S. RAMON Y CAJAL, R. G. HARRISON, AND THE BEGINNINGS OF NEUROEMBRYOLOGY

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Santiago Ramón y Cajal (1852-1934)

It is rare that the birth date of a branch of science can be determined rather precisely. The beginnings of modern developmental neurobiology can be traced to the eighties of the last centurv and to two eminent men: the German embryologist and anatomist Wilhelm His (1831-1904) and the Spanish neurologist Santiago Ramón y Cajal (1852-1934). Of course, the development of the nervous system had been studied before, but the foundations of our present view were laid by these men during the years 1886-1890.

Since our focus will be on Ramón y Cajal, he should be introduced briefly to nonneurologists. I rank him among the leading biologists of the last centurv, a peer to Darwin, Carl Ernst von Baer, Pasteur, Johannes Muller, von Helmholtz. He is the founder of modern neurology, which is also the basis of neurophysiology, neuropathology, and physiological psychology. Almost singlehandedly, he unraveled the design of the central nervous system of the vertebrates and man and traced its structure to the most intricate details. Some of his drawings, all of which bear the stamp of his originality, may still be found in modern textbooks, testifying to the amazing accuracy of his observations. His monumental *Histologie du système nerveux de l'homme et des vertébrés* (1904) is still a standard work.

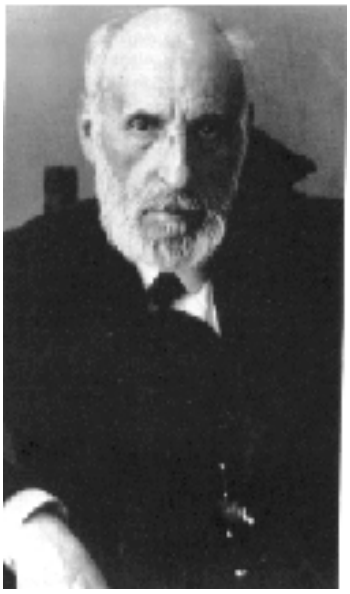

Figure 1. Santiago Ramón y Cajal

The combination of extraordinary conceptual insight and observational power which characterizes his genius were displayed right at the beginning of his work, around 1887 and 1888, in a breakthrough which liberated neurology from a fallacy that had hindered all progress and at the same time set it on the right track. The then-prevailing conception of the structure of the nervous system was embodied in the reticular theory. It envisaged the nervous system as a syncytial network of nerve fibers which were continuous with each other; the cell bodies were considered as trophic elements, at the intersection of the web. The fatal flaw of this theory is obvious: it obviates the establishment of specific pathways and connections, which are the necessary prerequisites of integrated function. Cajal revolutionized the concept of the nervous system by asserting&emdash;and demonstrating&emdash;that nerve fibers are not continuous but contiguous, that they possess terminal structures which contact other nerve cells but do not fuse with them. The contacts are now called "synapses." The hypothesis of contiguity had been proposed independently, then unknown to Cajal, by two Gerrnan investigators, A. Forel and W. His. But, as Cajal points out, their hypothesis, based largely on inferences, does not take us much farther than the reticular theory as long as the possibility of diffuseness of contacts is not ruled out. He states: "To settle the question [of contiguity vs. continuity] definitely, it was necessary to demonstrate clearly, precisely, and indisputably the final ramifications of the central nerve fibers, which no one had seen, and to determine which parts of the cells made the imagined

contacts" [1, pp. 337-338]. The momentous discovery of the synapse was made in l888. During an investigation of the structure of the cerebellum of birds, he observed that terminal branches of the axons of the so-called stellate cells "applied closely to the bodies of the cells of Purkinje about which they form a kind of complicated nests or baskets" [1, p. 330]. Other synapses of different types were observed in rapid succession, and synaptic contact was recognized as a basic phenomenon. Ironically, Cajal's success in demonstrating synapses was based on the method of chrome-silver impregnation of nerve fibers which had been introduced by the Italian neurologist C. Golgi, the major proponent of the reticular theory. The same method, later improved by Cajal himself, enabled him to identify specific nerve centers and specific connections of nerve centers on a large scale.

The idea that individual nerve cells, or neurons, are the basic units of the structure of the nervous system and that axons and dendrites are parts of the neuron became known as the neuron theory. For many years it was pitted against the reticular theory.

But why did Cajal turn to embryos? This was done by deliberate design. His motive is told best in his own words.

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. . . the great enigma in the organization of the brain was the way in which the
nervous ramifications ended and in which the neurons were mutually
connected. Repeating a simile already used, it was a case of finding out how
the roots and branches of these trees in the gray matter terminate, in that
forest so dense that, by a refinement of complexity, there are no spaces in it, so
that the trunks, branches, and leaves touch everywhere.
Two methods come to mind for investigating adequately the true form of the
elements in this inextricable thicket. The most natural and simple
apparently,but really the most difficult, consists ot expioring the tull-grown
forest intrepidly, clearing the glound of shrubs and parasitic plants. and
eventuallv isolating each species of tree, as well from its parasites as from its
relatives. Such was the approach emploved in neurology by most authors.
Such tactics, however, are inappropriate for the elucidation of the problem
proposed, by reason of the enormous length and extraordinarv luxuriance of
the nervous ramifications, which inevitably appear mutilated and almost
indecipherable in each section.
The second path open to reason is what, in biological terms. is designated the
ontogenetic or embrvological method. Since the full grown forest turns out to
be impenetrable and indefinable. whv not revert to the study of the young
wood, in the nursery stage. as we might say.  Such was the very simple idea
which inspired my repeated trials of the silver method upon embryos of birds
and mammals. If the stage of development is well chosen, or, more
specifically, if the method is applied before the appearance of the mvelin
sheaths upon the axons (these forming an almost insuperable obstacle to the
reaction), the nerve cells, which are still relatively small, stand out complete
in each section; the terminal ramificauons of the axis cviinder are depicted
with the utmost clearness and perfectlv free; the pericellular nests. that is the
inrerneuronal articulations, appear simple, graduallv acquinng intricacy and
extension; in sum, the fundamental plan of the histological composition of
the gray matter rises before our eYeS with admirable clarity and precision. As
a crowning piece of good fortune, the chrome silver reaction which is so
incomplete and uncertain in the adult, gives in embryos splendid
colourations, singularlv extensive and constant....
Realizing that I had discovered a rich field, I proceeded to take advantage of it,
dedicating myself to work, no longer merely with earnesteness, but with fury.
In proportion as new facts appeared in mv preparations, ideas boiled up and
josted each other in my mind. A fever for publication devoured me. [1. pp.
323-25]
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His "fever for publications-' produced 12 papers and monographs in 1889 and 16 in 1890, his most productive years. Very soon, what began as a "strategic subterfuge" became an endeavour in its own right, with intriguing problems of its own.

In 1890, Cajal was successful in obtaining splendid silver impregnations of the spinal cord of early (2.5-day) chick embryos [2]. They showed the early stages of differentiation of an embryonic neuron, or neuroblast; that is, cell bodies with a short outgrowth which was identified as the incipient axon. It terminated in a club-shaped thickening with short spikes. The latter were recognized later as filamentous pseudopodia. Cajal designated the terminal structure as the "growth cone." As was stated, the neuron theory asserted that the axon is part of the neuron. The discovery of the mode of origin of the axon was the categorical affirmation of this aspect of the neuron theory. Cajal observed that neuroblasts are polarized in the sense that the site of the outgrowth of dendrites is opposite to that of the axons, and he established the general rule that dendrites differentiate later than the axon. He found the clearest demonstration of neuroblast polarity in the earliest differentiation stages of spinal ganglion neuroblasts: they are at first bipolar, with two outgrowths at opposite ends of the cell. These extensions fuse later at their bases to form the single sensory fiber. The recognition of structural polarity later on became the basis of the theory of physiological polarity. But of all his observations in the field of neurogenesis, Cajal was most intrigued by the growth cone. We shall return to this point later.

To appreciate the fundamental importance of these discoveries, one has to place them in their historical setting. The axon outgrowth

theory had two formidable rivals: the *cell chain theory* of Schwann postulated that nerve fibers are produced by chains of Schwann cells which connect the nervous system with the peripheral organs. The nerve fibers are considered as products of these cells which fuse with each other and with the neuroblasts. More widely accepted was the *plasmodesm theory* of Hensen and Held. It was based on the ubiquity of protoplasmic bridges, or plasmodesms, resulting from incomplete cell divisions. In the original version of Hensen [3], some plasmodesms would be transformed into nerve fibers by functional validation. In the more sophisticated version of Held [4], an approach to the His-Cajal notion of axon outgrowth is evident. According to Held, the axon is built by two components. One is neurofibrillar material spun out by neuroblasts (demonstrable in Held's silver-impregnated material and distinguished by him from the protoplasmic outgrowth described by His and Cajal). The neurofibrils penetrate into plasmodesms, and these intraplasmatic neurofibrils are then transformed into nerve fibers by utilization and incorporation of plasmodesm material. Ramon y Cajal fought all his life battles on two fronts: for contiguity and against continuity in the structure of the nervous system; and for protoplasmic outgrowth, against cell chains and plasmodesms, in the origin of the nerve fiber.

During the crucial years in his career, from 1887 to 1892, Cajal was professor of histology in Barcelona. In this provincial place, he was remote from the mainstream of scientific research and not aware of the work of Wilhelm His, one of the leading German anatomists and embryologists of that time. In 1886 and 1889 [5, 6], His had given a very detailed account of the development of the spznal cord, first in human embryos and then in other vertebrate embryos. He had described the transformation of the neural epithelium into mantle and marginal velum. The neuroblasts were derived from mitotic cells at the inner lining of the central canal. Erroneously, he considered these proliferating "germinal cells" as a special strain of neuroblast precursors, and he derived ependymal layer and glia from the neural epithelium. We know now that the "germinal cells" are merely the mitotic phase of neuroepithelial cells which give rise to both neurons and glia. He coined the terms "neuroblast" and "dendrites." He was the first to describe the transformation of the postmitotic cell into a neuroblast and the formation of axon and dendrites as protoplasmic outgrowths from the neuroblast. Earlier than Cajal. he described the migration of neuroblasts to the periphery, where they form the mantle. He also noticed the originally bipolar configuration of the spinal ganglion cell.

In 1886 he stated the neuron theory very concisely: "I consider it as an established principle that each nerve fiber emerges as an outgrowth from a single cell. This is its genetic, trophic and functional center. All other connections of fibers are either indirect or secondary" [5, p. 5 l 3]. This statement includes implicitly the concept of contiguity as against a network. Cajal did not learn of these findings until 1890, when His sent him copies of his work. From then on, Cajal gives His full credit for his discoveries, but he states explicitly that his own discoveries were made independently of those of His. He adds: "This coincidence in thought on the part of the leading workers in the field, without any oral or written collaboration, constitutes the best moral encouragement and the strongest guarantee of the validity of the adopted interpretation" [7, p. 6].

Yet it is the merit of Cajal to have realized fully the dynamic implications of the outgrowth theory. In this he went far beyond His. I shall turn to this aspect and omit any further references to his substantial contributions to the development of many structures, such as retina, cerebellum, spinal cord, optic tectum [8]. The silver-impregnation method had permitted Cajal the observation of the growth cone which was not discernible in the material of His, treated with ordinary stains. Cajal describes it as a swelling with spiny extensions, sometimes triangular or lamellar, and ramified. In his treatise on histology he gives the following interpretation: "From the functional point of view the growth cone may be regarded as a sort of club or battering ram, endowed with exquisite chemical sensitivity, with rapid ameboid movements, and with certain impulsive force, thanks to which it is able to proceed forward and overcome obstacles met in its way, forcing cellular interstices until it arrives at its destination" [9, p. 599]. In this quotation, two points deserve attention: the uniquely dynamic interpretation of the static microscope slide picture; and the clear visualization of problems of pathfinding which are implicit in the outgrowth theory. Sherrington has an interesting comment on the first point:

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A trait very noticeable in him was that in describing what the microscope
showed he spoke habitually as though it were a living scene. This was
perhaps the more striking because not only were his preparations all dead and
fixed, but thev were to appearance roughly made and rudely treated&emdash;no
cover-glass and as many as half a dozen tiny scraps of tissue set in one large
blob of balsam and left to dry, the curved and sometimes slightly wrinkled
surface of the balsam creating a difficulty for microphotography. He was an
accomplished photographer but, so far as I know, he never practiced
microphotography. Such scante illustrations as he vouchsafed for the
preparations he demonstrated were a few slight, rapid sketches of points
taken here and there&emdash;depicted, however, by a master's hand.
The intense anthropomorphism of his descriptions of what the preparations
showed was at first startling to accept. He treated the microscopic scene as
though it were alive and were inhabited by beings which felt and did and
hoped and tried even as we do. It was personification of natural forces as
unlimited as that of Goethe's Faust, Part 2. A nerve-cell by its emergent fibre
"groped to find another"! We must, if we would enter adequately into Cajal's
thought in this field, suppose his entrance, through his microscope, into a
world populated by tinv beings actuated by motives and strivings and
satisfactions not very remotely different from our own. He would envisage
the sperm-cells as activated by a sort of passionate urge in their rivalry for
penetration into the ovum-cell. Listening to him I asked myself how far this
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capacity for anthropomorphizing might not contribute to his success as an investigator. I never met anyone else in whom it was so marked. [10, pp. xiii-xiv]

Indeed, the climbing fibers climbed and the synapses were "protoplasmic kisses, . . . the final ecstasy of an epic love story" [1, p-373]. Cajal's dynamic view was all-pervasive. For instance, it led him to postulate the polarization of impulse conduction, based solely on morphological data.

As to the second point, his immense intellectual analytical power equals his power of observation. In fact, both are two facets of his creative genius. Whatever he observed took on a meaning transcending the microscope picture. There are few problems on our present-day mind on which he did not reflect at one occasion or another. I shall elaborate on one example, his theory of neurotropism, which deals with the problem of how nerves find their way to their targets. This problem does not exist in the cell chain and plasmodesm theories. Cajal became aware of it when he discovered the growth cone. In his monograph on the retina he mentions for the first time a solution that had occurred to him, in terms of a chemical attraction of the growth cone by substances produced by the target structures (chemotropism):

> How does the mechanical development of the nerve fibers occur, and wherein lies that marvelous power which enables the nerve fibers from very distant cells to make contact directly with certain other nerve cells or the mesoderm or ectoderm without going astray or taking a roundabout course. His has concerned himself with this important question and is of the following opinion: The axis cylinder of the neuroblasts, whether in the medulla or in the mesoderm, always follows the path of least resistance. That resistance is offered by bone, cartilage, connective tissue, etc. which are found along the route of growing nerves. This accounts for the major part of the phenomenon.
>
> Without wanting to deny the importance of such a mechanical influence, especially in the growth of the nerve fibers from the retina to the brain and vice versa, I believe that one could also think of processes like the phenomenon called Pfeffer's chemotaxis, whose influences on the leukocytes was established by Massart and Bordet, Gabritschewsky, Buchner, and Metchnikoff....
>
> If a chemotaxic sensitivity in the neuroblasts is assumed, then it must be supposed that these cells are capable of amoeboid movement and are responsive to certain substances secreted by cells of che epithelium or mesoderm. The processes of the neuroblasts become oriented by chemical stimulation, and move toward the secretion products of certain cells. [11, p. 146]

The discoveries of plant physiologists concerning tropisms (chemotropism, geotropisms, etc.) and taxies figured prominently in contemporary thought. Taxies refer to directed movements of cells and organisms, and tropisms to directed outgrowth of parts, such as roots. Cajal refers specifically to the German plant physiologist W. Pfeffer, who among many other discoveries had described the attraction of sperm in mosses by malate produced by the ovary. Chemotaxis was suggested to Cajal for the first time when he observed the migration of granule cells from the superficial layer in the embryonic cerebellum to deeper layers [8, p. 291]. But the notion of chemotropism came to fruition onlv a decade later in the context of nerve regeneration.

A brief discourse on the status of nerve regeneration at the time when Cajal became active in this field (around 1905) is necessary. In the middle of the nineteenth century, Waller had discovered the degeneration of the distal stump, if regeneration after transection is prevented; and the central stump was recognized as the necessary "trophic center." It was also known that in the case of regeneration, Schwann cells in the distal stump proliferate and penetrate the scar between proximal and distal stump. Cajal encountered here again the unsettled controversy between those who, beginning with Waller and Ranvier, postulated that the regenerated nerve is an outgrowth from the proximal stump, and the adherents of the Schwann cell chain and plastnodesm theories who brought forth the same arguments as in nerve fiber origin in the embryo. In fact, the first decade of this century is marked by a remarkable revival of the old erroneous theories, combined with renewed attacks on the axon outgrowth theory, even in the face of Harrison's tissue culture experiment of 1907 (see, for instance, the treatise of Held [4]). Cajal, applying his silver-impregnation method, had no difficulty in finding growth cones both in the proximal stump and in later stages in the distal stump, thus bringing regeneration in line with axon production in the embryo. I omit again numerous other original findings by Cajal on nerve regeneration. I may mention the observation that, after nerve constriction, strings of beads are formed in the region proximal to the ligature. They were then rediscovered by Weiss and Hiscoe [12] and interpreted correctly as indication of axoplasmic flow.

In the meantime, the chemotropism theory had gotten a foothold in neurogenesis; and since, at the turn of the century, there were no methods available for testing it in the embryo, experimentation was carried out on regenerating nerves. Forssman [13], one of the experimenters in this field, believed that degenerating axons and Schwann sheath produced a chemotropic agent. He coined the term "neurotropism," which was adopted by Cajal, though he believed that Schwann cells (Buengner bands) rather than

degenerating material generate the tropic agent. A representative example of this type of experiment is the following, done by Cajal (fig. 1): The sciatic nerve of a kitten was split longitudinally. One half was transected once, the other half was transected at the same level and also at a more proximal level. In the latter half, the nerve sector between the two cuts degenerated. Six days after the operation, a strong bridge of nerve fibers connected the distalmost cut ends. They originated in the half that had been cut only at that level, and entered the degenerating tubes of the other half, where they grew in proximal direction. This and numerous similar experiments by Cajal, Tello, Forssman, and others were interpreted as evidence for neurotropism (see [14]). However, as was pointed out later by Weiss and others, they can be explained in a different way, that is, in terms of original random outgrowth of fibers in all directions, and survival of those which happened to grow in the direction of the other stump. In fact, at the right distal stump in figure 2, fibers do grow out in all directions.

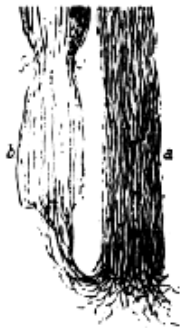

Figure 2. Nerve regeneration in sciatic nerve of a kitten (from [15, p. 317]).

At this point, I wish to follow the theoretical reflections of Cajal, which, though purely speculative, have a bearing on very recent developments. The simplistic statement of neurotropism in 1892, quoted above, was superseded by a very sophisticated version in 1913 [15]. He recognized three basic conditions for successful regeneration: "The nervous reunion of the peripheral stump and restoration, without physiological errors, of the terminal nerve structures, are the combined effect of three conditions: the neurotropic action of the sheath of Schwann and terminal structures; the mechanical guidance of the sprouts along the old sheaths; and, finally, the superproduction of fibres, in order to insure the arrival of some of them at the peripheral motor or sensory regions.&emdash;Of all these conditions the most essential, especially as regards the reconstruction of the terminal apparatus, is the trophism or neurotropism of the peripheral stump, motor plates, and sensory structures" [15, p. 371]. The strange juxtaposition of "trophism and neurotropism' will be commented on presently.

Furthermore, he distinguishes between general neurotropic action on the part of the peripheral stump, guiding nerve fibers toward the target, and specific action guaranteeing the appropriate connection with specific target structures: "The orienting chemical stimuli are probably, so far as their selective power is concerned, both generic and specific.&emdash; The at ractive substance elaborated by the embryonic connective cells and by the cells of Schwann of the peripheral stump have a generic character, acting without distinction on all sprouts; while the attractive substances given out by the spindles of Kuhne, motor plates, cutaneous sensory structures, etc., have a specific character, acting only on certain functional categories of regenerated axons" [15, p. 371]. Apart from neurotropism, his distinction between pathfinding toward the target and specificity of synaptic connections is now generally accepted. His notion of an overproduction of fibers, to insure the safe arrival of some of them at the target, in the earlier quotation has a very modern ring. In fact, he suggests that in synaptogenesis unsuccessful fibers and branches atrophy and unsuccessful neurons disappear, thus anticipating the phenomenon of naturally occurring neuronal death.

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There is no doubt that, at first. many imperfect connections are formed, and
that many duplicarions and errors of distribution occur. But these
incongruences are progressively corrected, up to a certain point, by two
parallel methods of rectification. One of these occurs in the periphery, and is
the atrophv through disuse of superfluous and parasitic ramifications, in
combination with the gro vth of congruent sprouts. The other occurs in the
ganglia and spinal centres; bv this there would be a selection, due to the
atrophy of certain collaterals and the progressive disappearance of
disconnected or useless neurones, of the sensorymotor fibres capable of being
useful. [15, p. 279]
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He even anticipates our present idea of a process of competition (for a synaptic site, or for a trophic agent) which figures prominently in our search for the explanation of naturally occurring neuronal death. ". . . It is only those expansions which are able to establish useful relations with afferent nerve fibres which survive in this contest for space and connections. In nervous regeneration this process of hyperformation is repeated" [l5, p. 278].

I was particularly intrigued by the refinement of the original notion of attraction at a distance. In his later view, the distal stump (and, more specifically, the Buengner bands) would release an agent whose funuion is to stimulate metabolism and assimilation in the sprouting axon growth cones. What I regard as a novel conceptualization is the combination of the idea of a trophic action with trotnsm&emdash;that is, directional growth&emdash;to which I had called attention in an earlier quotation. The following paragraph clarifies what he has in mind: "The neurotropic stimulus acts as a ferment or enzyme, provoking protoplasmic assimilation.... While in the present state of knowledge we cannot penetrate the mechanism of the neurotropic action, an analysis of all the facts of nervous reunion known to us suggest the hypothesis that the orienting agent of the sprouts does not operate thsough attraction, as many have supposed, but by creating a region that is favourable, eminently trophic, and stimulative of the assimilation and growth of the newly-formed axons" [15, p. 372]. In other words, he envisages the production, by the target, of a trophic agent which stimulates growth in the growth cone and then, so to speak, nurses the axon along toward the target. I shall come back to this point presently.

Ingenious as it was, the neurotropism theory has not fared well in recent decades. It is true that not very extensive efforts have been made to test it and that practically all experiments, both in vivo and in vitro, to that effect have given negative results. Admittedly, the regeneration experiments of Cajal, Forssman, and others are not conclusive, as I have pointed out above. But the negative results of Weiss and others are not a final verdict either. When such efforts are unsuccessful, one can always raise the question of whether the experimental design was sufficiently subtle. Anyway, neurotropism has been pronounced dead as recently as 1976 [16].

As it happened, the deceased was resurrected in the same book by R. Levi-Montalcini [17]. She had discovered a case of neurotropism in the central nervous system. In order to test the claim of Swedish investigators that transected axons of monoaminergic neurons in the brain stem of young rodents can be stimulated to sprouting by Nerve Growth Factor (NGF), a nerve-growth-stimulating protein, she injected NGF into the medulla of newborn rats, near the locus coeruleus. She observed a conspicuous enlargement of the sympathetic chain ganglia on the side of injection and a massive invasion of sympathetic fibers through dorsal roots to the site of injection (figure 3). Histofluorescence treatment demonstrated the passage of these fibers in the dorsal funiculus. They did not innervate any particular structure and disappeared when NGF injection was discontinued.

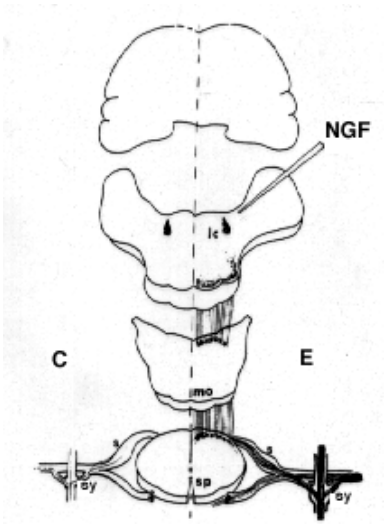

Figure 3. Chemotropic attraction of sympathetic fiber bundles to NGF injected intracerebrally into neonatal rats (from [17, p. 244]). C=control side; E=experimental side; lc= locus coeruleus; mo=medulla oblongata; s= sensory ganglia; sp= spinal cord; sy= sympathetic ganglia.

Of particular interest is her interpretation of this phenomenon which, *mutatis mutandis*, comes remarkably close to what Cajal had envisaged to be a tropic-trophic mechanism. I quote from a recent publication: "The entrance of sympathetic nerves into the CNS of neonatal rodents injected intracerebrally with NGF should however not be regarded as evidence of an attraction at a distance produced by the high NGF concentration gradient in the neural tube of the experimental mice and rats." (One remembers Cajal's statement that 'the orienting agent does not operate through attraction as many have supposed.") "The direct access of NGF to the sympathetic ganglia through the motor and sensory roots is clearly indicated by the hvpertrophic and hyperplastic effects elicited by the intracerebral NGF treatment. The same roots which served as transport channels and are presumably imbued with NGF provide in turn most convenient routes for the sympathetic fibers which engage in these paths and . . . gain in this way entrance into the CNS.... The axonal tip of the fibers moves along gradients of diffusion of trophic and tropic factors released by end organs.... Neurotropism would assist, rather than determining the course of nerve fibers toward their correct destination" [18, pp. 79-80]. The linkage of the words "trophic and tropic" by Cajal in 1913 and Levi-Montalcini in 1978 is startling; one is reminded again of what Cajal had to say of independent discoveries. What was envisaged by Cajal, by pure reflection, namely that a growth promoting agent, released by the target, imbues the intervening tissue and guides the growing tip of the axon along to its source, seems now to

be demonstrated in a controlled experiment. NGF has long been established as a trophic agent. If a tropic function can be added, then the revival of Cajal's idea that trophic and tropic may be two sides of the same coin could become an important new model in modern developmental neurobiology. Indeed, it is very difficult to be original in neurogenesis with Cajal looking over one's shoulder.

Ross Granville Harrison (1870-1959)

Figure 4. Drawing of R. G. Harrison published in Purves, D. and Lichtman, J. W. 1985. *Principles of Neural Development.* Sinauer Associates, Sunderland.

Harrison's major contribution to biology in general and to neuroembryology in particular is the invention of the tissue culture method. It will be remembered that in his classical experiment he isolated pieces of the neural tube of a frog embryo and reared it in frog's Iymph, in a depression slide. What links this experiment directly with Ramón y Cajal is the fact that it was motivated by the same concerns which preoccupied Cajal: to find a direct test for the axon outgrowth theory. Of equal importance is another major contribution of Harrison: Almost singlehandedly, he introduced the analytical experiment, that is, microsurgry on the embryo, as a tool for the exploration of neurogenetic problems. These two achievements would have been sufficient to rank him among the leading experimental biologists of the first half of the century. If one adds the solution of another fundamental problem, the origin of axial polarization and bilateral symmetry in vertebrates by ingenious experiments, then it is hard to understand that he did not share the Nobel Prize with Spemann, in 1935, as had been expected. In fact, he was proposed twice: first in 1914, but the prizes were suspended on account of the war, and then again in 1933. According to the official account of the Nobel Committee, in a special committee in 1933 "opinions diverged, and in view of the rather limited value of the method and the age of the discovery, an award could not be recommended" [19, p. 259]. What was actually of limited value was the judgment of the committee, and not Harrison's achievements. I knew Harrison well. He was a frequent summer visitor in the laboratory of his friend H. Spemann, in Freiburg, where I took my Ph.D. and then advanced to an instructorship. After a vacation in the Swiss mountains, he would occasionally spend a few weeks in Freiburg. Since the interest of Spemann in the nervous system ended with the closure of the neural tube and that of Harrison began at that stage, l got more help in my neuroembryological work from him than from Spemann.

Communication with Harrison was easy. He spoke German fluently, he had spent several pre- and postdoctoral years in Bonn, where he obtained an M.D. in 1899; he had published some of his early papers in German, he had a German wife&emdash;in short, he was at home in Germany. He was informal, unassuming, soft-spoken, and reserved; he had a good sense of humor. And he had an amiable human trait, the capacity for procrastination.

Harrison had been a graduate student in one of the best graduate schools for zoology of that time, at the Johns Hopkins University. W. K. Brooks was outstanding in embryology and remarkable for the number of prominent men who were his students. Among Harrison's fellow graduate students were T. H. Morgan and E. G. Conklin. He obtained his Ph.D. in 1894 and became then a staff member in the Anatomy Department under F. P. Mall, who was well known as a human embryologist. In 1907 he was called to Yale, and soon its Zoology Department became one of the most prominent in the country. Yale and Freiburg shared the reputation of being the leading centers of experimental embryology. For his students, most of whom became his friends, Harrison was "the Chief." He was influential in raising the standard of excellence at Yale, both in the sciences and in the medical school. For many years, he was the managing editor of theJournal of Experzmental Zoology, the most prestigious in its field. He was not particularly enthusiastic about teaching or administration. Most administrative chores were handled by his student, later colleague, and guccessor, J. S. Nicholas. Harrison's due place was in the research laboratory.

One of Harrison's first experiments was based on an experiment of a young German anatomist, G. Born. In 1894, Born had

discovered by chance that parts of frog embryos when cut apart could be.healed together again. Taking advantage of this extraordinary healing power, he had been able to fuse parts of embryos of different genera, such as frog and toad. This method of "xenoplastic" combinations, made possible by the absence of immunological barriers in embryos, was used widely by Harrison and Spemann. Harrison was the first to apply this kind of experimentation on the embryo to neurogenetic problems. In one of his earliest experiments, he employed the method of Born to demonstrate the mode of origin of the lateral line sense organs of aquatic vertebrates. These are sensors for water perturbations; they are evenly spaced in several rows in the head and along the trunk and tail. Each sense organ consists of sensory hair cells and supporting cells; those of the head are innervated by a branch of the facial nerve and those of the trunk and tail by a branch of the vagus nerve. Harrison ingeniously took advantage of species differences in pigmentation. He fused the darkly pigmented head of an early embryo of *Rana sylvatica* with the body of a lightly pigmented *R. palustris* embryo. He observed in the living composite tadpole the step-by-step deposition of dark spots, identified as embryonic lateral line sense organs, from tissue that emerged from the dark head and moved in several rows down the yellowish trunk and tail. He had thus uncovered a peculiar, unique long-range migration of cell clusters that followed prespecified paths, as was shown by variants of the experiment [20].

The major contributions of Harrison to experimental neurogenesis were motivated by the controversy between the axon outgrowth theory of His-Cajal and the cell chain and plasmodesm theories of the origin of the nerve fiber. In the first decade of this century, when Harrison became active, the plasmodesm theory had regained ground, and even support for Schwann's cell chain theory had not subsided completely. Harrison had become convinced of the correctness of the outgrowth theory in his earlier work on neuroblast differentiation in the salmon embryo and, like Cajal, he set out to put the competing theories to a test. It was clear to him that the best histological techniques could not solve the problem, so he took the crucial step of applying the powerful tool of experimentation to its solution. First, he took on the relatively easier task. He addressed the question, Is nerve fiber formation dependent on Schwann cells? Assuming that the Schwann cells originate in the neural crest, he removed the dorsal part of the neural tube and the adjacent neural crest, in early tail bud stages of frog embryos. He found that in the tadpole normal ventral roots and motor nerve fibers had developed which were naked and devoid of any cellular companions. Hence, the independence of nerve fiber formation from Schwann cells was proven. Furthermore, the then controversial question of the origin of Schwann cells was settled in favor of the neural crest, at least for frog embryos. These experiments date back to 1904 and 1906 [21] .

Harrison turned next to the problem of the role of protoplasmic bridges in nerve fiber formation. We remember the claim of Hensen that the substance of plasmodesms is actually incorporated in the formation of nerve fibers. As was mentioned, this theory had been revived by Held and others, and the opinion of leaders in the field was divided. Experiments by the German anatomist H. Braus, in which limb transplantations were used for the first time to address neurological questions, had been interpreted by the author in support of the plasmodesm theory [22]. In the spring of 1906, Harrison repeated the experiments; and his findings, which I shall not describe in detail, led him to a rejection of the claim of Braus [23]. Two points deserve mentioning. First, this was Harrison's first experience in the transplantation of limb primordia, an experiment which was to preoccupy him in his later work devoted to problems of regulation and determination of laterality. Second, Braus had found that the nerves in the limb transplants formed a normal pattern. This was to be expected if plasmodesms in the limb are transformed into nerves. But in Harrison's reinterpretation of limb innervation in terms of the axon outgrowth theory, the fact that ingrowing nerves from any source form a typical limb pattern can be interpreted only in one wav: "that the structures contained within the limb must have a very important directive action upon the developing nerve fibers, in that they determine their mode of distribution" [23, p. 276]. The contribution of the limb structures to pathfinding and patterning of their innervation is basic to an understanding of directional nerve outgrowth.

Yet, Harrison realized clearly "that in all of the first experiments the nerve fibers had developed in surroundings composed of living organized tissues, and that the possibility of the latter contributing organized material to the nerve elements stood in the way of rigorous proof of the view that the nerve fiber was entirely the product of the nerve center. The really crucial experiment remained to be performed, and that was to test the power of the nerve centers to form nerve fibers within some foreign medium which could not by any possibility be suspected of contributing organized protoplasma to them" [24, p. 790]. At another point? he said: "In order to reach a final settlement of this question, it thus became necessary to devise a method by which to test the ability of a nerve fiber to grow outside the body of the embrvo, where it would be independent of protoplasmic bridges" [25, p. 402]. The decisive step had been taken in 1907. Pieces of neural tube of frog embryos, prior to nerve outgrouth, were grown in a hanging drop of frog's Iymph. The outgrowth of individual fibers and their growth cones was observed under the microscope; the rate of growth was determined, and the important fact was established that nerves require a solid substrate for extension. Thus the plasmodesms, or, for that matter, any microscopic or submicroscopic materials in the embryo, are assigned their proper role: they serve for guidance but do not contribute materially to the formation of the nerve fiber.

It is clear that the design of the tissue culture method was the logical final step on the long road toward the solution of the problem of the origin of the axon. The immediate purpose was the crucial test of the plasmodesm theory. But, as the title of the detailed report of the tissue culture experiments in 1910 [24] indicates, the emphasis is shifted immediately from the critical to a positive aspect. The phenomenon of "the outgrowth of the nerve fiber as a mode of protoplasmic movement" is placed in the center of the scene. This, we remember, was a key element in Cajal's appraisal of the growth cone. Harrison states "the primary factor,

protoplasmic movement, must be regarded as definitely established and it will have to form the basis of any adequate theory of nerve development. The chief claim to progress that the present work has is that it has taken this factor out of the realm of inference and placed it upon the secure foundation of direct observation." And he goes a step further and fits this discovery in a broader frame of reference: "the first manifestations of activity observable in the differentiating nerve cell are of the same fundamental nature as those found not only in other embryonic cells but also in the protoplasm of the widest variety of organisms" [24, p. 840]. Thus, the tradition of Cajal's dynamic view of the growth cone was continued and it became a reality. The later discovery of axoplasmic transport [12] follows the same tradition.

It may seem strange that while the tissue culture method opened up a new field of knowledge and became an indispensable tool in a very broad range of biological endeavors, Harrison himself never made use of it again. The answer suggests itself to those who knew Harrison. The method was designed by him to solve a specific problem&emdash;which it did. The time was not ripe for analysis of protoplasmic movement in depth. He became intrigued by other fundamental problems and turned to their solution. His primary concerns were the basic theoretical issues in embryology and not the exploitation of what he called a technique. He made decisive contributions to the analysis of a key phenomenon in animal development, the "morphogenetic field" [26] and, as was stated earlier, he solved one of the most difficult problems in embryology, the origin of bilateral symmetry, which is a basic morphological attribute of vertebrates [27]. This led him to the consideration of the polarization of the three main axes, rostro-caudal, dorso-ventral, medio-lateral, in terms of molecular repeat patterns of protein molecules. He actually went to Leeds, to the laboratory of the great biophysicist V. T. Astbury, and they published jointly a paper on X-ray diffraction pictures of embryonic materials [28], a premature step in the direction of molecular embrvology. The fact that this enterprise was doomed to failure at that time is less important than the insight it gives in the train of thought of a truly great scientist who was far ahead of his time. His later achievements fully justify the abandonment of his gifted brainchild that was born in 1907 and is still very much alive and thriving.

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