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VIKTOR HAMBURGER AND RITA LEVI-MONTALCINI: The Path to the Discovery of Nerve Growth Factor

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■ **Abstract** The announcement in October 1986 that the Nobel Prize for physiology or medicine was to be awarded to Rita Levi-Montalcini and Stanley Cohen for the discoveries of NGF and EGF, respectively, caused many to wonder why Viktor Hamburger (in whose laboratory the initial work was done) had not been included in the award. Now that the dust has settled, the time seems opportune to reconsider the antecedent studies on the relation of the developing nervous system to the peripheral structures it innervates. The studies undertaken primarily to investigate this issue culminated in the late 1950s in the discovery that certain tissues produce a nerve growth–promoting factor that is essential for the survival and maintenance of spinal (sensory) ganglion cells and sympathetic neurons. In this review, the many contributions that Viktor and Rita made to this problem, both independently and jointly, are reexamined by considering chronologically each of the relevant research publications together with some of the retrospective memoirs they have published in the years since the discovery of NGF was first reported.

This review is dedicated to Viktor Hamburger on the occasion of his 100th birthday on July 9, 2000, and to Rita Levi-Montalcini to mark her 91st birthday on April 22, 2000, with admiration and affection.

INTRODUCTION

The announcement in October 1986 that the Nobel Prize for physiology or medicine was to be awarded jointly to Rita Levi-Montalcini and Stanley Cohen for their discoveries of nerve growth factor (NGF) and epidermal growth factor (EGF), respectively, was greeted by most developmental biologists with enthusiasm but also with some misgiving. On the one hand there was a general sense of elation that developmental biology had again been recognized in this prestigious and very public way. On the other hand, many developmental neurobiologists felt that by not including Viktor Hamburger, the Nobel committee had failed to appreciate the significance of his earlier contributions that had paved the way to the discovery

of NGF. As several of those familiar with the NGF saga pointed out, his direct involvement in the discovery appeared to have been overlooked: Not only was the work carried out in his department at Washington University, but it was through his efforts that Rita Levi-Montalcini, and later Stanley Cohen, were brought to his department. Moreover, his name had appeared as a coauthor on the first reports of a diffusible substance that promoted the growth of sensory and sympathetic neurons (subsequently identified as NGF). The comments of Dale Purves and Josh Sanes, two of Viktor's colleagues at Washington University, were typical of those who felt this way. "Many neuroscientists are puzzled by the omission of Viktor Hamburger from the prize," they wrote, "because his exclusion tends to obscure a line of research that now spans more than 50 years" (Purves & Sanes 1987). This feeling extended well beyond St. Louis: As reported in the *New York Times* of October 14, 1986, Dr. Jean Lauder of the University of North Carolina, at the time President of the International Society of Developmental Neuroscience, said that she and others were considering writing to *Science* and *Nature* to express their view that Hamburger should have shared the Nobel Prize.

The fact that prior to the announcement of the award, Viktor (as Hamburger is known to virtually everyone in developmental biology and neuroscience) and Rita (as Levi-Montalcini is generally referred to in the United States) had always been closely associated and mutually supportive made the Nobel committee's decision especially puzzling to their friends and colleagues in this country and abroad.[1] Before 1986, Viktor and Rita had written a number of reviews and personal memoirs in which they had each acknowledged their close scientific association and the importance of their independent and joint contributions to the work that preceded the discovery of NGF. For example, in a volume of essays published on the occasion of Viktor's 80th birthday, Rita wrote at length about her discovery during World War II of Viktor's seminal paper on the effects of early limb removal on the development of the motor columns in chicks (Hamburger 1934) and how this discovery had served as the impetus for much of her own work with her mentor Giuseppe Levi. She went on to describe how excited she had been when Viktor invited her to spend a year in his department and how graciously she had been treated on her arrival in St. Louis. She especially commented on how helpful Viktor had been in assisting her in writing her first papers in English. Touchingly, she ended her essay with the following token: "Viktor, we can look at this work, at our friendship, at the past we so much enjoyed, and at the future, that may or not materialize, in a *sub-specie-aeternitas* frame of mind, in a crystal-clear atmosphere uncorrupted by the turbulence of human passions and sorrow" (Levi-Montalcini 1981). The same good feeling is evident in Rita's dedication of her review (Levi-Montalcini 1982) entitled "Natural History of Nerve Growth Factor" that appeared in the 1982 volume of the *Annual Review of Neuroscience* "to Viktor Hamburger."

[1]That Stan Cohen was specifically recognized for the discovery of EGF was universally judged to be appropriate—as was linking his name with Rita's since he had also played a critical role in the isolation of NGF.

Viktor was equally complimentary in his remarks about Rita, acknowledging the combination of neuroanatomical expertise, experimental skill and intuitive insight that she brought to their joint work, and the energy and drive with which she pursued the isolation and testing of NGF.

Unfortunately, the omission of Viktor from the Nobel Prize resulted in a sea change in their relationship. This was marked by the appearance in the popular press and elsewhere of a number of unnecessarily critical and insensitive remarks by both Viktor and Rita. The resulting rift in their once harmonious and mutually supportive relationship has caused some of their colleagues to feel compelled to "take sides." This, in turn, has had the effect of clouding the actual history of the events that led to the discovery of NGF.

It is not my purpose to dwell on the rift between Viktor and Rita that developed following the awarding of the Nobel Prize. Rather, the purpose of this review is first to reexamine Viktor's contribution to the central problem of the relationship between the developing central nervous system and the peripheral structures it innervates, and second to consider the impact that Rita had on this work from the time she joined his laboratory in 1947 and culminating in the discovery of NGF and the elucidation of its role in the development of the sympathetic nervous system and the spinal (sensory) ganglia. To place their separate and joint work in its historic context, I have considered at some length each of the relevant studies and have included brief biographical accounts, which are largely based on their own published memoirs.

VIKTOR HAMBURGER: A Brief Biographical Background

Viktor was born in the small Silesian town of Landeshut on July 9, 1900. After graduating from the Gymnasium, he spent two summers at the University of Heidelberg, where his aunt was a senior assistant in the Zoological Institute. It was here that his interest in developmental biology was stimulated by taking an advanced course in experimental biology taught by Curt Herbst. This, in turn, led him to Freiburg (where, as he has remarked, the skiing and climbing were much better than at Heidelberg) and to the zoology department of the University of Freiburg. Here Hans Spemann, the head of the department, had assembled an outstanding group of students and junior faculty, including Otto Mangold, Hilde Proescholdt (soon to be Hilde Mangold), and Johannes Holtfreter. Not surprisingly, much of the interest in the department at the time was centered on the mechanism of primary induction and the role of the dorsal lip of the blastopore as the "organizer" (Spemann 1938).

Spemann discouraged Viktor from working on this problem, remarking that "there are already too many people hanging from the lip of blastopore" (Cowan 1981). Instead Viktor was assigned to reexamine an earlier report by Dürken to the effect that early eye removal in frog larvae could lead to a variety of limb abnormalities, which were thought to result from a cascading series of effects

consequent upon the denervation of the optic tectum (Dürken 1913). Though today this project may seem an unlikely beginning to a career, in the 1920s the possibility that eye removal could affect the development of the limbs seemed no less improbable than that the transplantation of a small piece of tissue from the blastopore could lead to the formation of a second embryo. In the event, Viktor showed that the limb abnormalities Dürken had observed were almost certainly due to the poor conditions under which the larvae were raised (Hamburger 1925). However, this experience later led Viktor to explore more fully the relationship between the developing nervous system and the limbs, and to the study of limb development following removal of portions of the spinal cord.

After completing his PhD in 1925, Viktor spent a year in Göttingen in the laboratory of Professor Alfred Kühn, who worked on pigmentary patterns such as the eye spots in butterflies and moths. Kühn suggested to Viktor that he examine color vision in fish with his senior assistant, Karl Henke This was to be one of Viktor's few departures from the study of limb innervation prior to 1960. It was during this time also that he became reacquainted with Marthe Ficke, whom he had first met in Freiburg and to whom he was married in 1928.

In 1926, Viktor was offered and accepted an assistantship in Otto Mangold's department of experimental embryology at the Kaiser Wilhelm Institute for Biology in Berlin-Dahlem. During his brief stay in Mangold's department, he carried out an extensive series of experiments on the development of the hind limbs in frogs (*Rana temporaria*) from which the relevant segments of the neural tube had been resected on one or both sides. Despite the problem of cord regeneration in the hemiextirpation experiments, and the problem of the swimming impairment in the animals with bilateral lesions, enough cases survived through metamorphosis to allow him to make a detailed analysis of the fate of the skeletal and muscular components of the nerveless limbs. In every case the skeleton, and (initially) the muscles, appeared perfectly normal. In those cases in which the spinal cord had partially regenerated, the peripheral nerves (to the extent that they were formed) seemed to follow the same basic pattern as in control preparations (Hamburger 1928, 1929). As Viktor was to return to this problem later, when he studied aneurogenic limbs in chicks, I defer further comment on these early studies except to note two things. First, the experiments were conducted with the same care and the results analyzed with the same thoroughness that were to be the hallmark of his later work. Second, these studies provided the definitive proof that Dürken's hypothesis—that the development of the limbs was in some way under the direct control of the central nervous system—was wrong.

In 1927, Spemann offered Viktor what was in effect an instructorship in his Institute, and it was with a sense of relief that he returned to Freiburg, even though it lacked the cultural amenities he had enjoyed in Berlin. His principal responsibilities were to teach introductory and advanced laboratory courses in experimental embryology. However, with the rather limited time he had available for research, he continued working on aspects of developmental genetics that he had begun in

Berlin, under the influence of Goldschmidt and his colleagues. It was his hope that it might be possible to apply the approaches of contemporary genetics to some of the problems of amphibian embryology, although he recognized that this was a formidable task: Amphibians were not exactly optimal for genetic studies since no mutants were available and the experimental generation of mutants was virtually impossible. He, therefore, turned his attention to making hybrids between different species of salamander—*Triturus cristatus* and *T. taeniatus*—whose forelimbs and digits grew at different rates. After slogging through several breeding seasons and constructing growth curves of the parental species and reciprocal hybrids, he finally abandoned the project and began the transplantation experiments he had earlier planned to carry out.

The autumn of 1932 marked a major turning point in Viktor's personal life and professional career. For some time he had hoped to visit the United States, and in particular to spend time at the University of Chicago, where Professor Frank R. Lillie, a long-term friend and admirer of Spemann, had established the best known and most successful experimental group studying chick development. Lillie's 1908 book *The Development of the Chick* (Lillie 1908) had set out many of the advantages that chicks offer for developmental studies. For example, he had shown that it was possible to destroy comparatively large portions of early embryos without adversely impacting their overall development, and furthermore that such experiments were not likely to be confounded by either regeneration or regulation. As early as 1909, one of his students, MC Shorey, had published an important paper on the effects of wing bud ablation on the development of the spinal cord and the neighboring sensory ganglia, which is discussed later (Shorey 1909).

In 1932, Viktor was awarded a Rockefeller Fellowship to work in Lillie's laboratory, but by the time he arrived in October, Lillie was no longer active in research, having taken on the responsibilities of Dean of Biological and Medical Sciences. He had been replaced as Professor of Embryology in the zoology department by Dr. Benjamin Willier [with whom Viktor and Paul Weiss were later to edit the influential volume *Analysis of Development* (Willier et al 1955)]. Knowing of Viktor's work on limb development in amphibians, Lillie reminded him of Shorey's study and suggested that Viktor repeat her experiments using the refined microsurgical techniques (using glass needles and hair loops) that Viktor had perfected in Spemann's laboratory, rather than the electrocauterization method Shorey had used. Viktor was of course aware of the experiments of Ross Harrison's student, Samuel Detwiler, who had analyzed the effects of limb removals, limb translocations, and other experimental manipulations on the development of the spinal cord and the sensory ganglia in *Ambystoma* (Detwiler 1920, 1924, 1927, 1936). And he was particularly aware of the discrepancy between Detwiler's findings and those of Shorey with respect to the motor columns of the cord. So repeating Shorey's experiments was not simply a repetitive and possibly futile exercise. Rather it held the prospect of clarifying a significant issue regarding the relationship between peripheral structures and the central nervous system. In a sense this was the reverse

of Viktor's earlier studies on the effect of the nervous system on the development of the limbs, a topic that he would again take up once he had transformed himself into a "chick developmental neurobiologist."

With the help of one of Willier's research associates, Dr. Mary Rawles, Viktor soon mastered the techniques of experimenting on chick embryos *in ovo* and before long he had succeeded in selectively ablating wing buds and in transplanting supernumerary wings to the flanks of host embryos. Thus was his career in the United States launched, and his fascination with center-periphery relations was to engage his interest for almost 50 years, as the following account documents. But before he could get fully started, the tranquility of his life was disrupted in April 1933 by a letter he received from the Dean of the Faculty at Freiburg informing him that under the recently promulgated law "for the cleansing of the professions," he had been dismissed from his assistantship in the zoology department. At about the same time he received a letter from Spemann pointing out that as the universities were state controlled, there was nothing he could do to circumvent this decision, and that he should try to find a position in the United States. Fortunately the Rockefeller Foundation responded quickly to the new Nazi policy and created an emergency fund to support displaced German scholars. Through this fund Viktor was assured of a further 2 years of support, which enabled him to complete his initial study in Chicago as an assistant in the zoology department. In 1934, he was offered an assistant professorship in zoology at Washington University, which he took up in the fall of 1935. He was to remain at Washington University for the rest of his career, becoming an associate professor in 1939 and, following the departure of FO Schmitt for MIT, a professor and chairman of the department in 1941. In 1969, Viktor was named Mallinckrodt Distinguished Professor Emeritus. Among his many awards and honors, I need mention only his election to the US National Academy of Sciences in 1953, and his receipt of the Wakeman Award in 1978, the National Medal of Science in 1989, the Ralph W. Girard Prize of the Society for Neuroscience in 1985, and the Karl Lashley Award of the American Philosophical Society in 1990. In 1950 and 1951, he served as President of the Society for Growth and Development (now the Society for Developmental Biology) and in 1955 as President of the American Society of Zoologists.

THE EFFECTS OF WING BUD EXTIRPATION ON THE DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM IN CHICK EMBRYOS (1934)

Viktor's first published study after moving to the United States (Hamburger 1934) was to set the pattern for much of his future work, and through its impact on Rita Levi-Montalcini, it was to have a profound influence on the future of developmental neurobiology (see Levi-Montalcini 1981). As indicated above, the

study was undertaken at Lillie's suggestion, but for the way in which the experiments were carried out and the careful way in which the results were analyzed, one need only look at Viktor's earlier work in Berlin (Hamburger 1925, 1928, 1929).

For this study, the wing buds of embryos, 68–72 hours after the onset of incubation, were removed using fine glass needles. At this stage, the core of the wing bud consists of an undifferentiated mass of mesoderm, and in the brachial segments of the spinal cord the earliest motoneurons are visible. Some motoneurons have sent out axons into what will later be recognizable as the ventral root. As Tello (1922) had pointed out, although some fibers have reached the base of the limb bud at this stage, none has yet entered it. Most of the embryos were allowed to develop for a further 4–6 days, some were fixed after 2–3 days, and two were allowed to survive for 9–10 days after the operation. In the best cases, the limb was completely missing at the time the embryo was fixed for histology; in a few cases a much reduced wing had formed, and in others, the wing bud and also part of the body wall were missing.

The analysis of the resulting changes was limited to the spinal cord and the brachial nerves. In a typical case killed 5 days after the operation, the most striking changes were seen in the anterior horn of the cord, where on the operated side, the large motoneurons in the lateral motor column (LMC) were reduced in number by about 60%. The medial motor column (that innervates the trunk muscles) was unaffected, but the volume and number of cells in the posterior horn were reduced by just over 20%. Only the volumes of the related spinal ganglia were measured: Ganglion 13 was reduced in volume by 18%, ganglion 14 by 47%, ganglion 15 by 39% and ganglion 16 by 35%. Sample cell counts for two ganglia (14 and 15) showed a reduction in number of about 28%. This was less than the observed reduction in volume, which Viktor attributed to the marked loss of neuropil in the ganglia. The ganglia at this age consist of two distinct groups of cells: a population of small, dorsomedially located neurons, and a surrounding population of distinctly larger cells. On the operated side the ratio of small to large cells was 1:1.35; on the control side 1:1.2. The initial paths taken by the peripheral nerves on the operated side were normal, but their distribution beyond the brachial plexus was grossly abnormal.

The changes in the other cases examined in comparable detail were qualitatively similar but differed in the degree of cellular "hypoplasia" seen in the LMC, depending on the amount of muscle tissue in the surviving portion of the operated limb. From reconstructions of the limb musculature, the loss of muscle tissue was found to range between 31% and 96%; the corresponding reduction in cell number in the LMC ranged from 22% to 60%, whereas in the posterior horn it varied from 14% to 21%. The average hypoplasia in the relevant spinal ganglia was more nearly constant, ranging from 37% to 54%. In light of later work it is important to note that it is specifically stated that "no degenerated neurones were found in the area affected." This critical (but, as we shall see later, mistaken) observation had a significant influence on Viktor's interpretation of his experimental findings.

The extensive and very detailed discussion of this 1934 paper is important in several respects, first and most significantly because of its conclusions that:

> The different peripheral structures while growing, are in some direct connection with their appropriate centers in the nervous system. Thus, they are enabled not only to control the growth of their own centers in general but even to regulate this growth in quantitative adaptation to their own progressing increase in size.

He goes on to say that

> [e]very structure within the growing limb, muscle as well as sensory organs, send[s] stimuli to the central nervous system. Each part of the peripheral field controls directly its own nervous center, i.e., the limb muscles affect the lateral motor centers, the sensory fields control the ganglia.

Second, the discussion gives appropriate credit to Shorey's earlier study (1909), in that it explicitly acknowledges that the central finding of a "hypoplasia" of the LMC was clearly anticipated in her work (p. 479). And because it specifically cites two cases in her study in which the spinal ganglia had been inadvertently damaged, she is also credited with having demonstrated that the loss of motoneurons is not attributable to some impairment of sensory input or to a mechanism that involves some form of reflex arc (p. 474). I stress this point because in most later reports, Shorey's work (although often cited) is rarely afforded adequate credit. The fact is she was the first to demonstrate that removal of a limb bud in chicks leads to a marked reduction in the LMC of the related brachial segments of the spinal cord, although as Viktor pointed out, the definitive observations in her paper derived from only three critical cases: "Miss Shorey's results are corroborated [by his findings] in every detail." Furthermore, he noted that she had established "by counting the anterior horn cells and measuring their sizes that the [hypoplasia of the LMC] is due to reduction in cell number and not in size of the single cell." He also agreed with her conclusion that because no degenerating cells were seen, the effect of the wing ablations was "a typical hypoplasia." He adds that "the same conclusion was reached in our studies." In this context it is important to note that Viktor was not uncritical of Shorey's speculations about the possible mechanism underlying the cell loss—including the notion that the products of muscular metabolism somehow filter into the lymph system and are carried to the spinal cord, where they act as a stimulus for motoneuron growth and maintenance.

The discussion section of Viktor's paper is important for a third reason, namely that it includes the first statement of Viktor's own view of the mechanism underlying the hypoplasia of the LMC. This is the notion that the first motoneuron axons to reach the periphery (which he called pathfinders) in some way sense the extent of the field to be innervated and signal this to the spinal cord:

> We must charge the end organs [i.e. the growth cones] of these first pathfinders with the double task of locating the peripheral field, and in some

> way 'reporting' back centripetally to the central organ [i.e. the developing motor column or sensory ganglion] the approximate size of the field to be innervated. The fibers would communicate the result of their exploration to their own cell bodies which would thus become the first relay station for the stimulus to be transmitted. . . . By such a kind of mechanism, or by transmission of true nervous excitations or of substances, stimuli must be transferred to the growing nerve centers. These centers on their part would be put in a state of corresponding physiological activity and *that condition would enable them to induce presumptive neuroblasts to join their group* [emphasis added].

Viktor would later allude to this discussion as anticipating the concept of the retrograde transport of growth factors (like NGF), which later became generally accepted. But more correctly it anticipated his subsequent, more fully developed notion that the periphery serves to induce the differentiation of motoneurons from a population of undifferentiated precursor cells (see below).

Lastly I must comment on the thoroughness and fairness of the Discussion. He mentions essentially every previous study on the effect of peripheral (and, in a few cases, intracentral) ablations on the development of the spinal cord and the sensory ganglia, and in several instances he comments at length on the critical findings. Moreover, he does not limit his discussion to similar work in birds but includes a discussion of many studies in anurans, urodeles, mammals, and even human cases with developmental limb anomalies. It is an unfortunate commentary on contemporary publications that such thorough reviews of the relevant literature appear to be anathema to modern editors.

THE EFFECTS OF LIMB TRANSPLANTATION ON THE SPINAL CORD AND SENSORY GANGLIA (1939)

Following the publication of his 1934 paper on wing bud ablations, Viktor returned to his earlier interest in limb development and published two interesting papers on this topic. The first (Hamburger 1938) concerned the morphological and axial differentiation of transplanted limbs—a topic that had been considered by his second embryological hero, Ross Harrison, some years earlier (Harrison 1921). The second (Hamburger 1939a) followed his own earlier work on the pattern of peripheral innervation of transplanted limbs in amphibia. I do not consider these studies here (important as they are for a more general account of Viktor's scientific contributions), but rather proceed to a consideration of another study he published in 1939, in which he examined the effects of transplanted limbs on the development of the spinal cord and sensory ganglia (Hamburger 1939b).

This study, which was the logical extension of his 1934 paper on wing extirpations, took advantage of the extensive number of wing and hind-limb bud transplantations that he had prepared earlier for the studies on limb axis determination

and the patterns of peripheral limb innervation. The supernumerary limb buds were transplanted into host embryos 60–70 h after incubation by making a small slit in the flank, near the somites and immediately adjacent to the host's fore– or hind–limb. The LMC of the spinal cord and the sensory ganglia were examined 8 or 9 days after the operation. In all the successful cases the transplanted limbs were innervated by nerves from the adjoining spinal segments that often formed plexuses comparable to those innervating normal limbs, but the numbers of nerve fibers entering the transplants were always appreciably smaller.

The enlargement of the sensory ganglia contributing to the transplants was often conspicuous, but in most cases comparatively few fibers entered the limbs, and the brachial and lumbosacral enlargements on the operated and control sides of the spinal cord were not noticeably different, even when the transplant was located very close to the host limb. When counts were made of the numbers of motoneurons in the two sides, the differences were striking in only a few cases. Thus, in the brachial enlargement, the numbers on the two sides differed only by 1.5%, 5.5%, and 8% in the three cases analyzed (the experimental sides always being the larger). In the lumbosacral region the differences ranged from −1.7% to 16.5% in the eight cases that were analyzed quantitatively. However, when counts were made over the length of every segment that contributed fibers to the normal and transplanted limbs, it was evident that the numbers of motoneurons in the segments related to the transplanted limbs were increased. For example, in one case of a wing bud transplant, the numbers of motoneurons in segments 15 and 16 exceeded those on the control side by 13% and 26.5%, respectively. And in the most successful case where a hind-limb bud had been transplanted just rostral to the host limb, the numbers of motoneurons in segments 23 and 24 were increased by 88% and 22%, respectively, compared with the control side. It is interesting that although the spinal ganglia were clearly enlarged when the transplants were in the trunk region, there was no detectable hyperplasia in the motor columns over the corresponding thoracic segments.

No cell counts were done to determine the degree of hyperplasia in the "overloaded" sensory ganglia, but an estimate of their respective volumes was made using a technique introduced for this purpose by Detwiler. This involved tracing the outline of sections through the ganglia onto paper, cutting out the tracings for each ganglion on the experimental and control sides, and then weighing them. This provided a reasonable measure of the relative size of the enlarged ganglia on the side of the transplant, compared with those on the control side. Although the degree of enlargement of the ganglia innervating the transplants varied considerably from case to case (the range was 15% to over 200%), in essentially every case the affected ganglia were increased in volume, including those that innervated the transplants inserted into the trunk region.

There had been a number of previous studies of this kind in anurans and urodeles, all of which had demonstrated an enlargement of the overloaded sensory ganglia. Detwiler, whose work on *Ambystoma* was perhaps best known, had claimed that there was no comparable hyperplasia in the spinal cord, including the LMC

of the anterior horn. However, Dürken (1911) and May (1930, 1933) (both of whom had used frogs) had observed a degree of enlargement of the anterior horn. Viktor's work was distinguished from these earlier studies by his careful attempts to quantify the effects of peripheral overloading, and in this he was greatly aided by the fact that the LMC are much better defined in chicks than in amphibians. His observation that the limb transplants did not result in a generalized enlargement of the LMC, but rather in a focal increase in cell numbers in those segments that contributed to the innervation of the transplant, was the first clear indication that the relationship between the limb musculature and the growth of the motor columns was highly specific. In keeping with his earlier work, he concluded that the simplest explanation for the findings of a motor hyperplasia was "to assume a growth-controlling agent travelling in centripetal direction from the periphery along the first motor fibers to the growing motor centers" (Hamburger 1939b:281).

THE EFFECTS OF PERIPHERAL FACTORS ON PROLIFERATION AND DIFFERENTIATION IN THE CHICK SPINAL CORD (1944)

Viktor's next contribution to the center/periphery relationship appeared in 1944, in a study with his student Eugene L. Keefe of the numbers of mitoses and estimates of overall cell numbers, in the brachial region of the cord in experimental and control embryos (Hamburger & Keefe 1944). In his 1934 paper, Viktor had concluded (in the absence of observable cell death in the LMC) that the hypoplasia that occurs after early limb ablation could be attributed to (*a*) an effect on the proliferation of motoneuron progenitors, (*b*) the failure of the young neurons to migrate into the motor column, or (*c*) the failure of the first motor cells whose axons reach the periphery to induce the differentiation of other motoneurons from a preexisting pool of undifferentiated cells. A test of the first of these possibilities, he argued, would be to determine if wing bud removals resulted in an observable reduction in the numbers of mitotic figures in the neuroepithelium lining the central canal of the cord at brachial levels (Hamburger 1934).

To establish the time course of proliferation in the brachial cord, a number of control preparations were made and an enormous number of mitoses counted. But for our purposes, the key observation was that none of the animals in which one wingbud had been removed showed a consistent difference in the numbers of mitoses on the operated and control sides. In the aggregate the numbers were 20381 and 20950, respectively. In 9 of the 11 animals examined on days 5 and 6 (when cell proliferation in the cord is at its peak), the numbers of mitoses on the operated side were lower than on the control side, but the differences were fairly slight, amounting at most to $\sim$10%. Since comparable differences were observed on the two sides of the animals in the control group, the significance of this finding is difficult to assess. In light of this finding, a series of estimates was made of the

total numbers of cells on the control and operated sides in a number of cases (some of which had been used in his 1934 and 1939 studies, and two of which had been prepared by Bueker for his 1943 study—see below) in which there was a marked loss of neurons in the LMC. Similar counts were done at the level of nerve 16 in one of the chicks used in Viktor's 1939 study that had shown an appreciable hyperplasia at this level, following the transplantation of a supernumerary wing. To reduce the amount of cell counting required, the estimates of cell number were limited to the ventral halves of the spinal cord.

Again, the results seemed clear. The total numbers of cells—including motoneurons and nonmotor cells—on the two sides were essentially the same in the animals in which there was a significant reduction in the motor cell column and in those in which the LMC was said to be "hyperplastic." To cite just one example, in an animal in which there were 1388 fewer motoneurons on the operated side (5 days after the removal of a wing) compared with the control side, this loss was almost exactly matched by an excess of 1403 nonmotor cells on the affected side.

Viktor was to return to the patterning of cell proliferation in the chick cord in 1948, but based on the findings of this study, he reaffirmed his earlier position that the hypoplasia seen in the motor columns was not due to an effect on cell proliferation but to a reduction in the inductive influence of the periphery on an undifferentiated pool of cells in the cord. And conversely, that the "hyperplasia" seen after supernumerary limb transplantation was due to a sensing of the expanded periphery by the axons of the first differentiated motoneurons. As he expressed it:

> We arrive then at the concept of a "histogenetic gradient field" which has its center in the small cluster of pioneer motor neurons and which spreads over adjacent undifferentiated cells, inducing them to differentiate. The newly recruited neurons are added to the lateral motor column and increase the strength of the field, that is the radius of its expansion. This process of augmentation is not a self-perpetuating mechanism, however, but under the "remote control" of conditions prevailing at the periphery.

While not entirely embracing the most elaborate hypothesis put forward in the previous year by Barron (1943), based on studies of motoneuron differentiation in sheep, Viktor was obviously much influenced in his thinking by Barron's ideas and especially by his suggestion that motoneuron dendrites play a role in the induction and recruitment of other cells to the motor column (Barron 1943). Summarizing his own views, based on his earlier studies and his findings with Keefe, Viktor stated:

> The entire mechanism of peripheral control has clearly three different components which we are trying to analyze separately: The setting up of a stimulus by the peripheral fields to be innervated; the changes occurring in the primary neurons as a result of this stimulus; and the inductive effect of the primary neurons on indifferent cells. Of the many detailed questions that remain unsolved, that of the primary stimulus is one of the most puzzling.

FURTHER ANALYSES OF MITOTIC PATTERNS IN THE CHICK SPINAL CORD (1948)

Viktor followed his study with Keefe with a long and detailed analysis of the patterns of distribution of mitotic figures in the chick spinal cord over much of its rostro-caudal length and at each of several stages from day 3 through the day 9 of incubation. In conception this study followed an earlier but essentially similar study by Coghill (1933) on cell proliferation in the spinal cord of *Ambystoma*, which had been done to determine whether there were dorso-ventral differences in proliferation over the extent of the hind-limb innervating segments that could be related to the individuation of specific reflexes. Since Viktor's (Hamburger 1948) study of cell proliferation in the chick cord does not bear significantly on the center-periphery issue, I need mention only a few of the more important findings, although this hardly does justice to the enormous amount of effort that went into the study.

The most relevant findings from the present perspective are the following: Although no clear rostro-caudal pattern in the numbers of mitoses was detectable (and, most interesting, the numbers in the brachial segments were not greatly different from those in the pre- and post-brachial regions), there was a very striking ventral-to-dorsal gradient. Thus, whereas the numbers of mitoses in the basal plate reach their peak on day 3, the peak period of proliferation in the alar plate occurs on day 6. In terms of the aggregate numbers of mitotic figures observed, the numbers in the alar plate exceed those in the basal plate by more than a factor of two. Some of the developmental factors that might contribute to the different proliferative patterns in the basal and alar plates are discussed at some length. However, the only points of interest in the present context are the references to Viktor's earlier experiments, in which the brachial segments had been isolated from the pre- and post-brachial regions by tantalum foil inserts, and to Visintini & Levi-Montalcini's (1939b) experiments, in which all long descending pathways to the limb segments had been interrupted. In neither instance were changes observed in the proliferative patterns in the cord. Of greater interest is the reference to a paper by Levi-Montalcini and Hamburger (at the time unpublished) with the following statement: "On the other hand, the same operation (i.e. early wing bud ablation) has a marked and permanent effect on the mitotic activity in the spinal ganglia."

RITA LEVI-MONTALCINI AND THE EVENTS THAT LED TO HER JOINING VIKTOR'S LABORATORY IN 1947

In October 1947, Rita Levi-Montalcini joined Viktor's laboratory from her native Italy. She had come at Viktor's invitation and expected to stay for just a few months or a year at most. In the event, she remained associated with Washington University for 30 years, and no one could have predicted just how momentous her arrival was to be for the future of developmental neurobiology.

Before considering their first joint research endeavor, I should say something about Rita, about her remarkable life in fascist Italy in the years leading up to and during World War II, and about her early ventures into neuroembryology. Rita herself has poignantly described this phase in her life in her autobiography *In Praise of Imperfection* (Levi-Montalcini 1988) and in a number of shorter articles (Levi-Montalcini 1975, 1981, 1982), so this account can be brief.

Born into an intellectual Jewish family in Turin in 1909, Rita, like most Italian women of her generation, had virtually no exposure to science until she and her cousin Eugenia decided that they wanted to study medicine. With the help of a private tutor, who put them through a crash course in mathematics and science, she gained admission to the University of Turin's medical school in 1930. Here she had the good fortune to get to know two other medical students, Salvador Luria and Renato Dulbecco (both future Nobel laureates) and, most important, to fall under the influence of the leading Italian neurohistologist of his generation, Professor Giuseppe Levi. Despite his notoriously ferocious manner, Rita found in Levi a brilliant and challenging mentor who shared with her the humiliation and personal abuse heaped upon Jewish academics by Mussolini's black-shirted followers. After graduating from medical school in 1936, Rita stayed on for further training in neurology and psychiatry until 1938, when Mussolini issued his *Manifesto for the Defense of the Race*, which prohibited Jews from studying and teaching in state schools and universities. This caused her to leave Italy in 1939, and for a short period she worked in a research institute in Brussels. Shortly after the outbreak of World War II she returned to Turin. As she tells it, she

> first met Viktor in a cattle car in northern Italy . . . on a day in . . . that fateful June of 1940 when Mussolini declared war on France . . . I was sitting on the floor of one of these railway cars . . . reading a reprint lent to me by Giuseppe Levi on the effects of wing bud extirpation on the development of the central nervous system of chick embryos. The article was dated 1934, and as Levi had informed me, it had been written by a pupil of Hans Spemann.
>
> (Levi-Montalcini 1981)

Determined to repeat this experiment, Rita set up a simple laboratory in her bedroom, obtained fertile eggs from a local farmer, kept them in a make-shift incubator, forged her own microsurgical instruments, and on completing the experiments removed and fixed the embryos for histological study, and then proceeded to eat the rest of the eggs! With Levi's help and encouragement, she did more than just repeat Hamburger's 1934 experiments; she extended them in three significant ways. First, she analyzed the effects of the limb bud removals (she chose to ablate the hind-limb bud rather than the wing buds), focusing as much on the spinal (sensory) ganglia as on the motor columns of the spinal cord. Second, she examined embryos over a wider range of survival periods (from days 4 through 20 of incubation). Third, in addition to conventional Nissl staining for cell bodies, she prepared many of the animals for staining by De Castro's modification of Cajal's silver method, which

she had learned from Levi. Facing continuous risk of discovery, and working under the most trying circumstances, she managed over the course of the next 2 years to complete this and another series of experiments, as well as to get her work published in the *Belgian Archives of Biology* (Levi-Montalcini & Levi 1942, 1943).

Her experiments on limb bud removals confirmed Viktor's observation that depriving the relevant spinal cord segments of their peripheral field results in a marked reduction in the lateral motor cell column. But more important, she observed that what Viktor had interpreted as a hypoplasia—and attributed to an inductive failure in the recruitment of motor cells—was in fact the direct consequence of the death of previously differentiated motoneurons. Even more striking was the observable degeneration of cells in the spinal ganglia, where cell counts showed that by day 12 of incubation and later, as many as 60%–70% of the neurons in ganglion 25 were lost on the operated side. And since in the silver-stained preparations it was possible to distinguish between fully differentiated neurons and undifferentiated cells, it was clear that the principal effect of early limb ablations was the degeneration of differentiated neurons. Rita's interpretation of her findings (no doubt aided by Levi's considerable experience) was thus quite different from Viktor's. The effect of removing the peripheral innervation fields of both motoneurons and sensory ganglion cells, she concluded, was not on their proliferation or differentiation but rather on their survival. This little-known study was to have a major influence on all future studies of center/periphery relationships, but it was not until after the war, when Viktor came across it, that its importance was recognized. His response on reading the paper was characteristic. As he later wrote:

> Of course, I accepted her version, but I felt that the analysis of the effect of limb extirpation could be carried further . . . I wrote to Dr. Levi and asked whether Dr. Levi-Montalcini would be interested in working in my laboratory for a year. She consented and arrived in St. Louis in the fall of 1947.
>
> (Hamburger 1996)

THE DEVELOPMENT OF SPINAL GANGLIA UNDER NORMAL AND EXPERIMENTAL CONDITIONS (1949)

Rita's arrival in St. Louis was a major landmark in the history of developmental neurobiology at Washington University. Although her earliest publications that appeared during the war were not widely known outside of Italy, it is clear on reading them that she had become an excellent neuroanatomist, had mastered the often capricious silver methods, especially De Castro's en-bloc staining technique, and had a sure grasp of most of the important issues in neuroembryology. In addition to her seminal paper on the effects of limb bud extirpation, she had published an excellent account of the early development of the accessory abducens nucleus in the chick (Levi-Montalcini & Levi 1942). And before that, she had

published two papers with a neurophysiologist in Turin's Clinic for Nervous and Mental Diseases, Dr. Fabio Visintini (Visintini & Levi-Montalcini 1939a,b). The first of these papers describes various aspects of the early morphology of the chick spinal cord and lower brainstem. The second illustrates the normal development of the cochlear and vestibular nuclei and the effects of surgical and cauterizing lesions at rostral brainstem and diencephalic levels on the development of the motor columns of the cord. She extended the analysis of the cochleo-vestibular complex in a paper published from St. Louis, but these studies with Visintini are noteworthy for their extensive use of silver-stained preparations and, in the case of the second paper, for its inclusion of a lengthy section describing various physiological and behavioral observations on the experimentally manipulated chicks (Visintini & Levi-Montalcini 1939a,b).

By both background and inclination, Viktor was at heart an experimental embryologist in the tradition of Roux, Spemann, and Harrison, so the appearance in his laboratory of an experienced neurologist and neuroanatomist opened up new possibilities for work on the central and peripheral nervous systems. Commenting on this, Hamburger once remarked:

> [Rita and I] came from entirely different backgrounds. I came from experimental and analytical embryology, of which Rita hadn't the foggiest idea. . . . Rita was a neurologist from medical school and knew the nervous system, of which I had only the foggiest idea. And she brought to St. Louis a most important tool, the silver staining method. [see McGrayne 1996].

Viktor was especially interested in reexamining with Rita the effects of limb ablations, and this formed the basis of their first joint study (Hamburger & Levi-Montalcini 1949). In retrospect it is not clear why they chose to focus on the sensory ganglia rather than on the motor columns of the cord since it was Viktor's observations on the motor system and his interpretation of the observed changes in the motor columns that had been called into question by Rita's study with Levi. Writing about their early interactions, Viktor recently stated:

> [We] agreed to repeat the limb bud extirpation experiment once more, and on the first step, to pay special attention to the finest details in the response of the sensory ganglia. *Fortunately we chose her preference; if my preference of the motor columns, which are more homogeneous then the ganglia, had prevailed, NGF would not have been discovered in my laboratory.* (Hamburger 1996, emphasis added)

As we have not found any comparable (or contradictory) statement from Rita, we may assume that Viktor's recollection of what happened is correct; it is certainly consonant with the fact that the spinal ganglia featured more prominently in her paper with Levi than did the motor columns, and it is perhaps for this reason also that Viktor later failed to recall Rita's having ever worked on the motor system (V Hamburger, personal communication).

Their 1949 paper on the development of the spinal ganglia is unquestionably one of the most important in all of neuroembryology. Its style suggests it was written by Viktor, but by his own admission, the experimental work and the initial analysis of the data were done by Rita. Again to cite Viktor's autobiographical memoir:

> The experiments and observations on the slides were done by Dr. Levi-Montalcini. [But he adds:] I followed her work and discoveries with intense interest, and we were in close communication all the time.

And as Rita looking back on this time wrote:

> What I liked most was the clarity of [Viktor's] thinking and his superb control of the English language. Writing a scientific paper was a new experience for me, and I concentrated on the effort of learning how to do it.

The paper itself included a detailed morphological description of the appearance of the lumbo-sacral sensory ganglia from days 3 through 20 of incubation, a careful analysis of a large number of cases in which either a wing or hind-limb bud had been extirpated at 2½–3 days of incubation, and a further group of 25 cases of wing or leg transplantations that had been carried out at the same developmental stage and allowed to survive generally to days 5 or 8 of incubation, but in some cases as late as days 9–17. In addition to staining with Heidenhain's hematoxylin, many of the preparations were stained according to a variant of De Castro's method.

The account of the normal development of the sensory ganglia followed closely that given in the paper by Levi-Montalcini & Levi (1943) in which they had recognized three developmental phases, the first beginning with the migration of the neural crest precursors (on day 2) through day 8 of incubation. This phase is marked mainly by the proliferation of ganglion cell precursors, the differentiation of the large sensory neurons in the ventrolateral part of the ganglia, and the appearance of substantial neuronal degeneration in the non–limb-related ganglia. By day 5, the central and peripheral processes of the originally bipolar neurons reach the spinal cord and dermis, respectively. The second phase continues through day 12 and is mainly distinguished by the appearance of the smaller dorsomedially located ganglion cells. The third phase extends beyond day 15, by which time it is difficult to distinguish the two populations of neurons. The first reflex responses to peripheral stimulation were observed on day 11, when the smaller cells were clearly differentiated and Tello (1922) had observed the innervation of muscle spindles.

Differences in the numbers of mitoses on the two sides could be seen in the experimental material as early as day 4 and were striking by day 5. In the case of the wing bud extirpations, the differences in ganglia 14–16 ranged from 11.8% to 37%; in the case of the supernumerary wing transplantations, the changes were less marked, ranging from +2.0% to +26.0% in ganglia 16–18. In some individual cases the observed differences were not statistically significant, but when all the

experimental data were pooled the findings were clear, leading them to conclude that

> the peripheral field controls the mitotic activity of the spinal ganglia; its reduction decreases the numbers of mitotic figures in ganglia which participate in its innervation, and its enlargement increases it.
>
> (Hamburger & Levi-Montalcini 1949:474)

In the cases of the wing transplants, these findings were indirectly confirmed by the observation that all the ganglia that contributed fibers to the transplants showed a distinct numerical hyperplasia on days 9–9.5, shortly after proliferation had ceased.

Their findings on the influence of the periphery on cell proliferation in the ganglia were in general agreement with the earlier studies of Detwiler (1920, 1936) and Carpenter (1933) in urodeles. But whereas the earlier studies had been based on counts of the numbers of surviving neurons in the ganglia, the influence of enlarging or reducing the periphery on cell proliferation was inferred rather than directly measured. The study by Hamburger & Levi-Montalcini (1949) provided the first convincing evidence, based on mitotic counts, for a direct effect of the periphery on cell proliferation in the sensory ganglia.

Since the effects of peripheral manipulations on cell proliferation were found to occur before the limbs are innervated, it was difficult to account for them on the basis of the mechanism that Hamburger (1934) and Hamburger & Keefe (1944) had suggested for the changes seen in the motor cell columns. Although this issue was not discussed at length, it was proposed that it might be due to some kind of "field effect," but the nature of the "field" and how it might be influenced by the periphery was left open (and presumably to the reader's imagination).

By contrast, the degenerative changes seen in the ganglia were considered at length and clearly represent the principal focus of the study. The key observations were twofold. First, relatively few degenerating neurons could be observed in the limb-related ganglia during normal development, whereas they were abundant in the upper cervical and thoracic ganglia. And second, following wing or hind-limb bud extirpations, large numbers of degenerating cells could be seen in the brachial and lumbo-sacral ganglia, where they were found to involve almost exclusively the large, differentiated neurons in ventrolateral parts of the ganglia. A particularly important observation was that the experimentally induced degeneration in the limb-related ganglia occurs at the same stages in which degenerating neurons were observed in the normal cervical and thoracic ganglia (days 5–7), as evidenced in both hematoxylin-stained preparations and ganglia treated with supravital trypan blue (which is especially useful for revealing degenerating cells). In addition to the marked degeneration affecting the large ventrolateral ganglion cells, the smaller, dorsomedially located cells were also affected. However, in the latter the effect of limb extirpation took the form of a slow progressive atrophy rather than an acute, sharply defined phase of cell death.

While there is much else of interest in this paper (including a lengthy discussion of the significance of the terms hyper- and hypoplasia, which were widely, and

often confusingly, used in the earlier embryological literature on this topic), the major findings, and those that were to prove to be of greatest importance for all later work on center/periphery relations, were the discovery of naturally occurring degeneration in the non–limb-related ganglia and the substantial degeneration of ganglion cells following limb bud extirpations. To account for the rapid degeneration of the large ventrolateral cells, the authors proposed two possible mechanisms: a breakdown in some essential metabolic or growth-related process [they mention as a possibility "axon flow," a process that had recently been described by Weiss & Hiscoe (1948)]; or alternatively, a disturbance in "a metabolic exchange between the growing neurite and the substrate on which it grows." In light of their future work, it is interesting to recall that they elaborated on this second possibility by stating that

> [s]ubstances necessary for neurite and neuroblast growth and maintenance would not be provided in adequate quantities when the limb bud is removed.

Later they added the following:

> Not the functional but the physical or chemical conditions at the periphery are ultimately responsible for the 'peripheral' effects on the development of nerve centers.

What is particularly significant about this study is not so much its recognition of neuronal degeneration as an important factor in neurogenesis, since this had been previously reported by Levi-Montalcini & Levi (1942, 1944). It had also been observed in the chick ciliary ganglion after early eye removal and in the trochlear nucleus [in a then unpublished study by Dunnebacke, one of Viktor's students (see Dunnebacke 1953)]. Rather, its significance lies in the extraordinary care and clarity with which the experimental findings were described and in the thoroughness with which they were discussed and evaluated in relation to virtually all the relevant literature. In these regards the paper was to serve as a model for all later studies on this topic. It also laid a sound foundation for much of the work that Viktor and Rita subsequently carried out. But before considering their later work, it is necessary to digress briefly to consider a surprising series of experiments by another of Viktor's former students.

ELMER BUEKER AND THE USE OF MOUSE TUMORS TO PROBE THE DEVELOPMENT OF THE NERVOUS SYSTEM (1943, 1945, 1948)

While working in Viktor's laboratory, Elmer Bueker had examined the effects of radical limb ablations and of supernumerary limb transplantations at different positions along the body wall. And, in a further series of experiments, he had grafted lengths of spinal cord about five segments long into the region adjoining the spinal cord, of host embryos (Bueker 1943, 1945). The radical limb

extirpations served to establish that virtually no neurons could be found in the related LMC by day 9 of incubation, but apart from this they added little to the findings of Hamburger and Keefe. Perhaps not surprisingly, his interpretation of these findings closely followed Viktor's. The experiments with transplanted cord segments were more difficult to interpret, in part because of the inevitable distortion of the tissue, the interference with the normal pattern of innervation from the host spinal cord, and the variable outgrowth of axons from the spinal cord graft into the host limb. However, these experiments are of historical interest because they suggested to Bueker an alternative approach to the problem of center/periphery relations that was to have lasting consequences. The reasoning behind Bueker's next series of experiments followed directly from his limb transplantation experiments: If providing a second, expanded area for innervation by the LMC increased the number of motoneurons found in the LMC, it might be possible, he reasoned, to achieve a similar effect by substituting some other rapidly growing tissue for the growing limb (Bueker 1948). The tissues he chose to explore for this purpose were a mouse mammary adenocarcinoma, a mouse sarcoma (referred to as sarcoma 180), and the Rous fowl sarcoma. All were known from previous studies to grow rapidly in chicks, and to maintain their histogenetic characteristics.

It is not clear what led Bueker to pursue this course, but the generally held view that this idea was suggested to him by Viktor is probably without foundation. By the time Bueker conducted these experiments, he had been away from St. Louis for some years and had held positions at the Medical College of South Carolina and at Georgetown Medical School. And as we shall see below, Viktor seems to have been unaware of the experiments until the work was published.

Most of Bueker's experiments involved removing the hind-limb buds of chicks or making a slit in the somatopleure lateral to somites 24–30, on day 3 of incubation, and then transplanting a small piece of the tumor into the exposed region; the tumors were allowed to grow in situ for periods ranging from 1 to 5 or 6 days. Most of the chicks bearing the Rous sarcoma died from extensive hemorrhages before reaching day 8, and most of the mammary adenocarcinomas failed to grow and were resorbed by day 8. In the few cases that survived to day 9, there was no evidence that nerve fibers had innervated the tumors, but in every case the lateral motor columns of the cord and the related spinal ganglia were markedly reduced in size. The findings in the animals bearing the sarcoma 180 transplants, however, were strikingly different. In nearly every case, some nerve bundles could be seen growing into the tumor mass (which had infiltrated the surrounding region), and as judged by the weight of cut-out tracings, the volumes of the spinal ganglia were increased by about 33%. Conversely, the numbers of cells in the related LMC were reduced, on average, by 35%. The findings that the spinal ganglia were increased in size in the animals with sarcoma 180 transplants (due, it was thought, to both a hypertrophy of individual ganglion cells and an actual increase in their number) was of particular interest. It suggested that the tumor could provide an effective growth-supporting periphery, at least for sensory neurons. Conversely, the loss of motor cells in the cord was

attributable to the extirpation of the developing limb and the inability of the growing sarcoma to provide "an alternative periphery" for motoneurons.

In retrospect it seems that Bueker did not fully appreciate the significance of the finding that sarcoma 180 was capable of selectively promoting the growth of sensory ganglion cells. At least there is no indication that he intended to follow up this study. Indeed, he published nothing further on this topic for several years, and nothing further might have come of the finding had Viktor not brought Bueker's paper to Rita's attention some months after its appearance.

FUTHER EVIDENCE FOR THE IMPORTANCE OF NEURONAL DEGENERATION DURING DEVELOPMENT (1949, 1950)

While carrying out the study of the effects of limb extirpation on the development of the spinal ganglia, Rita continued to work on the acoustic and vestibular centers of the brainstem that had interested her during her association with Visintini, and in 1949 she published a detailed analysis of the effects of early extirpation of the otocyst (Levi-Montalcini 1949). Although this study is somewhat tangential to the main thrust of the present review, it is worth recalling because it remains one of the best-documented analyses of the effects of depriving developing neurons of their afferent input. In brief, Rita was able to show that whereas the initial development of the acoustic centers is unaffected by the removal of the otocyst, in the period following the normal arrival of their afferent fibers, one of the deprived acoustic nuclei (the *nucleus angularis*) undergoes a very profound cell loss and a marked atrophy of the remaining neurons. A second center (the *nucleus magnocellularis*), while obviously affected, shows an appreciably less severe hypoplasia; the third center, the *nucleus laminaris* (which does not receive a direct input from the cochlear nerve but is in receipt of fibers from the nuclei angularis and magnocellularis), is not affected by removal of the otocyst (at least not until day 17 of incubation, which is as far as the study was continued).

We need not discuss this paper further except to note two things. First, we still do not know what factors afferent fibers provide for the trophic maintenance of their target neurons. And, second, Rita thanks Viktor "for his constructive criticism and help in editing the manuscript." Of more immediate relevance is another paper that Rita published the following year (Levi-Montalcini 1950), which provided the most striking evidence that substantial death occurs during the normal development of the spinal cord, as it does in the spinal ganglia.

This study involved a reexamination of the development of the nucleus of the origin of the preganglionic sympathetic fibers, usually referred to by chick embryologists as the *column of Terni*. Terni (1924) had claimed that from their first appearance the preganglionic sympathetic neurons occupied their position just lateral to the central canal of the cord, roughly midway between the dorsal and

ventral horns. Rita, on the other hand, by following the development of the neurons in a closely spaced series of embryos stained by the De Castro method, had concluded that the cells reach their definitive location by secondarily migrating in a dorsomedial direction from the region of the lateral motor column. There can be no question about the correctness of her conclusion. But what is of particular interest in the present context is that in studying the origin of Terni's column, Rita was obliged to reexamine the development of the entire rostro-caudal extent of the spinal cord.This brought to light the unexpected finding that substantial neuronal degeneration occurs at certain well-defined levels of the cord during normal development. As her findings made clear, at early stages there is a distinct visceral system (comparable to the nucleus of Terni and the associated rami communicantes) in the cervical cord. But between 4.5 and 5 days of incubation, the cervical visceral system undergoes complete disintegration such that "during this period the number of degenerating cells is so large as to obscure the presence of intact cells" (Levi-Montalcini 1950:266).

No secondary cell migrations nor degeneration was seen in the brachial or lumbosacral segments, but there was evidence for the appearance of a small sacral preganglionic (parasympathetic) column that developed in much the same way as the nucleus of Terni. That the mechanism underlying this developmental pattern is intrinsic to the spinal cord was evident from a single experiment in which the thoraco-lumbar region of stage 25 embryo had been transplanted between the brachial cord and the wing bud of a host embryo; in this case the segregation and migration of the visceral outflow followed the same pattern as in normal embryos.

In addition to clarifying the origin of the preganglionic sympathetic and the sacral parasympathetic outflow from a common viscero-somatic motor column, this largely neglected paper was important in providing the first clear evidence that large-scale neuronal degeneration occurs during the normal development of the CNS as it does in the sensory ganglia. It was important also in that Rita drew attention to earlier studies of what later came to be known as "naturally occurring cell death" during development. In particular Rita recalled the work of Collin (1906), who seems to have been the first investigator to report the presence of degenerating neurons in the spinal cord, and also the findings of Ernst (1926), who had concluded that cell degeneration was a general feature in the development of all organs.

Stimulated by Rita's findings on the nucleus of Terni, Paul Shieh, one of Viktor's graduate students, analyzed the development of the Terni's nucleus in segments of the cervical spinal cord transplanted to thoracic levels. Because this work is peripheral to my primary purpose, I shall not elaborate on it, except to note that Shieh observed the same pattern of cell degeneration in the transplanted cervical cord segments as Rita had described earlier. However, in the most caudal portions of the transplants there was evidence for a presumptive preganglionic sympathetic outflow, although not all the neurons involved followed the characteristic migratory pattern that Rita had described. The mechanism responsible for this transformation in the lower cord was left undetermined: It could (it was argued) be due to a specific

inductive influence operating at thoracic levels, or it might result from the removal of an inhibitory agent that normally prevents the appearance of a preganglionic system at cervical levels.

LEVI-MONTALCINI AND HAMBURGER REPEAT BUEKER'S EXPERIMENTS (1951)

Shortly after they had completed their study of the development of the sensory ganglia, Viktor and Rita carried out a series of experiments in which they had hoped to see if the transplantation of a more homogeneous mass of tissue than an entire limb could affect the development of the ganglia, the motor columns, and the associated peripheral nerves. The results of these experiments were never published, but they were referred to in the introduction of their next joint paper (Levi-Montalcini & Hamburger 1951). The transplanted tissues included portions of muscle, brain, skin, and liver that were introduced in place of a limb. As they stated:

> In this way, we hope to create specifically favorable conditions for the growth of one component. *Our preliminary results are not conclusive* [emphasis added].

Shortly thereafter, Rita later recalled, some time "in the fall of 1948, one year after my arrival in St. Louis . . . Viktor showed me a short article . . . which was to change entirely the direction of my research" (Levi-Montalcini 1975). The article in question was Elmer Bueker's paper describing the results of his experiments with various transplanted tumors. His success with sarcoma 180 was of special interest since not only were the tumor masses invaded by nerve fibers, but the nearby sensory ganglia were clearly enlarged. After writing to Bueker to request his permission for them to repeat (and expand) on his study, Viktor obtained mice bearing several different tumors from the Jackson Laboratories in Bar Harbor, Maine. In addition to sarcoma 180, they received a second mouse sarcoma (sarcoma 37), and two different mammary gland adenocarcinomas.

In repeating Bueker's experiments, Rita inserted small pieces of each tumor into a slit at the base of the limb bud (most transplants were at the level of the hind limb) in 3-day-old chick embryos that were allowed to survive for periods ranging from day 4 or 5 to day 17. In a parallel series of experiments, she transplanted portions of placenta from 15-day-old mouse fetuses. As before, the chick embryos were prepared for staining either with a variant of Heidenhain's hematoxylin or with De Castro's method. As Bueker had found, the transplanted adenocarcinomas were quickly resorbed (even though they had been found to grow well on the chorioallantoic membrane) and only the transplanted mouse sarcomas yielded useful results. For our purposes it is sufficient to summarize the data obtained from the 73 successful sarcoma transplants as follows.

Prior to the ingrowth of nerve fibers into the tumors, there was no evidence that they had affected either cell proliferation, early differentiation, or the initial outgrowth of sensory fibers from the ganglia, and no changes could be seen in the spinal cord. In some cases, the growth of peripheral nerves appeared to have been blocked by the tumor mass and the lateral motor columns, and the sensory ganglia were hypoplastic. Appreciable neuronal degeneration was observed among the large ventrolateral (VL) ganglion cells. From day 7 onward, however, large numbers of nerve fibers began to invade the tumors and this continued through day 17. In a typical case the LMC was markedly hypoplastic (due to the obvious obstruction to the growth of the limb nerves), but the adjoining spinal ganglia were greatly enlarged, as were the nearby paravertebral sympathetic ganglia. The ingrowth of sensory and sympathetic fibers into the tumors was especially clear in the silver-stained preparations, which often showed small bundles of fibers surrounding clusters of tumor cells.

The enlargement of the sympathetic ganglia was especially striking; in fact it often led to the apparent fusion of adjoining ganglia, some of which were six times the volume of the corresponding ganglia on the contralateral side. Cell counts at 11.5, 13, and 17 days showed striking increases in ganglion cell number compared with the controls (1.7x, 2.1x, and 3.07x, respectively). The fact that these numbers did not match the increase in the overall volumes of the ganglia was (as the silver preparations showed) due to the marked increase in the size of individual ganglion cells. Interestingly, the nucleus of Terni was unaffected in any of the experiments, but in cases in which the tumor had grown close to the suprarenal glands, large groups of sympathetic neurons were seen where normally only an occasional sympathetic ganglion cell was to be found. And in some cases sympathetic fibers from the contralateral side had grown across the midline to reach the tumor. In the spinal ganglia, the large VL neurons were severely depleted in number (presumably as a result of the blockage of access to the limb by the tumor mass), but the smaller dorsomedially located (DM) neurons were obviously increased in number and this was borne out by an observable increase in mitotic activity in the dorsomedial parts of the affected ganglia at day 7.

The discussion section of this paper is characteristically thoughtful and detailed, but only the following points merit comment. (*a*) The absence of any growth-stimulating effect from the transplanted fragments of E15 mouse placentas was taken as evidence that the action of the two sarcomas is quite specific and not simply a generalized growth-promoting mechanism. (*b*) This conclusion is strengthened by the findings that the VL sensory neurons and the motor cells in the LMC were not positively affected (as noted, their observed hypoplasia was attributed to the physical obstruction of their processes by the tumor masses). (*c*) The presence of the tumors strongly promotes the proliferation, differentiation, and growth of DM sensory neurons and sympathetic ganglion cells in the paravertebral ganglia. (*d*) The growth-stimulating effect of the tumor does not seem to be limited to neurons whose processes invade the tumors, but can also affect adjoining cells of the appropriate kind. (*e*) The effects do not depend on the establishment of

synaptic-type junctions or sensory receptor-type endings upon the tumor cells. In conclusion the authors stated that

> all [the] available data indicate that the sarcomas 180 and 37 produce specific growth promoting agents which stimulate selectively the growth of some types of nerve fibers but not of others . . . the effects are mediated by the nerve fibers to their respective centers.

EVIDENCE THAT THE NEURAL GROWTH-PROMOTING EFFECTS OF SARCOMAS 180 AND 37 ARE DUE TO A DIFFUSIBLE FACTOR (1952, 1953)

One of the more astute observations that Rita made during the work on sarcomas 180 and 37 was that sympathetic ganglia that had not sent fibers into the tumor mass, and other collections of sympathetic neurons that had no direct connection with the transplanted tumors were appreciably enlarged. This immediately suggested to both Viktor and Rita the possibility that the causative agent was a diffusible factor released by the cells of the tumor either into the surrounding tissue fluid or into the vascular system.

It was still their contention that the "growth-promoting factor" was taken up by the processes of the affected neurons and transported back to their cell bodies, but their earlier view that it was released only at the focal sites of interaction between the terminals of sensory and sympathetic fibers and the targets they normally innervated obviously needed to be revised if the factor released by the sarcomas could diffuse for some appreciable distance from its site of production.

Rita wrote about this finding (Levi-Montalcini 1975, original emphasis).

> It was a Spring day in 1951 when the block [to her acceptance that the findings of the tumor transplantation experiments could not be fitted into their previously held views] was suddenly removed, and it dawned on me that the tumor effect was *different* from that of normal embryonic tissue in that the tumor acted by *releasing* a growth factor of unknown nature rather than by making available to the nerve fibers a larger-than-usual field of innervation.

The fact that the sympathetic fibers had extensively invaded some of the adjoining viscera long before the onset of their normal innervation was for her proof positive that a diffusible factor must be involved. As it happened, her Italian mentor, Giussepe Levi, visited St. Louis at this time and when shown slides from some of the tumor transplant bearing chicks, "shook his powerful leonine head . . . and said 'How can you say such nonsense? Don't you see that these are collagenous and not nerve fibers?'" Rita was greatly relieved when shortly afterward Viktor reassured her that she was correct, and as she noted, he "immediately grasped the far reaching significance of these findings" (Levi-Montalcini 1975).

To establish that the factor involved was indeed diffusible, Rita then carried out a very extensive series of experiments in which fragments of the two sarcomas were implanted, not near the base of the limb bud but at three remote sites: into the coelomic cavity, onto the yolk sac (from which they became incorporated into the umbilical cord), and onto the allantoic vesicle. Many grafts from the latter two sites failed "to take," but those that survived were later found on the chorioallantoic membrane. Despite an unusually high mortality rate, the results from the successful experiments were unequivocal. Large numbers of sympathetic fibers grew into some of the developing viscera (the mesonephros was especially heavily innervated) and into veins; the sympathetic ganglia, including the superior cervical ganglion and Remak's ganglion in the lumbo-sacral region, which were well removed from the growing tumor mass, were greatly enlarged. In addition several unusual sympathetic ganglion-like masses were found behind the aorta and embedded in the adrenal gland. These effects did not extend to the parasympathetic ciliary ganglion or to the enteric plexuses.

From the enlarged sympathetic ganglia, large bundles of nerve fibers could be traced in silver preparations to various viscera that normally receive only modest innervation or, in the case of the mesonephros, no innervation at all. Among the organs affected by this hyperneurotization were the ovaries, spleen, thyroid, parathyroid, metanephros, and, to a lesser extent, the liver, thymus, bone marrow, and gut. However, in a few cases the nerve outgrowth was directed almost exclusively to the implanted tumor mass. One wholly unexpected finding was the invasion of small and medium-sized veins, which in some instances was so great as to completely occlude the vessel.

Since the extraembryonic transplants were far removed from the sensory and sympathetic ganglia and were connected with them only by way of the vascular system, the ineluctable conclusion to be drawn from these experiments was that the growth-promoting agent must be diffusible. And, furthermore, since its effects on its neuronal targets were so much greater than had ever been seen, even in the most successful supernumerary limb transplants, it must be extremely potent. Its effects, however, were not only quantitatively different from those seen when an enlarged "natural target" tissue was provided, they were also qualitatively different, since it resulted in the neoformation of ganglionic masses, the hyperneurotization of viscera, the invasion of blood vessels, and the rampant and uncontrolled growth of the sympathetic system.

These exciting findings were first presented by Rita at a meeting on *The Chick Embryo in Biological Research* held at the New York Academy of Sciences in the summer of 1951 and subsequently published in the Annals of the Academy (Levi-Montalcini 1952). Although this report included all the essential observations mentioned above, a more lengthy account, with Viktor as coauthor, appeared elsewhere (Levi-Montalcini & Hamburger 1953). The fact that Rita was the sole author on the initial report is understandable since by the time the work on the murine sarcomas began, Rita had taken responsibility for essentially all the experimental work and was responsible for most of the observations.

When, some years later, Rita was asked what part Viktor had played in the work, she pointed out that when the transplant experiments were carried out, Viktor was in Cambridge, MA, having previously committed himself to spending a semester at the Massachusetts Institute of Technology (MIT) to assist his former colleague FO Schmitt (who had left Washington University to become chairman of the department of biology at MIT) in the development of a new biology curriculum (Levi-Montalcini 1981, 1988). However, there is no suggestion that Viktor was deliberately excluded from the work. In fact, Rita specifically remarked that "I kept Viktor informed weekly of the progress of my studies and of my growing interest in this extraordinary effect." And further, "Upon his return to St. Louis in the Spring of 1950, Viktor shared my enthusiasm and my belief that the growth response elicited by the tumor differed in many respects from those called forth by supernumerary limbs."

It is also evident from the style and form of the 1951 publication on the murine sarcoma transplants that Viktor—although appearing as second author—was largely responsible for writing the paper. This is true also of their second (1953) paper on this topic, which clearly bears the stamp of Viktor's hand. It is long, detailed, and carefully argued. But much of the material it contains is just as clearly due to Rita; this is especially evident in the lengthy sections on the "neuronal development of the sympathetic system" and "the response of sympathetic ganglia to tumors." There is a hint, however, that Viktor was somewhat uneasy about Rita's independent report at the meeting of the NY Academy of Sciences, which is referred to in a footnote on the second page of their 1953 paper: "A preliminary report of this work has appeared in the *Ann. N.Y. Acad. Sci*. 55, 1952." Viktor's recollections of these exciting days was that he "actively participated in the early phases of this work [that led to the discovery of the nerve growth factor] and in the preparation of the first two publications [i.e. Levi-Montalcini & Hamburger 1951, 1953] but withdrew from the project in 1953 to pursue other interests" (Hamburger 1989).

In fact his name was to appear on two further papers on the subject in 1954, but it is evident that his role in these later studies was much less direct.

THE "GOLDEN HALO": A Bioassay for the Nerve Growth-Promoting Factor (1952–1954)

The initial excitement over the discovery that murine sarcomas 180 and 37 produce a diffusible factor that has a profound, but selective, effect on neurons in sensory ganglia and the sympathetic nervous system was soon tempered by the realization that if the discovery was to be taken further, two difficult problems would have to be confronted. The first and most obvious problem concerned the nature of the growth-promoting factor, and the second was its mode of action on the responsive neurons. Viktor and Rita's immediate reaction was to see if simple chemical extracts of the two tumors injected into embryos at the appropriate stages could replicate the effects seen after tumor transplants. It is not clear who suggested this approach,

how the extracts were made, or how many experiments of this type were carried out. There was no formal mention of this work in the papers published over the next few years, but it is mentioned in passing in Rita's autobiography.

> Two weeks later [after the NY Academy meeting], I was back in the lab attempting to reproduce the tumors' effects by injecting their extracts into embryos at early stages of development. Persistently negative results led me to resort to other techniques. (Levi-Montalcini 1988:152)

The obvious next approach was to see if the critical finding could be demonstrated in vitro, and in the fall of 1952, Rita set out to do just that.

While working in Turin, Rita had come to know Hertha Meyer, who had set up and maintained a tissue culture facility for Professor Levi's studies of axonal growth. Hertha (as Rita affectionately refers to her) had been trained and had worked in Germany, but when the Nazis seized power she moved to Italy. Later, when the Italian fascists began to flex their muscles she moved again, this time to Brazil, where she joined the Institute of Biophysics of the University of Rio de Janeiro, headed by Professor Carlos Chagas. Rita had kept in contact with her from time to time, and so when the next step in Rita's work called for a culture approach, it was natural that she should turn to her friend for help. Fortunately, Viktor was able to persuade the Rockefeller Foundation to provide Rita with a travel grant to enable her to visit Rio for 3 months. After returning to Italy for a brief visit to see her family, Rita traveled to Rio in late September 1952, accompanied by two white mice (each bearing a transplanted sarcoma) concealed either in her coat or in her purse (this small point varies in Rita's later accounts of her visit).

It was during the first 2 months of her visit that Rita discovered that when small fragments of the sarcoma were placed within 1–2 mm of explanted sensory ganglia from 6– to 7-day-old chick embryos, there was a striking outgrowth of neuronal processes, giving the ganglia a characteristic halo-like appearance. Throughout her stay in Rio, Rita conscientiously kept Viktor informed of her initial disappointments and later successes. She also sent him a series of pen-and-ink drawings she had made of the appearance of the stimulated ganglia, which he returned to her many years later when she was preparing to leave St. Louis for a new position in Rome. After returning to St. Louis in January 1953, Rita set up her own tissue culture facility and carried out an extensive series of experiments involving not only the two mouse sarcomas, but also adenocarcinoma and neuroblastoma cell lines. These and the initial series of experiments in Rio formed the subject of a full length paper that appeared the following year (Levi-Montalcini et al 1954).

This paper begins by setting out the rationale for the in vitro approach. Two specific reasons are given: (*a*) that the culture method obviates the possibility that the in vivo effects of the tumor on the nervous system are secondary to some generalized metabolic influence on the embryo; and (*b*) that this method might provide a useful bioassay for screening the action of the tumors (and, later, the active factor itself). The decision to focus on the sensory ganglia (rather than sympathetic ganglia, even though they had responded more vigorously to the

transplanted sarcomas) is said to have been based on the fact that in 1949 they had given a very detailed account of the development of the sensory ganglia, which could serve as a control. It may also have been influenced by the fact that Hertha Meyer had used sensory ganglia in a study she had done with Levi in Turin (Levi & Meyer 1941), although it should be mentioned that Levi & Delorenzi (1935) had earlier grown sympathetic ganglia in vitro. Probably the decisive reason was that it is considerably more difficult to dissect out sympathetic ganglia from 6- or 7-day chick embryos than the larger sensory ganglia. In any event, just over 100 sympathetic ganglia were cultured (42 with fragments of the mouse sarcomas) and a total of 668 spinal ganglia were cultured. In Rita's earliest experiments in Rio, the explanted tumor tissue was taken directly from the carrier mice; however, she soon found that such tissue fragments were considerably less effective than she had found before when the tumors were transplanted into chicks. Rita therefore tried passaging the tumor tissues through chick embryos before using them for her in vitro experiments; this proved to be very effective and so for all the later experiments such chick-passaged tumor tissue was used.

The results from the two sarcomas (180 and 37) were essentially the same. When small fragments were cultured within 2 mm of the explanted ganglia, they had a profound effect on the outgrowth of processes from the sensory neurons. As early as 16 h after coculture, large numbers of fibers had grown out of the ganglia, whereas in control preparations few or no fibers grew out at this time. The fiber outgrowth in the cocultures was always more conspicuous on the side facing the tumor, but by 24 h the entire ganglia were surrounded by haloes of nerve fibers. Conversely, the outgrowth of spindle-shaped cells (presumably fibroblasts or satellite cells) was suppressed in the presence of the tumors compared with the control preparations. This appearance persisted through 48 h of culture, by which time the haloes were very dense. Essentially the same pattern was seen when the sarcoma tissue was grown close to sympathetic ganglia (from 8– to 13-day–old embryos), the only differences being that the fibers were generally finer; in these experiments the halo, if anything, was more dense.

Additional experiments involving cocultures of the tumors with fragments of chick heart and spinal cord explants were uninformative, and to the extent they were analyzed, it seemed that the tumors had no effect on these tissues; it is particularly noteworthy that they did not increase the limited outgrowth of nerve fibers seen in control spinal cord explants. Three other tumor types were used: sarcoma 1, which when cocultured with spinal ganglia resulted in enhanced fiber outgrowth—but neither as consistently nor as markedly as with sarcomas 180 and 37; mammary adenocarcinoma DBRB, which did not provoke fiber outgrowth from the ganglia; and neuroblastoma C1300, which seemed to actually inhibit the outgrowth of both cells and fibers from the ganglia. The most important additional experiments reported involved coculturing chick spinal ganglia with fragments of heart tissue from embryonic, fetal, and newborn mice. Unlike chick heart explants that had earlier shown no effect on the ganglia, the mouse tissues stimulated fiber outgrowth from the ganglia within 24 h, and this was even more marked by the end of the second day. The appearance of the ganglia in these experiments was

different from that seen with the sarcomas, but the essential finding that the mouse tissues promoted fiber outgrowth was unquestionable and later proved to be of considerable interest (see below).

The conclusions to be drawn from these in vitro experiments were clear-cut. They confirmed that sarcomas 180 and 37 had a distinct, and evidently selective, influence on the outgrowth of nerve fibers from the ganglia and that this effect was mediated by a diffusible factor, as evidenced by the fact that it did not require the tumor and ganglion explants to be in contact. Furthermore, as the distance between the two explants was progressively increased, the neurite growth-promoting effect was proportionately reduced. The experiments served also to resolve an unanswered question from the prior in vivo studies, namely the possibility that the growth-promoting agent acted to "break down" some barrier that normally limited the degree to which organs and tissues can be innervated. Rather the in vitro experiments established that the product of the tumors acts directly on the ganglion cells to promote outgrowth of their processes. The coculture experiments using normal mouse tissues and chick sensory ganglia were also significant in suggesting that the growth-promoting factor was not the abnormal product of transformed tissues but might be produced and released by a variety of normal mouse tissues. But most important, these in vitro experiments raised the possibility, for the first time, that the growth-promoting factor might be isolated, and its presence assayed, at least semiquantitatively, by the use of the "halo effect." That this was a real possibility was soon to be demonstrated.

STANLEY COHEN AND THE ISOLATION OF THE NERVE GROWTH-PROMOTING FACTOR FROM MURINE SARCOMAS 180 AND 37

At some time during Rita's visit to Rio, both she and Viktor seem to have realized that if further progress were to be made and, in particular, if they were going to be able to isolate and characterize the nerve growth-promoting factor, they would need the assistance of a trained biochemist. Fortunately, exactly the right person was available: Stanley Cohen who was just completing his postdoctoral training and looking for a position and for a new challenge.

Stan (as he is generally known) was born in Brooklyn in 1922 of Russian immigrant parents. After high school he entered Brooklyn College, where he majored in biology and chemistry, and then went on to do a master's degree at Oberlin College. Transferring from Oberlin, he completed his PhD in biochemistry at the University of Michigan in 1948. This was followed by a few years as an instructor in the department of pediatrics and biochemistry, where he was engaged in metabolic studies of premature infants under the direction of Professor Harvey Gordon. In 1952, he moved to Washington University in St. Louis on an American Cancer Society fellowship, to work with Martin Kamen in the department of radiology. Here he came into contact with an intellectually stimulating and supportive group

of scientists associated with Carl and Gerti Cory in the department of biochemistry, and with Arthur Kornberg and the remarkable group of colleagues he had attracted to the department of microbiology. Over the years Viktor and Rita had been drawn into this circle, and it was through this association that Viktor learned that Stan Cohen might be available for the planned assault on the nerve growth-promoting factor. As Rita recalled, on hearing from Viktor that he had invited Stan to join them and had obtained funds from the Rockefeller Foundation to support his work, she wrote to Viktor from Rio: "From the way you describe him he seems the right person to tackle the difficult problem of identifying the factor released by mouse sarcomas." Later, when she had worked with Stan for a while, she was to say:

> I have often asked myself what lucky star caused our paths to cross . . . If I, in fact, knew nothing of biochemistry, Stan when he joined us had but vague notions of the nervous system. . . 'Rita,' Stan said one day, 'you and I are good, but together we are wonderful (Levi-Montalcini 1988)

Their immediate task was to prepare sufficient tissue from the two mouse sarcomas (after passage through chick embryos) and to set up the in vitro bioassay that Rita had developed in Rio. The work went surprisingly well, and by June 1954, Viktor was able to submit a paper to the Proceedings of the National Academy of Sciences describing the isolation of the growth-promoting factor (Cohen et al 1954). This was to be the fourth, and last, paper on this topic that bore Viktor's name. As he wrote in his autobiographical statement: "In the mid-1950's I withdrew from the project. I could no longer contribute to it because of its biochemical nature; but, of course, I followed its progress with keen interest" (Hamburger 1996).

The paper reporting the isolation of the nerve growth-promoting factor (it was not yet called nerve growth factor or NGF, for short) was brief, focused, and wholly convincing. "We have . . . found," it stated, "that cell free homogenates of the tumors [S180 and S37] can duplicate in culture, the effect of the actively growing tissue." From the initial tissue fractionations it was evident that biological activity was limited to the microsomal fraction that contained about 16% of the dry weight of the tumor. Further fractionation of the microsomal preparation using streptomycin to precipitate the highly polymerized nucleic acids and nucleoproteins yielded a fraction that possessed essentially all the activity of the whole homogenate. After treatment, a solution was obtained that showed a typical nucleoprotein absorption curve with a peak at 260 nm. The active material in this solution was heat labile and nondialyzable, and in the best preparation represented 3% of the dry weight of the tumor. It consisted of 66% protein, 26% RNA, and less than 0.3% DNA.

While convincing, the paper showed some signs of having been hurriedly written and without the usual attention to detail seen in most papers bearing Viktor's name. For example, each culture used for the in vitro assay was said to contain "a sympathetic ganglion isolated from a 10-day chick embryo," but the second group of four photomicrographs show only "silver impregnated sensory ganglia." Despite this caveat, from a historical point of view, this paper marked a critical

turning point and paved the way for the next surprising discovery and, ultimately, for the isolation of NGF from an unexpected source.

SNAKE VENOM AND MOUSE SALIVARY GLANDS (1956, 1960)

What followed was one of the most remarkably serendipitous events in the history of neuroscience. Since this aspect of the NGF saga has been recounted on many occasions I need only deal with it briefly. It began with a conversation between Stan Cohen and Arthur Kornberg, at that time head of microbiology at Washington University and already distinguished for his contributions to DNA replication. Stan was concerned to know whether the factor he had isolated from the two mouse sarcomas was simply a protein or a "nucleoprotein" (i.e. a protein bound to RNA or DNA). Kornberg suggested that he treat the preparation with an available snake venom that was known to be a good source of the enzyme phosphodiesterase, which would degrade whatever nucleic acids were present. If this treatment resulted in the loss of biological activity, it would strongly suggest that the active ingredient was in the nucleic acid fraction; on the other hand, if the preparation retained its activity, one could conclude that the active material was a protein (or a mixture of proteins). Stan promptly carried out the necessary experiment and gave the treated and control material to Rita to assay in her hanging-drop cultures. Within several hours Rita found that the preparation that contained the snake venom had produced an extraordinary halo radiating out from the ganglion. Since this preparation also contained the extract from sarcoma 180, it was not clear whether the observed result was due to the direct action of the snake venom on the ganglion cells, or whether some component of the venom caused the removal of a hitherto undetected inhibitory factor in the sarcoma extract. This issue was quickly resolved. The addition of a small quantity of snake venom by itself to a ganglion culture resulted in an equally dramatic outgrowth of nerve fibers. The conclusion was as unequivocal as it was surprising: The venom must contain a nerve growth-promoting factor either the same as or very similar to that in the original murine sarcomas.

These findings were reported in 1956 in a brief paper in the *Proceedings of the National Academy of Sciences* that was communicated by Viktor (Cohen & Levi-Montalcini 1956). The paper documents the methods and materials used (commercially available venom from two different species of snakes—the moccasin, *A. piscivorus*, and the rattlesnake, *Crotalus ademanteus*) and summarizes some of the properties of the active factor in the venom, including the fact that it was heat labile and nondialyzable. But the most important conclusion was that in each case the factor had a specific activity (on a protein basis) of at least 1000 times that of their best purified tumor fractions.

It is not clear from the published reports who first raised the possibility that it would be worth examining the salivary glands of mice (the mammalian homologues of the venom producing glands in snakes) to see if they too contained a nerve

growth-promoting activity. As was mentioned earlier, during Rita's stay in Rio she had carried out some in vitro experiments using both normal chick and mouse tissues and had found that whereas the chick tissues were ineffective, several of the mouse tissues examined caused a demonstrable outgrowth from the cocultured ganglia (Levi-Montalcini et al 1954). At the time this seemed to be just a curious, even uncomfortable anomaly (since it was then thought that the growth-promoting factor was most likely a feature of neoplastic tissues). As she later wrote:

> The mouse effect was a message I was not really capable of receiving, since I could not help thinking that it diminished—to the extent of annuling—the significance of the induction of the fibrillar halo by S180 and S37.
>
> (Levi-Montalcini 1988)

In a letter she had written to Viktor from Rio, she indicated that she was going to put aside for the time being the "mouse effect," describing it as "an unpleasant and complicated finding" (see Levi-Montalcini 1988). Regardless of whose idea it was, the decision to explore the issue was made, and in another publication, Stan reported the results of an extensive series of experiments aimed at isolating a growth-promoting factor from mouse salivary glands (Cohen 1960). In two companion papers published in the same volume, Rita and one of her graduate students, Barbara Booker, described the effects of the factor Stan had isolated and of an antiserum that he had raised against the protein (Levi-Montalcini & Booker 1960a,b).

Again it is unnecessary to describe the methods used to isolate the active fraction from the submaxillary glands of mice and the way it was assayed using sensory ganglia from 8- to 9-day-old chick embryos. However, several specific findings are worth noting. The first is that there was essentially no activity detectable in the fractions from the glands of young mice between birth and 17 days of age; thereafter the activity became increasingly evident and appeared to reach its maximum at about 50 days. It is interesting also that the specific activity of the factor isolated from the submaxillary glands of male mice was, on average, about fivefold higher than that from females. Comparable fractions from the submaxillary glands of hamsters and rats were also active, but at a level about a thousandth that found in adult male mice. The sublingual gland yielded a fraction with about a hundredth the potency of the submaxillary gland, and there seemed to be no detectable activity in the parotid glands. The activity in male submaxillary glands exceeded that in several other mouse tissues (heart, striated muscle, thymus, kidney, and serum) by a factor of approximately 5000.

Different modes of preparation of the submaxillary gland tissue yielded fractions of markedly different potency, but two in particular, identified as CM^2 and CM^3, yielded a substantial (3^+) response in the "halo" assay, at concentrations as low as 0.045 and 0.015 μg/ml, respectively. Like the factor isolated from snake venom, the submaxillary factor was heat labile, nondialyzable, and essentially removed by treatment with pepsin and chymotrypsin. Injections of the CM^2 and CM^3 factors into newborn mice resulted in a marked ($\sim$sixfold) increase in the protein

concentration of the superior cervical ganglia, and a two- to threefold increase in RNA and DNA, without affecting overall body weight.

Stan raised a polyclonal antiserum to the growth-promoting factor in rabbits. When introduced into their mouse bioassay system, the antiserum had the effect of completely blocking the biological activity of the mouse submaxillary factor; it also reduced the activity of the factor in snake venom, indicating that there must be some degree of cross-reactivity. (Conversely a commercially available antivenom did not affect the activity of the mouse factor.) Subcutaneous injections of the antiserum resulted in the rapid and near-total destruction of nerve cells in the sympathetic ganglia—a finding described more fully in the second paper by Rita & Booker (1960b).

This work was carried out at Washington University, but by the time the paper was published, Stan had taken up a position in the department of biochemistry at Vanderbilt University. According to Viktor, budgetary constraints made it impossible for him to offer Stan a faculty position in the zoology department. Stan and Rita were told of this decision in December 1958, and in the summer of 1959 Stan took up his new position. Viktor did, however, communicate Stan's paper to the National Academy, and on a personal level relations between them remained warm and supportive. In a sense, this marked the end of Stan's active participation in the work on what was becoming known as NGF, but he followed its further exploration with interest, albeit at a distance. Before he left St. Louis, Stan made another wholly unanticipated discovery. This was the finding that mice injected with a partially purified preparation of the mouse submaxillary factor showed premature opening of the eyelids (as early as 7 days rather than 12–14 days, which is normal) and precocious eruption of the incisor teeth (at 6–7 days instead of 8–10 days). A less astute observer, and especially one focused only on the changes in the nervous system, would have missed these findings and, in the process, missed the discovery of a second and in many respects equally interesting factor—epidermal growth factor (EGF). Stan described the isolation of this new factor in a paper in the *Journal of Biological Chemistry* in May 1962 (Cohen 1962). In 1986, when he shared the Nobel Prize with Rita, he was specifically cited for the independent discovery of EGF and its further development.

THE EFFECTS OF THE NERVE GROWTH-PROMOTING FACTOR ISOLATED FROM MOUSE SUBMAXILLARY GLANDS AND THE ACTIONS OF AN ANTISERUM DIRECTED AGAINST THE FACTOR ON THE SYMPATHETIC NERVOUS SYSTEM (1960)

The effects of injecting the submaxillary nerve growth-promoting factor on the mouse sympathetic nervous system that Stan had mentioned in his last paper on this topic, and the consequences of injecting the rabbit antiserum he had raised,

were only alluded to in a brief paragraph in his paper, but they were described more fully in the two papers by Rita & Barbara Booker (1960a,b). Rita also described them in a review she wrote in 1958 (Levi-Montalcini 1958).

The experimental section of the first paper begins with an account of the treatment of sensory and sympathetic ganglia isolated from four human fetuses (at 2.5 and 3.5 months of gestation). Using their usual hanging drop culture preparation, these ganglia were exposed to the mouse tumor extract, snake venom, and the purified submaxillary gland factor. They were found to respond in the same way as chick and mouse ganglion explants, producing a dense halo of outgrowing nerve fibers during the first 24 h in culture. Over the next 48 h the human sympathetic ganglion cultures underwent considerable liquefaction and were therefore discontinued.

Of greater interest were the experiments on newborn and adult mice injected with different submaxillary gland preparations. For these experiments large numbers of mice (10–50 in each treated group) were injected with differing concentrations of two of the fractions that Stan had isolated (fractions CM^1 and CM^3). The sympathetic chains were dissected out and usually stained as whole amounts; in some cases the superior cervical ganglia were sectioned and stained for histological examination and for counts of the numbers of mitotic figures. From a further group of 150 adult and 30 weanling mice, serum was isolated and tested (using 8-day-old chick sensory ganglion cultures) for the presence of the nerve growth-promoting factor.

It is hardly necessary to review all the experimental data analyzed in this paper. Suffice it to say that in all the mice injected with the submaxillary gland factors, the sympathetic ganglia were enlarged—up to six times in some cases. The degree of enlargement varied with the age of the animals at the time of injection (it was maximal in newborns), the amount of material injected, and the purity of the fraction used (the CM^3 fraction was consistently the most potent). The enlargement of the sympathetic ganglia was due to both the hypertrophy of individual ganglion cells and to an increase in mitotic activity (which was maximal at 5 days postnatally). Sympathetic neurons in male mice were, on average, larger than those in females; this appeared to be correlated with the appreciably higher concentration of the growth factor in the serum of adult male animals.

Although largely confirmatory of the results reported in brief by Stan, this study involved a considerable amount of work, and the documentation of the data is extremely detailed and compelling. It also marked a major departure for Rita from her previous work that had been almost exclusively focused on chick embryos and isolated chick sensory and sympathetic ganglia. From this time on, most of her work was carried out on mice (and to a lesser extent on other mammals, especially rats and hamsters). For many scientists who were trained in mammalian neurobiology, the documentation that the nerve growth-promoting material acted on mammalian neurons (as opposed to those of chicks) was considered especially important.

The second paper by Rita & Booker (1960b) dealt with the dramatic effects of injecting the antiserum that Stan had raised on the development and maintenance

of the peripheral sympathetic system. Most of these experiments were carried out on newborn mice, but a few similar experiments were performed on newborn rats, rabbits, a pair of kittens, and one 7-day-old squirrel monkey.

Again, the most important findings in this study can be briefly summarized. Mice that were injected with the antiserum each day from birth to 25 days of age developed normally and, on superficial inspection, were indistinguishable either from control animals injected with normal rabbit serum or from their untreated littermates (No attempt, however, seems to have been made to test the animals under conditions that would normally have stressed the sympathetic system.) On examination, the sympathetic chain and its associated ganglia were markedly reduced in size. Counts of the numbers of neurons in the superior cervical ganglion at 20 and 25 days showed that they were reduced to between 0.6% and 1.7% of the number seen in control mice. As early as day 4, the volume of the ganglia was no more than one sixth that in normal animals. Counts of the numbers of mitotic figures showed them to be clearly reduced in the youngest animals analyzed (just 1 day after beginning the antiserum injections) and by 2 and 3 days they reached a very low level; also at this time appreciable numbers of degenerating cells could be seen throughout the ganglia. Since small numbers of neurons were still present after 25 days, a few animals, injected for 8–20 days, were allowed to survive for periods ranging from 90 days to 4 months. In these mice the percentage of neurons that survived varied between 0.84 and 2.56, which suggested that beyond the first several days, no further neuronal loss occurred. No attempt was made to determine whether injections of even larger amounts of antiserum would completely eliminate all neurons from the ganglia.

Fewer experiments were attempted in other mammals, but the results all pointed in the same direction: After as few as seven daily injections (adjusted for body weight) the ganglia in the treated animals were reduced in volume by 90%–99% and the percentage of surviving cells was reduced to between 7% and 16%.

The interpretation of these findings remained open; as the authors discuss, the effect on the sympathetic ganglia could be due to the neutralization of a circulating growth factor (and the presence of the factor in the serum of male mice was considered consonant with this view) or to a direct cytotoxic action of the antiserum. However, the findings proved to be of considerable interest to neuroscientists. They raised a number of questions that would only be resolved several years later, such as the natural source of the nerve growth-promoting material: Was it produced in only a few select organs (like the submaxillary gland) or by most tissues innervated by the sympathetic system? Were the relatively rare cases of dysautonomia reported in the medical literature due to a comparable autoimmune mechanism or to some other selective, developmental disorder? But for the short-term future, the discovery of a practical method for immunosympathectomy provided developmental biologists with yet another useful tool.

Since by 1960 essentially all the ground work on the NGF saga had been done, this is a convenient point to bring this section to a close. The accompanying review

by Dr. Eric Shooter continues where this account leaves off and documents the ensuing decades of work on the chemistry and molecular biology of NGF and the many later discoveries bearing on its biological role.

VIKTOR'S FURTHER CONTRIBUTIONS TO CENTER/PERIPHERY RELATIONS AND HIS BELATED RETURN TO NGF

Although Viktor did not participate in the work on the growth-promoting factor once it had moved into its "biochemical phase," he continued for a while to be interested in the center-periphery issue, especially as it bore on the development of the LMC of the spinal cord. For a number of years he was principally engaged in studies of the ontogeny of behavior in chicks and rats (see Cowan 1981). Also while it is widely believed that Bueker had essentially lost interest in the problem after his initial observations on the effects of the murine sarcoma 180, he published half a dozen papers of interest after Viktor and Rita had taken up the subject (Bueker & Hilderman 1953; Bueker et al 1960; Bueker & Schenkein 1964; Schenkein & Bueker 1962, 1964). These papers have rarely been cited—indeed it is only in the extensive review by Rita and Pietro Angeletti that they are nearly all listed, but even here only the paper by Schenkein & Bueker (1964), which suggested that the active material might consist of two related components, is discussed (Levi-Montalcini & Angeletti 1968).

Before Viktor turned to the problems of early behavior, he made one further contribution of note to the periphery's influence on the development of the motor system (Hamburger 1958). This was essentially a follow-up of his work with Rita on the development of the sensory ganglia, but it focused specifically on the normal development of the LMC and the effects of early limb bud extirpations. The study confirmed that during normal development, cell proliferation in the ventral part of the cord is essentially over by day 4 of incubation and that by day 5.5 the LMC is fully assembled. The temporal separation of cell proliferation and migration and the subsequent outgrowth of motor fibers in some respects make the development of the LMC easier to analyze than the spinal ganglia. This point became especially clear when it was recognized that in normal development there is a considerable degree of cell death in the chick LMC between days 6 and 8, and when it was found that this degeneration is markedly accentuated following early limb extirpation. These observations finally settled the issue of the effect of the periphery on the LMC: Like sensory ganglion cells, motoneurons are dependent for their survival on the periphery; by contrast, the periphery has no effect on their earlier differentiation and migration. To this extent the study both confirmed and amplified Levi-Montalcini and Levi's earlier work and served to bring the motor system into line with the work on the sensory ganglia. While the essential findings are indeed confirmatory, Viktor's paper bears all the hallmarks of his other studies: It is carefully reasoned,

the methods and the findings are described in detail, and the general conclusions drawn are both clear-cut and convincing.

In the mid-1970s, some 17 years after this last study, and after publishing 10 research papers and a number of influential reviews on the ontogeny of behavior in chicks and rats, Viktor returned to the problem of cell death in the LMC (Hamburger 1975). Taking advantage of the fact that the motor cells in the columns are large and easily distinguished from the time the column is first recognizable, and since they are not so numerous as to make estimates of their number difficult, he undertook a systematic analysis of the numbers of motoneurons in the lumbar cord from day 5.5 of incubation to just after hatching. The importance of such systematic cell counts to determine the time course of what came to be known as "naturally occurring neuronal loss" had been pointed out in a number of previous papers and reviews (see, for example, Hughes 1961, Cowan & Wenger 1967, Prestige 1970, Cowan 1970, Rogers & Cowan 1973). In the absence of firm evidence about how long it takes for a neuron to die and for the resulting cellular debris to be removed, it was difficult to determine the real magnitude of the cell loss from counts of degenerating neurons. Therefore Viktor thought it important to document the scale and time course of the naturally occurring loss of motoneurons by serial cell counts at 5.5, 6, 7, 8, 9, 12, and 18 days of incubation, and on day 5 after hatching.

The principal observation in this study is that in normal chick embryos, the lumbar lateral motor column, when first fully assembled at day 5.5, contains approximately 20,000 motoneurons; this number persists at day 6, but by day 7 it is reduced to an average of about 18,400 cells and by day 8 to just over 16,500. By day 9 it is further reduced to about 13,000 and to just over 1200 by day 12. From then on the number remains constant until after hatching. The rapidity of the cell loss over just a 3–day period, its magnitude (~40%), and its timing (corresponding to the period between the arrival of motor axons at the periphery and the establishment of the initial innervation of the limb muscles, as was documented from an examination of silver-stained preparations) are all striking, and most easily interpreted in the following terms: There is an initial overproduction of neurons followed by the subsequent degeneration of roughly half the initial number of cells. The fact that this naturally occurring cell loss begins at the time the axons of the cells first reach their target field and ends about the time the innervation of the target field is complete suggests that the axons compete within their target field for a limited supply of an essential maintenance factor (which in the case of spinal sensory and sympathetic neurons would be NGF). The cells that are unsuccessful in this competition die while those that are successful survive throughout the life of the organism. Removing the target field (e.g. by early extirpation of the developing limb bud) leads to an accentuation of this cell loss that in radical cases may be total (for review, see Cowan 1970, Oppenheimer 1981).

While this general hypothesis was consonant with virtually all the available data at the time Viktor published his study, several key elements remained to be determined. Chief among these was the following question: Given that most muscle fibers are initially innervated by several axons (although generally only one

persists), why cannot all the axons compete equally for the available maintenance factor? And of course, the nature of the putative maintenance factors remained unresolved. By 1975 only NGF had been identified, but its selective action on spinal sensory and sympathetic neurons suggested that there might be a number of comparable trophic factors essential for the long-term survival of other classes of neurons (including motoneurons). There was also the question as to what maintains the neurons in the interval between the time they first differentiate and the time their axons reach their target fields. Prestige (1970) had postulated that neuroblasts are supplied ab initio with a supply of a maintenance factor that supports them until they innervate their targets; this may be so, but alternatively, it is conceivable that the cells draw on some other trophic support from their local environment or from the successive environments traversed by their axons. Lastly, there was the question of whether axons compete for specific contact sites, rather than for trophic substances. The example of NGF argued strongly for the latter view, but it was not clear whether this paradigm would hold for all classes of neurons. As Viktor stated:

> The available data do not permit a decision between the different alternatives. Once the analysis has been carried to the molecular level, the difference between a competition for contact sites and a competition for 'trophic' agents might disappear. (Hamburger 1975)

In the 1970s Viktor had a succession of postdoctoral fellows working in his laboratory, who, under his guidance, revisited a number of the issues that he and Rita had jointly or individually examined earlier. Among these fellows were Margaret (Peggy) Hollyday, Judy Brunso-Bechtold, and JW Yip, who between 1976 and 1981 published five important papers bearing on our present theme. While the fellows were generally responsible for the experimental work and the preparation of the material, Viktor actively participated both in the collection of the data (including often counting many thousands of neurons) and, most important, in its analysis.

The first of these studies (Hollyday & Hamburger 1976) was prompted by the suggestion, first clearly articulated in a review of the role of cell death in the regulation of neuronal number, that the so-called hyperplasia observed in the LMC and sensory ganglia after supernumerary limb transplants might be due, not to an increase in cell proliferation or neuronal differentiation, but to a reduction in naturally occurring cell death (Cowan 1970). To test this possibility, Peggy Hollyday repeated Viktor's earlier experiments with limb transplants (Hamburger 1939) but added an important dimension to the earlier work by systematically counting the numbers of motoneurons in the LMC in chicks with supernumerary hind-limb transplants before and after the period of naturally occurring cell loss, as defined in Viktor's 1975 paper.

The limbs were transplanted between stages 17 and 18 of the Hamburger & Hamilton (1951) series, and only those that looked morphologically normal at 6 days (stage 28) or exhibited normal patterns of motility at 11–12 days (stages

37–38) and again at 18 days were used for the analyses of cell numbers. The transplanted limbs were innervated from thoracic segment 22 and from lumbar segments 23–25. Corrected cell counts in the animals killed at 6 days (before the normal onset of cell death) showed no difference in the numbers of motoneurons in the LMC on the experimental (transplant) and control sides. In the 12-day-old animals, by contrast, the number of motoneurons was consistently higher on the side bearing the transplants, with the percentage increase ranging from 11% to 27.5%. In the two cases examined at 18 days, the numbers on the two sides were comparable to those seen at 12 days, with the transplant side having 11%–12% more motoneurons then the control side.[2] It is interesting that although one might have expected most of the increased number of cells to be limited to the rostral levels of the LMC (since it is from these levels that the transplanted limbs received their innervation), the data indicate that the increased motoneuron survival on the experimental sides was spread out over most of the rostro-caudal extent of the lumbar column.

The general interpretation of these findings was straightforward. Since the cell proliferation that gives rise to the lateral motor column extends from stage 17 through stage 24, it might have been argued that limbs transplanted at stage 17.5 could have influenced the genesis of the relevant motoneuron precursor pool. However, this interpretation is precluded by the finding that at day 6 (stage 28), when cell proliferation has ceased, the numbers of motoneurons on the control and experimental sides are the same. This leaves open only one plausible view, namely that the presence of the supernumerary limbs enables more motoneurons to survive than would occur normally. In other words, by expanding the "target field" of the motor column, the number of naturally occurring cell deaths is reduced.

As the authors point out, in light of this finding it is misleading to use the term hypoplasia for the change seen in the LMC after limb transplants (since it implies an increase either in cell proliferation or differentiation). Instead they suggested the terms neurothanasia for the process of naturally occurring cell death and hypothanasia for the reduction in cell death seen when the target field is expanded. To their disappointment, neither of these terms has come into general use. Their findings did not bear on the question of whether the survival of an increased number of motoneurons was due to an increase in the number of available innervation sites or to the increased availability of the postulated trophic or maintenance factor. In the absence of data about the actual location of the motoneurons that innervated specific muscles in the transplanted limbs, they could also throw no light on the unexpected finding that the hypothanasia extended over the entire length of the motor column.

The second study addressed the exact period during which motoneurons are generated in the brachial and lumbar segments of the chick spinal cord (Hollyday

[2]In his 1989 autobiographical review, Viktor noted that his colleague, Josh Sanes, had pointed out that if the comparison is made between the numbers of motoneurons before the onset of the naturally occurring degeneration at day 6 and after its termination at day 12, the actual increased cell survival in these experiments would be about 30%.

& Hamburger 1977). Previous evidence bearing on this point was equivocal. For the most part it was based on mitotic counts in the relevant regions of the cord, or on reports of the stage at which the first cytologically identifiable motoneurons could be recognized. By the 1970s the use of [^{3}H]thymidine autoradiography to determine the time of origin (or birth dates) of neurons had been well established (see Angevine 1965), and a variant of this approach had been used to study the birth dates of neurons in the spinal cord (Fujita 1964) and the multilayered optic tectum of chicks (LaVail & Cowan 1971). Since labeled thymidine introduced into the egg remains available for incorporation into DNA for a considerable period of time, the method used relies on the appearance of unlabeled neurons in the population (rather than labeled cells, as in pulse labeling studies as generally done in mammals).

The main finding of the study was that at least 95% of the motoneurons in the brachial cord are generated between stages 15 (2.5 days) and 23 (4 days) and in the lumbar cord between stages 17 and 23. There was also a clear medial to lateral (or inside-out) gradient in the time of appearance of the motoneurons in the LMC. And the paper also helped to clarify the origin and permanent location of early formed large cells in the alar region of the cord, which others had suggested might later migrate into the motor column.

Peggy Hollyday's third paper (with Viktor and Juanita Farris) examined the cells of origin of the fibers that innervate one specified muscle (the gastrocnemius) in the transplanted and normal control limbs, using as a marker the retrograde transport of horseradish peroxidase (HRP) injected into the muscle. This showed that in normal limbs the muscle is innervated by a central dorsal cluster of motoneurons in segments 26–29, whereas the gastrocnemius in the supernumerary limb consistently received its innervation from a medial cluster of neurons in segments 23–25. Although no attempt was made to determine which muscles are normally innervated by motoneurons in the latter region, the obvious conclusion was that the muscles in transplanted limbs are innervated by different cells than their normal counterparts, a finding that is of some interest for the question of neuronal specificity during development (Hollyday et al 1977).

As we have seen, when Viktor first studied the effects of early limb extirpations on the development of the LMC, he concluded that the first motor axons to grow out sensed in some way the overall extent of the field to be innervated and signaled this "estimate" back to the emerging motor pool to regulate the induction of more (or fewer) motoneurons from a population of as-yet-undifferentiated cells. Later, when it became clear from Rita's work with Levi (Levi-Montalcini & Levi 1942a,b; 1943) and his own studies with Rita (Hamburger & Levi-Montalcini 1949), that the hypoplasia that occurs in such experiments is due, not to a reduction in the inductive influence of the periphery, but rather to the death of previously differentiated cells, he accepted the view that the causative mechanism involved the availability of a trophic or maintenance factor in the target region. This led him some years later (Hamburger 1989) to re-interpret his 1934 hypothesis and to imply that the inductive signal he had originally postulated actually corresponded to the

retrograde transport of a trophic agent. In reality the evidence for the retrograde transport of such an agent from the periphery was largely indirect. For the most part it was derived from such observations as the quantitative relationship between the magnitude of the observed changes in the motor column and the extent to which the peripheral field was either reduced or enlarged. Interestingly, most of the work that had been done on NGF, in vivo, did not focus on this issue since it usually involved either tumor implants or the systemic injection of the growth-promoting factor. In 1978 Viktor revisited this issue with Judy Brunso-Bechtold, taking advantage of the availability of ^{125}I-labeled NGF prepared in the laboratory of Dr. Ralph Bradshaw.

Judy inserted small pellets of polyacrymalide gel that were impregnated with ^{125}I-labeled NGF, into the knee region of chicks at about stage 36 (day 10); and 8 h later sections of the lumbar region (including the spinal cord and spinal ganglia) were prepared for autoradiography. In the four successful cases, the ipsilateral lumbar spinal ganglia, especially ganglion 23, were intensely labeled, as were the peripheral nerves leading from the site of the ^{125}I-labeled NGF pellets. Labeling over the contralateral ganglia, the sympathetic ganglia, and the motor columns and alar region of the spinal cord never exceeded background levels.

In retrospect this simple experiment was the first clear demonstration that NGF could be selectively taken up by sensory nerves and retrogradely transported to their cell bodies in the spinal ganglia. To this extent it was valuable in providing one of the missing elements in the overall NGF saga.

Of the five papers from this period, the last, which it is worth noting was dedicated to Rita, is in some respects the most important. It is also noteworthy that Viktor appeared as the first author on this paper, and to those familiar with his work, the paper clearly bears the stamp of his mind and style (Hamburger et al 1981). The purpose of the study was to see if an exogenous source of NGF could prevent, or at least limit, the amount of naturally occurring cell death in the spinal ganglia. But the paper went well beyond this and, among other things, served to correct an error in what by this time was usually referred to as "the classic paper" by Viktor and Rita on the development of the spinal ganglia.

The design of the study was straightforward. Daily injections of NGF were made into the yolk sac between stages 21 (3.5 days) and 38 (day 12), and at appropriate intervals careful counts were done of the numbers of degenerating neurons in thoracic ganglion 18 and brachial ganglion 15. These counts were then compared with similar counts in the same ganglia from normal (untreated) animals. An important part of the experimental design was to separately examine the scope of the neuronal degeneration in the two subdivisions of the ganglia: the large-celled ventrolateral (VL) division and the smaller celled dorsomedial (DM) population.

The paper begins with a description of thoracic ganglion 18 as it appears at several stages [between day 4.5 (stage 24) and day 8.5 (stage 35)] and then proceeds to document the numbers of degenerating neurons seen in its VL and DM divisions over the period from day 4.5 to 12. As Viktor and Rita had reported previously,

cell death in the VL division reaches its peak at about day 5 and then declines to a fairly low level by day 6.5. In the DM division, the numbers of degenerating cells show a rather dramatic peak at day 8 and the numbers remain quite high through day 10. Following the administration of endogenous NGF, there is an appreciable reduction in the numbers of degenerating cells in both divisions of the ganglia. It is most marked in the DM division, where the number of such cells remains low throughout the entire period from days 5.5 to 12. In the VL division, the peak number of degenerating cells in the treated embryos is less than half that seen in the control preparations at day 5.5, but the number rises above the control level around day 8.

In interpreting the findings, Viktor pointed out that throughout the period studied, there is a level of what he terms sporadic cell deaths. These had been reported previously, and it was generally assumed that such deaths are caused either by errors in DNA replication or the later phases of the mitotic cycle, or by some intrinsic metabolic process. But the most important new finding was that there are distinct periods of cell degeneration in the two divisions of the ganglion with only minimal overlap between them. Whereas it had been assumed that no degeneration occurred in the brachial ganglia (Hamburger & Levi-Montalcini 1949), the new findings on brachial ganglion 15 showed unequivocally that here too there are distinct phases of cell death in the DM and VL divisions, although the levels are appreciably lower than those in thoracic ganglion 18.

Of equal importance is the clear evidence that exogenous NGF can supplement that normally produced within the target fields of the sensory neurons and can effectively eliminate most of the naturally occurring cell deaths in the ganglia. That this is true of the cells in the VL division (as well as the DM population) also disposed of the earlier notion that VL neurons are unresponsive to NGF. This view had been based on the early work with the implanted murine sarcomas (Levi-Montalcini & Hamburger 1951), but as Viktor points out in this last study, since the processes of cells from the VL division did not invade the tumor mass until day 7 (i.e. after the period of maximal responsiveness to NGF), they were not in a position to respond to the growth-promoting effects of the sarcomas. The same explanation could also account for the failure of the separated VL division to respond by forming a halo of outgrowing fibers in Rita's (Levi-Montalcini 1962) study that involved sensory ganglia from day-9 embryos (again, by which time the VL cells had lost their responsiveness to NGF).

The paper ends with the following concluding remarks, which may serve as a fitting summary of Viktor's career-long interest in this problem, for which he deserves the last word:

> We have demonstrated that NGF can rescue sensory neurons in the embryo at exactly the time they would have died without NGF supplementation. . . .These findings strengthen the notion that NGF is indeed the naturally produced trophic agent for sensory ganglia.

SUMMARY AND CONCLUSIONS

This review began with some comments about the reaction of the biological community to the announcement of the award of the 1986 Nobel Prize to Rita and Stan Cohen, and so it is perhaps appropriate to end by summarizing the antecedent history that may have led to the Nobel committee's decision to omit Viktor from the award. However, as was pointed out, the purpose of this review is not to challenge or call into question the committee's decision, but to indicate the contributions that Viktor and Rita made—both individually and jointly—to the problem of the relationship between the nervous system and the periphery that culminated, after more than 30 years, in the discovery of NGF.

What unquestionably emerges from this reexamination of the history is that Viktor's work on early limb development, and especially his studies of aneurogenic limbs, set the stage for much of what was to follow. In particular these early works paved the way for Viktor's study (Hamburger 1934) of the effects of early limb bud extirpation on the development of the motor columns of the spinal cord, which proved not only to be the impetus for Rita's first work on the center/periphery issue but also to set the standard by which all later studies of this issue have been judged. That Viktor's interpretation of the findings in his study was subsequently shown to be incorrect does not diminish its importance. In the context of its time, and especially given Viktor's background as "one of Spemann's students" (to use Rita's term), it is entirely understandable that he would consider the observed reduction in the number of motor neurons after limb removal in terms of the failure of an inductive interaction. But what is even more impressive is his insightful conclusion that whatever its ultimate cause, the effect must result from a signal detected at the periphery by the first outgrowing motor axons, which is then retrogradely transmitted to their cells of origin in the motor column. It would be more than 20 years before the nature of the "signal" was discovered (at least for the neurons in the neighboring sensory ganglia), but the basic idea was clearly articulated in Viktor's seminal paper (Hamburger 1934).

Viktor's erroneous conclusion, that the "hypoplasia" seen in the motor columns was due to failure of the first motoneurons to induce the differentiation of other such cells from a pool of uncommitted precursors, stemmed in large part from his examination of the LMC at only a few selected stages in development. Had he examined them at more closely spaced time intervals (as Rita and her mentor Giuseppe Levi did some 8 years later), he would have discovered that the LMC is fully assembled prior to the onset of the "hypoplasia" and that the role of the periphery is to maintain the survival of motoneurons, not to induce their differentiation.

Rita made this important discovery while repeating Viktor's study and working under the most appalling circumstances (Levi-Montalcini & Levi 1942). By showing that the periphery acts this way on both the motor columns of the cord and the sensory ganglia, she established that the regulation of neuronal growth and maintenance involves mechanisms operating within their projection fields, and suggested that this is probably a general phenomenon in neural development.

It is impossible to know what might have become of Rita's finding had Viktor not come across her papers in 1946. Since the paper had been published during the war and in a relatively obscure journal, it is understandable that it seems to have had little or no immediate impact. But when Viktor saw that her findings called in question his interpretation of the effects of limb extirpations, his immediate reaction was to invite her to join him in St. Louis to reexamine the issue. Because he made her visit possible (with the help of the Rockefeller Foundation), it is perhaps understandable that some of his colleagues believed that among Viktor's greatest discoveries was his "discovery of Rita." But to say this is to do Rita an injustice. As I have pointed out, when she joined his laboratory in 1947, she was not, as some have portrayed her, a naive, postdoctoral fellow: She was a well-trained neurologist, technically proficient in handling chick embryos, knowledgeable about the anatomy of the central nervous system, skilled in the use of the best available neurohistological methods, and already the author of half a dozen important (if little known) papers.

Her technical skill and neurological expertise were evident in the first papers she published after moving to St. Louis and most important, in her study with Viktor on the effect of limb bud extirpation on the spinal ganglia (Hamburger & Levi-Montalcini 1949). This paper is not only one of the classics of developmental neurobiology, it is also a landmark in the field. Among other things it established beyond question that cell death is a normal (and probably widespread) feature of neural development, and that although the periphery may have an effect on cell proliferation in some systems, its principal role is to regulate the numbers of neurons that survive. Inherent in this last conclusion is the notion that for their survival and maintenance, neurons are dependent on the availability of some form of trophic factor within their target field.

It is no exaggeration to say that this study set the agenda for much of neuroembryology for the next two or three decades, and that it set Rita and Viktor on the course that finally led to the discovery of NGF. That the discovery of NGF depended on a number of fortuitous events (and, at one point, a completely serendipitous finding) is too well known to be repeated here. Suffice it to say, it began with Viktor's receiving a reprint of Bueker's paper on the effects of the murine sarcoma 180 on the sensory ganglia, was followed by the more detailed analysis of the actions of this and a second sarcoma by Rita and Viktor, and led to Rita's discovery that the tumors had an even more profound effect on the sympathetic system—including sympathetic ganglia that were not in direct contact with the tumor mass. The striking demonstration that the implanted tumors release a diffusible factor that could act on the sympathetic system through the vascular system set the stage for an all-out effort to identify the factor involved.

Exciting though their in vivo experiments were, it was clear that if further progress was to be made, and especially if the active principle released from the tumors was to be isolated, a more manageable assay system would have to be developed. Furthermore, since neither Viktor nor Rita had the necessary biochemical expertise, they would need to recruit a well-trained biochemist. The first development was met by Rita's visit to Rio de Janeiro, where with the help of her

friend Hertha Meyer, she developed the in vitro coculturing system in which the outgrowth of fibers from sensory and sympathetic ganglia provided a semiquantitative assay for the presence of the nerve growth-promoting factor produced by the two murine sarcomas and certain other tissues. Although Rita was careful to keep Viktor informed about the progress of her work in Rio [and despite the fact that his name appeared on the paper reporting the use of the in vitro system (Levi-Montalcini et al 1954)], Viktor's role in this work was minimal. However, he played the key role in obtaining the necessary funds and in recruiting Stan Cohen to join the laboratory, which proved decisive as the work moved to the next important stage.

Again, although Viktor's name appeared as a coauthor on the paper that first reported the isolation of the growth-promoting factor from the murine sarcomas (Cohen et al 1954), by his own admission as the work became increasingly biochemical, he left the field to Rita and Stan and for the next decade or more devoted his efforts to the study of the ontogeny of behavior (see Cowan 1981). The most surprising discovery that snake venom (which had been used to remove nucleic acids from the partially purified preparations of the tumor-derived factor) possessed nerve growth-promoting activity soon led to the discovery that the same (or at least a very similar) factor was present in substantial quantities in the salivary glands of male mice. The generation of an antiserum against the factor (which by this time was referred to as nerve growth factor, or NGF) provided the first proof that the growth and survival of sympathetic neurons (and, by inference, sensory ganglion cells) was critically dependent during early development on the availability of NGF. As if that were not enough, while studying the effects of the partially purified factor derived from salivary glands, Stan made the equally exciting discovery of a second factor, soon to be known as epidermal growth factor (EGF), that caused precocious opening of the eyelids and eruption of the incisor teeth in mouse pups.

While Viktor was not directly involved in any of these last studies, he followed the work closely, as evidenced by his communicating a number of the papers to the Proceedings of the National Academy of Sciences. Finally, after a hiatus of almost 15 years, in the 1970s Viktor turned his attention once again to some of the as-yet-unresolved issues in the center/periphery problem and provided the first unequivocal demonstration that the administration of exogenous NGF to developing chicks can effectively eliminate naturally occurring cell loss in the spinal ganglia (Hamburger et al 1981).

By focusing narrowly on the discovery of NGF and EGF, one could reasonably conclude that the Nobel committee was correct in its selection of Rita and Stan for the 1986 prize. But viewed from a wider historical perspective, Viktor's contributions both prior to and following these discoveries, were both numerous and substantial. Fortunately, now that the "dust has settled" we can perhaps better recognize that the work which led to the isolation of NGF and EGF (and all the work that followed) is what really matters. In this conclusion Viktor and Rita would surely concur.

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