel Prize winner received some "25,000 duros" (125,000 pesetas, 20,833 €).

In *Recuerdos de mi vida: Historia de mi labor científica* (*Recollections of my life*, 1923), Cajal himself mentions that once he learned of the award of the Nobel Prize in Physiology or Medicine, he felt more fear than joy. He wondered how his foreign colleagues would react and what his adversaries would say. His apprehension was justified. He was the first Spanish scientist to receive the Nobel Prize in Physiology or Medicine (which he shared with the Italian Camilo Golgi) and as Cajal himself indicated (1923), he and Golgi were "like Siamese twins, joined by the back but looking in the opposite direction". So the award was not exempt from controversy (Armocida and Zanobio, 2006; Fishman, 2007; Grant, 2007; Fernández-Santarén, 2008). Indeed, there was also considerable controversy in the Nobel Committee's decision process, as it was divided between awarding a shared prize to Cajal and Golgi, and presenting it to Cajal alone (see Jones, 1999, 2011; López-Muñoz et al., 2006; De Carlos and Borrell, 2007; Mazzarello, 2007; Nieto, 2012). In the end, the committee voted that the Nobel Prize should be shared by Golgi and Cajal. Cajal was nominated for the Nobel Prize from 1901 to 1906 and thus, he competed with Pavlov for 4 years. The earlier prizes were awarded to von Behring (1901), Ross (1902), Finsen (1903), Pavlov (1904), and Koch (1905), but five nominations were submitted for Cajal in 1906, the year in which he was awarded the Nobel Prize.

In Cajal's Nobel lecture on December 12th, 1906 (Ramón y Cajal, 1907), he presented his work defending the neuron theory, referring only to facts and inferences. The speech was accompanied by many large polychrome images that graphically presented his findings to the profane. In his lecture, as expected, he praised the work of Golgi, the father of the technique with which Cajal himself had achieved so much: "He earned my admiration and all my books contain enthusiastic acclaims to the Wise Man of Pavia's initiatives. I was therefore entitled to expect from him an equally friendly treatment of his discourse on *La doctrine de neurons*" (Ramón y Cajal, 1923). Unfortunately, Golgi did not express himself in the same way. The day before, December 11th, he focused on dismissing the recent work of many European researchers while trying to rescue his almost forgotten theory of the diffuse nerve networks. In his lecture Golgi only cited Cajal when talking about the law of dynamic polarization and the work of the internal structure of nerve cells, completely ignoring the rest of his work, to the dismay of many European researchers who attended the ceremony (López-Piñero, 2000).

By contrast, Cajal, presented much evidence supporting his conclusions in his speech, the confirmation of his observations by others, the new technical resources developed, the advantages of the reduced silver nitrate process, the proof of Kupffer and His's neurogenetic doctrine, the evidence obtained from the regenerative mechanism of nerves, and the evidence from embryonic neurogenesis. He ended his speech with the following words: "In short, the set of observations just outlined, and many others of which I have not had time to speak, supports His's neurogenetic doctrine as an inevitable postulate, a doctrine formulated by that forgotten scholar whose eminent work has suffered the injustice of seeing a phalanx of young scientists describing his finest and greatest discoveries as mistakes in recent years" (Ramón y Cajal, 1907).

Cajal became immensely popular in Spain, making him a living legend. This enabled him to obtain the support of the government and crystallize important institutional projects that

were to have an important impact on science and Spanish scientists until the outbreak of civil war in 1936.

Concluding Remarks

In conclusion, Santiago Ramón y Cajal and Ivan Petrovich Pavlov were contemporary scientists and while they had a similar vision of science, they had completely opposite views on how to manage their particular schools and disciples. Pavlov exerted a hierarchical control over the work of his disciples, while Cajal offered his guidance, support and freedom for them to develop and expand their own research interests. While we do not know with certainty if they ever met in person, they certainly both attended the Congress in Madrid in 1903, and it is very likely that they were introduced to one another there. The 80th anniversary of Cajal's death on October 17th, 2014 coincided with the expiration of the copyright the family held over his legacy. This sets a new stage for research into different aspects of Cajal's work and personal life, and a further analysis of his personal correspondence with other scientists, such as Pavlov.

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Epilog: Cajal's unique and legitimated school

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Santiago Ramón y Cajal is recognized as the founder of modern neuroscience, his discoveries representing the fundamental pillars of our current understanding of the nervous system. As Cajal's career spanned a critical period in Spanish history, he witnessed strong social demands for progress in culture, education, and science. Indeed, the life of Santiago Ramón y Cajal can be considered to reflect the gradual development of Spanish science from the last third of the 19th century. Cajal promoted a national movement that had important consequences for Spanish science, mainly triggered by the creation of the "Junta para Ampliación de Estudios e Investigaciones Científicas," an instrument he established to enrich scientific research and that was later to bear such abundant fruit. The school generated by Cajal profited from this development, through which all Cajal's disciples received fellowships to train in laboratories across Europe. Unfortunately, the Spanish Civil War disrupted this revitalization of Spanish science and provoked the diaspora of many Spanish scientists. However, a political impulse, mostly following this spirit, was resumed in Spain during the eighties that successfully led to a renaissance in Spanish science.

Keywords: Cajal's school, Spanish neuroscience, growth cone carcinogenesis, JAE, plasticity, dendritic spine, synapse

Santiago Ramón y Cajal lived during difficult times in Spain, a period in which science was held in poor esteem. In that era there were only a few Spanish researchers, each of who carried out their work in quite isolated conditions. Cajal was aware of this and he tried to break out of this scientific isolation by attending as many international meetings as he could and by remaining up to date with the scientific literature, personally financing these activities (see De Carlos, 2001). The earliest and possibly the most fruitful meeting he attended was the congress of the German Anatomical Society held in Berlin in 1889, where he met the world-renowned scientist Kölliker. Talking to him and presenting his ideas on the organization of the nervous system proved to be a turning point in Cajal's career, resulting in his introduction to the international scientific community with which Cajal remained in permanent contact thereafter. The increasing popularity of Cajal that had been initiated abroad finally reached Spain in 1900, when the International Congress of Medicine (held in Paris) awarded him the prestigious Moscow prize. Thanks to this, the Spanish Government promoted Cajal, providing him with a laboratory and an endowment to support it, thereby dramatically improving his working conditions. Cajal worked in this laboratory for 33 years (the "Laboratorio de Investigaciones Biológicas"), over which time he was able to create his own scientific school. Further international recognition of his work came later, with the award of the highly prestigious "Gold Medal of Helmholtz" (1905) and the Nobel Prize (1906).

The state of science in Spain at the time of these developments appears to be a matter of some debate. The great Spanish philosopher and humanist Ortega y Gasset wrote in a newspaper article (El Imparcial, 10–08–1908): "*There is no science in Spain. our country should not be proud of Cajal's success but rather, it should be ashamed as it has come about by chance.*" However, there are other indications suggesting that the figure of Santiago Ramón y Cajal was not the exception that proves the rule but rather, the result, perhaps somewhat serendipitously, of the slow yet significant progress of science in Spain that commenced in the latter third of the 19th century. We cannot ignore that any scientific progress in Spain at that time was brought about by the tenacious individual will of those involved, so well incarnated by Cajal. He, like others, was able to compensate for the scarcity of resources through hard work, and this philosophy is strongly imbued in Spanish scientists who must now confront the current situation in Spain. Unlike foreign colleagues, Spanish scientists have progressed to a large extent thanks to their personal sacrifices, many times above what is humanly reasonable, a behavior bordering on stubbornness.

Cajal was aware of the limited capacity to perform science in Spain and although he initially carried out his studies in isolation, his ambition was to take advantage of his success: "*Although when I began my scientific career, both due to the force of habit and by necessity, I had to trust in the value of a solitary worker, I was always concerned with founding a school of histologists and biologists, above all once the State had entrusted me with a fine and well equipped laboratory.*"

By the last third of the 19th century, a kind of intellectual, scientific, and humanistic class had emerged in Spain, which included important personalities along with Santiago Ramón y Cajal. Indeed, the "Institución Libre de Enseñanza" (roughly translated as the "Independent Institution for Education"), was the driving force behind the cultural and social renewal of Spanish society

since its conception (1876). This movement brought with it certain consequences and for instance, and in response to the intense social demand, a new Ministry of Public Instruction and Fine Arts was created in 1900. This triggered the creation in 1907 of the "Junta para Ampliación de Estudios e Investigaciones Científicas," a board to foster scientific training and research that was chaired by Cajal. One of the main activities of this board was to sponsor the sojourns of younger investigators abroad, driven by the desire for better training, and likewise it set out to oversee and foment the building of new institutes and laboratories to host these researchers on their return. The histological school generated by Cajal profited from this structure, and all of his so-called disciples received training at top research centers in France, Germany, and England. Indeed, Cajal created a solid School of Histologists that flourished for quite a few years. Jorge Francisco Tello, Domingo Sánchez, Nicolás Achúcarro, Pio del Río-Hortega, Gonzalo R. Lafora, Fernando de Castro and Rafael Lorente de Nó, stood out among his disciples and they all made significant contributions to modern neuroscience, some of which are nowadays recognized as crucial milestones (see De Carlos and Pedraza, 2014). Unfortunately, the Spanish Civil War and the subsequent 40 year long dictatorship truncated this revitalization of Spanish neuroscience. Nevertheless, it is interesting to reflect on a similar political impulse that was appropriately resurrected in Spain during the 1980s, just after the return to democracy in Spain, giving rise to a flourishing renaissance of Spanish science that persisted for many years thereafter. Thus, although the scientific descendants of Cajal can hardly be traced, all Spanish neuroscientists feel as though they belong to Cajal's school, independent of the discipline followed, histological or not.

CAJAL'S MOST IMPORTANT MILESTONES

The field of neurohistology was revolutionized by Cajal's neuronal theory and indeed, this theory provided the conceptual framework on which modern neuroscience has since been built and developed. This doctrine was the result of countless observations that Cajal made during his lifetime and his interpretation of these (Ramón y Cajal, 1954). The concept of a synapse, for instance, is fundamental to the neuron doctrine, and it was Cajal who named "nervous articulation" and provided compelling evidence for its existence. Although, it was Sherrington who coined the name, it was Cajal who initially described the functional implications of this structure, which for many was unimaginable. The prediction of information flow in the brain, as illustrated by the Indian arrows he sketched in his drawings, indicates how comprehensively Cajal understood how the nervous system functions. Indeed, Cajal's illustrations depicted the way action currents propagate in neuronal networks. Clearly, the way in which Cajal so neatly described how information should flow in neural circuits (from axons to the dendrites or somas of other neurons) was to some extent obvious in some situations (e.g., the retina, olfactory bulb), yet it was certainly not that obvious in others (e.g., the cerebellum). Thus, Cajal not only correctly interpreted local relationships between neurons within a nucleus but also, long-range connections between nuclei (**Figure 1**). Combined with the postulate that electrical impulses propagate from dendrites to the cell body, then to the axon, Cajal could draw up what he called the *Law of Dynamic Polarization*, another fundamental contribution to neuroscience. Worth mentioning is that cortical pyramidal cells were conceptually advanced by Cajal as "psychic cells" in 1891 (Ramón y Cajal, 1891), an idea completed later on with the aid of his brother, Pedro, with whom reinforced this aspect by carrying out a comparative analysis in different species of vertebrates. As Patricia Goldman-Rakic discussed time ago (Goldman-Rakic, 2002) the name of "psychic" given by Cajal was entirely appropriate since pyramidal cells, particularly in the prefrontal cortex, process information from the outside world in the form of representation of on-going events and integrates it with previously stored knowledge, underpinning behavioral responses.

Cajal also made important advances in defining the concept of neuronal plasticity, as he claimed that areas of the brain used heavily would have richer connectivity, as their dendritic arborisation will grow with use. By contrast, he suggested that the connections in areas used less often would deteriorate and become functionally weaker. These concepts are familiar and fully accepted nowadays but amazingly, they were formulated by Cajal brain well in advance of their formal demonstration. Indeed, as pointed out by Kandel (1977), Cajal already conjectured this in 1894 on the occasion of the Croonian Lectures to the Royal Society: "*. . .it is possible to imagine that mental exercise facilitates a greater development of the protoplasmic apparatus and of the nervous collaterals in the part of the brain in use. In this way, pre-existing connexions between groups of cells could be reinforced by multiplication of the terminal branches of protoplasmic appendix and nervous collaterals. But the pre-existing connections could also be reinforced by the formation of new collaterals and protoplasmic expansions.*" In addition, the discovery of the dendritic spine as an anatomical and biochemically distinguishable structure was a fundamental milestone that paved the way for further studies demonstrating that this is indeed the structure where plasticity can occur, a substrate for learning and memory. Cajal was able to go further, warning that gross anatomy did not provide sufficient detail to understand mental activity. As quoted by Kandel (1977, p. 1138), Cajal wrote in 1911: "*No matter how excellent, every physiological teaching on the working of the brain based on localization leaves us ignorant of the mechanism of mental activity. These actions are certainly accompanied by molecular modifications in the nervous cells and preceded by complex changes in the relationships between neurons. To understand mental activity it is necessary to understand molecular modifications and changes in neuronal relationships. Of course one must know the complete and exact histology of cerebral centres, and their tracts, but that is not enough. It will be necessary to know the energetic transformations of the nervous system which accompany perception and thought, consciousness and emotion.*" One remains speechless upon reading these sentences, as they represent a large extent of what we currently consider to be the basis to explain learning and memory.

Another indisputable milestone derived from Cajal's work was the discovery of the growth cone (**Figure 2**). Cajal thought that during their migration, growth cones are orientated and attracted by specific chemical signals. Indeed, he used the term sniffing to illustrating how the growth cone navigates and leads the

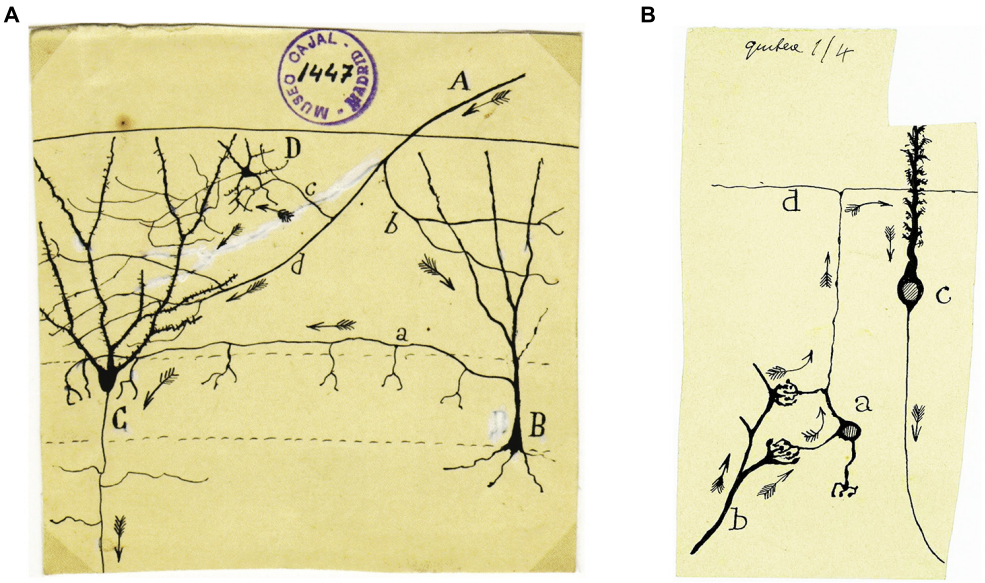

FIGURE 1 | (A) An original drawing by Cajal representing the circuit responsible for feed-forward inhibition in the dentate gyrus: A, afferent fiber; B, corpuscle of short axon terminating around the granules (i.e., basket cell); C, granule cell; D, small element of short axon. Cajal never understood this circuit as he ignored the existence of inhibitory neurotransmission, although he did apparently speculate about the utility of this "vain loop": "*In the figure, we show an example of the loop, apparently vain, described for afferent currents through the short axon cells.*" However, he added a few lines below: "*Not knowing the nature of the nervous movement well, it is difficult to understand how such elements increase the energy of the discharges*" (Ramón y Cajal, 1904). **(B)** In this drawing Cajal represents the cerebellar circuit in a very simple but accurate way, showing the direction of the nerve impulse with Indian arrows. Basically, from the pontine nuclei, the mossy fibers reach the cerebellum and transmit information to the granular cells. These cells conduct this information through their axons, the parallel fibers, towards the Purkinje cell dendrites, and finally, it is these cells that project the nerve impulse out of the cerebellum.

axonal fibers towards their targets. As a consequence, Cajal proposed the neurotropic theory with no more clues than a profound knowledge on how cytoarchitecture developed. As is now clear, attractive and repulsive molecules are responsible for this behavior and many such cues have now been identified, with a great deal having been determined about the signaling cascades they activate.

Cajal thoughtfully evaluated and commented publications by his contemporary colleagues and exposed his opinions on numerous aspects of science and life. Many of his thoughts and reflexions, as well as his scientific work, originally written in Spanish, have been translated into English (e.g., Ramón y Cajal, 1966; DeFelipe and Jones, 1988; Ramón y Cajal, 2002) opening paths for a general knowledge of Cajal's work.

IMPORTANT MILESTONES ESTABLISHED BY THE SCHOOL CREATED BY CAJAL

Cajal worked mostly alone. Perhaps his only life-long collaborator was his brother, Pedro. However, later on while having his own laboratory in Madrid, Cajal was able to create a good atmosphere in which neuroscience could progress, as can be seen if we examine some of the most representative findings of his main disciples. Cajal and Achúcarro had independent laboratories in the same building, which not only ultimately led them to collaborate but also, to share the library, instruments and technicians. Achúcarro had been trained academically abroad, occasionally visiting the clinic of Pierre Marie at the Salpêtrière, attending lectures by Babinsky and meeting neuropsychiatrists like Tanzi and Lugaro, who introduced him to the study of mental illnesses. Achúcarro had also worked in the laboratory of Alois Alzheimer, where he prepared his doctoral thesis. At that time, Cajal had just developed a new method of staining, the sublimated gold stain, that was very good to impregnate neuroglia and Achucarro had perfectioned the "Técnica de Achucarro" using tannin and ammonical oxide. In this environment and after having spent time in laboratories in Paris and Berlin, Pio del Río Hortega was accepted by Achúcarro to join his group. It was there that he was witness to the discussions between Cajal and Achúcarro (Río Hortega, 1986), who at that time insistently wanted to clarify the origin and meaning of two cellular formations close to the glial cells, which they called rod cells and granule-fatty bodies. Achúcarro died prematurely and Río Hortega took over his laboratory, from where he demonstrated the morphological characteristics of the interfascicular glia (i.e., oligodendroglia), describing this cell type as a variant of the neuroglia. He further demonstrated the mesodermal origin of the microglia, a candidate to be the third element of the nervous system. Cajal recognized these discoveries in his autobiographical book "History of my Scientific Work": "*The discovery of microglia in the nervous centers is one of the most valuable achievements of the Spanish school*" (Ramón y Cajal, 1981). Similarly, Gonzalo Rodríguez Lafora spent time in the laboratory of Cajal as an undergraduate student and after traveling to Germany, where he studied with Theodor Ziehen, Emil Kraepelin and Alois Alzheimer in the Neurological Clinic of Munich, and to Paris

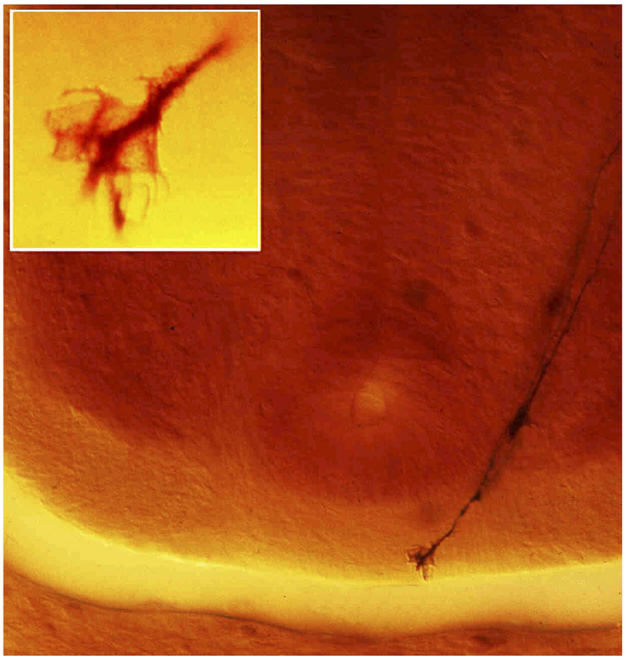

FIGURE 2 | Transverse section of the spinal cord of a day 4 Chick embryo taken by the authors from one of Cajal's original Golgi slides (preserved at the "Instituto Cajal" in Madrid) that led him to describe the growth cone in 1890; a crucial finding in the establishment of the neuronal theory and of the trophic theory. Cajal thought that developing neurons possessed chemotactic properties that would guide axons to their targets and he suggested that the growth cone would be guided by chemicals to find their secretory target. The figure shows two commissural axons running towards the floorplate to cross the midline. Cajal described this as follows: *"This fibre ends…in an enlargement which may be rounded and subtle, but that may also adopt a conical appearance. This latter we shall name the growth cone, that at times displays fine and short extensions…which appear to insinuate themselves between the surrounding elements, relentlessly forging a path through the interstitial matrix."* The insert shows a higher magnification of the growth cone of one of these fibers (from Nieto, 1996).

where he worked with Babinski, Magnan and Dupré, he returned to Spain in 1912 to work in the "Experimental Physiology of the Nervous System Laboratory," in the same building as Cajal's laboratory (Moya, 1986). Remarkably, he first described progressive myoclonus epilepsy in 1911, a disease that it is currently known as Lafora's disease (Lafora and Glueck, 1911), and that is histologically recognized by the large inclusions that accumulate inside the neuronal soma and dendrites, the so-called "Lafora bodies." It is now known that this is an autosomal recessive disorder caused by mutations in the EPM2A or EPM2B genes that lie on human chromosome 6. EPM2A encodes a dual-specificity phosphatase called laforin, while EPM2B encodes an ubiquitin E3 ligase, called malin. These mutations provoke seizures, drop attacks, ataxia, and the development of severe dementia (e.g., Gómez-Abad et al., 2005).

Fernando de Castro began to study histology with Achúcarro but he soon entered the laboratory of Cajal, which he never abandoned. In the 1920s, Fernando de Castro started to study the sensory innervation of the aorto-carotid region, where he described, anatomically, baro-receptors (that detect pressure changes in blood vessels) and chemo-receptors (that detect changes in the chemical composition of the blood). His histological research led to the location of the carotid sinus chemo-receptors in the "glomus caroticum" and of the baro-receptors in the walls of the large arteries arising from the carotid artery (De Castro, 1926, 1928). This finding might be considered his greatest scientific contribution, since this was the very first description of a chemoreceptor. Thus, De Castro laid the anatomical basis of cardiorespiratory reflexes, leading Corneille Heymans to study the "glomus caroticum" as a center of chemosensory reflexes. Indeed, De Castro was invited by Heymans to visit his lab in Gent, where he explained his theories and the surgical approaches he used to study the glomus. From then onwards, Heymans and his collaborators reorientated their studies in an attempt to understand the physiology of the carotid body, receiving the Nobel Prize in Physiology or Medicine for this work in 1938. It is not unfair to say that Heymans' discoveries were made possible through the studies of Fernando de Castro, which is why many members of the scientific community believed that Fernando de Castro deserved a share of the Nobel Prize awarded to Heymans.

Rafael Lorente de Nó was possibly the last direct disciple of Cajal and although he worked for the majority of his active life abroad, he did maintain a close relationship with Cajal and they frequently exchanged letters. Pedro Ramón y Cajal sent Lorente de Nó to Madrid to work in the laboratory of his brother Santiago, becoming Cajal's youngest pupil. He spent some time at the University of Uppsala (Sweden), working with Bárány on the vestibular system, from where he moved to Berlin and after a short period in Spain, to the USA (in 1931). His fundamental research on the structure and function of the mammalian cerebral cortex and brainstem (e.g., Lorente de Nó, 1934) allowed him to make important advances in drawing up the concept of columnar organization of the cortex (Lorente de Nó, 1949), and in defining the physiology of neurons and nerve fibers (e.g., Lorente de Nó, 1947, a cornerstone of modern electrophysiology). These studies have been of tremendous importance in neuroscience, securing Lorente de Nó a prominent place in the history of this field.

THE SIGNIFICANCE OF CAJAL'S FINDINGS IN OTHER FIELDS

The remarkable ability of Cajal to derive an understanding of function from the observation of histological preparations is particularly impressive. Most neuroscientists are familiar with his descriptions on the functionality of circuits and on the role of specific types of neurons. However, many neuroscientists are unaware of the importance of his observations in other fields. In addition to his well-known book "Textura del Sistema Nervioso del Hombre y los Vertebrados," published in Spanish in 1899 and 1904 (Volumes I and II, respectively), Cajal wrote other textbooks including the comprehensive manual of anatomic pathology ("Manual de Anatomía Patológica General"). In these, he produced all the histological slides and drawings to illustrate distinct pathological processes, in some cases with the help of his disciples, and he described the histopathology of many diseases in detail, including that of certain carcinomas (reviewed by López-Novoa and Nieto, 2009). The rational description of the cells present in mammary

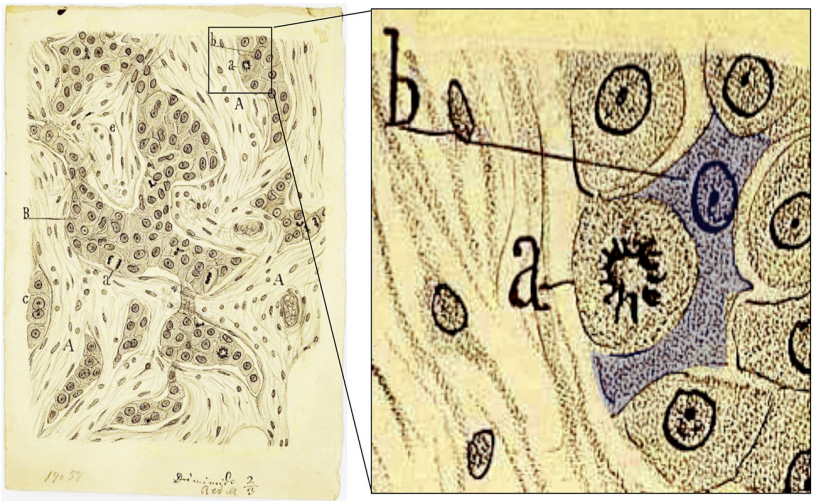

FIGURE 3 | Santiago Ramón y Cajal accurately drew and described the morphological appearance of a breast carcinoma more than 100 years ago. The drawing on the right is adapted from Figure 48 in Ramón y Cajal (1890). The morphology of the cell highlighted as "b" (blue shadow) is believed to be the first description of the epithelial–mensenchymal transition, a first step in the metastatic cascade: *"The cells are not attached to each other... This explains their invasive ability, since free of intercellular cement, they can migrate through the connective tissue"* (from López-Novoa and Nieto, 2009, with modifications).

tumors illustrates how far ahead of his time Cajal was, even in disciplines not related to neuroscience. Indeed, he drew the invasive cells associated with breast tumors and characterized them in detail: "*The epithelial islands are not surrounded by a basement membrane...We must mention the fusiform, pear-like and star-like forms*" (Ramón y Cajal, 1890). As many oncologists recognize, it is difficult to describe the epithelial cells that acquire invasive properties in any better terms. Indeed, it also seems that Cajal was the first to describe the so-called epithelial–mensenchymal transition (EMT) and to propose its underlying mechanisms, well before this phenomenon was implicated in cancer metastasis. Effectively, Cajal's description of breast tumors in his manual of anatomic pathology provides a premonition of this transition as the first step in the metastatic cascade (**Figure 3**). He described undifferentiated breast carcinomas as follows: "*The cells are not attached to each other...This explains their invasive ability, since free of intercellular cement, they can migrate through the connective tissue*" (Ramón y Cajal, 1890; quoted from López-Novoa and Nieto, 2009). It has since been demonstrated that what Cajal referred to as "intercellular cement" is E-cadherin, the molecule that holds epithelial cells together and that has been demonstrated to be the main target repressed to induce the EMT (Thiery et al., 2009).

EPILOG

Cajal not only left us with a huge scientific legacy but also a sociological one, with guidelines for the way we should head into the future. Both these legacies have yielded their fruit and they have to some extent been continued. Very few aspects of his work have undergone rectification after being revisited using modern approaches. His work has not only driven our understanding of the nervous system but it also blossomed into a healthy school of neuroscientists, particularly in Spain. For this reason, many Spanish scientists can be considered to be the heirs of Cajal's spirit, that which he imprinted on the "Junta para Ampliación de Estudios." Many of his ideas are distilled in his words in the last chapters of his book "Reglas y Consejos para la Investigación Científica" (Guidelines and Advice for Scientific Research). In the early 1980s, his idea of sending young researchers abroad for further training was re-adopted, and many of us who took this opportunity can be considered to have been molded as Cajal's would have wished.

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