**“THE GROWTH CONE AS SEEN THROUGH CAJAL’S ORIGINAL
HISTOLOGICAL PREPARATIONS AND PUBLICATIONS”**

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SUMMARY

During the development of the nervous system, each neuron must contact its appropriate target cell in order to establish its specific connections. More than a century ago, Ramón y Cajal discovered an amoeboid-like structure at the end of the axon of developing nerve cells. He called this structure the growth cone [cono de crecimiento] and he proposed that this structure was guided towards its target tissue by chemical substances secreted by the different cells that line its course. We have reviewed the discovery of the growth cone by Cajal using his original publications, his original scientific drawings and by studying his histological preparations conserved at the 'Instituto Cajal' (Madrid, Spain)^A. We found a very good correlation between the structure of the growth cone in the Golgi-impregnated and reduced silver nitrate stained material used by Cajal, and that which is revealed with present-day methods. Finally, Cajal's view of the function of the growth cone and his chemotactic hypothesis will also be considered in the light of present-day knowledge.

^A The Cajal Museum is situated within the Cajal Institute at Doctor Arce 37, Madrid, where most of the items that Cajal himself produced are conserved, such as: histological preparations; scientific drawings; his photographic collection; scientific manuscripts; scientific correspondence; artistic drawings and paintings (Freire, 2003).

INTRODUCTION

Santiago Ramon y Cajal (1852-1934) is one of the most outstanding neuroscientists of all time, to whom a great number of important contributions have been attributed including: the independence of neurons, the theory of dynamic polarization, the neurotrophic theory, the discovery of dendritic spines, the growth cone, the growth club, parallel and climbing fibres in the cerebellum, retinal centrifuges fibres, Cajal's interstitial cells, the Cajal body [cuerpo accesorio de Cajal] (Gall, 2003), etc. He also studied the cell-to-cell connections and basic circuits in practically all the known nervous centres. This immense and exceptional body of work was summarized^B by Cajal in his *opus magnum* "*Histologie du Système Nerveux de l'Homme et des Vertébrés*" (Cajal, 1909)^C.

Cajal started his scientific career in Zaragoza (1877-1883) where he worked as an ancillary professor at the Department of Medicine of the University while he was still studying at the Medical School. He later moved to Valencia (1883-1887) where he was appointed to the chair of Anatomy and finally, he occupied the chair of Histology and Pathological Anatomy at the University of Barcelona (1887-1892) and in the Central University of Madrid (1892-1922). Cajal's first publications on the nervous system appeared when he was in Barcelona and in 1890, he published an article entitled "*Sobre la aparición de las expansiones celulares en la médula embrionaria*" in the *Gaceta Sanitaria de Barcelona*, where he described the growth cone for the first time.

The goal of this article is to study the structure of the growth cone using the original slides prepared by Cajal, taking into account our current knowledge gathered through the use of electron microscopy and of specific fluorescent antibodies. We will discuss the function of the growth cone as proposed Cajal, as well as his chemotactic hypothesis.

^B Cajal tried to add novel information to each chapter. Indeed, in a letter he wrote to Retzius on the 23rd of January 1902 and in relation to the submission of a new version of chapter V of the *Textura* about the cerebellum, he commented "My desire to add some new detail to each chapter has meant that it has required more than 8 months of work for its writing" (C13789, Museo Cajal).

^C This is the second edition in French of the first Spanish edition "*Textura del Sistema Nervioso del Hombre y los Vertebrados*" (Cajal, 1899). For English speaking people there are currently two English editions. The first one comes from the French *Histologie* (Cajal, 1995) and the other from the Spanish *Textura*, adding the modifications incorporated into the French *Histologie* (Cajal, 1999).

THE GROWTH CONE DISCOVERY: CAJAL vs. LENHOSSEK.

The authorship of the discovery of the growth cone has become something controversial as some scientists attribute the discovery to Michael von Lenhossek. Throughout Cajal's literature, he admits that some of his observations or discoveries were also made independently at the same time by other contemporary scientists as the growth of axon sprouts in regenerating peripheral nerve centers, described also by Aldo Perroncito (Cajal, 1923). However, this was not the case with the growth cone which was, as he stated, first described by him.

"It is just to acknowledge that, with the exception of the growth cone, almost all those discoveries were also made independently by Lenhossék, although my communication saw the light of day before his" (Cajal, 1923).

Cajal described the structure, the components and the function of the growth cone, as identified using the Golgi method in the chick embryo, in an article published in Spanish in the "Gazeta Sanitaria de Barcelona" on Aug 10th, 1890 (Cajal, 1890a).

"This fibre is directed from behind in a forward direction... and it ends... in an enlargement that can be simple, rounded and not readily apparent, or a cone-like lump with a peripheral base. This terminal lump, which we shall name growth cone, displays short and thorny divergent processes at times that the silver chromate stains in cinnamon yellow; on other occasions it forms laminar triangular prolongations that seem to insinuate between the other elements, forging with its living force a path through the interstitial cement."^D (Cajal, 1890a).

The very same year (October 20th and November 21st, 1890), Cajal published a longer version of the Spanish article in French in the journal "Anat. Anz." (Cajal, 1890b). In this French

^D "Esta fibra dirígese de atrás adelante..., y se termina... por un engrosamiento ya simplemente redondeado y poco aparente, ya representado por un grumo cónico de base periférica. Este grumo terminal, que llamaremos *cono de crecimiento*, presenta, a veces, finas expansiones cortas, espinosas y divergentes, que el cromato de plata tiñe en amarillo de canela; otras ofrece prolongaciones triangulares, lamíneas, que parecen insinuarse entre los demás elementos, fraguándose a viva fuerza un camino por el cemento intersticial"

publication, a scientific drawing appeared for the first time that showed growth cones from an E4 chick embryo (Fig. 1c).

At the same time, Lenhossék hypothesized that “the ‘free-end’ of the growing nerve cell fiber itself contains the “miraculous energy” that allows the fibers to extend a long certain path” (Bulloch et al., 2002).

“...I would like to express myself in this regard for that hypothesis that situates in the free end of the growing main nerve cell fiber the enigmatic energy that enables to fibers not only to propagate from the limits of the medullar tube to the interior of the soft embryonic tissue by means of the fast capture of new material, but also to extend by this means - perhaps by unequal addition of new materials – along determined paths.”^E (Lenhossék, 1891).

These words appeared in an article of the proceedings of the 10th International Medical Congress celebrated in Berlin, 1890. Among the researchers that participated in this meeting were Lenhossék (Basel), Merkel (Gottingen), Weigert (Frankfurt), but not Cajal. On the 7th of August, in a practical section of the Congress, Lenhossék demonstrated his slides of embryonic chick tissue impregnated by the Golgi method and he used them to explain how the growth of the commissural neurons occurred. As we can see from Lenhossék’s words, there is no reference to any differentiated morphological structure like the growth cone.

GOLGI-IMPREGNATED HISTOLOGICAL PREPARATIONS USED BY CAJAL IN HIS STUDIES OF THE GROWTH CONE

There are 4.529 histological preparations from Cajal’s personal collection that are preserved at the Cajal Museum. Each of them was prepared personally by Cajal, and it was he who performed each of the steps (fixation, cutting, embedding, montage and the labelling of the slides in his own hand). Among these preparations there are examples of many different staining methods, some invented by Cajal such as the reduced silver nitrate (2.054)^F, sublimate gold chloride (200), uranium

^E “...ich möchte mich in dieser Beziehung für jene Hypothese aussprechen, die in das freie Ende des hervorsprossenden Ausläufers selbst die räthselhafte Energie verlegt, die die Faser befähigt, nicht nur durch rasche Aufnahme neuen Materiales über die Grenzen des Medullarrohres hinaus in die zarten embryonalen Gewebe hineinzuwuchern, sondern hierbei auch –vielleicht durch ungleiche Anfügung der neuen Stoffe – bestimmte Bahnen zu verfolgen”.

^F The number of slides is indicated between brackets.

formol-nitrate (29), trichromic methods (4), while others were modified by Cajal or adapted from the methods of others: Golgi-impregnation (809), Ehrlich method (108), ammoniacal silver oxide (160); and methods of other authors: haematoxylin-eosin (200), Nissl (160), carmine (144), eosin (40), Marchi (24), Bielschowsky (22), Loewitt (9) and Cohnhein (4).

Cajal used a great number of young animals and embryos, which can be explained by the fact that Cajal employed the Ontogenic Method since the nervous system of embryos and young animals displays less structural complexity than that found in the adult. Furthermore, he favoured these ages because complete impregnation of the axons of neurons can be obtained since myelin sheaths have still not formed.

We have found 8 histological preparations of the chicken spinal cord that originate from embryos that are 3 to 5 days old, and which gave rise to a total of 383 sections of the dorsal spinal cord (Fig. 1a). We have applied extended-focus digital microphotography to transverse sections of the spinal cord from these slides (Fig. 1b), which produced very similar results to those depicted in the first scientific drawings published by Cajal to show the growth cones of the commissural axons (Fig. 1c). Optic sections were taken from the preparation using a digital camera (DXM1200; Nikon, Tokyo, Japan), a motorized stage (ProScan H128; Prior Scientific, Rockland, MA), and a light microscope (Nikon Eclipse E600).

Structure, components and function of the Golgi-impregnated growth cone.

Cajal distinguished two types of growth cone appendages, the divergent thin short thorny processes today known as filopodia, and triangular laminar ones or lamellipodia (Cajal, 1890a). While silver chromate characteristically stains these processes cinnamon yellow, the axis of the growth cone stains black (Fig. 1d-p). This cinnamon yellow colour is due to the thinness of these processes. (Cajal, 1899).

Later in 1899, Cajal, on the 4th day of incubation, observed that the different shape that the axonal growth cone adopted corresponded to the region where they cross the spinal cord of chick embryo (Fig. 2, Cajal, 1899). In the gray matter, the borders of the growth cone were bristled with laminar appendages and they usually had a longer membranous process in their terminal portion (Figs. 1d-g, 2A). At the level of the ventral commissure where the cone encounters obstacles to its

progress, the base became wider (Figs. 11-p, 2B). The staining of growth cones becomes stronger and darker as they lose appendages when travelling close to the white matter of the ventral funiculus (Fig. 2C). According to his studies, Cajal concluded that the shape of the cone depended on the neighbouring interstices like *"the sealing wax to the relief of a seal"*. Some growth cones reminded Cajal of a webbed appendage, the thin cinnamon yellow lamellipodia corresponding to the interdigital membranes while the black axes of the growth cone were reminiscent of the webbed toes (Fig. 1k, m). From these studies using Golgi-impregnated material, Cajal considered the growth cone as *"a sort of living battering-ram with exquisite chemical sensitivity, rapid ameboid movements and certain propelling force"*. (Cajal, 1899)

REDUCED SILVER NITRATE HISTOLOGICAL PREPARATIONS

We have found 16 histological spinal cord preparations from 2 to 5 day old embryos stained by Cajal's reduced silver nitrate method. When Cajal employed this method to study the growth cone, he used both chick and duck embryos: see the slides of E2 (4)^G, E2^{1/2} (1), E3 (1), E4 (2), E4^{1/2} (1) and E5 (1) chick and E3^{1/2} (2), E3-4 (1) and E4 (3) duck embryos according to the slide's hand-written labels. Each histological preparation may contain between 12 and 38 transversal sections of the spinal cord.

Structure and components of the growth cone stained by the reduced silver nitrate method.

In 1903, Cajal developed a new method to stain neurons that selectively impregnates the neurofibrils of any type of nervous cell, the reduced silver nitrate method (Cajal, 1903). At the same time but independently, Bielschowsky developed a different method to stain neurofibrils based on ammoniacal silver oxide, which was particularly useful to observe these cells in pathological conditions (Bielschowsky, 1903). Earlier, Simarro and Fajersztajn had also developed silver based methods using the reduction of silver by light or with photographic developers, although they produced inconstant results (Simarro, 1900; Fajersztajn, 1901).

The Cajal's method is based in the reduction of a silver solution by a photographic developer. This represented a tremendous advance at that time because it permitted new

^G The number of the slides is indicated in brackets

observations to be made regarding the internal structure of the nerve cell. Moreover, it also overcomes the limitations of the Golgi method with respect to the age of the animal and the nerve cell type. However, this did not mean that the reduced silver nitrate method could substitute for the Golgi method but rather, both methods provide complementary information on neuronal structure.

Cajal also found that the growth cone of E3 commissural cell axons in the chicken spinal cord were stained by his reduced silver nitrate method but the structure was simpler than with the Golgi method (Figs. 3, 4). Neither filopodia nor lamellipodia are stained by the silver reduced nitrate method, and only the neurofibrillar bundle located in the axis of the cone can be seen (Figs. 3c-h, 4).

PRESENT-DAY INTERPRETATION OF GROWTH CONE STRUCTURE FROM CAJAL'S HISTOLOGICAL PREPARATIONS

Comparing the growth cone structure obtained with both the Golgi method and the reduced silver nitrate method, Cajal concluded that the growth cone has two components: the "*neurofibrillar bundle*" located in the axis of the growth cone and a "*special cytoplasm*" unstained by the silver nitrate but eager to take up silver chromate.

Electron microscopy studies revealed that the neurofibrils seen at the light microscope level using Cajal's reduced silver method correspond to both neurofilaments (100 Å diameter) and microtubules (MT, 200-260 Å diameter; Potter, 1971). Electron microscopy of growth cones in the embryonic chick spinal cord, shows that axonal microtubules may continue into the proximal part of the growth cone, but that they are usually absent in the distal segment where a fine network of filaments is located (F-actin, 50-65 Å diameter), which continues into the filopodial cytoplasm (Fig. 4; Skoff and Hamburger, 1974). When studied by electron microscopy, the thick lateral axonal filopodia of cultured dorsal root ganglion nerve cells sometimes contain a single microtubule (Yamada et al., 1971), in accordance with the invasion of the rich-actin-peripheral domain by microtubules described elsewhere (Tanaka and Kirschner, 1995; Sabry et al., 1991). Labeling growth cones with fluorescent antibodies that specifically recognize F-actin, tyrosinated MTs and acetylated MTs (Dent et al., 2003) demonstrates that the distal segment of the growth cone is made up of F-actin, while the proximal segment contains microtubules. Considering these data together, we

conclude that Cajal's reduced silver method only stains the microtubules in the proximal part of the growth cone meanwhile the Golgi method impregnates both the microtubules in the proximal part of the growth cone and the F-actin in the distal domain, although some microtubules may be present in the filopodial appendages (Figs. 2, 4). Interestingly, Cajal's reduced silver nitrate staining is very similar to the pattern observed upon fluorescent labelling of acetylated MTs in the proximal segment of the growth cone (Dent et al., 2003).

GROWTH CONE FUNCTION AND CHEMOTACTIC HYPOTHESIS

Cajal proposed his chemotactic hypothesis at the end of his monograph on the vertebrate retina (Cajal, 1893). This hypothesis was an attempt to explain how a nerve cell develops, forms its connections and displaces its cell body. Without denying the importance of any mechanical influences, Cajal considered that neuroblasts were capable of undergoing amoeboid movements and that they displayed a chemotactic response, activated by substances that are secreted by other nervous, epithelial or mesodermal cells.

When he first presented his chemotactic hypothesis, Cajal did not explicitly mention the growth cone, but in the *Textura* (Cajal, 1899) Cajal describes the growth cone at the tip of the axon of neurons as the part most sensitive to the chemical substances secreted by the spongioblasts. He considers the growth cone as a structure with chemotactic sensibility and rapid amoeboid movements.

According to Cajal, neuronal growth was due to three conditions: mechanical influences; the secretion of attractive substances; and chemotactic sensibility or amoeboidism due to chemical mechanisms. Cajal suggested that the definitive modelling of the dendritic and axonal trees was a dynamic process involving continuous growth and retraction of branches guided by different chemotactic signals:

"It appears therefore, that the innumerable processes and intercellular connections offered by the adult nervous system can be interpreted as the morphologic expression of the infinite routes traced in space by currents of inducing or positive chemotropic substances during the entire developmental period. Thus the total arborization of a neuron represents the graphic history of conflicts suffered during its embryonic life" (Cajal, 1899).

Distinct embryonic cells would successively secrete attractive substances during the earliest stages of their development. The attractive phase or the secretion of chemotactic factors occurs during a brief period in the spinal chord and it coincides with the emission of dendrites by the soma in all directions. Cajal also attempted to explain other particular circumstances that might arise during neuronal development. For instance, Cajal proposed that the initial tilt of commissural axons could be due to the production of attractive substances in the floor plate. Such attractive substances were finally identified as the Netrin family of proteins (Kennedy et al., 1994; Serafini et al., 1994). (Fig. 1, c).

"It would be explained by the production of inducing substances of great force at the level of the ventral half of the epithelial barrel". (Cajal, 1899).

Cajal's chemiotactic hypothesis has a remarkably modern flavour, not only due to the proposed neurotropic factors that are secreted by the targets of axons but also due to the sequential secretion of attractive molecules by different sources to guide axons to their targets. Indeed, these chemotactic factors may be either attractive or repellent, admitting the possibility of a negative chemotaxis (See box 1; Cajal, 1892, 1919). This Cajal's concept of neurotropic substance evolved to a concept of neurotrophic agents in his experimental studies of the degeneration and regeneration of the nervous system (Cajal, 1913, 1914) when he recognized the Schwann cells as the secretors of substances that create the trophic ambient for the growing of axonal sprouts.

"These substances have not only an orientating function, but they are also trophic in character, since the sprouts that have arrived at the peripheral stump are robust, show a great capacity for ramification and grow straight to their target without vacillations, as though they were following an irresistible attraction." (Cajal, 1913-1914).

Nowadays many neurotropic and neurotrophic factors have been described that might influence the outgrowth of axons, especially in the developing chick spinal cord, the same model as that studied by Cajal. (See Box1). The list of neurotrophic and neurotropic molecules has extraordinarily grown since the discovery of the first neurotrophic molecule, NGF: *Nervous growth factor*. (Levi-Montalcini, 1952, Cohen et al., 1954). This list includes neurotrophic factors such as BDNF: *Brain derived neurotrophic factor* (Davies et al., 1986, Barde et al. 1987), NT-3, NT-4/5; *Neurotrophins*, NT-3, and NT4/5 (Berkemeier et al., 1991; Maisonpierre et al., 1990) and their receptors (the trk receptor tyrosine kinases and the p75 neurotrophin receptors) (Kaplan and Miller,

2000; Patapoutian and Reichardt, 2001) and neurotropic substances such as: Netrins (Sestan et al., 1999; Kennedy et al., 1994) /DCC-Unc (Hedgecock et al., 1990; Ishii et al., 1992), semaphorins (Kolodkin et al., 1992; Luo et al., 1993)/plexins-Neuropilin (Chen et al., 1997; Fujisawa and Kitsukawa, 1998; He and Tessier-Lavigne, 1997; Windber et al., 1998) and Slits (Brose et al., 1999; Kidd et al., 1999) /Robo (Kidd et al., 1998; Zallen et al., 1998).

CONCLUSIONS

The structure of the growth cone revealed by the Golgi method and with Cajal's reduced silver nitrate method can now be interpreted in terms of microtubules and actin filaments. The dark central axis of the Golgi-impregnated growth cone is mainly comprised of microtubules while the cinnamon yellow peripheral part and filopodia mainly consists of actin filaments. Cajal's reduced silver nitrate method stains only the microtubules of central part of the growth cone. However, although many stable microtubules remain in the center of the growth cone, this distribution is not so fixed and a population of dynamic microtubules can actively explore the periphery (Kabir et al., 2001; Zohu et al., 2002; Schaefer et al., 2002). These microtubules penetrate the filopodia where they can interact with signaling pathways linked to the cytoplasmic domains of the receptors of guidance cues. The interaction between actin filaments and dynamic microtubules in the peripheral domain may play a role in the motility of the growth cone during axon guidance.

Cajal's exceptional scientific intuition combined with his power of generating working hypotheses from scientific facts make his publications the most interesting reading. In the literature there is usually a tendency to establish a relationship between the growth cone and Cajal's chemotactic hypothesis. For Cajal, this hypothesis was more general and it was an attempt to explain how nerve cell processes develop, how they establish their connections and how they displace cell bodies. All parts of the embryonic neuron are sensitive to attractive factors, although the growth cone might be the most sensitive to such chemical substances. In addition to these chemotactic signals, he also proposed that neuronal activity was one of the principal factors in the modelling of the dendritic tree (Cajal, 1899).

Tremendous advances have been made in this field of the Neurosciences during the 20th century. However, a revision of the original publications of Cajal and testing his original working hypotheses still proves to be a profitable task. Regarding the chemotactic hypothesis he stated:

“It appears that with this hypothesis we have shed light into a dark cave, when in reality we have explored only the entrance, from which its imposing abyss appears even more distant and black. On what bases are mechanical influences guiding the created ameboid streams? Which is the cause of certain preferences of time and location in the distribution of secretory phases? Why does the chemotactic sensitivity cease of decrease in certain periods? These are questions that present day Science can only pose: their clarification, i.e, their total reduction to physico-chemical mechanism, will be the work of the future” (Cajal, 1909).

Box 1 | Pathway of chick spinal cord commissural axons

Commissural axons in the dorsal region are repelled by the *bone morphogenic proteins* (BMPs) secreted by the roof plate (Augsburger et al., 1995; Butler and Dodd, 2003). Accordingly, their axons project ventrally to the mid-line towards a chemo-attractive substance produce by the cells of the floor plate, *Netrin-1* (Kennedy et al., 1994). Another morphogen helps netrin-1 to guide the axons to the mid-line, *Sonic Hedgehog* (Shh: Charron et al., 2003). Once the axons have crossed the floor plate they are repelled by Slit and the ephrins (Brose and Tessier-Lavigne, 2000; Guan and Rao, 2003)

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Figures

Figure 1 | Growth cones in Cajal's histological preparations of the embryonic chick spinal cord. (a), Cajal's original histological preparation impregnated by the Golgi method with the label handwritten by Cajal (Instituto Cajal): "† comisurales completas conos bbb" ["† complete commissural cones bbb"; "b" means "bien" in Spanish, "good" in English], "Pollo 5 días 2 comisurales buenas" ("Chick 5 days 2 good commissurals"). (b), An extended-focus microphotography of two transverse sections of the spinal cord from an E5 chick embryo: growth cones (G) of the comisural neurons, dorsolateral (DI), ventrolateral (VI) and floor plate (Fp). Cajal's first drawing (c) of growth cones (G) in the spinal cord of an E4 chick embryo (published in *Anat. Anzeiger* 5, Fig. 1, p. 610, 1890) commissural neurons (D). (d-p), Extended focus microphotographies of growth cones taken from Cajal's slides. d-g, DI growth cones; h-k, VI growth cones, and l-p, Fp growth cones. The drawing is reproduced with the permission from the heirs of Santiago Ramón y Cajal.

Figure 2 | Growth cones of spinal cord axons from E4 chick embryos visualised with the Golgi method. **A**, cones advancing through the gray matter. **B**, cones located in the ventral commissure. **C**, cones circulating through the white matter of the ventral funiculus. Cajal's drawing published in *Textura del Sistema Nervioso del Hombre y de los Vertebrados*, Volume I, Fig. 186, p. 515, 1899. It is reproduced with the permission from the heirs of Santiago Ramón y Cajal.

Figure 3 | Growth cones in Cajal's histological preparations of the embryonic chick spinal cord. **A**, Cajal's original histological preparation stained with his reduced silver nitrate method as indicated on his hand-written labels: "de 4 días 1/2. medula obliqua simple" ("from a 4 1/2 day-old simple oblique spinal cord"). **B**, Extended focus microphotograph of an oblique transverse section of the spinal cord from a 4-day-old chick embryo showing the growth cones (**G**) of the commissural neurons: dorsolateral (**DL**), ventrolateral (**VL**), and floor plate (**FP**); **C-H**, growth cones (axonal tip at the bottom).

Timeline | The structure of growth cone in the Cajal’s scientific work and its present interpretation.