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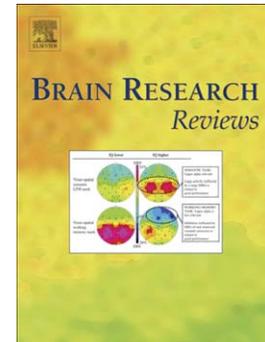
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**Camillo Golgi and Santiago Ramon y Cajal: the anatomical organization of the cortex of the cerebellum. Can the neuron doctrine still support our actual knowledge on the cerebellar structural arrangement?**

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**Abstract**

Camillo Golgi and Santiago Ramón y Cajal were the two main investigators that revealed the morphological organization of the cerebellar cortex, although they never shared the same basic concepts. While for Golgi all axons fused into a large syncytium (the diffuse nerve network), for Cajal they had free endings and communication between neurons was done by contiguity not by continuity. The classical diagrammatic representation of the cerebellar circuitry shown by Cajal in his Croonian lecture (1894), although still valid, has drastically change by the accumulation of the great amount of data generated from 1894 to our days. The topic of this review is to briefly summarize this new knowledge, and to confront it with Cajal's concepts, to determine whether or not the added complexity to the circuit invalidates the Cajal's principles. Our conclusion is that although most of these principles are consolidated, the applicability of the law of dynamic polarization does not adapt to some of them.

**Key words:**

Cerebellar circuit; Lugaro cells; unipolar brush cells; neuromodulatory systems; interneuronal gap junctions.

The recent celebration of the 100 years anniversary of the Golgi/Cajal Nobel prize has given rise to a new revival of the old polemics between reticularism and neuronalism. It is true that in one century Neurosciences has grown to become one of the most prosperous parts of today's biological sciences. During this time many important discoveries have challenged somewhat part of the principles put forward by Cajal in his neuron doctrine and law of dynamic polarization. But it would be an extreme oversimplification to summarize the achievements of the 20<sup>th</sup> century just by saying that the new information gathered has provided evidence indicating that the approach of Cajal was reductionist while the approach of Golgi was holistic and more adapted to recent concepts of the nervous system functional organization, because we are using words in a 21st century contest, far from that of the two anatomists trying to unravel the composition of the brain with quite primitive methods.

During these last two years a large number of publications have appeared either to defend the solid arguments of Cajal or trying to convince the reader that the neuron is already an old fashion concept that although it has been of great help, it is already outdated and belongs to the past. As the stubborn neuroanatomist I am, I still consider that for the time being the simple concept that the nervous system is composed by billions of independent and highly specialized cells forming precise networks through which the nerve impulses are channeled according in most instances to the law of the dynamic polarization can still be of great help for the next decades. At least until the time when we can adapt our morphological searching methods to the anatomical study of organized neuronal assemblies, with their significant aspects of neural activity in the global brain, future times that are not too far away. The purpose of this review is to comment about the work of Golgi and of Cajal on the cerebellum, particularly the cortex, and to discuss which of their discoveries remain still valid today and which ones have been already overruled.

**The cerebellum and the history of the neuron doctrine.**

The cerebellum has been the battlefield of the neuron doctrine. It was in this nervous center that Johannes Evangelista Purkinje got the first glimpse on the presence of nerve cells composing the brain (1837), named after him Purkinje cells. But the most important advancement for understanding the complexity of the CNS structural organization was done many years later by Camillo Golgi with his "*reazione nera*" (1873), a new and revolutionary method for the impregnation and visualization of the nervous constituents, nerve cells and macroglial cells particularly astrocytes. The great advantage of this method is its selectivity (only one out of hundreds or even thousands of neural cells was impregnated) allowing the visualization of the black silhouette of entire cell, perikaryon and processes (axon and dendrites) contrasting within a perfectly clear background. Many years of work with his method, allowed Golgi (1903) to gather an impressive collection of data, which were published in an extensive monograph devoted to the structural organization of the nervous system, where the cerebellum –together with other centers- was analyzed in detail.

It is important to remember what was the general intellectual framework, during the second half of the 19<sup>th</sup> century, when Golgi started his studies with his own silver method (see in Sotelo, 2003). After some years of attempts, the triumph of the "Cell Theory", initiated in 1838 by Theodore Schwann and Matthias Schleiden, arrived with Rudolph Virchow (1855) who proposed that the cell was the basic functional unit of all living things. Even though this fundamental concept changed completely the biological sciences (it has been repetitively said, the Cell theory is to biology as Atomic theory is to physics), it was not believed to apply to the nervous system. The reasons for this exception were probably two fold: i) anatomically, the softness of the nervous tissue made very difficult its handling, sectioning and preparation of thin sections allowing the direct microscopic visualization of its structure, for disclosing its complexity; ii) physiologically, the discovery of the electrical nature of nerve impulses (Galvani, 1791). Because in the absence of valuable tools to examine the histology of the brain, and with the fundamental idea that electrical nerve impulses should navigate without obstacles through out the CNS, the morphologists accepted the concept postulated by the German anatomist Joseph von Gerlach (1872) who considered that the two classes

of nerve processes, the axon conveying information to other nerve cells and the dendrites receiving information from other nerve cells, were fused and formed a large interlocking plexus or syncytial network that occupied the whole of the grey matter. In Gerlach's view, nerve fibers emerged from this plexus and were directed towards the white matter. This simplistic explanation, named the 'reticular theory' or 'reticularism', was almost unanimously accepted.

Golgi (1883) in his studies on the cerebellum was able to see that the dendritic process of the Purkinje cells ended in a completely free manner (Fig. 1A) and therefore, he postulated that dendrites did not participate in the formation of the dense grey matter network. On the contrary, since dendrites were often associated to blood vessels, for Golgi they played a nutritive function. But the mistake of Golgi was to consider that the functional processes, the axons did not end free, and formed an extensive syncytium as the result of the fusion of all axonic processes (Fig. 1C). This way, Golgi adopted the notion of the central nervous system constituted as a complex, diffuse nerve network providing connections among all the elements eliminating this way the requirement for "hard wiring", favoring the validity of the reticular theory.

Many other, to my viewpoint, more positive discoveries were done by Golgi in the cerebellum, and other central regions. He was able to see that there were two distinct categories of neurons, which later were coined Golgi Type I and Golgi Type II. In the Golgi Type I cells, the axon is long, poorly branched, and enters the white matter to become a myelinated fiber, exemplified by the Purkinje cells (Fig. 2A). In contrast, in the Golgi Type II cells the axon ramifies profusely at once, close to the cell body of origin, as if these cells had a distributive function by bringing a single cell into physiological contacts with many others (Fig. 2A, B). Therefore, Type I cells correspond to projection neurons, whose axons will run for long distances to interconnect different nervous centers. Whereas, Type II cells correspond to local circuit neurons or interneurons, whose axons will form terminal bulky and short plexuses in their vicinity, as clearly shown by Golgi in his own drawings of the interneurons of the cerebellar cortex, molecular and particularly granule cell layer where he described a cellular population that later on was named by Cajal (1894)

the “Golgi cells” (Fig. 2B). For Golgi, the axon of the Type II cells *“loses its individuality, and takes part in toto in the formation of a neuronal reticulum which traverses all the layers of the gray matter”*.

One important problem with the Golgi method is that it was extremely capricious, frequently providing very poor impregnations. Santiago Ramon y Cajal learned the Golgi method from Luis Simarro, a Spanish psychiatrist student of Louis Ranvier in Paris. Cajal was able to improve the method (see “rapid Golgi method” in Valverde, 1970), making it much more reliable. Using his improved Golgi method with its variables single, double or triple impregnation, and applying his excellent idea to combine the study of mature with immature nervous tissue because of its much simple arrangement and easier to stain, Cajal selected for his first investigations on the CNS the cortex of the cerebellum, particularly the avian cerebellum. This choice was extremely lucky. He corroborated that the dendrites of cerebellar neurons ended free and extended Golgi’s observations with the important discovery of dendritic spines: the distal branches of Purkinje cell dendrites were studded with spines (Fig. 1B). More importantly, he was able to obtain morphological evidence that axons also had free endings (Fig. 1D). Indeed, in his papers on the avian cerebellum (Cajal 1888a; 1888b; 1889; 1890), he presented the first evidence in favor of his “neuron doctrine”, including the laws that govern the morphology and the connections between nerve cells.

### **The Nobel debate: lessons from the cerebellum, the “diffuse nerve network” versus “neuron doctrine”.**

In 3 years of work, Cajal had in his hands all the elements that comprise the organization of the cortical circuitry of the cerebellum and the routes followed by the impulses reaching this cortex. But, as previously commented, this study had much more general implications than those confined to the cerebellum itself. Indeed, the occurrence of basket cells, with their peculiar pericellular baskets and pinceaux (brush-like) formations ending freely around PC bodies and axonal initial segments (Fig. 1D), together with the correct interpretation of the developmental history of the climbing fibers, and their ultimate disposition coiling up along the

main branches of the PC dendrite (see in Sotelo, 2008a), prompted Cajal to conceive that the diffuse nerve network, as speculated in the conception of Gerlach and Golgi did not exist. On the contrary, the free terminations of the basket cell axons and the climbing fibers provided him the solid morphological evidence required to postulate that the cell theory also applied to the nervous tissue. However, even if he convinced many anatomists, physiologists and pathologists of the reality of his discoveries, he was unable to convince the father of the “reazione nera” Camillo Golgi, and up till his death he was obliged to fight against other scientists who, with not very reliable new techniques (I am thinking on Held, Apathy and others), were constantly decrying Cajal’s ideas.

Eighteen years after Cajal’s studies on the cerebellum, during the Nobel ceremony in 1906, as it has been often commented, Golgi (1967) -who was the first speaker- addressed his objections to Cajal’s “neuron doctrine” using his studies, particularly those on the cerebellum, to try to destroy Cajal’s conclusions about its organization and circuits. Thus, Golgi said speaking on basket fibers: “*Similarly, I cannot accept as a good argument in support of the theory (“Neuron doctrine”) the statement... that says that the processes of the cells of the molecular layer of the cerebellum terminate by forming endings on the bodies of the cells of Purkinje, for I have verified that the... bundles of fibrils coming from the nerve process of the small cells of the molecular layer, and which ought to form the alleged ending on the surface of the cells of Purkinje, can be seen to continue by an infinite number of subdivisions into the nerve network.*” More importantly, Golgi insisted that he could not accept the neuron doctrine: “*because I was confronted by one concrete anatomical fact; this was the existence of the formation which I have called the diffuse nerve network. I attached much more importance to this network, which I did not hesitate to call a nerve organ, because the very manner in which it is composed clearly indicated its significance to me... I have demonstrated its existence in the chief areas of the nervous system, in the spinal cord, in the cerebellum, and in the cerebral cortex, realizing that it is a little modified from site to site.*”

I do not want to close this historical incident without adding a wonderful sentence included by Cajal (1967) in his Nobel Prize lecture. The passage reads: “*Besides, we*

*believe that we have no reason for skepticism. While awaiting the work of the future, let us be calm and confident in the future of our work... If future science reserves big surprises and wonderful conquests for us, it must be supposed that science will complete and develop our knowledge indefinitely, while still starting from the present facts*". The required future science finally arrived 50 years after the "Stockholm controversy" and 23 after the publication of Cajal's scientific testament (Cajal, 1933) where he summarized 46 years of continuous work on his "Neuron doctrine", and this new science did indeed corroborate Cajal's facts. This resounding success was the fruit of a new and powerful technique, the electron microscopy by allowing the visualization of the plasmamembrane, could provide the final demonstration that neurons were independent cells, with contiguity among each other because there was "*a discontinuity between the cytoplasms of the pre- and postsynaptic elements, each of which surrounded by its own cell membrane*" (Palay, 1956). The excellent ultrastructural work carried out by Palay in his later analysis of the cerebellar cortex, making of this circuit the first central circuit to be unraveled at the synaptic level (Palay and Chan-Palay, 1974), has corroborated almost entirely the cerebellar circuit proposed by Cajal more than 100 years before. For me, there is no doubt that from an anatomical viewpoint Cajal conceptions have prevailed and that the "diffuse nerve network" has been invalidated in favor of the "neuron doctrine".

This drastic conceptual change, considering that between the nerve cell processes there was no continuity, only contiguity, provided the basis upon which modern neuroscience stands: the nervous system is composed of billions of independent, richly and precisely interconnected neurons, organized into specific functional assemblies. But fortunately for us, the Neurosciences did not stop with Cajal. His work has provided the foundations upon which all modern Neurosciences are built. The topic of this paper will be to briefly summarize the new knowledge accumulated between the end of the 19<sup>th</sup> century to our days, and to confront the new advances obtained in the study on the structural organization of the cerebellum with Cajal's concepts, to convince the readers, I expect, that although the complexity of the structural circuits of the cortex of the cerebellum has fortunately greatly expanded, the added complexity does not invalidate the Cajal's

principles but on the contrary consolidate most of them.

**The law of dynamic polarization. The circuitry of the cerebellar cortex.**

One inconvenient aspect of the reticularist concept is that the nerve impulses should travel in all directions within the broad network that occupies the whole grey matter. This randomness of nerve impulses was not easily acceptable neither by physiologists nor by anatomists, for whom the occurrence of independent units forming specific circuits suggested that the nerve impulses were orderly oriented. Cajal and some of his contemporaneous colleagues (particularly van Gehuchten, 1891 and van Gehuchten and Martin, 1891) postulated that the receptive surfaces of the neuron are the dendrites and somata, whereas the axon bears the effector mechanisms. In other words that nerve impulses traveled within the neuron in a unidirectional manner from the receptive pole, the cell bodies and dendrites, to the effective pole, the axon. Such an important principle was coined by Cajal “law of dynamic polarization”, and was rapidly accepted because it has the great advantage to allow the withdrawal of the all-directional chaos imposed by reticularism. This principle was found wrong by taking into consideration that axons and dendrites can transmit their impulses in the two opposed directions: cellulifugally as well as cellulipetally (ortodromic and antidromic directions). Nevertheless, it resulted to be right when Sherrington discovered the valvular action of the synapses, allowing the transit of information in only one direction, from presynaptic to postsynaptic elements. It is important to recall that the triumph of neuronalists ideas opened a great avenue for future adventures, particularly for neuroanatomists, that during the 20<sup>th</sup> century, created what has been called functional neuroanatomy. This explains how Golgi Type-I neurons are connected, and has been so important for establishing morphofunctional correlates, helping to the understanding of the brain.

Cajal, in his Croonian Lecture (1894), has not only completed the work of Golgi on the cerebellum, but by applying his “law of dynamic polarization” has provided the basic functional circuitry of this cortex, that even today remains valid: the cortex contains five types of neuronal populations with only one kind of Golgi Type-I

neurons, the Purkinje cells. The four others are Golgi type-II and correspond to the stellate and basket cells in the molecular layer, and the granule and Golgi cells in the granule cell layer. There are two extracerebellar afferent fibers, the climbing and the mossy fibers. The first class represents a very convergent input, since only one climbing fiber climbs all over the principal branches of the dendritic tree of each Purkinje cell. In contrast, the mossy fiber afferent system is rather divergent. First these fibers synapse on numerous granule cell dendrites forming the glomeruli, and the axon of these relay Golgi Type-II neurons ascend to the molecular layer, where they divide in T to form the parallel fibers, which will extend through out the lobule synapsing finally on each Purkinje cell (synapses en passant) found within the folium. Cajal also described precisely how stellate, basket and Golgi cells contributed to the circuit. It is of interest to remark that Cajal with his precise description of the way the descending branches of the basket cell axons terminated, forming the “pinceaux” around the initial segments of the Purkinje cell axons, accepted one of the first exceptions to his law of dynamic polarization. The free endings of these descending axons closely surround the initial segments of Purkinje cells anticipated the occurrence of axo-axonic contacts, as revealed much later by electron microscopy (Palay and Chan-Palay, 1974; Somogyi and Hámori, 1976; see below).

But the main problem for a correct interpretation of the circuit was that Cajal did not recognize the functional dichotomy of neurons, since he never considered the possibility of non-excitatory influences in the CNS. After the work of Sherrington (1897), and for the cerebellum the work of his pupil Eccles (Eccles et al., 1966a, b), the importance of inhibition became evident and, even though the anatomical diagram of Cajal remained untouched, the neuronal circuitry of this cortical structure took into consideration the importance of the balance between excitatory and inhibitory inputs for the fine tuning of cerebellar functions and their development (Sotelo, 2008b). Therefore, since the sixties it is known that the two afferent systems convey excitatory influences to Purkinje cells, which are inhibitory Type I neurons (Ito et al., 1966), even that climbing fibers are the highest excitatory inputs in the whole brain, and that stellate, basket and Golgi cells are GABAergic inhibitory neurons (Oertel et al., 1981; Ottersen and Storm-

Mathisen, 1984) which axons impinge on either Purkinje cells (stellate and basket cell) or on granule cells (Golgi cells). Adapting this new knowledge to the wiring reported by Cajal, granule cells receive through their dendrites excitatory inputs from mossy fibers as well as inhibitory inputs from Golgi cells, which in turn receive excitatory parallel fiber synapses on their dendrites. This Cajal's circuit corresponds, therefore, to the feedback inhibition observed by electrophysiologists (the mossy fibers activate granule cells that through their axons the parallel fibers will activate Golgi cells; the latter finally inhibit granule cells, closing the loop) (Eccles et al., 1966b; Eccles et al., 1968). Cajal was also interested by phylogenetic problems, and devoted part of his studies to the analysis of the cerebellar circuits from humans to inframammalian vertebrates, reaching the conclusion that the cerebellar circuit he described was present in all studied species. The only observed changes were quantitative but not qualitative, even when considering amphibians, particularly frogs, where the existence of inhibitory interneurons was questioned by certain investigators (Glees et al., 1958; see in Sotelo 1969).

This basic circuit reported by Cajal (1894) has been progressively modified during the last century as the result of important new discoveries that have revealed new complex anatomical situations never foreseen in Cajal's concepts. This new basic circuit is schematically depicted in Figure 3. The questions that arise here are: Do these new complex situations revealed by post-Cajal studies challenge the classical circuit of the cortex of the cerebellum introduced by Cajal (1894)? Do these discoveries invalidate the neuron doctrine?

### **New classes of cerebellar interneurons.**

#### ***i) The Lugaro cells:***

Improvements to the circuit began to appear very early, almost simultaneously to the publication of the circuit. These improvements consisted either in the discovery of new categories of Golgi Type II cells or of new classes of extracerebellar fibers. Ernesto Lugaro graduated in Medicine in 1894, the same year he published an important paper on the cerebellum analyzed with the Golgi method, where he reported the presence of a new class of Golgi Type II, the

“Lugaro cells”. These cells are located just beneath the row of Purkinje cell bodies and are characterized by their fusiform cell body, with thick proximal dendrites extending horizontally into the Purkinje cell layer. These bipolar dendrites spread like a flattened X centered by the cell body, implying that Lugaro cells are arranged in a flat lattice just beneath the Purkinje cell layer (Sahin and Hockfield, 1990; Lainé and Axelrad, 1996). The axons of Lugaro cells either enter the molecular layer branching profusely in a territory just above the parent cell body, or in some other cases they descend into the granule cell layer, and even enter the white matter axis of the folium. Lainé and Axelrad (2002) reported, using Golgi impregnation, the occurrence of a subset of Lugaro cells, the globular cells. The latter can be distinguished from the classical Lugaro cells because they have three to four long radiating dendrites dispersed through the three layers of the cortex. Moreover, the axon projects more or less directly into the molecular layer, where it expands in a local parasagittal plexus of twisted thick collaterals ascending through most of this layer. Occasionally, the axon gives off a collateral that runs in the transverse direction, just above the Purkinje cell bodies, parallel to the parallel fibers, some of these axons are myelinated. Lugaro and globular cells express a distinct package of markers than Golgi cells allowing their characterization (Simat et al., 2007). It has been recently established that these interneurons are inhibitory and colocalize GABA and glycine, using both as neurotransmitters, and that each axon would contact about 100 Golgi cells (Dieudonné and Dumoulin, 2000; Dumoulin et al., 2001). Even though, these inhibitory interneurons are not very numerous (in the rat 1 out of 15 Purkinje cells, according to Diedonné and Dumoulin, 2000), they play an important role that will be discussed further down in this review, after the mention of the serotonergic afferent system. These interneurons receive an important synaptic input from recurrent collaterals of Purkinje cell axons (subganglionic plexus) and provide innervation to stellate, basket and particularly Golgi cells, at their dendritic level.

***ii) Unipolar-brush cells (UBC):***

The second new class of interneurons of the post-Cajal period, the “unipolar brush cells”, had a difficult and slow birth, extending over more than 15 years. Altman and Bayer (1977) reported, in Nissl stained preparations, the occurrence in the

granule cell layer of a new class of Golgi Type-II that they called “pale cells” because of the low heterochromatin content of their nuclei. These cells, in addition, were characterized by their spherical shape and their intermediary size, being larger than granule cells and smaller than Golgi cells. However, the most important feature of the pale cells was their peculiar location, instead of being ubiquitously dispersed throughout the cerebellar cortex as it is the case for all the other classes of cerebellar neurons, they were confined mainly to the vestibulocerebellum (nodulus, ventral uvula, flocculus, and parts of the paraflocculus) and with less cellular density in the anteroventral folia like the lingula, indicating that they should be related to vestibular afferents. Ten years later, they were rediscovered by Susan Hockfield (1987) using monoclonal antibodies generated by a procedure for rapid immunization and selective immunosuppression of antibody responses. The Mab Rat-302 visualized together with Purkinje cells throughout the cerebellar cortex, other very peculiar cells that were only present in ventral regions of the cerebellum, like the pale cells they were particularly confined to the vestibulocerebellum. The Rat-302 cells had only one dendrite (monodendritic neurons) that can be quite long, ending into a spray of thinner branches as an efflorescence issuing multiple stubby projections, as they were described three years later in the human cerebellum immunostained with an antibody against chromogranin-A (Muñoz, 1990). But the study of the axon of these neurons and their connections started in 1993. Mugnaini and his collaborators (Harris et al., 1993; Mugnaini and Floris, 1994) coined these monodendritic neurons unipolar brush cells (UBC) because their stubby dendrite ended forming a tightly packed group of branchlets resembling a paintbrush. In Golgi impregnated preparations, the axons of UBC exhibited a tortuous course, provided with collaterals. Most of the latter terminated by characteristic expansions resembling the rosette excrescences of the mossy fibers (Berthié and Axelrad, 1994; Mugnaini and Floris, 1994. Nunzi and Mugnaini, 2000), revealing the presence of intracortical mossy fibers to be added to the well-known extracortical ones. Three distinct UBC subsets have been identified in the mouse cerebellar cortex depending on the panel of markers expressed (one subset expresses calretinin (Mugnaini et al., 1994), a second expresses both the metabotropic glutamate receptor (mGluR)1 $\alpha$  (Nunzi et al., 2002) and phospholipase C(PLC) $\beta$ 4, and the last one only expressing

expressing PLC $\beta$ 4) (Chung et al., 2009), suggesting possible variability in the cerebellar function.

Electron microscopic and electrophysiological studies (Rossi et al., 1995; Jaarsma et al., 1995) revealed the synaptology of these interneurons. Each dendritic brush-like ending was in synaptic contact with a mossy fiber of vestibular origin and, like real glomeruli, with varicosities belonging to Golgi cell axons (Nunzi and Muganini, 2000; Nunzi et al., 2001). The characteristics of the large synaptic interfaces between mossy terminals and brush dendrites precisely correspond to those named “synapses en marron”, clearly suggesting that the latter were wrongly interpreted as mossy fibers synapsing on Golgi cell dendrites (Chan-Palay and Palay, 1971). In a way, UBC and granule cell receive similar inputs, since both are stimulated by the glutamate released from the mossy fibers, while the GABA/glycine combination emerging from the Golgi cell synaptic boutons would serve to balance this excitatory drive. In addition, they also share their own transmitter, because UBC as granule cells are glutamatergic excitatory interneurons. They synapse by way of their mossy-like terminal rosettes on granule cell dendrites, as extracerebellar mossy fibers do, but also on the brush dendritic endings of other UBCs (Nunzi et al., 2001). Mugnaini and his collaborators have considered that UBCs would contribute to spread in a precise patterned manner the vestibular information. Thus, if as discussed above, Golgi cells were wired to convey feedback inhibition to granule cells, UBCs would in turn convey a feed-forward excitation to potentiate the vestibular information (Diño et al., 2000), by generating an intracortical mossy fiber system to spread the extracerebellar mossy fiber information to a much larger population of granule cells. The characteristic large synaptic contact established between UBC dendrites and mossy fibers has as a functional consequence that the response to a single presynaptic mossy fiber stimulus provokes a long-lasting train of action potentials (Kinney et al., 1997). Therefore, the granule cell firing is under the control of a fine tuned network of excitatory and inhibitory interneurons dispersed within the whole width of the granule cell layer.

***iii) Are there other new classes of local circuit neurons?***

In addition to these two types of post-Cajal added neurons, some other types of interneurons have been reported, but with the exception of a third type, the candelabrum cells (Lainé and Axelrad, 1994), they are still not well characterized and could just be subtypes of Golgi cells (Geurts et al., 2003). The somata of candelabrum cells lie between PC bodies and from their apical poles, as is the case for Golgi cells, dendrites spread through the entire molecular layer. However, their axons follow a quite different track; they wind horizontally above the Purkinje cell layer giving off vertically oriented beaded branches ascending through the major part of the molecular layer. It is probable that the postsynaptic elements of these vertically oriented axons forming a candelabrum like image are PC dendrites, but since neither their physiological functions nor the type of used neurotransmitter are known, I will not consider them further on here. In any case, the occurrence of two, three or more classes of new interneurons in the cortex of the cerebellum does not challenge the principles of the neuron doctrine; they only provide more complexity to this circuitry (Fig. 3), and will help to brighten up the synaptic and cellular processes responsible of the important synchronization and oscillations of the cerebellum (see below).

**Synaptic connections: classical axodendritic and axosomatic chemical synapses:**

The synapses are the modern equivalents of Cajal's neuronal contacts or articulations. They are junctional zones of close apposition or contiguity between neurons responsible for the high adhesion existing between pre- and postsynaptic elements, and correspond to specific membrane patches for functional communication and transfer of information between neurons. According to the nature of the dispatched messages, there are two types of interneuronal communicating contacts: either through chemical neurotransmitters in what is named chemical synapses, or through electrotonic coupling at precise regions bearing membrane patches of low resistance or electrical synapses. The morphological correlate of the membrane junction that characterizes chemical synapses, the synaptic complex of Palay (1956), resembles a modified attachment plate occurring between epithelial cells. The presynaptic membrane or "active

zone” of Couteaux (Couteaux and Pécot-Dechavassine, 1970) has been remodeled into a presynaptic grill to allow the fusion of synaptic vesicles to the axolemma and the release of the neurotransmitter. The postsynaptic membrane with its postsynaptic density corresponds to the scaffolds present in the membrane to anchor the receptor molecules for the neurotransmitter. The morphological correlate for electrical synapses also corresponds to a membrane junction, the gap junction, very frequently encountered between epithelial and other classes of cells (see below).

According to the neuron doctrine and its law of dynamic polarization, synaptic contacts among neurons could only be possible between axons and dendrites or cell bodies (axo-dendritic and axosomatic synapses, and these are by far the vast majority of the synapses encountered in the CNS, particularly in the cerebellum. From the very beginning of the neuron doctrine, Cajal’s investigations on the retina and olfactory bulb made it clear that some neurons were devoid of axons (what Cajal named amacrine cells) and, therefore, that part of their dendrites should work not as receptive areas but as effectors involved in the transmission of information as axons do. The important studies of Rall et al. (1966), with the discovery of dendrodendritic synapses in the olfactory bulb, have revealed that some neurons, in the case of olfactory bulb not only amacrine-like neurons, the granule cells, but also mitral cells in the case of the reciprocal synapses (mitral and granule cell dendrites establish at the same interface two way synapses, an excitatory one from the mitral dendrite to the granule cell dendrite and an inhibitory one from the granule cell dendrite to the mitral dendrite) are able to communicate using dendritic profiles as presynaptic elements. In addition, with the advent of electron microscopy and the detailed definition of the features of the membranes engaged in active zones or on postsynaptic densities, it was possible to identify axonal profiles establishing synaptic complexes with other axons on either their initial segments (see above for the pinceaux formations of Cajal) or on other axonic synaptic boutons (serial synapses of Gray, 1962). The conclusion of this kind of study is that each of the neuronal compartments, dendrites, axon and soma, may be presynaptic or postsynaptic one to another. From a functional viewpoint, it can be speculated that neurons are not the smallest and simplest functional units

composing the CNS, and that within single neurons, each compartment or even part of the compartment may have some kind of functional independence, and participate in the circuit as effector elements of synaptic transmission.

### **Unusual classes of cerebellar synapses.**

#### ***i) Axoaxonic synapses: electrical inhibition.***

The synaptology of the cortex of the cerebellum appears to be devoid of dendrodendritic as well as somatodendritic synaptic contacts; whereas, axoaxonic synapses were already apprehended by Cajal in his description of pinceaux. However, quantitative electron microscopic studies of the pinceaux as well as the ultrastructural analyses of the synaptology in agranular cerebellum disclosed interesting aspects of these uncommon synaptic contacts. Concerning the pinceaux formations in the rat, they developed rather late (Sotelo, 2008b), the descending branches of the basket cell axons initiated their wrapping of the Purkinje cell bodies by postnatal day 7 and about P 9, they reached the level of the Purkinje cell axons' initial segments. It is remarkable that the majority of the scanty synaptic complexes observed in adult cerebellum (no more than two synaptic contacts per pinceau, Somogyi and Háromi, 1976) are formed at the onset of the development of the vortex-like arrangement of the basket cell axons encircling the initial segment of the Purkinje cell axon. Therefore, even though the precise location of the pinceaux is optimal for their powerful inhibitory action on Purkinje cells, the amount of released GABA would be too small to accomplish this task. Many years ago, we found (Sotelo and Llinas 1972) that the descending basket axons forming the tightly packed meshwork around the axon initial segment were bound to each other by numerous septate-like junctions and were almost completely separated from the initial segment by intervening astrocytic processes, which in addition encapsulated the entire pinceau arrangement. The establishment of septate-like junctions between basket axon terminals (Sotelo and Llinas, 1972) by creating high-resistance cross-bridges between basket cell terminals, together with its glial encapsulation could compartmentalize the extracellular space and this way, regulate the extracellular current flow. The anatomical organization of the pinceaux supports the electrophysiological suggestion that this unique structure

could be involved in the generation of an inhibitory electrical field imposing a passive hyperpolarizing potential on the axon initial segment (Korn and Axelrad, 1980), as it was clearly demonstrated many years before for the electrical inhibition at the axon hillock of Mauthner cells (Furukawa and Furshpan, 1963). Therefore, our results indicate that in addition to the classical chemical synaptic inhibition, other classes of neuron-to-neuron interactions might occur at the pinceaux formations, interactions able to provoke inhibitory electrical fields.

In conclusion, although electrical field interactions or ephaptic effects (Arvanitaki, 1948) are, at present, not easy to fit with the neuron doctrine, the very special electric field generated by basket axons around the initial segment of the Purkinje cell axon should be considered as part of some very particular cases, where the need of a specific complex environment is essential to compartmentalize the extracellular space, and to distribute the outward terminal action currents of the terminal segments of the basket axons, as a vertical sleeve around the Purkinje cell axon. It would thus function as an anodal blocking device similar to that subserved by the axon cap in the electrical inhibitory system of the teleostean Mauthner cell (Furukawa and Furshpan, 1963). The late development of this complex environment (during the second postnatal week of rat pups; Sotelo, 2008b), the anatomical independence of basket axons and the initial segment of the Purkinje cell axon, the correlation between septate-like junctions and the electric field provoking solely the unidirectional inhibition from the basket axons to the Purkinje cell axon seem to me excellent arguments in favor of the validation of the neuron doctrine. Indeed, the electric field does not randomly spread throughout the tissue, but only affects the initial segment of Purkinje cell axons.

***ii) Dendrodendritic and somatodendritic synapses.***

Despite the widespread location of dendrodendritic synapses in the CNS, from the spinal cord to the thalamus, the cortex of the cerebellum is devoid of such unusual type of synapses. However, during my many years of study of the phenotypes of the cerebella of mice affected by spontaneous mutations, I have repetitively observed the occurrence of dendrodendritic and even occasionally somatodendritic synaptic complexes (Fig. 4A, B) in agranular cerebella such as the

*weaver* and *reeler* mice (Fig. 4 B) (Sotelo, 1975, Mariani et al., 1978; Sotelo, 1990), as well as in agranular cerebellum obtained in the rat by repetitive neonatal X-irradiation (Fig. 4A) (Sotelo, 1977a). The presynaptic elements were either the few remaining granule cells or the GABAergic interneurons, while spines of Purkinje cell dendrites were in the vast majority of the cases the postsynaptic elements. My interpretation of these results is that neurons have the inner capability of establishing synapses through their axons and presynaptic dendrites, but the latter is repressed during normal synaptogenesis. In contrast, this capability is revealed in agranular conditions, where innumerable free postsynaptic receptor sites of Purkinje cells are calling for innervation. Hence, lack of competition between afferent inputs and the presumptive attraction exerted by non-innervated postsynaptic sites seem to be responsible for the phenotypic expression of these unconventional synaptic connections. Hámori and Somogyi (1982) have reported that, after isolation of the cerebellar cortex in adult rats, local circuit neurons of the granular layer respond to mossy fiber denervation by developing presynaptic sites on their somata and dendrites. These findings corroborate the above-mentioned hypothesis and, furthermore, indicate that cerebellar local circuit neurons retain their multipotentiality during all or most of their life span. In any case, these observations plead in favor of a potentiality of cerebellar local circuit neurons to transfer information within neuronal networks, not only through axon terminals, but through presynaptic somata and dendrites as well. Although their presence is difficult to conciliate with the law of dynamic polarization, it is most probably that in these synapses their valvular effect persists, and the directionality of the nerve impulses remains intact, even if electrophysiological data on the function of unconventional synapses in agranular cerebella do not exist, and that in special classes of synapses, the postsynaptic elements can influence the presynaptic ones, as it is the case for endocannabinoid systems (Heifets and Castillo, 2009). The occurrence of the unusual dendrodendritic and somatodendritic synapses has enlarged the concept of functional units in the CNS that does not longer apply only to neurons (Shepherd, 1972). In agreement with Shepherd's proposal that the organization of functional units is rather diverse "*the nervous system is built of overlapping assemblies and hierarchies of such units of increasing extent and*

*complexity*", the synapses between whatever involved neuronal compartments should be the equivalent of the functional units (Shepherd, 1972).

### **The neuromodulatory system: monoaminergic and cholinergic fibers.**

#### ***i) Noradrenergic innervation.***

The development of new techniques is always the starting point for new discoveries. The finding of the histofluorescence method by Hillard and Falck, the first published method allowing the microscopic visualization of a putative neurotransmitter within neurons and along their axons, opened a new era in neurohistology (Falck et al., 1962). The Swedish histologists took profit of the method and published a large number of important papers reporting the monoaminergic (noradrenergic, NAergic and serotonergic, 5-HT) innervation of the CNS, including the cerebellum of the rat. This histochemical approach allowed Fuxe (1965) and Hökfeld and Fuxe (1969) to discover that noradrenergic (NAergic) fibers emerging from neurons in the brainstem –it was later found that they arose from the locus coeruleus (Olson and Fuxe, 1971; Pickel et al, 1974)- entered the cerebellum through the superior peduncle and spread within the cortical layers, particular the Purkinje cell and molecular layers. NAergic fibers were thin and varicose. In the granule cell layer they branched in the sagittal plane forming randomly organized plexuses. The fibers outlined the perikarya of Purkinje cells as they ascended perpendicularly into the molecular layer, up till the pial surface (Bloom et al., 1971). These radially oriented axons had T-shaped divisions at several depths of the molecular layer, mimicking parallel fibers (Mugnaini and Dahl, 1975).

#### ***ii) Serotonergic innervation.***

Similarly, 5-HT fibers originated in the caudal raphe nuclei (mainly raphe obscurus, pallidus, magnus, reticularis gigantocellularis, and reticularis paragigantocellularis; Bishop and Ho, 1985) reached the cerebellar cortex and scarcely spread all over the three layers, and were relatively abundant at the Purkinje cell layer (Hökfeld and Fuxe, 1969; Beaudet and Sotelo, 1981; Bishop and Ho, 1985). In the vestibulocerebellum 5-HT markers labeled in addition some

mossy fibers, but since they did not express tryptophan hydroxylase, the rate-limiting enzyme in the biosynthesis of serotonin, they were not considered as belonging to the serotonergic system.

### ***iii) Dopaminergic innervation.***

The last catecholaminergic afferent system to be discovered was the dopaminergic one (DAergic). This late discovery was due to the facts that: first, the cerebellar content on DA has been always very low, considered to correspond only to that used as precursor for the NA; second, the first selective binding of DA receptors in the cerebellum was observed rather late (Martres et al., 1985); and third, the anatomical correlate suggesting the occurrence of a direct cerebellar projection from mesencephalic DA neurons, particularly in the ventral tegmental area, was lacking up till the work of Ikai et al. (1992). Moreover, since the expression of tyrosine hydroxylase and other DAergic markers (D3 DA-receptor, DA-plasma membrane transporter and vesicular monoaminergic transporter; Kim et al., 2009) was observed in Purkinje cells particularly of lobules IX and X of the posterior vermis has made difficult to determine if the scanty extracerebellar tegmental projection (Ikai et al., 1992) does provide a DAergic innervation to all the cortex of the cerebellum. In any case, if a clear and genuine DAergic innervation exists, it must be of very low density and more abundant in the molecular layer than in the granular and Purkinje cell layers.

### ***iv) Cholinergic innervation.***

Finally, cholinergic fibers were also detected in the cerebellum, mostly in a subset of glomeruli formed by acetylcholine containing mossy fibers, identified either after subcellular fractionation (Balazs et al., 1975) or by immunohistochemistry with antibodies against choline acetyltransferase (ChAT) (Kan et al., 1978). These ChAT- immunopositive mossy fibers were mainly concentrated within the vestibulocerebellum. The cell bodies of origin of these fibers were traced to the caudal medial vestibular nucleus and to the nucleus prepositus hypoglossus, both containing ChAT-positive neurons (Barmack et al., 1992), and their terminal mossy fibers synapsed upon granule cells and UBCs. In addition to these mossy fibers there was also a low-density cholinergic innervation, consisting of thin,

varicose axons, spreading in both the granule cell and the molecular layers. These thin modulatory fibers originated in the pedunclopontine tegmental nucleus, the lateral paragigantocellular nucleus, and to a lesser extent in various raphe nuclei (Jaarsma et al., 1997).

**“Global” versus “point-to-point” systems: Is the occurrence of modulatory systems controversial to the neuron doctrine?**

From an anatomical and functional viewpoint, the CNS is constituted by two different overlapping systems (Sotelo and Alvarado-Mallart, 1986; Agnati et al, 1986). The first one is the “global” system (diffusely projecting regulatory pathways) characterized by the fact that a low number of neurons innervate very extensive terminal domains, as it is the case in monoaminergic systems (Mobley and Greengard, 1985). The most peculiar ultrastructural feature of these axon terminals is that many of them are deprived of the membrane specializations required for the establishment of synaptic junctions (Beaudet and Descarries, 1978). The proportion between junctional and non-junctional boutons varies according to the neurotransmitter, the species under consideration, and the domain of innervation. Although, this proportion can change from 90% to only 7% of junctional boutons, the non-junctional boutons are often the vast majority (see table 1 in Descarries and Mechawar, 2000). This morphological arrangement suggests that monoaminergic axon terminals are able to release their neurotransmitter in a paracrine fashion. Once in the extracellular space, the transmitter would diffuse as far as several hundreds of microns, affecting all neurons equipped with adequate receptors that are present within this tissue volume. The global system corresponds, therefore, to modulatory systems. The second and more extended systems are the “point-to-point” systems, which correspond to those in which each nerve cell contacts no more than a few target neurons. In these cases, as happens in more sensory and motor systems, the neurons are highly specifically connected by specific synaptic contacts. Even though these two ordering systems are very differently organized, the first is able to regulate the action of the second. Using a simple metaphor, the communication within the CNS occurs at two distinct levels: in one the transfer of messages results

from an extremely precise cabled arrangement between neurons (their hard wiring system) similar to a giant telephone central interconnecting neurons in the spinal cord and the brain. The second level, at the top of the previous one, consists on the diffusion of messages on the air, as in a radio station. The news travels throughout the extracellular space but is only heard by those neurons equipped with the specific radio receiver.

Our studies on the NAergic and 5-HT innervations of control and agranular cerebellum showed that both are organized like global systems. Indeed, we were unable to find junctional NAergic varicosities in our sample of wild-type mouse (Fig. 5A) cerebellum (Abbott and Sotelo, 2000), indicating that they should not exceed 3 to 5 %. Moreover, in the posterior vermis of the rat cerebellum less than 9% of the varicosities were junctional (Fig. 5C) (Beaudet and Sotelo, 1981), revealing that in both innervations, the release of neurotransmitters is done in a paracrine manner, as real modulatory systems. A comparative study done in agranular cerebella (the weaver mutant mouse for NA, Fig. 5B, and the neonatal X-irradiated rat cerebellum for 5-HT) has provided an unexpected conclusion. In the agranular cerebellum, with thousands of free postsynaptic sites (particularly the Purkinje cell distal spines), only the 5-HT system is able to change modality passing from non-junctional to junctional (Fig. 5D) (Beaudet and Sotelo, 1981). Therefore, the non-junctional to junctional switch of the 5-HT system, with the underlying formation of heterologous synapses, does not seem to be part of an unspecific remodeling, but points to a hierarchical affinity process providing indirect evidence in favor of some degree of specificity in the formation of heterologous synapses in agranular cerebella. Therefore, the different behavior observed between 5-HT and TH-positive fibers in agranular conditions further corroborates and expands the concept of hierarchical affinity previously reported for the mossy fiber system (Sotelo, 1990), as part of the mechanisms used to create specificity among newly formed synapses during cerebellar development.

### **Serotonin and cerebellar function.**

5-HT fibers are disposed in a very specific way, particularly at the molecular layer where they are in apposition to Purkinje cell dendrites as well as dendrites of

inhibitory interneurons (stellate, basket and Golgi cells) and at the Purkinje cell layer where they are frequently apposed to Lugaro cell dendrites. The studies of Stephan Dieudonné and collaborators (Dieudonné and Dumoulin, 2000; Dieudonné, 2001) have elucidated the role of the 5-HT innervation in the cerebellar cortex. The stimulation of 5-HT fibers with the resulting paracrine release of 5-HT provokes an excitatory response on Lugaro cells, and through their parasagittal GABAergic and glycinergic axonic plexuses synapsing on Golgi cells (about one Lugaro cell could provide innervation to more than 100 Golgi cells), Lugaro cells could be responsible of the synchronization of the activities of Golgi cells. This way 5-HT and Lugaro cells, via Golgi cells, control the spatial and temporal firing pattern of granule cells, in particular of the processes underlying the widespread oscillations and the synchronization in the cortex of the cerebellum, which are essential for its function (Geurts et al., 2003).

It is true that the existence of neuromodulatory systems in the brain was known neither by Golgi nor by Cajal, and that it poses problems to the law of dynamic polarization. Even some investigators have tried to equate the diffuse nerve network of Golgi with these global systems. However, it is not conceivable for me that paracrine cellular intercommunication could be an obstacle for keeping the neuronal identity as independent cells. Indeed paracrine intercommunication is a general process in many different tissues and organs, and could be somewhat equivalent to a local endocrine system that does not use blood supply to reach their targets. Moreover, even though the released messengers have the capability to move for short distances through the extracellular space in all directions, as already discussed, they cannot affect neurons devoid of their specific receptors, preserving in a way an important selectivity of action, which was not predictable in Golgi's concept of the diffuse network. Neuromodulatory systems are of great importance for brain operability, and they greatly enhance the behaviors of neuronal circuits, as reported above for the 5-HT system in the cerebellum.

**Electrical synapses, “gap junctions” between neurons, in the cortex of the cerebellum.**

Gap junctions were discovered by Revel and Karnovsky (1967) between parenchymal liver cells and cardiac muscle cells. The gap junction hemi-channels or connexons, built up with six molecules of connexins, bridge the plasmalemma of the two linked neurons, forming low resistance channels for ions and small molecules up till a molecular weight of 1000 daltons (Loewenstein, 1966; Rose et al., 1977). In recent years almost complete information on the molecular organization of gap junctions has been obtained either by analysis with the atomic force microscopy (Sosinsky and Nicholson, 2005) or by crystallization of human connexin 26 (Cx26) (Maeda et al., 2009), revealing this way the structural determinants of solute transport through the connexon channel. Owing to the inherent properties of the gap junction channels, unlike chemical synapse where the attachment plates are transformed into synaptic complexes, a structural remodeling of the gap junctions is not required to function as electrical synapses. Their presence between two neurons is sufficient for the electrotonic coupling of the linked neurons. But while in the chemical synapses communication follows a strict direction (from presynaptic active zone to postsynaptic densities) in the vast majority of electrical synapses the communication is bidirectional (with exception of the electrically rectifying synapses, also present in vertebrate CNS; Auerbach and Bennett, 1969).

For years it was considered that electrical transmission was a rare kind of synaptic mediation almost exclusively found in phylogenetically primitive forms (Eccles, 1964). However, the discovery of electrical synapses in the mammalian CNS (the first description was obtained in the lateral vestibular nucleus of the rat, Sotelo and Palay, 1967; 1970) drastically changed this erroneous idea. More recently the cloning of connexin 36 (Cx36), a specific connexin for interneuronal communication (Condorelli et al., 1998), has facilitated the search of electrical synapses that before cloning were only identifiable at the electron microscopic level, and were considered to be very rare in the mammalian CNS, with exception of some regions such as the inferior olivary complex and the retina (Sotelo et al., 1974; Raviola and Gilula, 1975). After the cloning of connexins, and the possibility to obtain specific antibodies to each class of connexin, by applying either the

freeze-fracture replica immunogold labeling (FRIL) or using immunofluorescence labeling, it was possible to visualize the subcellular and regional localization of Cx36 in rat brain and spinal cord and to determine that neuronal gap junctions occur in sufficient density to provide widespread electrical and metabolic coupling in adult CNS (Rash et al., 2000). Therefore, even though they are much less abundant than chemical synapses, electrical synapses constitute an important system of fast interneuronal communication practically ubiquitously distributed within the mammalian CNS, including the cerebellum.

Cooperative activity between neurons frequently appears in the way of network oscillations. Over 10 years, it is known that the mechanisms that underlie oscillations in the allocortex and neocortex is the result of synchronization through gap junctions of GABAergic interneurons. These oscillations have been proposed to establish transient temporal correlations between spatially distributed neurons with a temporal resolution of less than ten milliseconds (Tamas et al., 2000). The discovery of gap junctions between dendrites of hippocampal GABAergic interneurons was done by Kosaka (1983), and the electrophysiological corroboration reporting the high-frequency oscillations was published 15 years later (Draguhn et al., 1998). Twenty-seven years were necessary between the description of gap junctions linking stellate and basket cells, the GABAergic interneurons of the molecular layer in the cat and rat cerebella (Sotelo and Llinas, 1972), and the electrophysiological corroboration finally reporting that also in this cortex molecular layer interneurons were electrotonically coupled forming networks to generate synchronized activity (Mann-Metzer and Yarom, 1999). In addition, gap junctions were also found between basket fibers at the “empty baskets” of the adult nervous mutant mouse, indicating that axo-axonic electrical synapse do also occur (see Figs. 25 and 26 in Sotelo and Triller, 1979).

Very recently, Dugué et al. (2009) have completed the work by revealing the mechanisms that underlie oscillations in the cerebellum, and by showing that Golgi cells, which gate information transfer in the granule cell layer, were also coupled through electrical synapses. These electrophysiological results prompted me to consider that some of the dendrodendritic gap junctions I observed with freeze-

fracture method in the lower half of the molecular layer (Fig. 2C, D) could belong to the ascending dendrites of Golgi cells. If we now consider what has been said in the previous section about serotonin, and granule cell layer GABAergic interneurons, specifically Lugaro cells which became depolarized by 5-HT, and that owing to the peculiar transverse organization of their axons, they will be able to inhibit the electrotonically coupled network of Golgi cells. This way, Lugaro cells playing a role in Golgi cells synchronization along the same parallel fiber beam, will be able to organize the widespread oscillations recorded from the granular layer in the awake animal at rest. Consequently, it has been proposed that the Lugaro cell is a 5-HT driven intracortical switch involved in the processing of mossy fiber information (Geurts et al., 2003).

Although it has been reported, with the dye coupling approach, that postnatal Purkinje cells might also be linked through gap junctions in slice cultures taken from P1 and P10 rat cerebellum, and maintained in vitro for up to 15 days (Meller et al., 2005), such a class of interneuronal communication has not been yet encountered in adult cerebella. In any case, despite the fact that Purkinje cells are also GABAergic, exerting an inhibitory action of their postsynaptic targets, mostly the deep nuclear neurons, they are as the glutamatergic pyramidal cells in the allocortex and neocortex (the projection neurons of these cortices) subjected to the oscillatory control of GABAergic interneurons.

### **Does the occurrence of interneuronal networks between GABAergic cells linked through gap junctions invalidate the neuron doctrine?**

From the discovery of the presence of interneuronal gap junctions in the mammalian CNS, a debate started among some neuroscientists who considered that this discovery partially invalidated the Cajal's neuron doctrine. I remember a meeting organized in 1977 (Sotelo, 1977b) by János Szentágothai in the marine biological station of Tihany at the Balaton Lake (Hungary), where I heard for the first time that electrical synapses will provide valuable arguments against the principles of the neuron doctrine. Szentágothai made fun of me saying: how is it possible that a disciple of Fernando de Castro (my undergraduate mentor and last

pupil of Cajal) can defend the idea of the occurrence of gap junctions in the mammalian central nervous system, because its presence testifies for the reality of reticularists concepts? From that period of my life I frequently used the same contradictory arguments to propose that the occurrence of interneuronal gap junctions does not change the concept that neurons are independent cells but, on the contrary, increases their functional modalities.

In favor of my first assertion, that electrotonic coupling through gap junctions does not invalidate the cellular independence of each linked neuron, I advance the similar classes of arguments that those used by Cajal in his defense of the neuron doctrine: ontogenetic, anatomo-functional and trophic arguments. First, the coupled neurons originate from different precursor cells. Moreover, despite the fact that gap junctions during early embryogenesis are essential elements for temporal synchronization and metabolic coupling, my own studies on the development of electrical synapses in the lateral vestibular nucleus (unpublished) and in the inferior olive of rats (Bourrat and Sotelo, 1983; Gotow and Sotelo, 1987) have disclosed that the formation of interneuronal, adult remaining, gap junctions is a relatively late process that happens once the majority of the chemical synapses have been established. For instance, during the development of the inferior olivary glomeruli –the location of most of the inferior olive interneuronal gap junctions– the formation of protoglomerular structures takes place around P10, and it is only between P10 and P15 that the final stage of maturation takes place with the acquisition of the adult-like pattern of the GABAergic innervation and the appearance first of small kissing junctions (minute gap junctions) followed, somewhat later, by adult-sized gap junctions between dendritic appendages of olivary neurons at the central core of the glomerulus. Therefore, the formation of electrical synapses follows a characteristic temporal course different from the one of chemical synapses. But in both instances the process consists in building up specific membrane junctions between two independent cells allowing their communication. Therefore, the ontogenetic argument, one of the three feet upon which Cajal based his neuron doctrine, is not invalidated by the occurrence of electrical synapses.

The only disputable feature of electrical synapses is that, like sites of low electrical resistance, ions and small particles can passively flow from one neuron to another. However this peculiar feature, to my sense, does not transform the network of coupled neurons into a syncytium, because: first as discussed above the network is not the result of incomplete cell division during which several nuclei are formed but without subsequent cytokinesis. Second, it is also not the result of cell fusion. Third, to me the most important point, the connexon hemi-channels on each cell membrane delimit a tiny funneling tunnel that is not passive but that, on the contrary, has a proper chemical regulation controlled by intracellular (second messengers,  $\text{Ca}^{2+}$ , pH) and extracellular signals (mainly neurotransmitters and nucleotides) (see in Giaume et al., 2010), which allows its opening and closure accordingly to a specific pharmacology. In conclusion, the gating behavior of the connexins dictate the properties of the gap junctions, which are different from those expected for a syncytium. Therefore, the anatomical integrity and independence of the coupled neurons is maintained, constituting the second pillar of the tripod upon which Cajal (1933) supported the neuron doctrine. An ultimate and important argument to clearly differentiate the networks of GABAergic interneurons of the cerebellar cortex from the diffuse nerve network of Golgi is that in the former the electrotonic spread of current is confined to gap junctions that is to say to very specific areas of the plasmamembrane provided with hemichannels of connexin, keeping a strong specificity (for instance, in the cerebellum it only affects interneurons), while in the diffuse nerve network the direction of impulses is always random and can affect Golgi type I as well as Golgi type II neurons.

Finally the trophic argument, the third foot of the above mentioned tripod, although not fully demonstrated for electrical synapses may be considered as almost proved. August Forel (188 ), a Cajal precursor, was one of the first that challenged the reticular theory on trophic grounds, based on retrograde degeneration of axotomized neurons during development. Today, it is a solidly established fact that the axon requires the presence of its own cell body to remain alive. Therefore, after axotomy, the distal segment –the one separated from its cell body– degenerates. This anterograde degeneration is one of the major arguments

of the neuron independence (trophic argument), because in the case of syncytia it would never degenerate after such kind of lesion. Working on the frog (*Rana esculenta*) vestibular nerve, which primary fibers ending on cell bodies and dendrites of lateral vestibular neurons are linked to their postsynaptic partners through mixed synapses (a term used by Sotelo and Palay, 1970 to name the common occurrence in axon terminals of synaptic complexes and gap junctions side by side), I was able to verify that the degenerative course of these axon terminals separated from their own cell bodies was much longer in the presence of gap junctions (Sotelo, 1977) than in their absence. Although they survive for much longer periods of time, nevertheless after a long post-lesional survival, up till two months, this type of axon terminal provided with mixed synapses eventually degenerate. These results indicate that despite the fact that gap junctions are metabolic communicating channels, allowing the passage of nutrients from postsynaptic to presynaptic elements, prolonging this way their survival period, they are in the long run unable to avoid their ultimate degeneration and disappearance. Whereas, when syncytial elements are partially lesioned only the locally affected part degenerates.

### **Conclusions.**

The morphological advances accumulated since the Golgi and Cajal studies on the anatomical organization of the cortex of the cerebellum have concerned the two following aspects: i) the discovery of new interneuronal cell types and of new extracerebellar afferent fibers with ajunctional endings releasing their neuromodulatory transmitters in a paracrine manner, and ii) the occurrence of unusual classes of synaptic connections (axoaxonic, dendrodendritic), and the electrical synapses between inhibitory interneurons in the molecular and granule cell layers.

One of the main criticisms raised about the neuron doctrine is its reductionist approach ignoring the global organization of the CNS into functional assemblies. Concerning the cerebellum, knowledge gathered during the last decades has shown that its cortex is organized into narrow parasagittal zones, the microzones

(Oscarson, 1979; Ito, 1984), which are comparable to the modular columnar organization of the neocortex. Therefore, the neuronal assemblies forming each microzone are responsible of the cerebellar function. The functional organization of these microzones would be better understood in a near future owing to the application of already existing technology based upon the use of cell-biological principles, which will allow the expansion of fast optogenetic technologies into suitable methods able to analyze intact-systems biology towards the understanding of brain information processing (Gradinaru et al., 2010).

The viewpoint defended in this review is that, for the time being, the new acquired information has neither drastically changed Cajal's cerebellar circuit nor its neuronal organizational principles. To further sustain this opinion, I should like to finish this review with a short summary of what is known on the development of microzones, to try to demonstrate that their formation is based on the cell theory, because Purkinje cells are absolutely required for the formation of the microzones: they support the neurogenesis of granule cells (Wechsler-Reya and Scott, 1999; Sotelo, 2004), and the formation of projection maps, particularly olivocerebellar maps (Sotelo and Wassef, 1991). Moreover, the inactivation of the *Ptf1a* (*pancreas transcription factor 1a*), a bHLH transcription factor that is required for the production of GABAergic neurons in the cerebellum, induces a phenotype characteristic of a spontaneous mouse mutation called *cerebelless*, a *Ptf1a* mutation affecting the cerebellum but not the pancreas, which prevents the formation of all GABAergic cerebellar neurons, including Purkinje cells, provoking the agenesis of the cerebellum (Hoshino et al., 2005). These data indicate that phylogenetically as well as ontogenetically, the cerebellum appeared as the conjunction of Purkinje cells and extracerebellar afferent fibers, especially climbing fibers. In other words, microzones are dependent of the articulation (in Cajal's sense) of two independent cells: the distal process of the axon of an inferior olivary neuron, and a developing Purkinje cell dendrite. In conclusion, although the newly acquired knowledge has greatly improved our conception of the cerebellar circuit, by adding more complexity to it, I still believe that the so-called holistic interpretations of Golgi with his "diffuse nerve network" were wrong, and that Cajal's law of dynamic polarization still prevails.

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**LEGENDS FOR FIGURES**

**Figure 1:** Drawings by Golgi (A, C) and by Cajal (B, D) for comparison of details and accuracy.

(A) This Golgi drawing illustrates a Purkinje cell with the free termination of its dendrite. Note that the axon, during its course through the granule cell layer, gives rise to at least six recurrent collaterals.

(B) The Cajal's Purkinje cell exhibits two important different features: The distal branches of its dendrite (d) are studded with spines (see the enlargement of the boxed area at the lower right corner), and its axon (a) has only one recurrent collateral (b). Today, it is well established that these neurons only have one to three recurrent collaterals, most often one, which will form the supra- and infraganglionic plexuses lining the Purkinje cell layer, as indicated in Cajal's drawing.

(C) Golgi's interpretation of his diffuse nerve network in the granule cell layer. The descending collaterals of the axons drawn at the top of the figure belong to basket fibers, go beyond the initial segments of the Purkinje cell axons, and fuse with all axons present in the granule cell layer, Golgi cell axons, mossy and climbing fibers.

(D) Composite figure taken from two different drawings of Cajal (the separation between them is marked by the broken line). The one at the upper third illustrates basket cells in the molecular layer and their descending axons in the Purkinje cell layer. Note the brush-like free terminals of these axons, the pinceau formations, which are completely independent from axons in the granule cell layer. The lower two-thirds represent at the right site the mossy fibers and at the left side the relay neurons, the granule cells with their characteristic claw-shaped dendritic endings, specialized to adapt to their presynaptic elements, the mossy fibers.

**Figure 2:** Cerebellar Golgi cell.

(A) Schematic drawing by Golgi of the cortex of the cerebellum illustrating Purkinje cells (brownish color), Golgi cells (black neurons with somata at the Purkinje cell layer) and relay granule cells (the small black neurons over the pink granular layer). The axons of the Purkinje and Golgi cells descend within the granular layer but their trajectories are shortly interrupted. In the white rectangle,

a more detailed Golgi cell is depicted. Note that its axon spreads close to its emergency as interneurons do.

(B) Golgi cell taken from the cerebellum of a heterozygous GAD65-GFP transgenic mouse pup of 16 days of age illustrating its axon (arrow), and its intense branching within the nearby granule cell layer. Note that the axon mimics the disposition indicated by Golgi in his drawing. Scale bar = 20  $\mu\text{m}$ .

(C) Electron micrograph of a freeze-fracture replica of the deeper half of the molecular layer, illustrating a gap junction plaque (asterisk) between the plasmamembranes of two apposed dendrites, which could belong to Golgi cell dendrites (den). Numerous axon terminals (Ax) are surrounding this dendrite. Taken from Sotelo (1977b). Scale bar = 0.5  $\mu\text{m}$ .

(D) Higher magnification of the boxed area in (C) illustrating the gap junction plaque. The fracture plane passes from the P face at the periphery, which contains the clustered intramembrane particles, to the E face with their corresponding pits. Scale bar = 0.1  $\mu\text{m}$ .

**Figure 3:** Cellular and synaptic organization of the cerebellar cortex. The schema, slightly modified from Chédotal (2010), not only includes the two new types of interneurons, Lugaro cells (LC) and the unipolar brush cells (UBC), but also the neurotransmitter used by each class of neurons, and the monaminergic extracerebellar afferent fibers.

**Figure 4:** Electron micrographs illustrating somatodendritic synapses in the cortices of agranular cerebella.

(A) Deeper half of the cerebellar cortex in a rat X-irradiated at birth to destroy all or almost all granule cell progenitors in the external granular layer. The large profile at the left of the micrograph belongs to an interneuron (In), most probably to a Golgi cell perikaryon. The arrow points to a synaptic complex between the interneuronal perikaryon (note the active zone with the presynaptic dense projections and the cluster of synaptic vesicles) and a giant spine (S) of a Purkinje cell dendrite (PD). Taken from Sotelo (1977a). Scale bar = 1  $\mu\text{m}$ .

(B) Cerebellar cortex of the *reeler* mutant mouse. In this micrograph, the perikaryon of a granule cell (GC) is in synaptic contact with a large dendritic

profile most probably belonging to a Golgi cell. The arrow point to the synaptic complex established between the soma and the dendrite, which corresponds to the Gray Type I. The large dendritic profile is also contacted (asterisk) by a non-identifiable axon terminal (Ax). Taken from Mariani et al. (1978): Scale bar = 0.5  $\mu\text{m}$ .

**Figure 5:** Noradrenergic and 5-HT axons innervating normal (A, C) and agranular cerebellum (B, D).

(A) Electron micrograph illustrating two immunocytochemically labeled axon with an antibody against tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis. Both varicosities lack synaptic complexes, even though one of them is apposed (arrow) to small dendritic profile (den) of a molecular layer interneuron, which also receives a synaptic contact (arrowhead) from an unlabeled neuron. Normal mouse cerebellum. Taken from Abbott and Sotelo (2000). Scale bar = 0.5  $\mu\text{m}$ .

(B) Same immunocytochemical staining than in (A), but from the cerebellum of an adult *weaver* mutant mouse. This micrograph illustrates a labeled varicosity between a stellate cell soma (SC) and a Purkinje cell dendritic spine (asterisk). This labeled varicosity lacks synaptic complex despite its apposition to a free spine. Taken from Abbott and Sotelo (2000). Scale bar = 0.5  $\mu\text{m}$ .

(C) Electron microscope radioautograph from the molecular layer of a normal rat cerebellum, which posterior vermal lobe has been topically superfused with a solution of tritiated 5-HT for 3 h. The neuropil contains numerous cross-sectioned parallel fibers, as well as two axon terminals (PF) synapsing on Purkinje cell spines (S). The center of the micrograph contains a labeled varicosity which exhibits the cytological features of 5-HT afferents: mitochondria, large core vesicles (arrowhead), numerous elongated synaptic vesicles and some profiles of the smooth endoplasmic reticulum embedded in a moderate electron dense axoplasmic matrix. Note the absence of synaptic complex between this varicosity and the surrounding elements. Taken from Beaudet and Sotelo (1981). Scale bar = 0.5  $\mu\text{m}$ .

(D) Electron microscope radioautograph from the agranular cerebellar cortex of an X-irradiated rat treated like the control posterior vermis. In this cerebellum, the

labeled varicosity which shares all its inner cytological features with the one illustrated in (C), is in synaptic contact (arrow) with a Purkinje cell dendritic spine (S). Taken from Sotelo and Beaudet (1979). Scale bar = 0.5  $\mu\text{m}$ .

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Figure 1

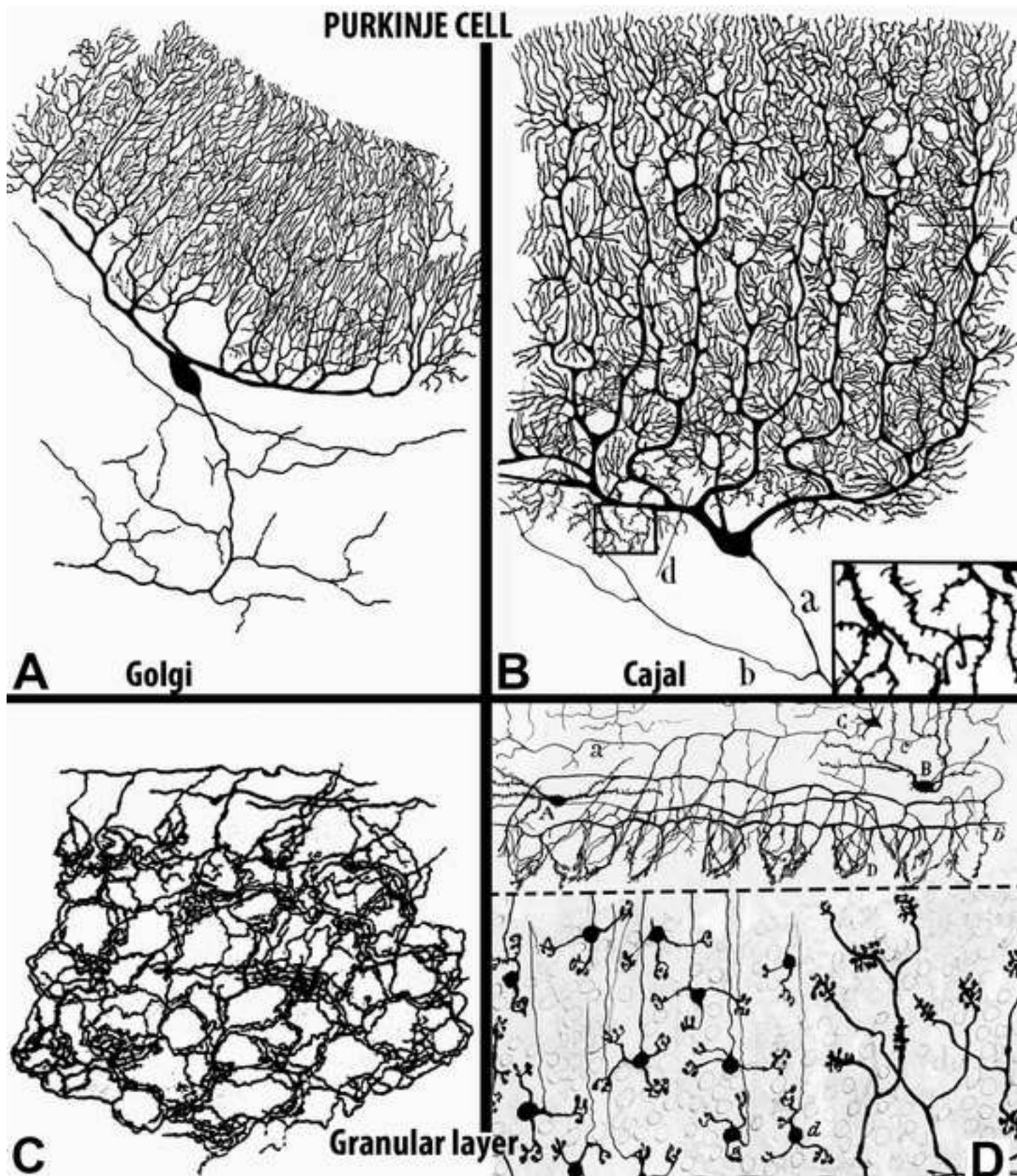


Figure 2

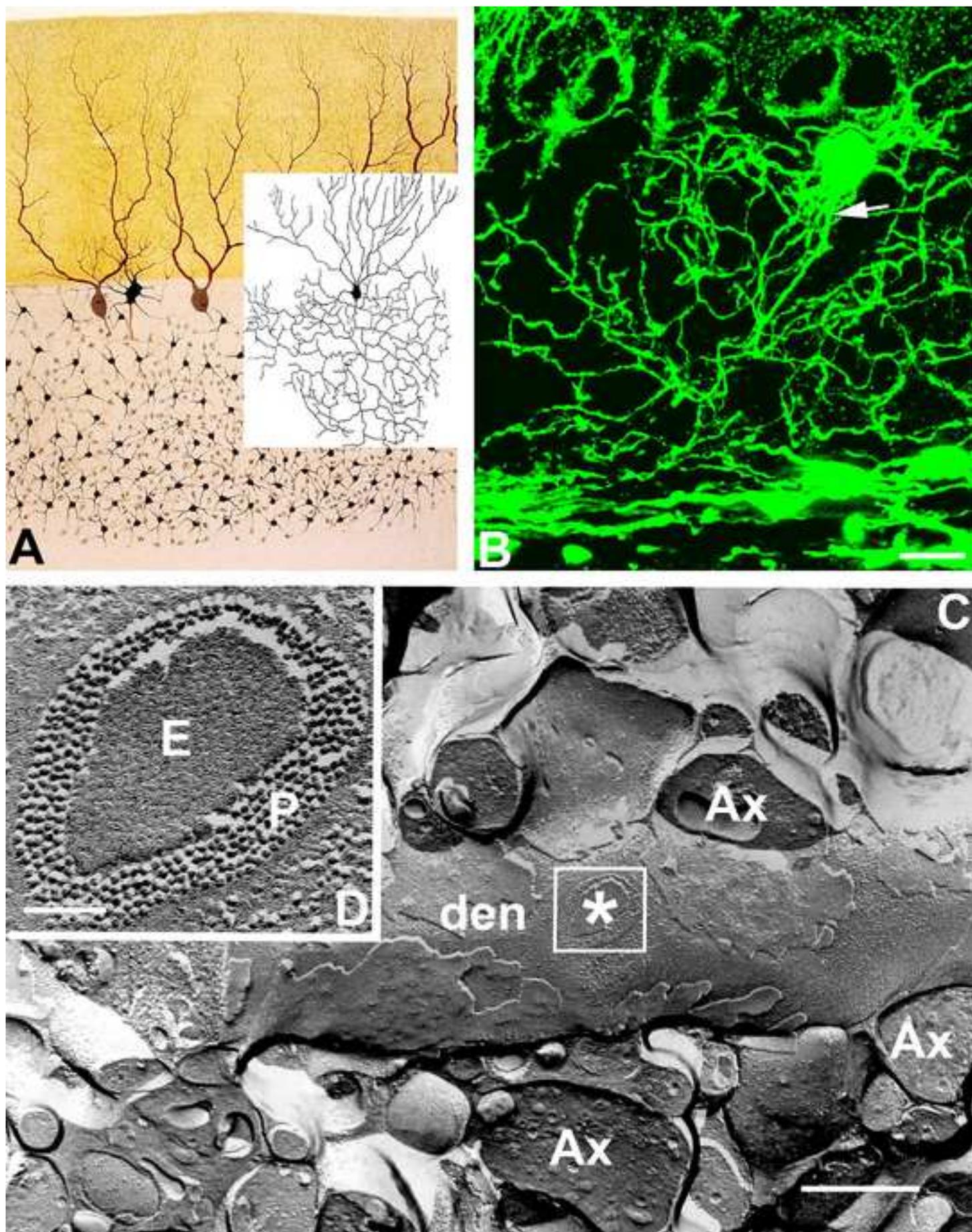


Figure 3

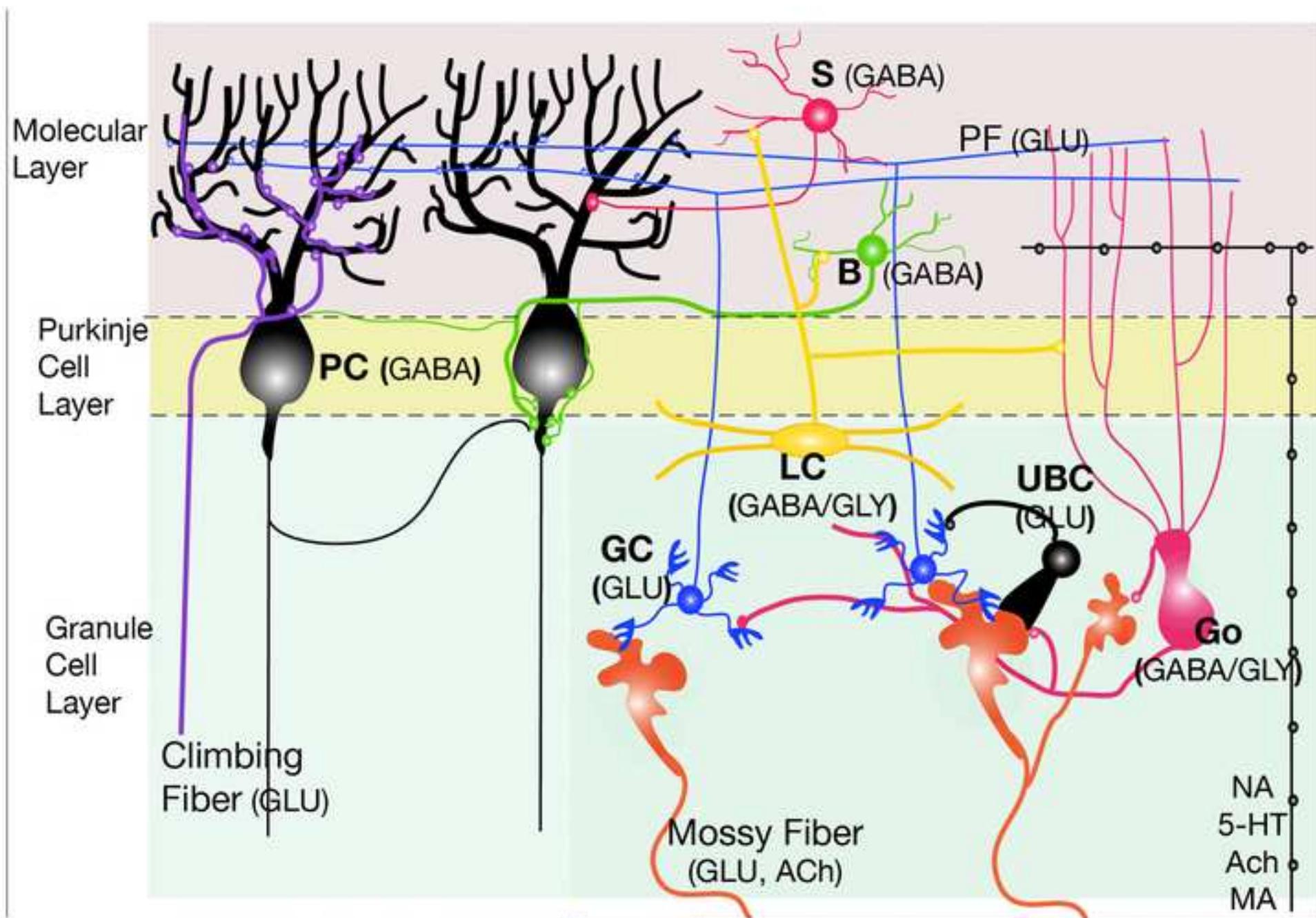


Figure 4

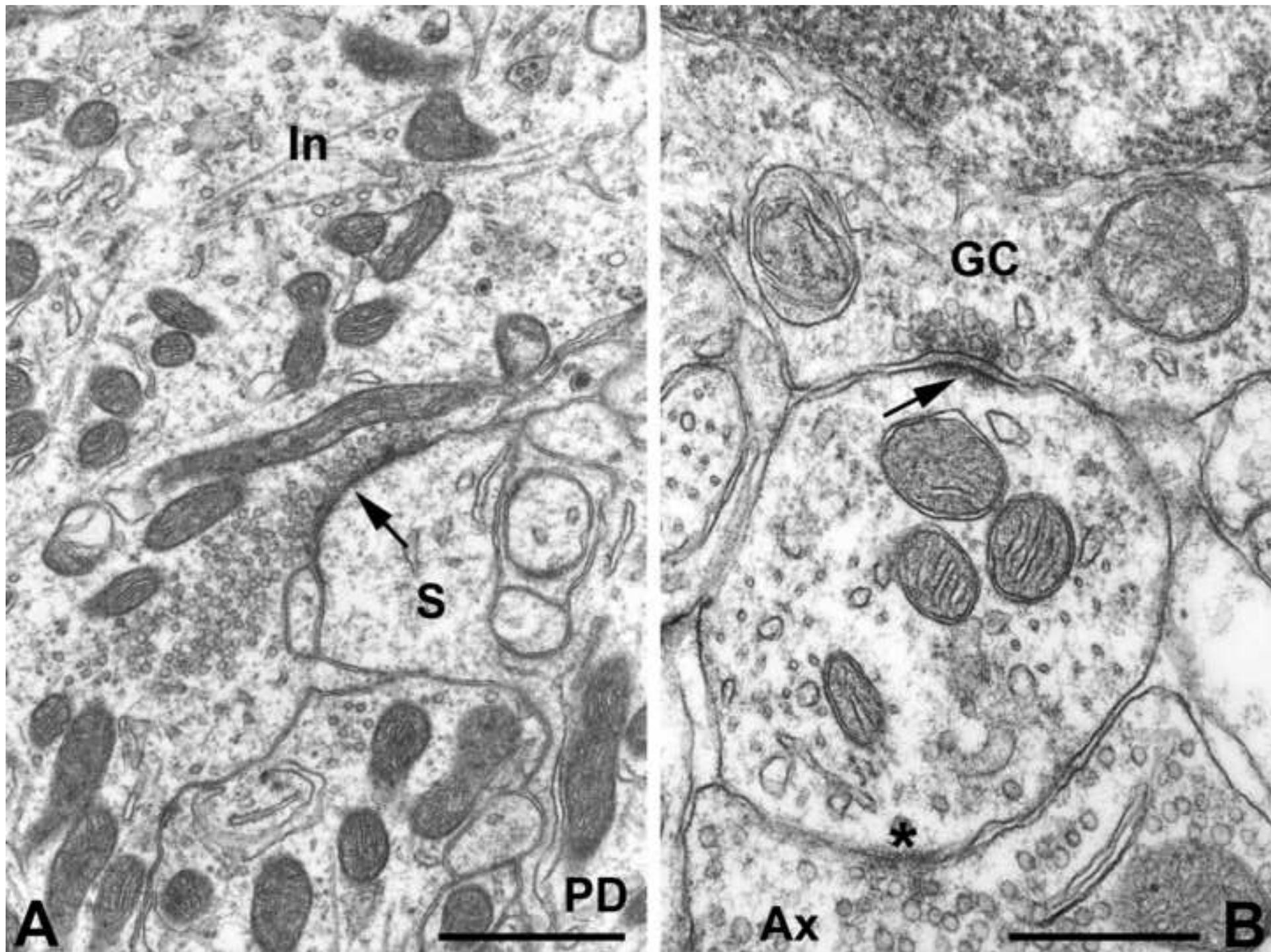


Figure 5

