Immunosympathectomy as the first phenotypic knockout with antibodies

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Edited by Solomon H. Snyder, and approved February 5, 2013 (received for review October 26, 2012)

In a PNAS Classic Article published in 1960, Rita Levi-Montalcini offered formal and conclusive proof that endogenous NGF was responsible for the survival of sympathetic neurons in vivo. Thus ended an experimental tour de force lasting a decade, starting with the demonstration that a humoral factor, produced from a tumor transplanted in a chicken embryo, was responsible for stimulating outgrowth of nerve fibers from sympathetic and sensory neurons. From a more general methodological point of view, this work provided a breakthrough in the quest to achieve targeted loss of function and experimentally validate the function of biological molecules. Finally, this work provided an example of the ablation of a specific neuronal subpopulation in an otherwise intact nervous system, an immunological knife of unsurpassed effectiveness and precision. The novelty and the importance of the PNAS Classic Article is discussed here, collocating it within the context of the particular moment of the NGF discovery saga, of Rita Levi-Montalcini’s scientific and academic career, and of the general scientific context of those years. This seminal work, involving the use of antibodies for phenotypic knockout in vivo, planted seeds that were to bear new fruit many years later with the advent of monoclonal antibodies and recombinant antibody technologies.

The path to the discovery of NGF was a story of creativity and endurance, alternating with moments of frustration, delusion, and the excitement of many unexpected and theatrical coups de foudre.

All this is beautifully documented in the letters Rita Levi-Montalcini wrote from St. Louis to her mother and twin sister, Paola, in Italy, which provide a vivid and unique description of the joy and pleasure she derived from her scientific discoveries, written with the familiarity of everyday words to entertain her family (1).

(December 4, 1949) I think there are few things in the world so delightful as giving birth to new ideas and nurturing them...this is one of the aspects of my work that I find captivating, a bit like a truffle dog searching for truffles, even if they are not to be found its smell in the air is very exhilarating. I believe to have a very good sense of smell and...I hope to sniff out a few more truffles...

(January 15–16, 1961) Unlike yours, our work is full of hitches, strokes of luck, futile experiments alternating with fruitful experiments. Usually they come when you least expect it, and the unexpected is precisely the “thrilling” part of the game.

The summer of 1959 was undoubtedly one of the high points in the scientific quest to discover the function of NGF.

(June 21, 1959) My dear Mamma and Pa [her nickname for her twin Paola], I wrote in a recent letter that my work has not been very productive this year...three days ago...the situation has taken a turn. An experiment...has given results that lead me to believe in a new gold mine. For three days Stanley and I have both been working fervently to analyze data and to know the extent and consequences of this discovery. Of the discovery we have no doubts; but we cannot yet predict if it is of general value or restricted to the species we are studying, that is mice. If it is of general value, this would have a great impact not only in biology, but also at the clinical level. ...As in the past, the discovery came entirely unexpectedly, and left us with bated breath all day. Today we are still in a euphoric state, as if intoxicated. But what is this discovery?

It is difficult to explain in a few words, although you, mother dearest, are by now well aware of the work I am doing, having read my presentation in Paris. I never told you how moved I was by your interest in my work. The essential fact (started in 1951) was the possibility of using various substances to provoke a growth in volume in the component of the peripheral nervous system of which the sensory and sympathetic systems are part. The discovery, made initially in the chick embryo, was extended in the past year to the nervous system in young and adult vertebrates of mammals. A few months ago we found that also the sympathetic nervous system of humans reacts in the same way when using these substances. The discovery, made two days ago, gave exactly the opposite result: an “anti-substance” that causes the total disappearance of the sympathetic nervous system in the mouse. This opens a new field of research, whose outcome, as I said, we cannot yet evaluate, but is certainly of great interest. As soon as I return from my vacation I will ask Washington for more funds, and I am certain they will be granted. ...

(August 8, 1959) ...our work is at its peak and the possibilities for development are such as to make us dizzy. My euphoria is evident to all of the entourage, we are all happy and excited, as if we are about to have a party. (1)

The discovery described by Levi-Montalcini is immunosympathectomy, the destruction of sympathetic ganglia after injection of anti-NGF antibodies in mice and other mammalian species, and is described in the 1960 PNAS Classic Article “Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein” (2).

This result, which represents a pioneering example of the use of antibodies to neutralize an endogenous protein—a very effective knockout ante litteram, and a remarkable example of selective cell ablation—has been somewhat less celebrated in the many accounts of the NGF discovery. It was considered significant in its demonstration of the role of NGF but was overlooked as an advance in a more general sense.

However, this was truly a landmark breakthrough in its own right. This demonstration offered formal proof that endogenous NGF (as opposed to exogenously added NGF) was

Author contributions: A.C. wrote the paper.

The author declares no conflict of interest.

This article is a PNAS Direct Submission.

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 Profile on page 4873.

The English translation of the letters, which are written in Italian, is by the author. The help of Mrs. Pina Moliterno in revising the translations is warmly acknowledged.

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responsible for the survival of sympathetic neurons in vivo, closing an experimental tour de force that spanned a decade, beginning with the demonstration that a humoral factor, produced by a tumor transplanted in a chicken embryo, was responsible for the massive outgrowth of nerve fibers from sympathetic and sensory neurons.

From an experimental point of view, this work provided a breakthrough in the quest for methods to achieve targeted loss of function to validate the function of biological molecules. Finally, this work provides an example for the ablation of a specific neuronal subpopulation in an otherwise intact nervous system—an immunological knife of unsurpassed effectiveness and precision.

In this Perspective, I will discuss the novelty and the importance of the PNAS Classic Article, placing it within the context of the NGF discovery saga, of Levi-Montalcini’s scientific and academic career, and of the general scientific context of those years. I will discuss how this seminal work, involving the use of antibodies for phenotypic knock-out in vivo, planted seeds that would bear fruit many years later, with the advent of monoclonal antibodies and recombinant antibody technologies.

**Background: Snakes, Tumors, and Nerve Cells**

The PNAS Classic Article is the third of three companion articles presented by Viktor Hamburger to the Proceedings of the National Academy of Sciences on January 4, 1960. In the first one (3), Stanley Cohen reported the results of an extensive series of experiments aimed at isolating the nerve growth-promoting factor from mouse salivary glands. In the following two companion articles (2, 4) Levi-Montalcini and her technical assistant, Barbara Booker, described the effects of this isolated factor on the sympathetic ganglia of mice and the in vivo effects of an antiserum that Cohen had raised against the purified protein.

The three 1960 PNAS articles constitute a single body of work that represents the completion of an incredibly productive scientific cycle. This also coincided with Cohen’s departure from Washington University (which he had joined in 1953), to take up a new position. The interaction between Cohen and Levi-Montalcini had been remarkably productive and focused, based on the perfect timing of their collaboration and their different but complementary backgrounds and expertise (Levi-Montalcini reports Cohen saying, “Rita, you and I are good, but together we are wonderful.”).

In the 4 years previous to the publication of the 1960 trilogy articles, after the publication of two milestone articles in 1956 (5, 6), Cohen and Levi-Montalcini had not published any major articles. It is quite interesting to study in detail what happened in those intervening years.

By transplanting sarcomas 180 and 37 onto the allantoic membrane of the chicken embryo, Levi-Montalcini demonstrated that the neural growth-promoting effects of the tumors were due to a diffusible factor (7). Afterward, Levi-Montalcini demonstrated that these critical in vivo findings could be replicated by developing an in vitro assay (8) in which neurons, in the presence of an NGF-secreting tissue, sprout a rich halo of neurites from ganglia grown in tissue culture. The parallel effects of the tumors in vivo and in vitro suggested that the same agent was involved in both cases. After the success of these experiments, performed in Rio de Janeiro, Victor Hamburger recruited a trained biochemist, Stanley Cohen, to isolate and characterize the factor. The “halo effect” was then used as a bioassay to purify and identify the factor (9). In the course of this purification work, a serendipitous discovery was made that snake venoms were a very rich source of the same nerve growth-stimulating agent (5, 6). Initially intended to degrade NGF activity (and only later a cause of great celebration), this finding enabled the factor to be purified with a specific activity at least 1,000 times greater than that of their best purified tumor fractions. When injected in vivo, the snake venom and the protein purified from it produced the same neural growth-promoting effect as the transplanted tumors, which constituted a validation of the in vitro bioassay as a means to purify the protein.

These articles confirmed the scientific success of the collaboration between Levi-Montalcini and Cohen in their quest toward the purification of the growth-promoting agent:

*(July 27, 1956)* Two papers have come out, that I sent for publication in July … I received many letters from people that had read them and “they were speechless” … it is still a mystery how the venom acts and I don’t know if we’ll be able to find out …

With success came a sense of excitement and accomplishment:

*(June 18, 1956)* I’m still in a state of euphoria which set in after the initial enthusiasm. One discovery seems to lead to the next one; it’s like eating cherries. After the first one there were other little ones, and so I spent the entire week as if on hot coals. I was at the lab at all hours …

However, the work was also a source of restlessness and anxiety. The timing of Levi-Montalcini’s experiments was now dictated by the pace of the biochemical purification work by Cohen:

*(July 27, 1956)* The work that is most important for me, on snake venom, is fairly static at this moment. The biochemist [Stanley Cohen] is working on it and I’m waiting until he has improved the purification of the agent so that I can start to study its biological effects … *(1)*

It is interesting to note that although all of the articles by Levi-Montalcini typically conclude with a list of a few clearly defined and well-formulated questions for future investigation, with a clear agenda, her article on snake venom (6) ends more vaguely than usual: “… the ready availability of different snake venoms will facilitate the further analysis of the phenomena reported above.” The purification of NGF from snake venom and the demonstration of its equivalence to the factor released by the tumor marked a definitive conclusion, closing a scientific cycle that can be considered a true achievement. The research had started by studying the importance of extrinsic factors in the growth and differentiation of nerve cells. This had now been demonstrated and an important biological principal established. A well-defined chemical substance outside the nervous system and present in two totally unrelated sources (sarcomas and snake venom) was now convincingly shown to stimulate neuronal growth.

The focus, then, was not whether the extrinsic factors were of any physiological relevance. Indeed, the rather exotic sources of the factor itself (“Snakes, tumors, and nerve cells” was the typical title of lectures that Levi-Montalcini would give in that period) did not yet allow the question about the other side of the equation: Are there equivalent factors to be found in more physiological or biologically plausible tissues in a developing or adult organism? This fundamental question was simply not yet on the agenda, because the focus had shifted to the purification and characterization of the factor, which was an experimentally useful “tool.” The tumor and snake venom were experimental “artifacts” that taught something profound about the processes underlying the growth and differentiation of neurons.

With the 1956 articles came academic success and wider recognition. Nonetheless, the restless universe of Levi-Montalcini’s imagination was now driving her toward new areas of research, alongside her mainstream NGF work. She decided to pursue new projects, setting up a new electrophysiological laboratory with a young Chilean
colleague, where she divided her time with the work with Cohen.

(October 27–28, 1957) Viktor has proposed my promotion to full professor, that is the highest rank. This doesn’t mean that the University will ratify the proposal, but it’s possible. ... I had mentioned to you in another letter that I received an invitation to attend a symposium in Baltimore next spring. It’s an important and challenging invitation that will keep me still tied to tumors, poisons and mice. It’s funny that I should receive this recognition now and not when I had hoped for it a few years ago (although, I did not long for it back then either), making it difficult for me to cut the ties with my past activities. I accepted because it’s an invitation you simply can’t refuse, and I asked them if Stanley Cohen could be invited as well.

(October 7, 1957) My attraction for electrophysiology continues to increase and will not be discouraged by the pile of books on my desk and the countless things that I do not know and should know. I spend most of my day at the Medical School with Nando...to follow electrophysiological experiments.

She continues:

...work is even more intense than usual because of the simultaneous activity in two laboratories. I try, as much as possible, to work in one or the other on different days of the week, but it is not always easy to respect a schedule. The experiments are going well in both of the laboratories and when I work with Nando, Stanley calls me to discuss one of the experiments (he is on the third floor) and when I am working with Stanley, Nando calls me to show me one of his latest recordings. And when Stan and Nando do not call me, Viktor asks me to conduct an experiment for him. There is enough work to fill a consecutive 24-hour shift.

However, the events took an unexpected twist, as so often in the NGF saga:

(December 31, 1957) ...a very surprising discovery that changes our ideas on the effect of tumors and snake venom and persuades me to continue my research in an area that I was about to abandon. The results are very interesting and will be presented...at the Symposium in Baltimore in March. (1)

Cohen and Levi-Montalcini reasoned that because snake venom is produced in a modified salivary gland, then mouse salivary glands could be another possible source, and indeed, they discovered it to be an extraordinarily rich source of the same nerve growth agent. The demonstration of the equivalence of the factor isolated from tumor, snake venom, and mouse salivary gland brought back to mind something that Levi-Montalcini had kept silent and deliberately hidden for years: the so called “mouse effect.”

While developing the bioassay in Rio de Janeiro, Levi-Montalcini found that several normal mouse tissues caused a small but significant outgrowth of fibers from the cocultured ganglia (8). At the time this seemed just a curious, even uncomfortable, anomaly, because it was then thought that the growth-promoting factor was most likely a feature of neoplastic tissues, a useful artifact for the main object of study, which was the neuron. As related in her autobiography (10):

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 annulling—the significance of the induction of the fibrillar halo by S180 and S37.

In a letter written to Hamburger from Rio de Janeiro, she indicated that she was going to put the “mouse effect” aside for the time being, describing it as an “unpleasant and complicated finding.” It was only as a consequence of the discovery of NGF in the mouse salivary gland that the “mouse effect” was reevaluated and the question of the physiological relevance of the NGF effect became a priority. This was vividly and explicitly accounted for by Levi-Montalcini in her lecture at the Baltimore Symposium on The Chemical Basis of Development, in March 1958, which she concluded as follows (11):

...perhaps the most remarkable feature of this investigation was to prove the possibility of a peaceful collaboration between an embryologist and a biochemist. Such a possibility seems to have been questioned by some participants at the Symposium.

Finally, we should like to say that if two of the outstanding aspects of this phenomenon are its specificity and the magnitude of the effects, its most mysterious and challenging aspects are its significance and the mechanism of action of this protein agent.

What is the function—if any—of this agent present in the salivary glands of some vertebrates on the differentiation and growth of embryonic nerve cells under normal conditions? What is its function—if any—on mature nerve cells? It is conceivable that the salivary glands play a role neither on the differentiation of embryonic nerve cells nor in the maintenance and growth of differentiated nerve cells. If so, does this protein with such a potent effect have any function?

The lack of information about the functional significance of such an agent need not detract from its biological interest, which resides in the discovery of a clear-cut response of given nerve cells to well-defined chemical agents.

The Baltimore lecture turned out to be a great success:

(March 23, 1958) It’s one a.m. and I write only a few words because I promised to write after my presentation. Viktor, Mel and I, and other friends, had a toast to my successful afternoon and to my appointment to Full Professor. Viktor
informed me of my appointment this evening.

The presentation went very well. Even Weiss shook my hand and congratulated me. (1)

The salivary gland discovery thus represented a turning point in the research, beyond the obvious and crucial fact of providing a handy and rich source for the factor. It determined a major shift from work on chicken embryos to mammals, including human tissue, and laid the ground for directly addressing the crucial question about the significance and function of the factor under study. This is the main common thread, and the novelty, of the 1960 PNAS trilogy articles, and the well-coordinated crescendo marks a high point in the ongoing story of the NGF discovery. For the first time, the factor is not a tool but the actor, and its function is the question. Incidentally, it is in the 1960 trilogy (and more consistently in ref. 12) that the “nerve-growth factor” wording, as we know it today, starts prevailing over many others used in previous papers, such as nerve growth stimulating agent, nerve growth promoting protein, growth factor, and nerve-growth agent. The NGF acronym is first found in a 1959 letter:

(October 5, 1959) Now that Stan has left, I and my two young assistants, Barbara and Jean, are left with a heavy load; we must continue the chemical extraction of the nerve-growth agent (NGF) and study its biological properties. (1)

1960 PNAS Trilogy

The first article of the 1960 PNAS trio (3) describes the purification of NGF from mouse salivary glands (100-fold purification, 10–20% yield, active at 15 ng/mL). The purified protein was injected into mice (described in ref. 4) and used to raise a very highly neutralizing rabbit antiserum. In the course of this purification, Cohen discovered another growth factor, which he would later identify and characterize as epidermal growth factor (13). The consequences of injecting the salivary-derived factor and the rabbit antiserum are alluded to in this article, serving as an introduction to the two following companion articles.

In the second article (4), the active fraction isolated from the mouse salivary glands was tested in vitro on ganglia of human fetuses, revealing that it elicits the same effects as previously observed for ganglia of other species. This finding represented a remarkable step toward demonstrating the generality of the phenomenon under study. The active fraction was then injected in newborn and adult mice, resulting in a marked increase (up to sixfold) of the sympathetic ganglia. The article also provided evidence for the presence of NGF in the serum of adult and weanling mice. Finally, the physiological function of the factor was investigated by surgically removing the submaxillary and sublingual glands in adult and weanling mice. If these glands are the...
only source of NGF, one would expect that the sympathetic ganglia would be reduced after gland excision. On the contrary, no size difference was observed, and the conclusion was made that the nerve growth agent is present in mice even in the absence of the salivary glands, synthesized by other tissues or cells and thus supporting the survival of the sympathetic ganglia.

The ground was now ready for the direct functional question: because such a factor is present in the general circulation, would it be able to inactivate the sympathetic system and that the antibody against the factor is sufficient to destroy 97–99% of the sympathetic nerve cells, and the damage is irreversible. The injected newborn and adult mice showed no adverse effects on other systems. The results provide the best possible evidence that "in the normal animal a factor circulates which is necessary for growth and maintenance of the sympathetic system and that the antiserum inactivates this factor" (2). The universality of the conclusion was proven by confirming the results in a wide number of species: mice, rats, rabbits, kittens, and squirrel monkeys. From the point of view of the biology of NGF, the immunosympathectomy result marks the highpoint of a decade-long scientific tour de force, offering the conclusive proof that the factor is normally present in the developing and the mature organism, where it plays a physiological role in the maintenance of sympathetic nerve cells. The circle was closed.

The article also had far-reaching conceptual consequences and predictions. As Levi-Montalcini noted a few years later, "...there is reason to believe that each nerve cell type might be receptive to only one specific factor. ... These results give evidence for the essential role of this particular protein in the growth differentiation and maintenance of sympathetic nerve cells. They also suggest that other nerve cells might also depend upon specific factors for their differentiation and growth." (14)

It was almost 20 years before the prediction was demonstrated true and the second neurotrophin was discovered (15). As her letters home reveal, these results stirred great excitement in Levi-Montalcini, clearly aware of their importance and of their potential clinical relevance, and anticipating an enthusiastic response from the scientific community.

(July 12, 1959) The discovery has been finally confirmed and the intense activity of these days is concentrated on clarifying some aspects of the phenomenon and writing up a paper before we leave for our summer vacation in mid August. We will send two manuscripts, one in Stan’s name and the other in mine, to the Academy of Sciences. This is an important discovery and I have no doubt that the two manuscripts will be of great interest. Therefore, in this short time we must work hard and focus on a few points that are still obscure in this phenomenon.

(November 29, 1959) Some good results validate our previous data and confirm that the first observations were correct. In doubt, I held off sending the two manuscripts that were ready. They will be sent this week.

(March 20, 1960) ...when I got home at three I received a phone call from the New York Times. They had read about my publications and Stanley’s (I have not yet seen them) in the Proceedings of the Academy of Sciences and want to publish an article. The telephone interview lasted about half an hour. I think I managed to persuade them not to publicize our work. These past few months there has been too much talk and I prefer to avoid publicity. However, no doubt that the articles published in the Journal of the Academy of Sciences, which has an excellent reputation, will arouse some interest in the scientific community... (1)

**Immunosympathectomy: Phenotypic Knockout Before Gene Knockout**

What is striking about the immunosympathectomy article is the natural and highly understated way in which the experimental strategy is presented. Today the approach of using antibodies to probe the function of the target antigen would indeed be straightforward, but how obvious was it at the time? In a way, the anti-NGF experiment is similar to the "artificial passive immunization" or "antibody therapy" that immunologists had been practicing since the beginning of the 20th century, following Roux, von Behring, Ehrlich, and others, using antitoxin antiserum to provide immunity to diphtheria or tetanus. von Behring was awarded the first Nobel Prize in Medicine in 1901 for his discovery and development of an antibody therapy for diphtheria. However, in those cases, the antigen targeted by the antisera was an exogenous pathological antigen, whereas in the immunosympathectomy approach the anti-NGF antibodies target the endogenous factor, providing a form of "passive autoimmunity." It was indeed a conceptual leap to turn from immunization to functional studies, using antibodies to target an endogenous protein.

We get a glimpse of the state of the art of the functional use of antibodies at the time, by looking at the proceedings of a Conference on Immunology and Development (16) that brought together in 1956 top members of both scientific communities and was referred to by Levi-Montalcini in her PNAS Classic Article (2). The question on the "essentialness of antigens in growth and development" was addressed at the conference: "Are there antigenic elements in a cell so essential to its continued existence and development that combination with antibody results in their inactivation and leads to death of the cell?" (16). However, the experiments reported are vague and ambiguous, because the "antigens" were unknown mixtures with unknown properties, and therefore the antisera were diverse and ill-defined. The conclusion of the meeting was that "None of the questions discussed during this conference has reached a definitive answer" (16).

Geneticists had been studying loss-of-function mutations for many years. The immunosympathectomy method induced a loss-of-function phenotype. However, compared with the randomly generated "loss-of-function" methods used by geneticists at the time, the antibody-mediated knockout constituted a targeted loss of function, more analogous to what today is achieved by current gene targeting techniques.

It is fair to conclude, therefore, that although the concept of the experimental approach to probe the function of an endogenous protein using antibodies might have been among the objectives of the scientific community of the time, the effectiveness and selectivity of the "knockout" achieved in this PNAS Classic Article by Levi-Montalcini is totally unprecedented. The article represents a proof of concept of a unique method. Among the reasons for the effectiveness of the method was certainly the purity of the antisera that, owing to the purity of the antigen, was virtually monospecific. This methodological advance, as such, has not been sufficiently acknowledged by the scientific community at large, partly because it was not underlined by the authors themselves, and partly because the methodological innovation might...
have been overshadowed by the wealth of biological results that came with it.

On the other hand, in the NGF and neurotrophin field, antibody application has since been widely used, and the immunosympathectomy experiment has inspired generations of neuroscientists. Decades of work have assessed the effects of blocking the biological activity of NGF in vivo with anti-NGF antibodies, extending our knowledge of the diverse actions of NGF in different systems. For instance, when anti-NGF antibodies are delivered to embryos, rather than postnatally, a more complex neuroendocrine syndrome is observed (17). Sensory neurons, which are remarkably spared when anti-NGF antibodies are delivered postnatally, are destroyed by exposure in utero to anti-NGF antibodies (18–20). In a reciprocal phenomenon, NGF has the opposite effect in the visual system. The application of NGF-neutralizing antibodies to the developing avian retina reduces normally occurring cell death, demonstrating a killing action by NGF as part of normal development (21). Furthermore, delivery of anti-NGF antibodies during the critical period of development of the visual cortex blocks the synaptic plasticity processes involved in postnatal development of the visual system (22).

As soon as gene knockout by homologous recombination became feasible in mice (23), neurotrophins and their receptors Trk and p75NTR were among the first targets for which knockout mice were derived (24). Both the NGF and trkA knockouts result in dramatic phenotypes (25, 26), entirely predictable on the basis of earlier observations in animals exposed prenatally to anti-NGF antibodies (17, 19, 27). For the other neurotrophins, the evolutionary conservation across species for BDNF and NT-3 meant that blocking antibodies have been difficult to generate, and prevented the examination of the physiological functions of these neurotrophins in a manner analogous to NGF. For this reason, the gene targeting knockout studies for BDNF, NT-3, and their receptors have been more informative (24).

**Immunosympathectomy: Example of Selective Cell Ablation in the Nervous System**

The selective ablation of defined cell populations in the nervous system represents an experimental objective that is essential in current approaches in systems neurosciences for analyzing in vivo the functions of cells and of circuits in otherwise intact systems. The immunosympathectomy experiment offered a working example of the possibility of achieving this goal (Fig. 1).

The importance of this strategy for specific functional studies on the sympathetic system and on its role in the homeostasis of the organism was immediately recognized and became the object of a major line of research in subsequent years (28). This was noted in the conclusion of the PNAS Classic Article, where attention was called to the “high tolerance of the organism for such deviations from normality as the six-fold increase in volume of the sympathetic ganglia, or their near-total extinction.” In particular the anti-NGF–treated animals are comparable to controls in growth and in other respects, so that the lack of any sympathetic control seems to be compatible with a normal life, “at least under the sheltered conditions of the laboratory.” With the growing interest in the mechanisms and pathways on how information about the body’s state and subjective feelings are represented in the brain (29, 30), the immunosympathectomy model could be of greatest importance, as a selective way of altering homeostatic mechanisms. The well-known emotional asymmetry in humans, in which the left and right halves of the forebrain differentially associate with particular emotions and affective traits, has been anatomically linked to an asymmetrical representation of homeostatic activity originating from asymmetries in the peripheral autonomic nervous system. In this respect, immunosympathectomized animals represent a unique model, in which an irreversible destruction of the sympathetic system can be triggered by a delivery of anti-NGF antibodies within a limited time frame. Thus, immunosympathectomy achieves a remarkable asymmetric disconnection of the brain from the body proper and the “emotional,” affective, and cognitive responses in immunosympathectomized animals could be studied in the absence of other confounding elements.

The central role of the NGF-TrkA system in mediating interoceptive representations is highlighted by genetic studies of CIPA [congenital pain with anhidrosis, or hereditary sensory and autonomic neuropathy (HSAN) type IV], an autonomic recessive genetic disorder characterized by insensitivity to pain, anhidrosis, and mental retardation. CIPA results from loss-of-function mutations in the NTRK1 gene encoding for TrkA NGF receptor (31, 32). As a result, CIPA patients lack NGF-dependent neurons. Recent results have shown that mutations in the NGFB gene encoding for NGF protein cause a form of congenital insensitivity to pain (HSAN V) that lacks autonomic deficits and shows no major neurological symptoms (33). The HSAN V mutated NGFR100W protein (in which residue R 100 is changed to W) differentiates the neurotrophic actions of NGF (including those on sympathetic neurons) from the pain-sensitizing actions on sensory neurons, explaining the clinical phenotype (34). Combining the analysis of immunosympathectomy models (living without a sympathetic system but with sensory functions) with HSAN V models (living without sensory and nociceptive functions, with a normal sympathetic system) should allow study of the role of the NGF-TrkA system in the establishment and function of the networks for interoception, homeostasis, and emotional responses.

From the methodological point of view, the exquisite precision and efficacy of the immunosympathectomy procedure, in terms of a selective cell ablation of sympathetic neurons, is due to the selectivity and specificity of the anti-NGF antiserum, the strong dependence of the target cells on NGF, and the perfectly chosen time window. Indeed, the sympathetic ganglia go through a period of NGF dependence at a much later stage than do dorsal root ganglia sensory neurons, which explains why no sensory deficit was found in the PNAS Classic Article but is found when anti-NGF antibodies are delivered prenatally. These ingredients are similarly crucial in current cell ablation approaches, which instead rely on the transcriptionally regulated cell-specific expression of genes encoding toxins or death-inducing proteins. More refined methods for cell silencing, in an otherwise intact neuronal circuit, involve the use of suitably targeted excitatory or inhibitory optogenetic probes. In any case, from the conceptual point of view, the genesis of these cell-specific ablation or silencing approaches can be traced back to the immunosympathectomy experiment (Fig. 1).

**Anti-Growth Factor Therapeutic Antibodies**

The potential clinical applications of anti-NGF antibodies had not escaped the attention of Levi-Montalcini:

(7/19/1959) I spent almost all day in a deserted and silent laboratory—seems logical on a beautiful, warm Sunday in July. Just me, the mice, bunnies, newborn (or still hatching) turtles, and the chameleons. The chick embryos for some time now come second.

New results in these last two days have given us a second wind and a new charge of euphoria. When these results will become public in two or
three months, I don’t think we will be able to work with all the publicity as we were able to do these past years. This last discovery…which has important therapeutic implications will certainly catch the attention of the clinicians and I’m sure that the pharmaceutical companies will be contending with each….

Neither Stan nor I however, have any intention of exploiting the output from the discovery in terms of clinical consequences. We will therefore limit ourselves in communicating the results and let others do the clinical development. There is also the possibility to use this discovery to treat hypertension and vascular spasm…besides the pathological and therapeutic importance, there is also the biological interest in the phenomenon.”

This expectation has indeed been recently fulfilled, even if not related to the sympathetic system. NGF has emerged in recent years as a crucial mediator of intractable forms of chronic and inflammatory pain (35). Indeed, NGF and its receptor TrkA are strategically positioned in controlling both the neuronal and the inflammatory part of the chronic pain process, acting both on nociceptor sensory nerve endings and on inflammatory cells, sensitizing the neuron to potentially painful stimuli. This contributes to a feed-forward process that leads to chronic sensitization. Human genetic studies further validate this target. For this reason, the pharmaceutical industry has looked with great interest at inhibiting the NGF/TrkA system as a target system for a new generation of analgesic drugs. The forerunner anti-NGF antibody tanezumab (36, 37) reached phase III clinical trials for the treatment of osteoarthritis and other intractable forms of chronic pain, and was soon followed by four other anti-NGF antibodies currently under clinical testing in humans (38), and one anti-TrkA antibody is also approaching the clinical trials (39). The analgesic properties of these antibodies in a large variety of animal models for pain, as well as in man, are remarkable. There are great expectations for this class of new analgesics. However, safety concerns, owing to unanticipated side effects, led the US Food and Drug Administration (FDA) to halt, at the end of 2010, the clinical testing of the anti-NGF programs under development. In March 2012, the FDA Arthritis Advisory Committee recommended unanimously that clinical research be resumed, provided attention is paid to some critical issues (40).

Given that currently available painkillers have limited efficacy and/or severe side effects, the hopes for this new class of analgesic drugs, based on the inhibition of the NGF/TrkA system, are very high. This is yet another legacy of the anti-NGF immunosympathectomy experiment.

More generally, growth factors and their receptors have become a preferred target for a new class of clinically approved therapeutic antibodies. Therapeutic antibodies (such as Herceptin, Erbitux, and others) targeting the EGF receptor or the related HER2/neu protein are used for the treatment of various forms of human cancer. Even more directly relevant to the NGF case, the discovery by Ferrara and colleagues of VEGF as the major mediator of angiogenesis (41, 42) has led to the development of a very effective therapy with anti-VEGF antibodies (Avastin, Lucentis) for wet macular degeneration, a leading cause of blindness in the elderly, and for various forms of cancer (41, 42) (Fig. 1).

Using Antibodies as Genes for Protein Silencing

Recent developments in antibody technologies, inspired by the immunosympathectomy experiment, allow researchers to interfere with a protein target with a high spatiotemporal precision using ectopically expressed antibodies (Fig. 2).

The advent of monoclonal antibodies and phage display antibody libraries (43) allow for the isolation of genes that code for specific antibodies. Antibody genes can thus be ectopically expressed via gene-transfer techniques (reviewed in ref. 44). Depending on the localization of the target protein of interest (extra- or intracellular), the antibody, suitably engineered, can be expressed as a secreted or as an intracellular protein and can be targeted to different subcellular compartments.

After the demonstration that antibodies could be ectopically assembled in nonlymphoid cells and secreted with particular efficiency by neuronal cells (45), the concept of achieving a phenotypic knockout in the nervous system (neuroantibody approach) by recombinant antibodies was demonstrated by targeting of the neurokinin substance P neuropeptide with a recombinant antibody expressed in the adult brain of transgenic mice (46). The neuroantibody approach was also instrumental in deriving the AD11 mouse model, in which the postnatal expression of an anti-NGF recombinant antibody in transgenic mice results in a progressive Alzheimer’s-related neurodegeneration, characterized by cholinergic deficit, tau and amyloid pathology, as well as synaptic plasticity and behavioral deficits (47, 48). A transgenic mouse expressing the neutralizing mAb MNAC13 anti-TrkA antibody recapitulates the neurodegenerative phenotype of the AD11 model (49).

This antibody-mediated protein silencing approach, based on the expression in transgenic mice of the genes coding for an antibody directed to a protein antigen of interest, was exploited for immunological inhibition of prion disease in vivo (50). Expression of anti-prion protein antibodies in transgenic mice prevented pathogenesis of prions introduced by i.p. injections in spleen or brain.

The “antibody protein silencing” concept was extended to intracellular antibodies targeted to different intracellular compartments of mammalian cells (51, 52) (Fig. 2). The intracellular antibody (intrabody) approach is a gene-based strategy that relies on the expression of recombinant antibodies (or antibody domains) directed to subcellular compartments, to block or modulate the function of target molecules. Because the Ig effector functions are not required, nor useful, inside the cell, simpler antibody domains are used for intracellular use, based on variable V regions only (reviewed in ref. 44).

In the two decades following the description of the use of intrabodies in mammalian cells (52), and the initial proof of concept functional studies by us and others (53–55), several examples of intracellular antibodies effectively inhibiting the function of intracellular targets have been reported (reviewed in refs. 56–58). Intrabodies can provide very effective inhibition of protein function in widely diverse cellular contexts, subcellular compartments, and intracellular processes (signaling or transcription pathways, protein trafficking, and viral assembly and replication) and in organisms, including transgenic mice (59).

Initially, genes coding for intrabodies were derived from hybridoma or phage-display technology (43) by a labor-intensive procedure. Methods have now been developed that allow the fast, effective, and user-friendly isolation of functional intrabodies. Antibodies are selected directly on the basis of their ability to bind antigen in vivo (60) in a modified version of the two-hybrid method for protein–protein interactions. This method allows the direct screening of yeast cells expressing antibody libraries from different sources (61–63). These “single-pot libraries of intrabodies” have greatly facilitated the selection of antibody fragments for downstream use as intrabodies in functional studies, providing a user-friendly and robust source of stable antibodies.

RNA interference methods are currently used for knock-down studies. Although effective and relatively simple, there are many questions that cannot be addressed by RNA-
based methods. Compared with RNA interference methods, intrabodies can, in principle, address the full diversity of the protein space, including quaternary states (with antibodies selective for a specific oligomeric form of a given protein), misfolding states of a given protein, and posttranslational modifications of a protein, which RNA targeting methods cannot. Moreover, intrabodies can target proteins in a subcellular compartment while not affecting the pool in another compartment, a property that can be useful in polarized cells such as neurons. Finally, intrabodies allow interfering with intracellular protein networks, by selectively targeting protein–protein interactions, leaving the protein itself unaffected (edge vs. node interference). From the point of view of interference or silencing techniques, the distinction between nodes or edge removal is important. Indeed, nucleic acid-based approaches (gene knockout or RNA interference) are typically node-removal approaches. No other general technique is readily available to specifically interfere with edges in a protein network of interest. In conclusion, intrabodies can mediate effective protein silencing, addressing questions that gene- or mRNA-targeting approaches cannot deal with (Fig. 2).

The mode of action of an intrabody, upon binding to its target protein in the cell, may involve several possibilities (reviewed in ref. 44): the intrabody may be intrinsically neutralizing, or it may act as a retargeting agent. Effector functions can be added to the antigen binding variable domains for live imaging purposes, or to cause either the induction of cell death upon intracellular antigen binding (64) or proteolysis of the intracellular target protein upon intrabody binding (65). This switch for protein degradation provides a tool for reversible protein silencing on a relatively fast time scale of minutes, which cannot be achieved with RNA interference methods because they require much longer times.

Protein silencing with subcellular precise targeting of recombinant antibody domains is emerging as a powerful technology (Fig. 2). The intrabody approach combines the molecular binding diversity of the antibody repertoire with the precision of subcellular targeting. We envisage three aspects of the intrabody approach as promising: (i) the possibility of targeting specific protein–protein interactions, while sparing other interactions engaged by the same protein; (ii) the possibility of selectively targeting posttranslationally modified proteins, with respect to the unmodified protein; and (iii) the possibility of selectively targeting a subcellular pool of a given protein.

In the PNAS Classic Article, Levi-Montalcini provided more than 50 years ago evidence for a phenotypic knockout with an anti-NGF antisera. We can now harness the power of in vitro immune repertoires and of selection methods to achieve tightly regulated protein silencing with antibodies inside cells and in organisms.

Conclusions

The experiment described in the PNAS Classic Article was the conclusion of a heroic cycle and the start of a long journey, centered on NGF and its antibodies. The “NGF agenda” is constantly being updated with new unexpected findings and changing priorities. The recent identification of NGF with the ovulation inducing factor and the characterization of its role in regulating gonadotropin release and ovarian function (66) is just the most recent example, very much in line with Levi-Montalcini’s recent perspective insights into the possible roles of NGF in reproduction and fertilization and in the very early phases of development: “we know that NGF has an active, vital role from early ooocyte, sperm, zygote, from very early on” (67). For Levi-Montalcini, NGF has been a close companion in life, as she so eloquently wrote in her autobiography (10):

On 1986 Christmas Eve NGF appeared in public under floodlights, amid the splendour of a vast hall adorned for celebration, in the presence of Royals of Sweden, of princes, of ladies in rich and gala dresses, and gentlemen in tuxedos. Wrapped in a black mantle, he bowed before the king and, for a moment, lowered the veil covering his face. We recognized each other in a matter of seconds when I saw him looking for me among the applauding crowd. He then replaced his veil and disappeared as suddenly as he had appeared.

Has he gone back to an errant life in the forests inhabited by the spirits who drift at night along the frozen lake of the North, where I spent so many solitary, enchanted hours of my youth? Will we see each other again? Or was that instant the fulfilment of my desire of many years to meet him, and I have henceforth lost trace of him forever?

ACKNOWLEDGMENTS

I thank Rita for being a constant source of scientific inspiration throughout my scientific life, for being an example of creativity, imagination, and endurance against all odds throughout her life, and for having shared with us her scientific inquisitiveness.