CLASSIC PROFILE

Rita Levi-Montalcini: NGF, the prototypical growth factor

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In the fall of 2007, 2 years shy of her 100th birthday, Nobel laureate and National Academy of Sciences member Rita Levi-Montalcini yearned to start a new research project. Her idea stemmed from studies she had carried out more than half a century earlier, through which she unveiled and characterized the function of NGF. Time had taken its toll on her sight and hearing, but her keen observation skills and scientific insight were as strong as ever.

To embark on this project, she solicited the help of her colleague Antonino Cattaneo, a neuroscientist at the European Brain Research Institute (EBRI) in Rome, which Levi-Montalcini founded in 2002. "Although she's a person who looks forward, she still goes over and over what she did in the past and looks at it from different angles," Cattaneo says. "She said, 'When I did my experiments I showed that sympathetic and sensory neurons were dependent on NGF at relatively advanced stages of development. Yet, it is known in the literature that NGF and its receptors are expressed much earlier in development,'" Cattaneo continued. "She was well aware that this data was present in the literature, and during one of our discussions she remarked, 'There is a question here. If the receptors are present much earlier in the embryo, is it possible that NGF, acting through these receptors, has some effects in early development that are different from the effects of NGF I had discovered years ago?'"

To find out, Cattaneo assembled a group of scientists from his laboratory, each with different areas of expertise. Levi-Montalcini proposed that the group use the technique she first described in the 1960 PNAS Classic Article, "Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein" by Levi-Montalcini and Barbara Booker (1). In this work, Levi-Montalcini uncovered convincing evidence of NGF's function in vivo by injecting newborn animals with antibodies against NGF to eliminate its activity. This pioneering and creative use of antibodies to probe the function of a protein—a type of phenotypic knockout—revealed a nearly complete destruction of the animals' sympathetic nerves, which regulate blood pressure, heart rate, and myriad other bodily functions. The finding not only led to a better understanding of the function of the peripheral nervous system but also solidified the conceptual basis for the now widely studied group of growth-regulating chemical messengers known as growth factors. The work furthermore provided an experimental blueprint for subsequent studies of growth factors and other proteins by the use of antibodies to block protein function.

Levi-Montalcini knew that Cattaneo's group possessed the techniques and reagents to inject anti-NGF antibodies into the early chicken embryo but lacked experience working with chicken embryos. So she demonstrated how to do the injections, as early as possible in the living embryo, and during her daily visits to the institute to discuss the forthcoming data, taught the researchers what to look for under the microscope (Fig. 1).

"Her enthusiasm was very, very contagious," says Annalisa Manca, a postdoctoral fellow in Cattaneo's laboratory and one of the researchers who worked on the project. "What was also impressive was that almost every day she was here in the Institute, in the lab, and every day she had a new idea, a new insight. She was very curious about new technologies, new techniques, and of everything we did. She wanted to come into the lab to see the experiments with the embryos using the microscope."

The team started out by scanning the embryos for signs of massive nerve cell death, an effect that Levi-Montalcini observed in the 1950s when cells are deprived of NGF. "This was something that was worrying me in the analysis, because it was not there, but it was something that Professor Levi-Montalcini was looking for," says Simona Capsoni, a former EBRI postdoctoral fellow and now a neurobiology professor at Scuola Normale Superiore in Pisa. To her surprise, Capsoni observed localized cell death at the level of somites, but also noticed a more significant effect: the embryos injected with anti-NGF antibodies displayed an abnormal 3D shape within 48 hours of injection, which the team determined was caused by a defect in a crucial rotation process that the embryo undergoes during development (Fig. 2) (2). This rotation is the result of a coordinated wave of proliferation that seems to be regulated by NGF. "We were all surprised about these results," Capsoni says. "We were not thinking about an effect on rotation."

"Rotation is actually important for the final fate and development of nervous system, and so it is not just a matter of geometry," explains Francesca Paoletti, a postdoctoral fellow in Cattaneo's laboratory and coauthor of the paper. "It's really important for the

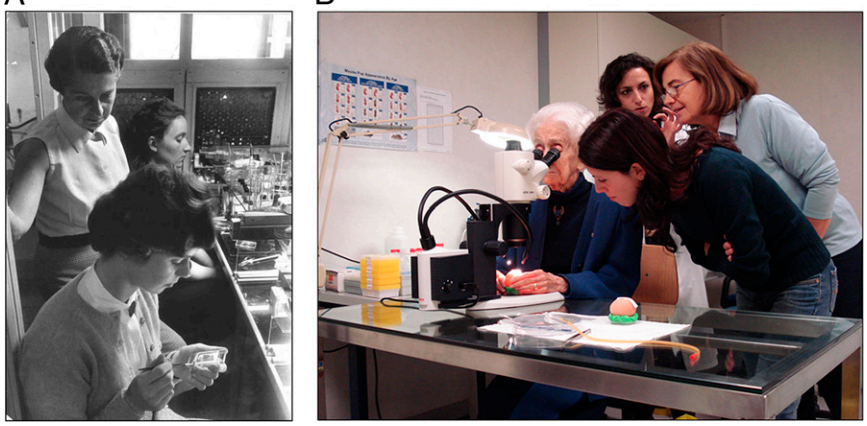

Fig. 1. Rita Levi-Montalcini supervising laboratory experiments (*A*) at upper left in 1963 (Washington University, St. Louis, MO), and (*B*) at left in October 2007. Images courtesy of the EBRI Foundation.

See Classic Article "Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein" on page 384 in issue 3 of volume 46.

See Retrospective on page 4862.

See Classic Perspective on page 4877.

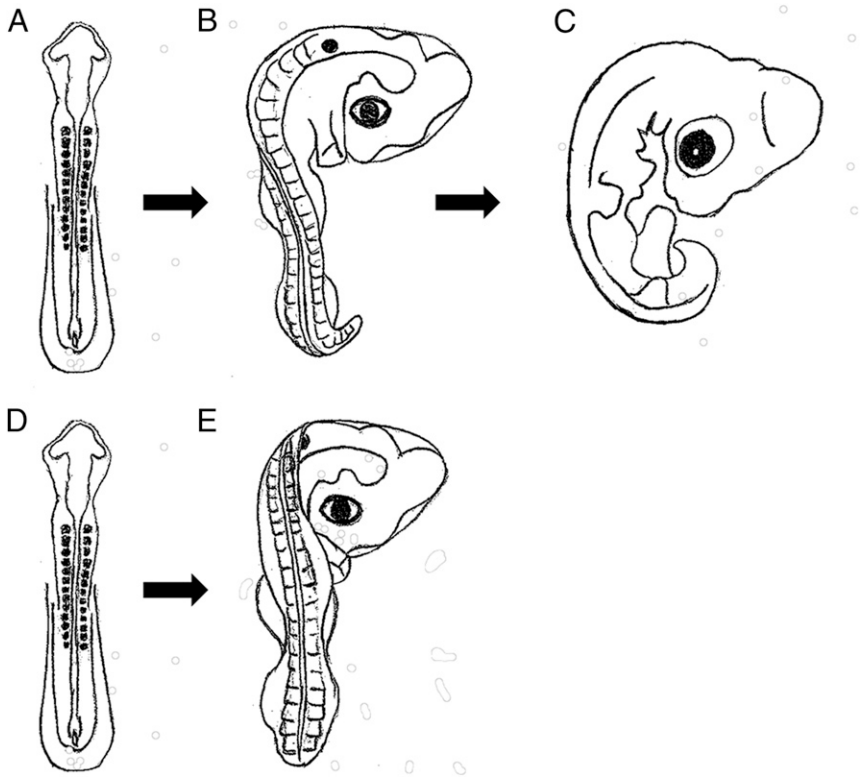

Fig. 2. Axial rotation in chicken embryos. Normally the embryo initially lies with its ventral side on the yolk sac, with the head facing down (*A*, stage 11). By stages 12–13 the head starts to rotate so that its left side comes to lie against the yolk sac with right side upward. At this stage the trunk has not yet turned, but gradually the rotation spreads down to the body (*B*, stage 18), and it is completed at approximately stage 20 (*C*), when the entire embryo lies on its left side. In embryos injected with anti-NGF antibody (*D*, stage 11) the rotation of the head occurs normally in the following stages, whereas the rest of the trunk fails to rotate, leading to the formation of an abnormal torsion between the neck and thorax (*E*). Images courtesy of Antonino Cattaneo and Simona Capsoni.

symmetric development of the organs." The findings not only extend the action of NGF into earlier stages than previously recognized but provide the latest evidence for what Levi-Montalcini calls "the vital role of NGF," opening up yet further avenues of inquiry into the biological roles of this growth factor.

Nerves of Steel

Levi-Montalcini was born Rita Levi (as an adult, she appended her mother's maiden name, Montalcini) in 1909, and grew up in a well-off, intellectual Jewish family in her native city of Turin, Italy (3, 4). Her father, an engineer by trade, subscribed to the traditional, Victorian era beliefs regarding the status of women and decreed that his daughters were not to pursue higher education or professional careers that would interfere with their marital and maternal obligations. However, once her beloved governess fell ill, her body riddled with tumors, Levi-Montalcini resolved to become a medical doctor. She summoned the courage needed to persuade her father to allow her to attend medical school and entered the University of Turin School of Medicine in 1930.

There, she began working on a research project under the guidance of the legendary histologist Giuseppe Levi (of no familial relation to Levi-Montalcini), and alongside fellow students and soon-to-be friends Renato Dulbecco and Salvador Luria, both future Nobel laureates (4). Levi-Montalcini's assigned task—to find out whether the number of nerves was rigidly fixed or influenced by environmental factors—required tedious counts of the nerve cells sprouting from the spinal cords of mice. To do this, she perfected the elegant silver staining technique for nerve cells invented by the Italian histologist Camillo Golgi and developed further and more famously by the Spanish neurologist Santiago Ramón y Cajal. The technique, simple but sometimes capricious, caused nerve cells to stand out against surrounding tissues and enabled her to clearly identify nerve cells under the microscope, which would later prove crucial to the success of her future work.

After graduating from medical school in 1936, Levi-Montalcini began advanced training in neurology and psychiatry, uncertain whether to practice medicine or pursue basic research in neurobiology (4). She continued her research using the silver staining technique to study peripheral nerve formation in chicken embryos until 1938, when the Fascist regime prohibited Jews from studying and working in state schools and universities in Italy. To escape the increasingly anti-Semitic environment, she moved to Brussels in March of 1939 and continued her research at a neurological institute there. During that time she frequently visited Levi, who had taken refuge in the nearby city of Liège and who had established a tissue culture center, having recognized the burgeoning potential of in vitro studies.

Just before the German invasion of Belgium at the end of 1939, Levi-Montalcini rejoined her family in Italy (4). Prohibited from working in public, she decided to conduct her interrupted research at home in a secret bedroom laboratory. She procured a microscope, created a makeshift incubator, and forged surgical instruments from sewing needles. She set out to study how developing nerve cells made their way from the motor column in the spinal cord to innervate their target organs, skeletal muscle in the body. She was inspired by a recent article (5) that Levi had recommended to her, by the embryologist Viktor Hamburger, at Washington University in St. Louis, MO. Hamburger, who was exploring how developing limbs shape the formation of the central nervous system, reported that when he excised the developing wing tissue from chicken embryos, he witnessed a dramatic decline in the size of structures called spinal ganglia—bundles of nerve fibers located outside the central nervous system—and a reduction in the number of nerve cells on the operated side. He hypothesized that signals from the developing limb tissue influenced the differentiation of immature nerve precursor cells and recruited the nerves to innervate the target tissue.

With the assistance of her mentor Levi, Levi-Montalcini repeated the experiment, applying the silver staining technique to get a closer look at what was happening to the developing nerves upon removal of the target limb tissue. She carefully examined embryos over a wide range of time intervals after limb excision. Like Hamburger, she also observed that removal of the developing limb ultimately triggered a marked reduction in the spinal ganglia. However, Levi-Montalcini recognized that a different mechanism might be responsible for the effect: despite the removal of the developing limb, she observed that nerve cells continued to proliferate and differentiate but later died upon reaching the stump of the amputated limb. Levi-Montalcini suspected that the shrinkage that Hamburger had identified was due to a degenerative process, rather than a failure to recruit and differentiate nerve cell precursors (6, 7). She proposed that the nerve cell death was caused by the absence of a factor that influenced the survival and maintenance of differentiated nerve cells, and not, as Hamburger had suggested, by the

absence of a factor that induced the proliferation of neuronal precursors.

As conditions in Turin steadily deteriorated during the second half of 1942, Levi-Montalcini and her family fled from the bombing raids to a rural cottage. She reestablished her clandestine laboratory and continued her research, biking from farm to farm, purchasing the fertilized eggs that she needed for her experiments and using the edible remains to prepare family meals (4). However, shortly thereafter, Mussolini was overthrown and German troops invaded Italy. Levi-Montalcini abandoned her laboratory, and escaped with her family to Florence, where they lived underground until the end of the war.

Insightful Journeys

After the war, Levi-Montalcini returned to Turin and resumed her previous position at the university (4). In the summer of 1946, Levi received a letter from Professor Viktor Hamburger, the author of the article that had inspired the neuroembryology experiments that Levi-Montalcini had carried out in her makeshift laboratory. Hamburger had read Levi-Montalcini's publications and was intrigued by their results. He invited Levi-Montalcini to spend a semester at his laboratory in St. Louis to repeat and expand the research further with him. Levi-Montalcini accepted, and in the fall of 1947 embarked for St. Louis, unaware that her stay was to last for 30 years.

In addition to reexamining the effects of limb removal on the development of peripheral nerves in the chicken embryo, Hamburger and Levi-Montalcini also transplanted a developing wing bud from one embryo onto another (8). Levi-Montalcini observed that the limb-related ganglia degenerated after limb removal and proliferated in the presence of the limb transplant, providing convincing evidence that the growth of sensory nerves depends on a signal from the developing limbs; without this signal, the innervating nerves die. In their article, they concluded: "The periphery provides for conditions necessary for continued growth and maintenance of neurons in stages following the first outgrowth of neurites." As Levi-Montalcini carried out these experiments, she also observed that large numbers of nerve cells died in regions of the embryo that had not been affected by the surgeries, suggesting that neuronal cell death was a natural part of development. The two set out to learn more about the agent that was responsible for maintaining the growth and survival of nerve cells.

Shortly after completing these experiments, Hamburger received a letter and a manuscript from his former graduate student, Elmer Bueker, who had been carrying out his own limb transplantation experiments (9). Bueker thought that perhaps any rapidly growing tissue might attract nerve fibers the way a growing limb does, and he had transplanted small pieces of proliferating tumors onto chicken embryos. He discovered that one of the tumors that he grafted, known as mouse sarcoma 180, had proliferated and had become infiltrated by nerves, and that the nearby spinal ganglia of the embryo had enlarged. Intrigued, Levi-Montalcini repeated his experiment right away using Cajal's silver stain technique. She not only corroborated Beuker's findings but astutely observed that the nerves penetrating the tumor did not make direct connections with the cells of the tumor, as happens when nerves innervate normal tissues (10). Furthermore, tissues far removed from the tumor became flooded with large numbers of nerves, long before the same regions became even sparsely innervated in control embryos. When Levi-Montalcini noticed that some of the nerves bored into blood vessels that drained the tumor, she began to suspect that the tumor secreted a diffusible, growth-promoting factor (11). To establish that the factor was a diffusible substance, she transplanted the tumors onto the thin, external membranous layer that envelops the chicken embryo; thus, the tumor was not in direct contact with the internal embryonic tissues but shared the same blood supply. The tumor still exerted its dramatic effects on nerve growth, providing unequivocal evidence that the tumor cells released a soluble, nerve growth-promoting factor and setting the stage for a vigorous effort to identify this mysterious factor.

After failing to reproduce the tumors' effect by injecting tumor extracts into embryos, Levi-Montalcini realized that in vitro cultures of nerve tissue might provide a faster and easier path to identifying the factor compared with the time-consuming and difficult experiments using fertilized chicken embryos. In 1952 Levi-Montalcini boarded a plane to Brazil with two tumor-bearing mice concealed in her handbag to visit her friend Hertha Meyer, a former student of Levi who had since established a culture facility at the University of Rio de Janeiro (4). There Levi-Montalcini learned to culture isolated chicken embryo nerve tissue in a dish and discovered that a fragment of mouse sarcoma placed next to, but not in direct contact with, the isolated tissue elicited the outgrowth of a dense halo of nerve cells within 10 hours.

Serendipity and Preparedness

By the time Levi-Montalcini returned to St. Louis armed with a powerful and efficient in vitro bioassay to monitor the factor's biological activity, a talented young biochemist named Stanley Cohen had joined the laboratory. Within a year, their team succeeded in purifying from mouse sarcomas a cell-free solution of proteins and nucleic acids that duplicated in culture the halo-producing effect of growing tumor (12). To determine whether the factor was a protein or a nucleic acid, Arthur Kornberg, an enzyme biochemist at Washington University, suggested that they incubate the solution with snake venom, known to be a rich source of phosphodiesterase, an enzyme that degrades nucleic acids. To their surprise, the snake venom not only did not destroy the nerve growth-promoting effect of the tumor extract, but rather enhanced the effect. In fact, their carefully controlled experiments—influential PNAS articles in their own right—revealed that snake venom alone caused nerves to grow in culture (Fig. 3) and in embryos, and it was much more effective than tumors or tumor extract (13, 14).

"This was of course serendipity," says Cattaneo, "but as it is often said, serendipity helps the prepared mind. One would have thrown the experiments down the bin because this result was totally unexpected, and maybe something had gone wrong. But instead they pursued this." After laboriously testing progressively purified fractions of proteins from snake venom in Levi-Montalcini's bioassay, Cohen soon identified a fraction containing a protein with nerve growth activity, which they called nerve growth factor (NGF). Cohen and Levi-Montalcini longed do more extensive analyses of NGF's action, but they realized that isolating sufficient NGF from snake venom would have been tedious and expensive. They began systematically searching for other tissue sources of the factor, when it occurred to Cohen to test mouse salivary glands, the mammalian homolog of snake venom glands. He soon isolated from mouse salivary glands an NGF that was approximately 10 times more active than that from snake venom (15). "This was the

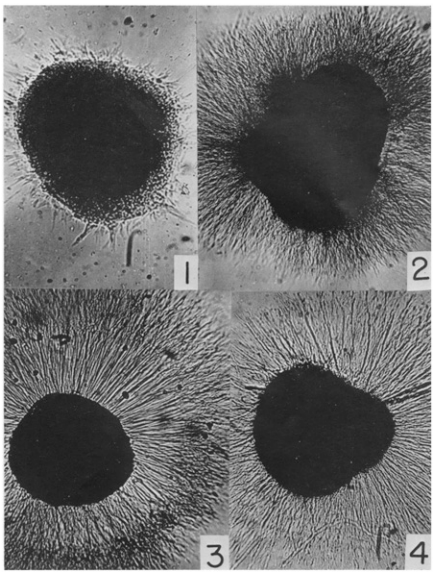

Fig. 3. Chicken embryo nerve tissue cultured with (*i*) control medium (*Upper Left*), (*ii*), crude snake venom (*Upper Right*) (*iii*), a partially purified protein fraction of snake venom (*Lower Left*), and (*iv*) the protein fraction purified from sarcoma 180 (*Lower Right*). Image reproduced from ref. 13 with permission from Stanley Cohen.

crucial step for the biochemical purification of the protein, because with such a rich source, this allowed the biochemistry to be done much more easily," Cattaneo says.

A partially purified fraction of NGF from salivary glands, injected into newborn mice, produced the same enlargement of peripheral nerves that they had witnessed in chicken embryos with mouse tumors (16). Cohen also observed that the treatment caused the mouse pups' eyes to open earlier and their teeth to emerge sooner compared with controls, but the effects on the eyes and teeth disappeared when he used a more highly purified fraction of NGF. Cohen suspected that the crude extract contained not only NGF but perhaps another factor that was responsible for the nonneuronal effects on the newborn mice. A few years later, Cohen identified this second factor as epidermal growth factor (EGF), a discovery for which he shared the 1986 Nobel Prize with Levi-Montalcini.

Despite the purification of NGF, its dramatic activity and unusual sources—mouse tumors, snake venom, and mouse salivary glands—left many scientists skeptical of its physiological relevance, says Pietro Calissano, a neuroscientist at the Italian National Research Council, Vice President of EBRI, and close friend of Levi-Montalcini. To determine whether NGF was physiologically important, Levi-Montalcini and graduate student Barbara Booker injected newborn mice with an antiserum against NGF. The treatment almost completely deprived the animals of a sympathetic nervous system but did not otherwise interfere with any other organs or tissues (1).

"This was the most convincing evidence that NGF was a physiological molecule," Calissano says, adding that it was also the first experimental "knockdown" of a protein to determine its function. "When I met Levi-Montalcini in 1965 and she showed me these experiments, I was so impressed that I immediately accepted her offer of a fellowship position."

"This was the first proof that there was a very important developmental function of this protein in newborn animals," adds Moses Chao, a molecular neuroscientist at the Skirball Institute of Biomelecular Medicine at New York University in New York City. "Paradoxically, decades later, when you look at mice that have a knockout of NGF, you get the same effects. It's pretty remarkable, that without sophisticated tools, she was able to figure out what the effect of this protein is during development."

Lasting Influence

Not only did this Classic Article begin to convince skeptics, the technique, known as immunosympathectomy, became a valuable tool for researchers to study how the lack of sympathetic nerves interferes with a variety of physiological functions, says Solomon Snyder, a neuroscientist at Johns Hopkins Medical School in Baltimore, MD. "Immunosympathectomy was even more elegant and powerful than gene knockout is today," he says. "Hence, it was widely used to characterize functions of the sympathetic nervous system," the hottest area of biomedical research in the 1960s, he adds.

"The discovery of NGF really changed the world in which we live, because now we knew that cells talk to other cells, and that they use soluble factors. It was hugely important," says Bill Mobley, Chair of the Department of Neurosciences at the University of California, San Diego School of Medicine. As it became clear that NGF only acted on a few specific types of nerve cells, many researchers became engaged in the search for additional factors that might influence the growth and survival of other types of nerves. However, the hunt for additional factors proved to be long and arduous, and it was not until the 1980s that Yves Barde, Hans Thoenen, and colleagues isolated a factor from the brain, which they called brain-derived neurotrophic factor. Soon, NGF was also found to act in the brain, and two additional neurotrophins were later discovered.

The discovery of growth factors in the brain ushered in a second wave of excitement for growth factor biology and spurred research to determine their role in neurodegenerative diseases and psychiatric disorders, Mobley says. Recent studies have shown that NGF plays a role in learning and memory, and experiments conducted by Cattaneo's and Calissano's research groups demonstrate that removal of NGF in either in vivo or in vitro experimental models results in the onset of an Alzheimer-like molecular syndrome, which leaves many hopeful that NGF and other neurotrophins may be useful for treating Alzheimer's disease. Furthermore, anti-NGF antibody-based therapeutics have shown promise in recent clinical trials for the prevention of pain, further proof that Levi-Montalcini's pioneering techniques and experimental findings are just as relevant today as they were half a century ago. However, perhaps the most recent evidence of NGF's many-faceted role can be found in a 2012 PNAS article that reported that this protein factor is present in semen and modulates ovulation, linking NGF to reproductive function and suggesting that it may one day be useful for treating infertility (17).

Like NGF, Levi-Montalcini never ceased to surprise and amaze. Her most recent experiments were published in January 2012 (2), which made her the oldest member of the National Academy of Sciences to publish in PNAS. Levi-Montalcini is no longer alive, but she has transferred her knowledge and enthusiasm to a cadre of talented young scientists, who undoubtedly will continue to carry on her search for the biological functions of NGF.

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