



# Hans Thoenen

## **BORN:**

Zweisimmen, Switzerland  
May 5, 1928

## **EDUCATION:**

University of Bern and Innsbruck, Certificate of Medicine (1953)  
University of Bern, Doctorate in Medicine (1957)  
University of Basel, "Habilitation" in Experimental  
Pharmacology (1969)

## **APPOINTMENTS:**

Staff Scientist, Department of Experimental Medicine,  
Hoffman-LaRoche, Basel (1961)  
Visiting Scientist National Institute of Mental Health  
(1968–1969)  
Research Group Leader Neurobiology, Biocenter, University of  
Basel (1971)  
Director Max Planck Institute of Neurobiology, Munich (1977)  
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## **HONORS AND AWARDS (SELECTED):**

Member Deutsche Akademie der Naturforscher, Leopoldina  
(1979)  
Feldberg Prize (1980)  
Cloetta Prize (University of Zurich, 1985)  
Wakeman Award (Duke University, 1988)  
Honorary Doctorate, University of Zurich (1992)  
Ipsen Prize (1994)  
Charles A. Dana Award (1994)  
Ralph W. Gerard Prize (Society for Neuroscience, 1995)  
Foreign Associate National Academy of Sciences, United States  
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*Hans Thoenen elucidated the mechanism of action of 6-hydroxydopamine that led to the serendipitous detection of trans-synaptic enzyme induction.*

*Subsequently his laboratory made crucial contributions to the field of neurotrophic factors, including the cloning of brain derived neurotrophic and ciliary neurotrophic factor and the analysis of their physiological functions.*

# Hans Thoenen

**W**hen I was invited to contribute an autobiographical chapter to the *History of Neuroscience in Autobiography* I was very hesitant about it. My scientific career, at least at the beginning, was tortuous and certainly anything but a model career. Ideally, the description of such a career should set an example to young scientists how to organize their scientific life to become successful. However, in this respect I can barely provide a positive example, more likely how not to do it.

My main motivation for doing research evolved from a combination of profound curiosity and a taste for adventure, to embark to new territories with all its excitements and risks.

## A Boy from the Swiss Mountains

In the biographies of many successful scientists, the wish to become a scientist was already apparent in their early childhood. Barely out of their diapers, they had their own laboratory and were reading the biographies of their scientific idols. My laboratory was the natural environment of a mountain valley in the Swiss Alps. We observed plants and animals in all seasons, and our imagination was stimulated, for example, by finding the remains of a hare together with the tracks of a fox in the snow. Our taste for adventure was satisfied by exploring rock caves with self-made carbide lamps that not too seldom failed. And when not outdoors, I became acquainted with many different occupations such as carpenters, plumbers, coopers, ski and sledge makers, and many more. When I visited their workshops they were more amused than annoyed as I pestered them with questions why they were doing what in which way.

Before I went to school my reading and mathematical abilities were very limited. When I participated in my first ski race I had a high number that was beyond the limits of my numerical knowledge so the amused starter had to say, "Come on Hansi, it's your turn now." I did not shine in this first race. Later on I did much better and won many races in different age categories.

Very soon I also made my first attempts to climb the rocky bastions surrounding my home valley, partly under the supervision of experienced mountaineers but to an increasing extent independently. For quite a while I envisaged the possibility to earn my living as a ski instructor and mountain guide. However, I then decided, with the encouragement of my parents, to invest some time in preparation for entering high school in Burgdorf,

a small town at the entrance to the Emmental. The preparation was minimal. It consisted largely of a crash course in Latin, given by the local reverend, and some extra lessons in geometry and algebra.

## Ancestors and Family Background

A school teacher traced our family back to the thirteenth century, when my paternal ancestors moved from the neighboring Valais to the Simmental. The Simmental, like virtually all the valleys of the Swiss Alps, became overpopulated. The young people were forced to leave, especially those who did not fit into the society, did not go to church, or otherwise did not behave in an orderly way. For a long time virtually the only option was to become a mercenary. Many returned from their exploits as cripples, if at all; only very few came back with a modest fortune.

After mercenaries were no longer in demand, young men still had to leave their home valley, had to emigrate when still teenagers. They were often sent with a one-way ticket to the United States, preferably Wisconsin. Not too seldom these ne'er do wells returned as "the rich uncles from America" and were then courted by their respectable relatives at home. More recently a small proportion of the younger generation had the privilege of going to high school and university. My paternal ancestors enjoyed this privilege. My grandfather was a judge at the castle of Wimmis at the entrance to the Simmental. My father was an M.D. who, initially, had envisaged an academic career. The prerequisite for this was a substantial fortune. The economic crisis after World War I forced him to change to practical medicine. Here, it should be recalled that at the beginning of the twentieth century Switzerland was strongly oriented toward Germany. The main commander of the Swiss Army was related to the Bismarck family and, because my father had invested his money in German stocks and bonds, he lost everything.

However, the strong orientation toward Germany had also its limits. My grandfather, as the representative of the Bernese government, had to welcome the German Emperor immediately before World War I, when Wilhelm II came to the Simmental to shoot chamois. My grandfather was not only supposed to welcome him but the rigid protocol demanded him also to bow low. My grandfather strictly refused to do so, considering this to be an unbearable humiliation. Maybe my limited belief in authority has some genetic roots.

## High School Education in a Small Town

I entered the high school in Burgdorf with minimal preparation, as I mentioned earlier. The entry criteria for boys—seldom girls—from the mountains were very generous. We were given a year to catch up to the required level. It was well known that the children from the mountains were not

lacking in motivation. For the majority of their parents this was a heavy financial burden. Often they and their relatives had to pool their resources to send just one student to high school and university.

At the age of 14 I was in Burgdorf completely on my own. I had my own room, and nobody checked my coming home or supervised my homework. This promoted my independence and sense of responsibility.

The time in Burgdorf was an enjoyable and very fruitful one. We were taught in small classes and had outstanding teachers, highly motivated to give us the best possible education. Some of them were university professors, but their salaries were so miserable that they had to work as high school teachers to make ends meet.

## University Education

After obtaining my *Maturitätszeugnis* (school-leaving certificate) in 1947, I went to the University of Bern to study medicine. I originally wanted to study biochemistry and medicine in parallel. Unfortunately this was not possible at that time. The first year at medical school perfectly met my gusto. We had very small classes and received our basic training, including practical courses, together with physicists and chemists. The rest of the education at the medical school corresponded to the general contemporary standard. It was common practice to study for part of the time at another university. My choice of Innsbruck was governed more by the prospect of mountaineering in the Austrian Alps than by specific aspects of medical training that were offered there and not in Bern. The time in Innsbruck was, nevertheless, very instructive. Most of the students were much older than I was. They had been drafted at an early age, before they had finished high school. A large number had been awarded their school-leaving certificate for destroying tanks or shooting down planes. At the end of the war many of them spent years in Russian prisoner camps in Siberia. An Austrian fellow-student who introduced me to the climbs around Innsbruck was one of the only five survivors of a *Gebirgsjäger* (mountain troop) regiment. His reaction to the war experiences was rather exceptional. He remained an enthusiastic rock climber and loved strenuous outdoor activities. The attitude of the majority of the students with similar war experiences was completely different, along the lines of "For years we have been freezing, starving, and lying in the mud; enough is enough."

## From Clinical to Experimental Research

During my medical studies it became clear that practical medicine was not my professional goal. I was interested in basic physiological functions including pathogenic mechanisms. I was not ready to blindly accept the "rigid therapeutic recipes" that were not infrequently controversial and the subject of "religious wars" between different "clinical schools."

After a period of military service and a year of training in general pathology at the University of Bern, I was accepted as an assistant in the Department of Internal Medicine led by Hans Staub in Basel. He was originally a pharmacologist and in spite of not being a full-blown clinician became head of the Department of Internal Medicine. This was a very unusual setup, to some extent way ahead of its time, particularly for the rather conservative University of Basel. Although there were therapeutic schemes for given clinical situations, Staub expected a rational justification for the treatment of every individual patient.

By the time I joined his department he had already suffered a serious heart attack. Although he encouraged his assistants to pursue clinical research projects, he no longer had the strength to supervise them adequately and to ensure they had sufficient time to work in the lab besides their heavy caseload of 25 patients each. It became clear to me that the quality of research I could accomplish under these circumstances did not meet my expectations. Moreover, even under optimal conditions, I would very soon encounter the ethical and technical barriers that are familiar to those carrying out clinical research with humans. A problem could become really interesting, but it was not possible to take it as far as I would have wanted. Although I would have had the opportunity to pursue a promising clinical career, I decided to change from clinical to pure experimental research.

My first position in experimental research was mediated by Alfred Pletscher, the medical research director of Hoffman La Roche in Basel, who, as a former assistant, still had contacts with the Department of Internal Medicine. I approached him for advice and with the best of intentions he recommended me for a position in a small, newly founded research center, mainly supported by Hoffmann La Roche. My experience was so disappointing and even embarrassing that I refrain from giving the details of the research projects I was supposed to pursue. This first position ended one Christmas morning when I was fired for reasons of “scientific incapability and insubordination.” The label of “incapability” was based on the fact that I did not find what my boss expected. The label of “insubordination” resulted from my rather undiplomatic way of explaining to him why I could not satisfy his expectations. Although it was a lost year, I learned a very important lesson for my whole life, namely how not to treat my collaborators.

## To be Married to the Right Wife

When I informed my wife what had happened on this memorable Christmas morning, she just burst out laughing and told me that she had been expecting such a dramatic end for a long time. Not a word of reproach with respect to my responsibility for my family. Also in the preexperimental period she supported me in every possible manner and encouraged me to participate in a mountaineering expedition to the Himalaya (Everest-Lhotse) when she

was pregnant. Because of serious health problems of my father I had to cancel my participation in this expedition. Again, at the end of my clinical time, when I was selected for an expedition of the Swiss Alpine Club to the Peruvian Andes, my wife supported me in every possible way, even though by then we had our two little boys.

The importance of my wife was not confined to the beginning of my scientific career. Later on she had a central function in my research group, first at the Biocenter in Basel and then at the Max Planck Institute in Munich. She was looking after our foreign scientists and their families, helping them to find accommodation, to overcome difficulties with awkward officials at the immigration office, and to cope with everyday problems. For instance, she had to convince desperate Midwest girls that it is perfectly possible to survive without the brand of cornflakes available in the United States from Alaska to Key West and that cheese does not have, at any price, to match the geometry of "Wonder Bread." In fact my wife became the mother of my department, comforting collaborators when they had personal problems, listening to them and giving advice only when asked. Today young scientists may have difficulties to imagine the existence of such a function. However, at that time, a scientific career was a family enterprise and the function of the wife was clearly understood to be as important as that of the husband.

### Basic Research in a Drug Company

In spite of my "scientific incapability and insubordination" label I was offered a position in the Department of Experimental Medicine at Hoffmann La Roche. This position gave me the opportunity to do basic research under much more favorable conditions than would have been possible at the University of Basel. There, the quality of research in the areas I was interested in was rather mediocre.

In the early 1960s Roche started to harvest the fruits of their blockbuster drugs Librium and Valium, and it was the policy of the firm to invest the money, as far as possible, in (basic) research. At the end of the decade this led to the foundation of the Roche Institute of Molecular Biology in Nutley (New Jersey) and the Basel Institute of Immunology. Before these large institutes were founded, a few small basic research groups were created within existing research units. These small groups were expected to do qualified (basic) research without being directly involved in the routine screening. Again, this was initiated by Alfred Pletscher and his "right hand" Alfred Studer. I joined a small research group consisting initially of Albert Hürlimann (with a background in experimental cardiovascular pharmacology) and Willy Haefely (with a background in bacteriology). Before starting my active research in Basel, Roche gave me the opportunity to spend short periods in different laboratories, including the Heymann Institute in Ghent, Belgium, under the directorship of I. Leusen, and the laboratories of H. Blaschko, E. Bülbring, and W. Feldberg in England.

The general topic of our research was the analysis of the physiological function of the sympathetic nervous system as a basis for rational pharmacological modifications. On my return to Basel I began to develop a preparation of the isolated perfused cat spleen. This preparation enabled me to compare the mechanical response of the spleen (perfusion pressure and volume change) to electrical stimulation of the splenic nerves with changes in the norepinephrine output in the perfusion fluid. We found that compounds with anti-depressive properties, like Imipramine and Desimipramine, enhanced the volume changes and the perfusion pressure of the spleen in response to the electrical stimulation of the sympathetic splenic nerves. At the same time these compounds increased the concentration of norepinephrine in the perfusion fluid of the spleen. Other drugs, in particular phenoxybenzamine, reduced the mechanical response but nevertheless enhanced the norepinephrine output. Here, I missed an essential mechanism by which phenoxybenzamine enhances the norepinephrine output. I thought that phenoxybenzamine enhanced the norepinephrine output exclusively through an inhibition of reuptake. However, under physiological conditions phenoxybenzamine enhances the norepinephrine output also through a negative feedback mechanism that is mediated by norepinephrine and affects a subpopulation of alpha-adrenergic receptors on the sympathetic nerve terminals.

## False Adrenergic Transmitters

After these initial experiments on the importance of the reuptake of endogenously secreted norepinephrine, I shifted my attention to the analysis of false adrenergic transmitters. It is important to recall what the state of knowledge was at that time. Immediately before World War II, Hermann Blaschko had already proposed how catecholamine synthesis might proceed from phenylalanine to norepinephrine. When I started my investigations there was only fragmentary information on the individual enzymes and the corresponding cofactors. The enzymes were neither purified nor were antibodies against them available.

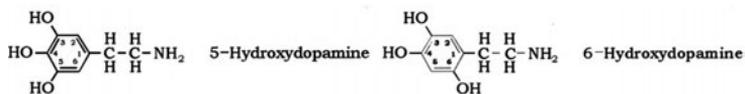
When I began my experiments, E. Muscholl and coworkers in Mainz had just demonstrated that alpha-methyldopa, in clinical use as an antihypertensive drug, is metabolized to alpha-methyl-norepinephrine and that this metabolite is released by electrical stimulation of sympathetic nerves. I wanted to investigate the concept of false adrenergic transmitters in more general terms, and Hoffmann La Roche was the ideal place to pursue such a project. A number of young, talented chemists were enthusiastic to synthesize compounds for a rational concept rather than for the purpose of broad general screening, with a "lucky shot" as the only attractive prospect. The sympathetic neurons can be "cheated" by means of false transmitters because the enzymes involved in the synthesis of the adrenergic transmitter are not absolutely specific. The same is true for the amine transporters of the plasma membrane and the storage vesicles. The effects of the proposed false transmitters

or corresponding precursors were first analyzed by their depleting action on norepinephrine in sympathetically innervated organs. We analyzed the functional consequences in the isolated perfused spleen, the nictitating membrane of the cat, and the changes in blood pressure of unanesthetized rats. Based on this information we decided whether it was worth initiating a more detailed analysis. If this was the case, the corresponding precursor molecules or the false transmitter itself were labeled with tritium. This enabled their metabolism to be analyzed. I identified these various metabolites by paper chromatography with solvent systems established with the help of our chemists, in particular Albert Langemann. The rate of separation by these solvent systems was very much dependent on temperature. It is amusing to recalling that I spent many (hot) summer nights on an air mattress in my lab—no air conditioning was available—to catch the optimal time point in the separation process.

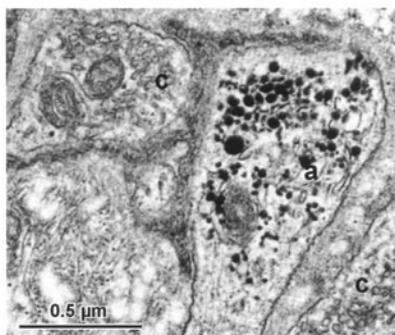
An important change in the direction of my research took place when Jean-Pierre Tranzer joined Hoffman La Roche. He was an outstanding electron microscopist and decided to leave academia in France to provide to his wife and his five children an appropriate standard of living. He took the opportunity of joining Roche with particular pleasure in view of the prospect of doing, nevertheless, predominantly basic research. Jean-Pierre and I joined forces immediately. We felt that advanced methods of electron microscopy (EM) could help us to arrive at a better, comprehensive understanding of the replacement of the physiological transmitter norepinephrine with false transmitters than biochemical analysis alone.

Only a few years before Jean-Pierre started his work on false adrenergic transmitters, De Robertis in Argentina had identified synaptic vesicles as probable transmitter storage organelles. In the few EM studies of sympathetic nerve terminals the presence of a “dense core” was very variable, depending on the fixation procedure used. Jean-Pierre first improved the fixation and contrasting methods for norepinephrine at the EM level. By contrast with previous findings, he demonstrated that virtually all synaptic vesicles had a dense core and that the intensity of the labeling could be increased by preincubation in medium containing norepinephrine. For false transmitters we could predict whether they could be visualized at the EM level by mimicking the fixation and contrasting procedure in the test tube. In this way we identified 5-hydroxydopamine (5-HODA) as a very useful marker for adrenergic neurons. All synaptic vesicles were densely labeled and the picture was particularly impressive when adrenergic and cholinergic nerve terminals were located in close proximity to each other (Figure 1).

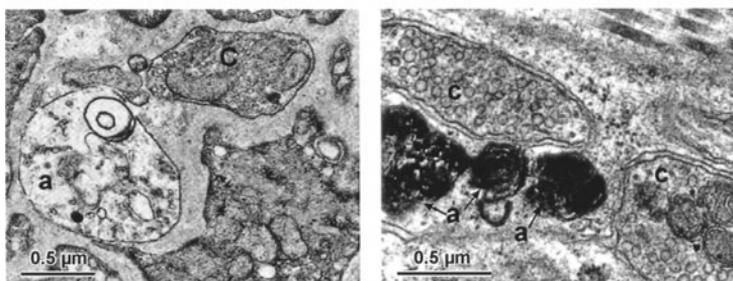
The experiments with 5-HODA also revealed other interesting aspects that it was difficult to interpret correctly at the time. “Reticulum-like” structures were labeled in addition to the synaptic vesicles, although less intensively. We thought that besides the synaptic vesicles there might be an “additional storage compartment” for the adrenergic transmitters. Most likely



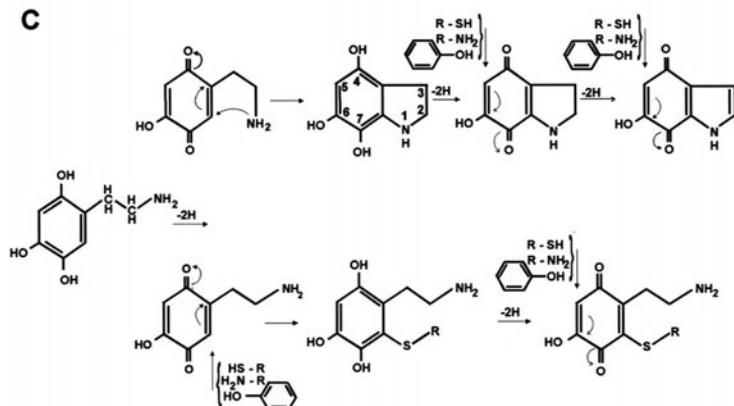
**A**



**B**



**C**



**Fig. 14.1** Comparison between the replacement of the physiological transmitter *norepinephrine* by 5-HODA and 6-HODA. After treatment with 5-HODA all the synaptic vesicles (small and large) are filled with electron dense material (A). Immediately adjacent cholinergic nerve terminals (c) remain empty. After treatment with 6-HODA (B) the cholinergic nerve terminals (c) remain intact, do not contain electron dense material, whereas the adrenergic nerve terminals (a) are in different stages of degeneration. (C) Oxidation products of 6-HODA and covalent binding to nucleophilic groups of macromolecules (proteins, nucleic acids).

we identified the precursor compartment of the (small) synaptic vesicles. In contrast to the large dense core vesicles, which are produced in the perikaryon, the small synaptic vesicles are produced as tubulo-reticular precursors. Mature vesicles are formed in the nerve terminals. Such a precursor compartment was recently identified by Wieland Huttner and coworkers. In this precursor compartment the amine transporters are most likely "diluted," and after one or possibly several cycles of exo/endocytosis the mature synaptic vesicles are formed. This concentration process is linked with the sorting of other specific constituents of the (small) synaptic vesicles. The details of these sorting mechanisms are still not completely understood, for example, how the amine transporters of the plasma membrane and the synaptic vesicles are separated from each other.

### Detection of The mechanism of Action of 6-Hydroxydopamine (6-HODA)

In parallel to the analysis of 5-HODA, we also investigated its isomer 6-HODA. It also fulfilled the requirements of a false transmitter to be visualized at the EM level in the storage vesicles. From previous experiments by Marthe Vogt in Babraham/Cambridge (U.K.) and the Merck Laboratories in the United States it was known that 6-HODA produced a long-lasting depletion of norepinephrine. We evaluated a broad spectrum of dosage schedules leading to a maximum long-term depletion of norepinephrine in different species, in particular rats and cats (see Thoenen and Tranzer, 1968; Tranzer and Thoenen, 1968). Because we knew that 6-HODA is easily oxidized, we saturated the solutions to be injected intravenously with Argon. We expected that after such treatment the storage vesicles of the adrenergic nerve terminals would be filled with electron-dense material and that this material would remain there for a much longer time than after treatment with 5-HODA.

I will never forget the moment when Jean-Pierre rushed into my lab and said in his beautiful Alsatian dialect "*Du müesch sofort cho lüege die si alli futü*" ("Come and look quickly, they are all gone"). We rushed to the EM and spent a very long time at the screen. The degenerating adrenergic nerve terminals were located next to intact cholinergic nerve terminals (Figure 1). We repeated the experiments several times and also established the time course of the degeneration. We thought that all this information would be sufficiently interesting for publication in *Nature*. We were wrong: The paper was rejected out of hand and qualified as a "fixation artifact." We also received fatherly advice how to fix tissues for EM analysis. Although we had emphasized the selectivity of the effects, the reviewer ignored our statement and our clear experimental evidence for the selectivity of the destructive effect. We were disappointed and angry. Indeed, nobody was qualified to teach Jean-Pierre Tranzer how to prepare tissues for EM examination. Jean-Pierre absolutely refused to contest this decision and to embark on a

“discussion with idiots.” We sent the paper to *Experientia*, a journal that, at that time, had a reputation not very far behind that of *Nature* and *Science*. This paper, together with a more extensive paper in *Naunyn Schmiedeberg's Archives*, became citation classics and the “fixation artifact” of 6-HODA has been confirmed many thousand times by hundreds of laboratories all over the world.

After having established that the reason for the long-lasting depletion of norepinephrine by 6-HODA was the selective destruction of adrenergic nerve terminals rather than a long-term storage of 6-HODA, the question arose what was the molecular mechanism responsible for this destructive effect. Albert Langemann, who was in charge of the large-scale synthesis of 6-HODA, was continuously confronted with its extreme susceptibility to oxidation. He suggested the formation of a para-quinone derivative and its possible further transformation into a trihydroxyindole (Figure 1). Both of these oxidation products can easily form covalent binding with a variety of nucleophilic groups such as SH, NH<sub>2</sub>, and phenolic HO-groups. This assumption was first supported by preliminary observations made by Fritz Bigler, who found that after administration of H<sup>3</sup>-6-HODA a substantial proportion of the radioactivity was covalently bound in sympathetically innervated tissues, whereas 99% of the radioactivity could be extracted after injection of labeled norepinephrine. Thus, the specificity of the destructive effect of 6-HODA seemed to result from its efficient accumulation in adrenergic nerve terminals. As such the destructive effect of 6-HODA is nonspecific as impressively demonstrated when 6-HODA was accidentally injected subcutaneously and produced a local necrosis.

In experiments performed after my return from the laboratory of Julius Axelrod (see below), Alfons Saner and I directly identified by spectroscopy the metabolites of 6-HODA suggested by Albert Langemann.

The extreme reactivity of the oxidation products of 6-HODA had, most probably, disastrous consequences for those scientists who were dealing with large quantities of these oxidation products. It is otherwise hard to understand why all the scientists who were involved in such experiments died a few years later. Albert Langemann died of thyroid cancer, Jean-Pierre Tranzer from acute bleeding into a brain tumor, and Fritz Bigler died of leukemia. I probably escaped the fate of my colleagues because, in experiments I performed with my own hands, I took every possible precaution to prevent 6-HODA from oxidation before I injected it intravenously.

### Detection of the Trans-Synaptic Induction of Tyrosine Hydroxylase, a Further Consequence of the “Magic Brown Powder”

Just after the detection of the mechanism of action of 6-HODA, I joined the laboratory of Julius Axelrod at National Institutes of Health in Bethesda, Maryland. I arrived with my family in June 1968 at a time of unrest with violent riots in Washington, D.C., and it took some extra time to get settled.

During this period, when I was not yet working full-time in the lab, I noticed with great interest that Bob Mueller, a young postdoc, had just established an assay for tyrosine hydroxylase (TH). Because I had to give a survey lecture on the occasion of the International Congress of Physiology I thought it would be attractive to document biochemically the effect of 6-HODA not only by a depletion of norepinephrine but also by a drastic reduction of the rate-limiting enzyme of its synthesis. The TH assay was sensitive enough for the high TH concentrations in the adrenal medulla. However, when determining TH activity in sympathetically innervated organs we were sometimes working at the sensitivity limit of the assay. Bob Mueller and I managed to determine TH activity in the highly concentrated homogenates of rat hearts. The supernatants had to be passed over a molecular sizing column to eliminate "low molecular inhibitor(s)." These inhibitors, most likely, reflected the high concentrations of endogenous tyrosine that simply diluted the relatively low specific activity of H<sup>3</sup>-labeled tyrosine used for the assay. When the first 6-HODA treated rats were ready to be analyzed, Bob left for a short vacation. I was pleased to see that the TH activity in the heart homogenates was drastically reduced. I used the adrenals as controls for the (tricky) assay in the heart. To my great surprise there was a more than 2-fold increase in TH activity. In this context it is important to remember that 6-HODA neither destroys adrenal medullary cells nor the cell bodies of adult adrenergic neurons. The destructive effect is restricted to the sympathetic nerve terminals. I repeated the experiments several times and confirmed the initial observation. Julius Axelrod, who went by the name of Julie, was as keen as we were to pursue this unusual finding. Bob and I joined forces and left our original projects. We assumed that the increase in TH resulted from general stress and activation of the pituitary-adrenocortical axis. Here, we were strongly influenced by the discovery made not long before by Dick Wurtmann and Julie that phenylethanolamine-N-methyltransferase (PMNT) was regulated by glucocorticoids. In fact, besides the marked increase in TH activity there was also a small (about 15%) increase in PMNT activity. To evaluate the importance of the pituitary-adrenocortical axis more specifically as the cause of the 6-HODA-mediated TH induction, we repeated our experiments with hypophysectomized animals. However, the TH induction remained unchanged. We then denervated the adrenals unilaterally and demonstrated that we were dealing with a neuronally mediated effect. On the denervated side there was no increase in TH. The enzyme kinetic analysis supported the assumption of an increase in enzyme protein rather than the activation of a given quantity of TH. This view was further supported by the fact that cycloheximide treatment impaired the increase in 6-HODA-mediated TH induction.

In subsequent experiments we found that other drugs like reserpine and phenoxybenzamine, which interfere with the postganglionic sympathetic transmission, had an effect similar to that of 6-HODA. However, the

increase in TH was not restricted to the adrenal medulla but also occurred in sympathetic ganglia, and the TH induction was prevented by transection of the preganglionic sympathetic nerves. Moreover, the very small increase in PMNT activity in the adrenal medulla was shown to be mediated by neuronal activity, that is, it could be blocked by denervation of the adrenals.

Our results were published in *Science*, *Nature*, and other prestigious journals and attracted the interest of the scientific community. Other laboratories, which had been working for years on the hydroxylation of phenylalanine and tyrosine, were somewhat annoyed that outsiders were the first to demonstrate the selective induction of a macromolecule through neuronal activity. It was indeed a fortuitous observation that deserves the label of "serendipity." We can, however, claim the merit of not discarding this "odd observation," just being satisfied with the drastic reduction of TH in the rat heart by 6-HODA treatment. Trans-synaptic enzyme induction was not the result of an investigation designed to establish it, it resulted from an observation made in a small side project that simply served the purpose of satisfying my pride to present some new unpublished data in a survey lecture.

The period I spent working in Julie's lab was a very important and productive one. I concentrated almost exclusively on the experiments and hardly ever went to the library. I followed Julie's advice: "Don't let yourself be distracted by the literature, read when you've done the experiments." However, if experiments permitted, I attended evening seminars on subjects that included the regulation of enzymes in bacteria. I was impressed to learn that the enzymes involved in a given metabolic pathway were often regulated as functional units, forming so-called operons. I was attracted by this idea and wondered whether such a mechanism might also come into play in mammals for the regulation of the enzymes involved in norepinephrine synthesis.

After my return to Switzerland I took my investigations further and demonstrated that physiological stimuli such as exposure to cold and the stress of swimming also resulted in an induction of TH in adrenal medulla and sympathetic ganglia. After establishing the assays for the other enzymes involved in the synthesis of the adrenergic transmitters (norepinephrine and epinephrine) I was delighted to see that all the experimental conditions that led to an induction of TH also produced an increase in dopamine- $\beta$ -hydroxylase (DBH). I was not too much disturbed that dopa-decarboxylase (DDC) was not specifically increased because it was already known that this enzyme is also expressed in nonneuronal tissues.

## First Experiments with Nerve Growth Factor (NGF)

By the time I was working on trans-synaptic enzyme induction I was aware of the existence of nerve growth factor (NGF). I knew the story of its fortuitous detection, the rich source of the male mouse salivary gland, and the spectacular effects on sensory and sympathetic neurons *in vitro* and *in vivo*.

I was also aware of the production of an anti-NGF antiserum that provided the first direct evidence that NGF had a physiological function.

I first became more directly interested in NGF through a seminar given by Pietro Angeletti, a senior collaborator of Rita Levi-Montalcini. I wondered whether the dramatic increase in the size of sympathetic ganglia was also reflected by a corresponding increase in TH and DBH as in trans-synaptic enzyme induction. So far, the analysis of the biological effects of NGF had virtually exclusively been confined to the morphological analysis. Pietro Angeletti was as keen as I was to work on this. He sent me large quantities of 2.5 S NGF that enabled newborn rats to be treated with 10 mg/kg of NGF a day for 10 days. Pietro carried out the morphological analysis, that is, he determined the total volume of the superior cervical ganglion, the number of neuronal cell bodies and their diameter. I carried out the biochemical analysis, that is, determined the enzyme levels involved in norepinephrine synthesis. I was thrilled to see that the pattern of NGF-mediated enzyme induction was very similar to that seen in trans-synaptic enzyme induction, including the enzyme kinetic data providing evidence for an increase in the quantity of TH and DBH protein rather than an activation of these enzymes. I imagined two signal transduction pathways funneling into a common end point at the transcriptional level. It is no wonder that, given the scarcity of information available on signal transduction in general, this (naïve) hypothesis proved to be wrong. However, this kind of day-dreaming is an essential part of the day-to-day fun in research.

## From Industry Back to University

During my time at NIH, Julie Axelrod told me on several occasions that my future should be in academia. I suspect that he himself took active steps in this direction. Indeed, after my return to Basel I received attractive offers from U.S. universities and research institutes. Although tempted to return to the United States, I had serious concerns due to the ongoing Vietnam War. My teenage boys would have risked being drafted within the next few years. On the other hand, on my return from NIH the atmosphere at Roche had changed. There was increasing pressure on me from intermediate hierarchical levels to become more directly involved in general screening and administration. This would have meant a considerable increase in my salary. Even my wife was approached, but her answer was straightforward: "Do you expect me to choose more money in return for an unhappy husband?" It became clear that I had to make a decision while I was still receiving attractive offers. The productive activities in the Axelrod lab proved to be important when a new multidisciplinary institute, called Biocenter, was founded at the University of Basel. The Biocenter, which represented a bold leap forward, was expected to become a first-class international research institute. I was offered a position as head of a neurobiology research group,

first as associate and 2 years later as full professor, accommodated in the Department of Pharmacology. However, my teaching duties in pharmacology were confined to a few lectures in neuropharmacology.

A new curriculum had to be designed to provide the students with an appropriate training. In the first 2 years the main focus was on mathematics, physics, biophysics, and chemistry. This was interspersed with survey lectures in cell biology and neurobiology at the level of *Scientific American* to encourage students, who were predominantly interested in biology, to accept the initial mathematically oriented training as a prerequisite for the high standard of biological research they were aiming at. These 2 years of basic training were followed by so-called block courses in which the students became acquainted with contemporary methods in genetics, biophysics, biochemistry, cell biology, and neurobiology. The classes were small, about 30 students per year. This permitted a very intensive and individual training, also serving the purpose of showing the students that even the most sophisticated and specialized methods are “doable.”

In 1971–1972 not all the research groups of the Biocenter had been set up. The newly founded Friedrich Miescher Institute of Ciba-Geigy was therefore accommodated in those parts of the Biocenter that were not yet occupied, until the Friedrich Miescher Institute had its own facilities. The Miescher Institute was led by Hubert Bloch, an eminent science leader with a visionary capability to identify gifted young scientists. He encouraged his staff members to participate in the neurobiology block course, and very soon fruitful scientific collaborations developed with Irwin Levitan, Ron Lindsay, Denis Monard, and Frank Salomon. An additional benefit of the contacts with the Friedrich Miescher Institute was the large number of male mice that became available to my research group. They originated from toxicological control groups of Ciba-Geigy. This enabled us to purify our own NGF in large quantities and to expand the spectrum of our research, in particular to the autoradiographic localization of labeled NGF at the light and EM level and a more detailed analysis of the mechanism of action of NGF.

## NGF Research at the Biocenter

### *Detection of the Specific Retrograde Axonal Transport of NGF*

After having established the similarity between the selective induction of TH and DBH by NGF and enhanced preganglionic activity, I became interested in the physiological functions of NGF in general. I started to read the original publications of Victor Hamburger and Rita Levi-Montalcini and also became interested in the cocultivation experiments of Geoffrey Burnstock. He had demonstrated that there was a correlation between the density of innervation of the target tissues of sympathetic neurons and the extent of fiber outgrowth these tissues elicited from sympathetic ganglia when cocultivated.

This fiber outgrowth could be abolished by the administration of anti-NGF antibodies.

Just after my move to the Biocenter I met a young Australian Ph.D. student, Ian Hendry, who was attending the annual winter school in Zuoz in the Canton Grisons. He expressed interest in working in my lab after finishing his Ph.D. with Les Iversen, Cambridge, U.K. It became never clear to me whether he was more interested in my research or in my skills as a potential ski instructor in the Swiss Alps.

After finishing his Ph.D. Ian came to Basel and put forward the hypothesis that NGF could replace sympathetic target organs. If this hypothesis was correct, NGF had to be transported retrogradely to the cell bodies because the regulation of TH by NGF occurred at the transcriptional level. We therefore injected J<sup>125</sup> NGF into the anterior eye chamber and determined the rate of accumulation of radioactivity in the superior cervical ganglion of the injected and contralateral side. There was in fact a greater accumulation of labeled NGF on the injected side and a much lower accumulation on the contralateral side resulting from J<sup>125</sup> NGF escaping into the general circulation. This interpretation was supported by autoradiographic experiments showing a weak diffuse labeling of the superior cervical ganglion on the contralateral side. By contrast, on the injected side, the radioactivity was concentrated in a relatively small number of neuronal cell bodies corresponding to the neurons innervating the iris. This interpretation was further supported by the fact that this accumulation could be blocked by transection of the postganglionic axons or by interference with the axonal transport through local administration of colchicine.

After the departure of Ian Hendry, a young Ph.D. student, Martin Schwab, approached me to ask whether he could perform birth-dating experiments with H<sup>3</sup>-thymidine in my lab. Martin pursued his thesis in an institute outside the Biocenter where he could not perform these experiments. He finished his thesis very rapidly and became more and more interested in the ongoing projects of my research group. With his basic training in developmental neuroanatomy he was well qualified to take over the analysis of the retrograde axonal transport at the EM level and to include in this analysis also methods of morphometry. In a very short time Martin had acquired all the necessary skills for conventional EM, autoradiography at the EM level, and determination of the size of the different cell compartments of neurons by stereological methods.

### *Specificity of the Retrograde Axonal Transport of NGF in Sensory Neurons*

We now expanded the analysis of the retrograde axonal transport to the sensory neurons by injecting labeled NGF unilaterally into the forepaws.

We then analyzed the rate and specificity of the retrograde transport to the corresponding dorsal root ganglia C6–C7. As with injection into the anterior eye chamber, there was a highly selective accumulation of radioactivity in the ganglia of the injected side. The accumulation of  $J^{125}$  NGF was again confined to a relatively small number of heavily labeled neurons. As controls for the specificity of the retrograde axonal transport of  $J^{125}$  NGF we injected labeled proteins with differing physico-chemical properties. Of particular interest was cytochrome C, which has a high isoelectric point like NGF and virtually the same molecular weight. No evidence for a specific retrograde transport of cytochrome C could be demonstrated.

### *Routine Control Procedure Opens up a New Field of Research*

To evaluate the possibility of a specific retrograde axonal transport of NGF in motoneurons, we injected  $J^{125}$  NGF unilaterally into the deltoid muscle of the rat foreleg. However, this did not result in an accumulation of radioactivity in the corresponding motoneurons (C6–C8). To decide whether this was due to insufficient penetration of  $J^{125}$  NGF to the nerve terminals in the motoneuron end plate, we injected  $J^{125}$ -labeled tetanus toxin, which was known to propagate from the periphery via the axons to the motoneuron cell bodies. There was clearly a greater accumulation of  $J^{125}$  tetanus toxin in the motoneurons of the injected side. In subsequent experiments at the EM level, Martin Schwab demonstrated that tetanus toxin was not only transported to the motoneuron cell bodies but also transferred trans-synaptically to (inhibitory) interneurons. The time period of this trans-synaptic transfer coincided with the appearance of tetanic rigidity. These investigations also ended a dispute as to whether tetanus toxin was transported within or along the axons. To our astonishment, tetanus toxin was also transported retrogradely in sensory and sympathetic neurons. We then expanded our analysis to the central nervous system (CNS) and demonstrated that labeled tetanus toxin, when injected into a projection field, was transported retrogradely to the corresponding cell bodies. Tetanus toxin thus became a very useful tool for analyzing the projection fields of all peripheral and central neurons. In view of the already known high affinity of tetanus toxin to the trisialoganglioside TGT1, we investigated whether the simultaneous administration of TGT1 or treatment with neuraminidase, an enzyme that degrades gangliosides, interfered with the retrograde transport of tetanus toxin. This was indeed the case. By contrast, the retrograde transport of NGF was not affected. We then also included cholera toxin in our studies, which was known to have a high affinity to the monosialoganglioside GM1, and wheat germ agglutinin, a lectin with a high affinity to glycoproteins with N-acetyl glucosamine residues. All these molecules were transported retrogradely in all the populations of neurons investigated in the peripheral and central nervous system.

*More Detailed Analysis of the Retrograde Axonal Transport*

The EM studies with  $J^{125}$  NGF provided evidence for the association of the radioactivity with tubulo-vesicular compartments within the axons, which are indistinguishable from smooth endoplasmic reticulum. This cautious formulation reflects the level of knowledge in the late 1970s, when neither luminal nor specific membrane markers for smooth endoplasmic reticulum were known. After injecting  $J^{125}$  NGF into the anterior eye chamber or the submandibular gland (another target organ of the adrenergic neurons of the superior cervical ganglion), about 20% of the radioactivity was localized in secondary lysosomes (dense and multivesicular bodies), but the majority of the radioactivity was again localized in smooth endoplasmic reticulum-like compartments. There was no radioactivity (above background level) in the nucleus, mitochondria, and the Golgi cisternae. In view of the limited resolution of EM autoradiography we sought to produce a coupling product between NGF and horseradish peroxidase to achieve direct cytochemical localization. The coupling procedure not only had to preserve horseradish peroxidase activity but, most important, also had to preserve the biological activity of the coupled NGF. Kitaru Suda, a very gifted Japanese chemical engineer, managed to create such a coupling product by oxidation of the carbohydrate moiety of horseradish peroxidase to aldehyde groups that then were reacted with the free amino groups of NGF. The direct cytochemical localization of the reaction product of horseradish peroxidase confirmed the subcellular localization suspected in axons and the perikaryon from autoradiographic studies. In previous experiments, retrograde axonal transport of horseradish peroxidase alone had been demonstrated by injection into the anterior eye chamber of cats and hamsters. However, the concentrations of horseradish peroxidase used were several hundred times higher than those present in our coupling product. When used in these (lower) concentrations no retrograde axonal transport was detectable.

Coupling products of horseradish peroxidase were also used for the more direct localization of the retrograde transport of tetanus toxin, cholera toxin, wheat germ agglutinin, phytohaemagglutinin, and ricin. All these molecules showed the same subcellular localization as NGF. The only exception was tetanus toxin, which, as in motoneurons, was also transferred trans-synaptically to the presynaptic cholinergic nerve terminals of the superior cervical ganglion. There, it was localized in a vesicular compartment of 600 to 1000 Ångstroms diameter.

*Attempts to Elucidate the Mechanism of Trans-Synaptic and NGF-Mediated Enzyme Induction*

Our efforts to obtain more detailed information on the mechanism of trans-synaptic and NGF-mediated enzyme induction led to a clear identification of

the unresolved questions, but we were hampered by the fact that the necessary tools were simply not (yet) available. Nevertheless, I think it might be informative to briefly summarize our way of thinking and to present the steps we took to get at least some information. As already mentioned, neither the amino acid and complementary deoxyribonucleic acid (cDNA) sequences of TH and DBH were known nor their genomic organization. Even for cyclic adenosine monophosphate (cAMP), the details of its signal transduction were only rudimentary.

To determine the duration of enhanced preganglionic neuronal activity necessary to initiate TH and DBH, we exposed rats to short repetitive bouts of swimming stress. We then determined the period of time during which the induction of TH and DBH could be blocked by the administration of actinomycin D and cycloheximide, inhibitors of transcription and translation respectively. Because the treatment of rats with maximally effective doses of cycloheximide could not be extended beyond 10 hours, we investigated whether trans-synaptic induction initiated *in vivo* could be extended in organ culture. This was indeed the case, provided glucocorticoids were added to the culture medium. The development of organ cultures also proved to be important for the analysis of the incorporation of radioactive amino acids into DBH as soon as polyclonal antibodies against DBH became available (see below).

The evaluation of the importance of cAMP in trans-synaptic enzyme induction was the subject of heated debate between the laboratory of Erminio Costa and my group. We agreed that the administration of reserpine initiated a marked increase in cAMP in the adrenal medulla. However, the point of controversy was whether the brief marked increase in cAMP was the crucial mechanism responsible for the trans-synaptic induction of TH. Bob Mueller, who spent a sabbatical with me at the Biocenter in Basel, demonstrated together with Uwe Otten that the TH induction could be prevented by unilateral transection of the branches of the splanchnic nerves (supplying the adrenals), even if the transection was performed after cAMP had returned to control levels. Conversely, a steady, marked increase in cAMP over a long period of time resulting from the administration of phosphodiesterase inhibitors did not result in TH induction. These data did not support a direct involvement of cAMP in trans-synaptic TH induction.

#### *Purification of Dopamine- $\beta$ -Hydroxylase (DBH) and Production of Anti-DBH Antibodies*

Claude Gagnon, a Canadian Ph.D., joined my laboratory with the goal of purifying DBH and producing antibodies against it. Claude isolated chromaffin granules from bovine adrenal medulla. After lysis of the chromaffin granules and several subsequent column steps, all the DBH activity was located in one band of approximately 75,000 in a one-dimensional sodium

dodecyl sulfate (SDS) gel. The antibodies were produced in rabbits using standard procedures. The antiserum produced a single precipitation band against crude and purified DBH. To label DBH with radioactive amino acids, we injected rats with large quantities of  $H^3$  leucine. However, the incorporation into DBH was barely detectable. We therefore applied our knowledge of the time course of trans-synaptic and NGF-initiated DBH induction in vivo and the continuation in organ cultures in vitro. We compared the rate of DBH induction in the adrenal medulla and the superior cervical ganglion. In the adrenal medulla the maximum rate of synthesis, reflected by the incorporation of  $H^3$  leucin, was already attained when the organ culture was started one hour after the intravenous injection of NGF. In the superior cervical ganglion there was a relatively small initial increase that was followed after 4 hours by a further continuous increase, so that the whole process lasted 24 hours. This time course of DBH induction in the superior cervical ganglion is reminiscent of the  $J^{125}$  NGF accumulation after intravenous injection. After a very rapid, relatively small accumulation within the first 15 min a much larger accumulation followed after 4 hours, reaching a maximum after 8 hours. The rapid initial increase reflects the direct supply of  $J^{125}$  NGF to the neuronal cell bodies. The delayed, protracted accumulation reflects the retrograde transport of NGF from the periphery.

#### *Differences in the Susceptibility of Sympathetic Neurons in Newborn and Adult Animals*

The affinity purification of very large quantities of polyclonal anti-NGF antibodies after the immunization of sheep and goats enabled us to carry out experiments in which we compared equivalent quantities of anti-NGF antibodies in newborn and adult animals. The injection of a single dose of antibodies in newborn animals virtually completely destroyed the whole peripheral sympathetic nervous system. The TH levels in the superior cervical ganglia were reduced to less than 10% of those in the controls and remained at this reduced level until adulthood. If a corresponding quantity of antibodies was injected into adult animals, this resulted in only a transient reduction of TH and DBH. The reduction of DBH was much larger than that of TH, reflecting the higher turnover of DBH. At intermediate ages the reduction of both enzymes was larger than in adult animals, and the return to control levels was much slower than in adults. These experiments clearly demonstrated that the sympathetic neurons of newborn animals are much more sensitive to NGF deprivation than those of adult animals. They also provided a plausible explanation for the complete destruction of sympathetic neurons by 6-HODA in newborn animals. In fact, in complementary experiments in collaboration with Rita Levi-Montalcini we demonstrated that in newborn animals treated with an optimal destructive dose of 6-HODA, the cell bodies of sympathetic ganglia could be protected by

the simultaneous administration of NGF. In contrast the sympathetic nerve terminals could not be protected.

### *First Steps Toward a Reliable Immuno-Assay for NGF*

My group at the Biocenter was already well established when a young scientist from Geneva, Yves-Alain Barde, visited me. He was seeking advice about how to optimally denervate the brown fat pad of rats with 6-OHDA. We discussed in detail the procedure I had worked out with Jean-Pierre Tranzer and the main purpose of the visit was rapidly settled. We talked mainly about ongoing research projects, and I felt that Yves-Alain was becoming increasingly interested. After a few additional meetings he expressed his intention to accomplish the Swiss Certificate of Molecular Biology. This certificate involved completion of a specific range of courses and experimental work corresponding to a master's thesis. The 3rd-year block courses in the curriculum of the Biocenter met these course requirements in an optimal manner. Out of all the possible topics for the experimental work Yves-Alain chose a very demanding one, namely the determination of the levels of NGF in sympathetic target tissues in relation to the density of their adrenergic innervation.

After a few exploratory attempts to study the incorporation of radioactive amino acids into endogenous NGF in the rat iris, we came to the conclusion that the development of a sensitive immuno-assay would be a more suitable way of approaching this question. First, working together with Kitaru Suda, Yves-Alain evaluated the suitability of the NGF competition assay. For this assay, a limited quantity of anti-NGF antibodies was adsorbed to the wall of a polystyrene tube and a quantity of J<sup>125</sup> NGF, sufficient to saturate these antibodies, was incubated together with the serum or tissue homogenate to be assayed. The more NGF there was present in the sample, the less J<sup>125</sup> NGF should be bound to the adsorbed antibody. Yves-Alain and Kitaru detected that in the serum samples the results were falsified by the presence of a macromolecule, later shown to be predominantly macroglobulin II. This molecule, present in large quantities, bound the labeled NGF and prevented its association with the anti-NGF antibodies adsorbed to the tube. This was the explanation for the excessively high levels of NGF determined by this assay. Nor could these excessively high levels of NGF be confirmed by the classical neurite outgrowth assay developed by Rita Levi-Montalcini in the early 1950s. This biological assay has its own pitfalls and is in any case not sensitive enough to detect the very small quantities of NGF present in sympathetic target tissues. Yves-Alain and Kitaru therefore developed a two-site assay. In contrast with the competition assay a large quantity of anti-NGF antibodies was adsorbed to the bottom of a polystyrene tube in which the samples were incubated. After thorough washing, J<sup>125</sup>-labeled anti-NGF antibodies were added to the tube as "detector molecules." This assay was

sufficiently sensitive to demonstrate unambiguously that the values determined by the competition assay were artifacts. However, to obtain a sufficiently high sensitivity for the determination of NGF levels in small tissue samples, the antibodies had to be more strongly linked to the incubation vessel to withstand the washing steps with detergents necessary to reduce the background. Moreover, with respect to the general reproducibility of the assay, monoclonal antibodies would have been preferable. This approach became realistic as Milstein and Köhler had just published the procedure for their production. However, its implementation had to wait until our move to Munich (see below).

### *Dark Clouds Over My Activity at the Biocenter in Basel*

My time at the Biocenter was characterized by a very good and friendly relationship with my colleagues. Unfortunately, this was not the case with the officials of the government of Basel, who had a very direct influence on our research activities. Each individual appointment of a staff member—technical, administrative, or academic—had to be approved by the government. The government could also cancel the agreements that formed the basis of our appointments. Because I expected a considerable increase in animal experiments in the future, I had negotiated a position for a veterinarian. As I did not fill this position immediately it was axed because “such a position was apparently not really necessary.” Another particularly painful regulation concerned the quota of working permits for foreign scientists. At that time only a limited number of foreign scientists were allowed to work in Switzerland. From the financial point of view it was understandable why the government of Basel wanted to reserve the quota, as far as possible, for the pharmaceutical industry. This policy kept the taxes flowing into the notoriously empty cash box, whereas staff positions granted to us meant spending tax money. In this situation I could not make any reliable long-term plans with my staff positions because the contracts for foreign scientists were only approved a few weeks before the persons concerned were expected to arrive in Switzerland. When I handed in an application earlier I received, in answer, a list of unemployed Swiss biochemists or biologists who were simply not qualified for such positions (returning hippies or recently released inmates of psychiatric clinics).

The tension with the government came to a head when Karl Bucher, Professor of Pharmacology, was approaching retirement. The government was not willing to guarantee his replacement by the time of his retirement. I was now in danger of having to take over all the teaching of medical and pharmaceutical students, including all written and oral examinations. This would have ruined my research. In addition, a planned cellular EM group, to be led later on by Martin Schwab after his return from a postdoctoral training at Harvard Medical School, was not guaranteed either. In this situation I started to look with different eyes at offers from outside. I had already

received several offers from England, the United States, and Germany. The offer from the Max Planck Society was by far the most attractive. It included the reorganization of an institute and also involved the construction of a new research building. The offer was very generous, and there were absolutely no restrictions to the employment of foreign scientists. The only restrictions applied to technical and administrative positions.

It was an extremely difficult decision to leave the Biocenter as I had to leave excellent colleagues and good friends. They made every effort, together with leading scientists from other Swiss universities, to convince the government of Basel to fulfill my requirements. They did not succeed. My departure from Basel also had grotesque consequences. Just at the time I decided to accept the Max Planck Society's offer I learned that I had been selected for the Marcel Benoit Prize, the most prestigious Swiss award for achievements in the area of biology. However, this award was cancelled by the Swiss Minister of Education and Research to demonstrate his "loyalty" to the government of Basel. I was more amused than bitter about this small-minded provincial reaction.

## The Move to Munich

My decision to move to Munich was a difficult and painful one. However, I never regretted it. The contrast with the conditions imposed by the government in Basel was striking. The administrators of the headquarters of the Max Planck Society, in particular Edmund Marsch, gave me the reassuring feeling that their exclusive goal was to support our institute and to promote the research in the best possible way.

After my formal appointment in spring 1977, I had to organize my move to Munich. I designed the new department of neurochemistry to include three *Nachwuchsgruppen* (independent junior research group) corresponding to assistant professors with their own budget. I did not intend to have a large group of my own, but to complement my own spectrum of research with that of these groups.

By contrast with the appointment of the academic staff, I had to take over the technicians already in employment. They were generally well qualified and also motivated to learn the methods necessary to work in my research group. The Max Planck Society gave me the opportunity to train them for their new tasks in Basel.

I was also very pleased when all the scientists who were essential for pursuing ongoing or planned new projects agreed to move with me to Munich. These collaborators were Yves-Alain Barde, David Edgar, Greg Harper, and two graduate students, Felix Eckenstein and Theo Schäfer. Martin Schwab was joining us a few months later after the end of his post-doctoral period at Harvard Medical School. Until the new research building was ready to move into, we were accommodated in free lab space of the Max Planck Institute of Biochemistry in Martinsried.

Initially the new building was supposed to be located at the Kraepelinstrasse, in downtown Munich. However, it very soon became clear that this would be extremely difficult due to the strict German building regulations stipulating that the maximum size of a new building had to be in proportion to the “green area.” The decision of the Administrative Board of the Max Planck Society to put up the new building in Martinsried next to the Max Planck Institute of Biochemistry was very fortunate for me but hard to accept for the colleagues already appointed. They all had strong emotional ties to the “Kraepelin Institute,” not only linked to the name of Kraepelin, but also other famous scientists like Alzheimer, Numa, Page, Spielmeyer, and many more. For me the decision of the Administrative Board was a big relief because the scientific contacts with the Max Planck Institute of Biochemistry became increasingly important. Moreover, it was also much easier to supervise the construction of the new building I was in charge of. This new building had to match the general design of the Max Planck Institute of Biochemistry and, accordingly, we could incorporate improvements into the planning of our new institute. I was also very fortunate to have a qualified administrative help with the practical realization of the construction of the new building: Gisbert Nowozcek. He was an organic chemist and strongly technically minded, and he supervised the day-to-day progress of the construction work in a competent manner. He took care that the architects did not deviate too much from the original plans interfering with the requirements of experimental research. This policy paid off very well, and finally also my colleagues Herz, Kreutzberg, and Lux with their strong emotional links to the Kraepelin Institute were very happy to work in the new research facilities in Martinsried.

### *New Institute, Major Decisions*

The move to Munich coincided with a period when important new techniques became available, in particular the production of monoclonal antibodies and the synthesis of oligonucleotides. The latter opened up the possibility of progressing from a partial amino acid sequence of a protein to the determination of the corresponding cDNA sequence and establishment of the genomic organization. In the following I outline those individual projects that, at least retrospectively, are the most important ones. Many of the projects described separately influenced each other conceptually and methodologically. I paid particular attention to those aspects that were not mentioned in our original publications but were nevertheless of utmost importance for success or failure.

### *Purification of Brain Derived Neurotrophic Factor (BDNF)*

In initial experiments, still performed in Basel, we observed that C6 glioma-conditioned medium contained survival activity detectable in dissociated

embryonic (E10–12) chick sensory neurons. It was different from NGF because it could not be blocked by an excess of affinity-purified anti-NGF antibodies.

With the move to Munich, we took advantage of the opportunity to pursue long-term (high-risk) projects without the continuous pressure to publish. Nevertheless, we put ourselves under pressure insofar as the selected approaches had to have a rational basis for success. We very soon abandoned the idea of purifying a factor from glia-conditioned medium. Assuming that the biological activity of this new factor would have properties similar to NGF, 5–10,000 liters of conditioned medium would have been necessary to purify one nanomol. At that time this was the minimum quantity necessary for the determination of a partial amino acid sequence of a protein. We reasoned that the factor produced by glial cells might also be present in brain tissue. Indeed, in brain homogenates of different species there was a comparable survival activity. The decision to use pig brains was determined by the very short interval between killing and removal of the brains and the virtually unlimited quantities that could be collected in the slaughter house within a short time. The brains were frozen at  $-70^{\circ}\text{C}$  until use. All the following steps were performed at  $4^{\circ}\text{C}$ , that is, in a refrigerated centrifuge or in a cold room. After two ammonium-sulfate precipitation steps and carboxymethyl cellulose chromatography, the final purification was accomplished by two-dimensional gel electrophoresis. The whole activity was localized in one single spot. The apparent molecular weight was about 13,000 as compared with marker molecules. After publishing the first purification procedure in 1982, we were urged to coin a name for this factor. Because we knew that the names initially given to newly detected molecules were often wrong or even misleading, we chose the most noncommittal name we could think of: *brain derived neurotrophic factor* (BDNF).

Although the quantities of BDNF isolated by the first purification procedure were very small, we nevertheless thought that they would be sufficient to determine at least a partial amino acid sequence. Unfortunately the N-terminus was blocked and the quantities of BDNF were too small to permit the production of proteolytic fragments. After this disappointing outcome, Yves-Alain Barde redesigned the whole purification procedure with great dedication and imagination. He replaced the two-dimensional electrophoresis by two hydrophobic separation steps on octyl and phenyl sepharose columns and a final C8 microbore reversed phased column. In this way sufficient quantities of BDNF became available for the production of proteolytic fragments and the determination of their amino acid sequence.

The quantities of pure BDNF also enabled us to analyze its survival effect on a broad spectrum of placode- and neural crest-derived sensory neurons, such as the neurons of the nodose, vestibular, petrosal, geniculate, and trigeminal ganglion. These experiments were partly carried out in collaboration with Alun Davies and Ron Lindsay. The effect of BDNF *in vivo* was

also evaluated by Yves-Alain and his graduate student Magdalena Hofer. For these investigations they chose quails, which are interchangeable with chicks but much smaller, so that less BDNF was necessary to perform these experiments *in vivo*. Finally Jim Johnson demonstrated that BDNF also had a survival effect on isolated rat retina ganglionic cells in culture.

At this stage efforts had already been made to immunize mice with the aim of producing monoclonal antibodies against BDNF. It became apparent that, by contrast with NGF, BDNF was a very poor antigen. The production of monoclonal antibodies against BDNF had to wait for many years until large quantities of recombinant BDNF were available and fish BDNF was cloned and produced in recombinant form.

### *Cloning of BDNF*

The new purification procedure enabled the production of proteolytic fragments and the determination of their amino acid sequences by the gas phase procedure, a method that had, in the meantime, been established by Friedrich Lottspeich. In this way about one third of the amino acid sequence of mature BDNF could be determined and compared with that of NGF. The homology between BDNF and NGF was about 50%, suggesting that they were members of a common gene family. With the availability of the partial amino acid sequence and the rapid progress made with the automatic synthesis of oligonucleotides, we thought that the cloning of BDNF could be accomplished in no time. This, however, was not the case, and difficulties that could scarcely have been foreseen arose from the different genomic and cDNA libraries used. The genomic libraries contained at least three equivalents of genomic DNA and, accordingly, at least three positive clones were to be expected. However, no positive clones were detected in any of the genomic and cDNA libraries, although we made use of all the oligonucleotide technology available at that time for the screening of libraries. On rescreening after BDNF cloning, we confirmed the negative results. Some mysterious forces seemed to be out to thwart us. Unfortunately we did not use the polymerase chain reaction (PCR) method at the earliest possible time. We had been discouraged to do so by a scientist at our institute who was reputed to be the expert in genomic analysis. He was a specialist in the forensic exploitation of single nucleotide polymorphism and declared PCR to be a useless, artifact-ridden method. After all the disappointing negative results with the screening of our libraries, we finally decided, nevertheless, to try the PCR method. Because we had good reasons to believe that the genomic organization of NGF and BDNF, as members of a common gene family, would be very similar, we concluded that it should be relatively easy to obtain PCR products coding for BDNF from genomic DNA. At that time the first contacts with Regeneron had already been initiated and Eric Shooter, a main promoter of this start-up firm (see below) had a young Polish postdoc, Piotr Masiakowski,

who was very familiar with the PCR method. With his help, including the selection of the appropriate equipment, we had the first genomic PCR product coding for BDNF in a very short time. These genomically determined sequences then formed the basis for the cloning of the full length sequence of BDNF cDNA and the identification of other members of this gene family.

I consider the cloning of BDNF to be the most important contribution of my laboratory to the field of neurotrophic factors. The credit goes to Yves-Alain Barde who succeeded in this monumental task, never being discouraged by any setbacks, however serious they were.

The cloning of BDNF represented a turning point in the field of neurotrophic factors. In fact, the field exploded, and a great number of laboratories moved into it. It promised to become a gold mine with respect to new attractive directions of research and new therapeutic perspectives.

### *First Contacts with Biotech Firms*

In the late 1980s, when the cloning of BDNF was within our grasp, we were flooded with offers from established and start-up biotech firms in the United States. In contrast, companies in Germany showed absolutely no interest. It has to be remembered that this was a time when molecular genetics was demonized. To bring a plasmid to Germany was as criminal as smuggling heroin. At best the German firms “encouraged” us with taunting remarks such as “when you have something that works orally, let us know, then we will consider the possibility of your working for us on a contract basis.”

Although I had worked in a pharmaceutical firm, I had no experience with patents and even less with commercial negotiations. I was also not willing to spend too much time on this kind of thing. We were more than happy to hand over all these tasks to Garching GmbH, the institution created by the Max Planck Society for commercially exploiting the results of basic research. Although commercial exploitation was encouraged, the interests of the scientists had absolute priority. Accordingly, we had an essential influence on the selection of the firm to cooperate with. Out of the large number of firms and individuals approaching us during this period I chose the start-up firm Regeneron. This decision was based on my longstanding friendship with Eric Shooter, in whom I had unlimited confidence and who, together with Len Schleifer, was the driving force behind this new biotech firm. The relationship with Regeneron did not develop as expected. It became clear that the main goal of Regeneron was to become a leading power in basic research on neurotrophic factors and, thus, they became our competitors. This precluded an open exchange of information and endangered the careers of our postdocs and graduate students. Moreover, it proved to be impossible to delineate in a reliable manner the projects to be followed in Tarrytown and Munich. After some disappointing experiences Yves and

I resigned from the advisory board of Regeneron, and the relationship with this firm remained almost exclusively in the hands of Garching GmbH.

### *On the Search for Neurotrophic Factors Supporting Motoneurons*

At the same time as Yves-Alain Barde was working on the cloning of BDNF and other members of the neurotrophin family, David Edgar, together with Ulrike Dormann, embarked on the purification of neurotrophic factors present in embryonic skeletal muscle extract, which were thought to regulate the survival of motoneurons during embryonic development. In contrast to the well-established culture systems for autonomic and sensory neurons, no corresponding method for motoneurons was available. Although survival effects on motoneurons in mixed cultures of spinal neurons had already been reported, it could not be decided whether the survival effect resulted from a direct action on motoneurons or an indirect one via other spinal neurons or nonneuronal cells. David and Ulrike developed a culture system for chick spinal motoneurons. It was based on the following main steps: dissection of the ventral lumbar and brachial spinal cord, digestion by trypsin, trituration, filtration, and a final metrizamide gradient step. The motoneurons, retrogradely labeled by previously injected rhodamine-isothiocyanate, were localized in one fraction of uniformly large neurons. The time when this motoneuron purification could be carried out proved to be very limited, that is, restricted to E-6. Before this stage the differentiation was not sufficiently advanced and later on the isolation of motoneurons became more difficult, necessitating higher trypsin concentrations and more vigorous trituration. This unavoidably damaged the motoneurons. In an initial paper it was demonstrated that extracts from chick embryonic muscle had a survival effect, present in the 25% to 70% ammonium sulfate fractions. The work on the purification of this survival activity was not continued, since Ulrike Dormann left our laboratory for personal reasons to move to London.

This original preparation of motoneurons was then substantially improved by Yoshi Arakawa, a visiting scientist from the Japanese drug company Esay. He wanted to purify a new factor he suspected in muscle and skin. He thought that he could immediately start with the purification. However, Michael Sendtner and I convinced him that the prerequisite for a successful purification was a reliable, optimal assay and that it was clear that our assay had not yet reached this level. He accepted this with some reluctance but was then very successful in improving the motoneuron assay. He did not change the original concept but made many modifications, such as reducing the dissection time, reducing the trypsin concentration, and using a simpler metrizamide gradient. With this improved purification procedure for motoneurons, Yoshi Arakawa analyzed the survival effect of neurotrophic factors that were already available. To our great surprise, none of the already purified and cloned neurotrophins (NGF, BDNF, and NT-3) had

a survival effect on this preparation of motoneurons. The same was true for a great variety of mitogens and cytokines and various interleukines. However, ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor had a very strong survival effect. They both kept motoneurons surviving for more than a week, and their effects were cumulative, in combination virtually 100% (see Arakawa et al., 1990).

The results obtained with the improved preparation of chick motoneurons were nevertheless in many respects misleading because they only related to a small time span. This became apparent as soon as Michael Sendtner and Tony Hughes introduced the cultivation of rat motoneurons based on the binding of motoneurons to solid phase anti-p75<sup>NTR</sup> antibodies, a system developed by Chris Henderson in Marseille. In this culture system more than 50% of motoneurons survived at low concentrations of BDNF. NT-3 and NT-4/5 also showed substantial survival activity. The survival effect of IGF-1 was also much stronger because we used a serum-free medium, avoiding the high concentrations of IGF-1-binding proteins that are present in the horse serum used for the cultivation of chick motoneurons. The neurotrophic support of motoneurons by a great variety of molecules from different gene families suggested multifactorial support. The prevalence of the supportive function of these factors changed during the various developmental stages. It might also be possible to exploit this multifactorial support for therapeutic purposes to reduce the possible side effects of individual trophic factors. At least in vitro, the combination of border-line effective doses of individual molecules resulted in a virtually 100% survival. The potential exploitation of this concept for therapeutic purposes has been extensively discussed by Michael Sendtner and myself in a review of *Nature Neuroscience* (see Thoenen and Sendtner, 2002).

## Purification and Cloning of Ciliary Neurotrophic Factor (CNTF)

The purification, cloning, and evaluation of the physiological functions of CNTF are closely connected with the name of Michael Sendtner. Michael joined my laboratory with the goal to be trained for 2 to 3 years in contemporary methods of cellular and molecular neurobiology to pursue experimental clinical research in the Department of Neurology of the Technical University of Munich.

When Michael joined my laboratory he had virtually no experimental experience. However, from the very beginning it was apparent that he had an exceptional talent for experimental research. He adopted very soon all the necessary methods and designed his experiments like a scientist with many years of experimental experience. In his very first experiments, Michael resolved a controversial question as to whether the initial step of NGF-mediated signal transduction was the activation of Na<sup>+</sup>K<sup>+</sup>-ATPase, as

reported by the laboratory of Silvio Varon. Michael demonstrated that this was the case only when chick sensory neurons used for this assay were severely damaged and consistently died within 24 hours. The activation of  $\text{Na}^+\text{K}^+$ -adenosine tri-phosphate (ATP)ase was not observed in more carefully treated neurons, which survived in the presence of NGF. This conclusion was further supported when no  $\text{Na}^+\text{K}^+$ -ATPase activation was initiated by NGF in chick sympathetic neurons and calf adrenal medullary cells.

Michael Sendtner's dedication to neurobiological research was coupled with many other talents worthy of a "Renaissance man." At high school he also studied music and obtained a diploma in classical lute, winning prestigious prizes at an early age opening up a promising career in music. Occasionally he was unsure whether music or neurobiology should have priority. Finally it was possible to convince him—my wife played an essential part—that he could continue music as a hobby while pursuing a scientific career, but the reverse was simply not possible. The initially planned 2 years became around 10 years, and he then moved to the University of Würzburg where he became head of an independent clinical research group. He made essential contributions to the elucidation of the pathogenetic mechanisms of amyotrophic lateral sclerosis (ALS) and corresponding animal models and signal transduction mechanisms essential for the survival effects of neurotrophic factors. He also contributed crucially to the molecular understanding of the pathogenetic mechanisms of spinal muscle atrophy.

After the departure of Ulrike Dormann, Michael Sendtner took over the identification and purification of motoneuron survival factors. His approach was much broader in that he did not confine his analysis to embryonic muscle extract but also included other tissues. In this way he found a strong survival activity in extracts of chick eyes, where the Varon group had identified a potent survival factor for parasympathetic neurons of chick ciliary ganglia. A similar activity, in particularly high concentrations, had been identified in the rat sciatic nerve and partially purified by G. M. Barbin, a French postdoc in the Varon lab. In addition to the survival effect on chick ciliary neurons, they had also demonstrated a survival effect on sensory and sympathetic neurons. However, they did not include the analysis of motoneurons. Although we assumed that the Varon laboratory would work intensively on the further purification and cloning of CNTF, we decided to do this ourselves to remain independent in every respect in the future.

Michael Sendtner managed to purify CNTF to homogeneity in a very short time. He modified the preparative gel electrophoresis procedure described by Barbin and used additional chromatographic steps, in particular hydrophobic columns. The rapidity of purification took also advantage of the experience of Yves-Alain Barde to purify BDNF. The partial amino acid sequence of CNTF was determined from fragments obtained by cyanogen bromide cleavage and tryptic digestion using the gas phase microsequencing procedure. The nucleotide sequence of full-length cDNA was determined by PCR from messenger ribonucleic acid (mRNA) isolated from cultures of rat

astrocytes shown to produce substantial quantities of CNTF. At the same time Patrick Carroll identified a genomic clone of CNTF that confirmed the cDNA sequence.

Contrary to our expectations, the Varon lab had not continued with the purification and cloning of CNTF. However, a biotech firm, Synergen, cloned CNTF from rabbit sciatic nerve. The two papers were published almost simultaneously in *Science* and *Nature* in 1989.

Our further investigations demonstrated that CNTF was certainly not involved in the regulation of motoneuron survival during embryonic development. The expression of CNTF (mRNA and protein) was not detectable during embryonic development, neither in the periphery nor in the central nervous system. By contrast, the CNTF receptor cloned by George Yankopoulos was already expressed during embryonic development, indicating that CNTF was not the only activating ligand of this receptor, and a series of other ligands have in fact been identified, which belong to other gene families.

In motoneurons, the embryonic period of target-dependent regulation of neuronal survival is followed by a period of increased sensitivity to axonal injury. This sensitivity decreases with increasing postnatal age and, remarkably, is inversely related to the increase in the levels of CNTF in the axon-sheathing Schwann cells. This led us to speculate that CNTF, as a nonsecretory molecule, might act as a "lesion factor." In fact, after transection of the facial nerve in newborn animals it was possible to prevent the degeneration of motoneurons in the facial nucleus by local administration of CNTF. The function of CNTF as a lesion factor was also compatible with the subcellular localization in the sciatic nerve in adult animals after nerve lesion. In intact sciatic nerves CNTF is equally distributed in the cytoplasm of the ensheathing Schwann cells. However, after lesion, besides the downregulation of the CNTF mRNA distal to the lesion site, CNTF protein was localized with patchy distribution in the extracellular space. The reappearance of CNTF mRNA and protein in Schwann cells distal to the lesion site occurs parallel to the outgrowth of regenerating axons. After the production of CNTF KO-mice (see below), additional evidence for the function of CNTF as a lesion factor was provided in the mouse mutant pmn (progressive motoneuronopathy). In this autosomal recessive mutant, the first symptoms of paralysis occur in the hind legs by postnatal week 3 and then progress rapidly to the anterior parts of the body. Between week 7 and 8 all the animals die. By the 6th postnatal week about 40% of the motoneurons of the facial nucleus have degenerated. However, when the facial nerve is transected at the 4th postnatal week there is a dramatic reduction of the degenerating neurons. This rescue effect is absent when pmn mice are crossed with CNTF KO mice.

### *Production of CNTF KO Mice*

The gene targeting method, which had just become available, seemed to be just what we needed to help us understand the physiological functions of our

newly detected neurotrophic molecules. Because it proved to be impossible to obtain embryonic stem (ES) cell lines from the very few laboratories that had already reported on the production of KO animals, I decided to go the autodidactic way. This was possible because Rolf Kemmler provided us with an ES line (D3) he had developed for other purposes, that is, not for gene targeting experiments. As absolute beginners in this field we had to focus all our efforts. The physiological function of CNTF was less predictable than that of all the other neurotrophic molecules we had cloned. CNTF was not a secretory molecule and, additionally, had a rather mysterious pattern of expression, as described above.

Together with a technician I established the cultivation of the D3 cells and their transduction by electroporation with the target constructs that were produced by a very gifted Japanese molecular biologist, Yasuo Masu. The positive clones were handed over to Eckhard Wolf, a Ph.D. student in the laboratory of Gottfried Brehm. Eckhard had already extensive experience with the production of conventional transgenic mice through pronucleus injection. The injection of ES cells into blastocysts was thus rapidly implemented. Very soon we had the first chimeras, and the germ line transfer also worked reasonably well. The homozygous CNTF KO mice were fertile and did not show any behavioral peculiarities. We nevertheless decided to subject their motoneurons to a more detailed analysis, particularly in view of the expression of CNTF postnatally and in adulthood suggesting an involvement in maintenance functions. In spinal and facial motoneurons there was in fact a small but statistically significant reduction in the number of cell bodies. The residual neurons showed differing signs of atrophy and degeneration, and there were reactive astrocytes and an augmented number of microglial cells in their vicinity. These morphological changes were reflected by a slight but statistically significant reduction in muscle strength.

Soon after the publication of the consequences of the CNTF gene targeting in mice, Takahashi and coworkers reported that a relatively high (2%) proportion of the Japanese population has a homozygous CNTF mutation leading to a complete inactivation of the biological activity of CNTF. Interestingly, Takahashi and coworkers did not observe any neurological defects in this population. However, meanwhile a similar proportion of total CNTF defects was detected in other ethnic groups and there, the absence of CNTF activity resulted in a much earlier beginning and more rapid progress of a specific form of a familial ALS resulting from a specific mutation in copper/zinc superoxide dismutase 1 (SOD-1). This form of familial ALS results from a (toxic) gain of function rather than a reduced activity of superoxide radical scavenging activity. In a mouse model of this SOD-1 mutation the ALS manifestations were much more severe and occurred at an earlier age when the ALS mice were crossed with CNTF KO mice. Moreover, also in patients suffering from multiple sclerosis there is evidence for a modifier function

of CNTF. Again, also here the manifestations of experimental multiple sclerosis in mice were more serious on a CNTF KO background.

All these observations that were reported in the last few years are in line with the evidence that the survival and the maintenance of specific functions of motoneurons is determined by a variety of different molecules. The question arose as to whether the absence of CNTF might not make motoneurons more sensitive to the absence of other trophic factors or the action of toxic molecules, which alone would not have any clearly detectable effects. Because leukemia inhibitory factor (LIF) is up-regulated in the peripheral nerves after axotomy and LIF also has a survival effect on motoneurons *in vitro*, we analyzed in collaboration with Philip Brûlet in Paris the consequences of the production of CNTF/LIF double KO mice. By contrast with CNTF KO mice, no morphological changes in motoneurons could be detected in LIF KO mice up to an age of 12 months. However, in CNTF/LIF double-KO mice the signs of motoneuron degeneration occurred much earlier and were more extensive than in CNTF KO mice alone. Correspondingly, there was also a much stronger reduction in muscle strength at an earlier age than in CNTF KO mice.

#### *Development of a More Sensitive Two-Site Enzyme Immuno-Assay for NGF*

Just before the move to Munich, Yves-Alain Barde and Kitaru Suda had identified the artifacts inherent in the NGF competition assay. However, the reliable two-site assay they developed using  $J^{125}$ -labeled affinity-purified polyclonal anti-NGF antibodies was not sufficiently sensitive to determine NGF levels in sympathetically innervated tissues and sympathetic ganglia. Yves was fully taken up with the purification of BDNF. Greg Harper, who moved with us to Munich, was still involved in NGF research (purification and cloning of bovine NGF) but could not be convinced that it would be possible to develop an assay more sensitive than the classical neurite outgrowth bioassay introduced by Rita Levi-Montalcini in the early 1950s. Sigrun Korshing, a graduate student with a good background in chemistry, then agreed to take over this demanding project. In the initial experiments the first antibody did not bind strongly enough to the polyethylene assay tubes to permit the necessary thorough washing with detergents, the prerequisite for reducing the background and increasing the sensitivity. Specially treated assay plates, binding the first antibody in a "pseudo-covalent manner," were not yet available. Sigrun decided to covalently bind the first antibody to uniform, small (1 mm diameter) glass beads. After replacing the  $J^{125}$  labeling of the second (detector) antibody by coupling with  $\beta$ -galactosidase, it was possible to increase the sensitivity of the assay 500-fold.

The two-site immuno-assay developed in Basel was based on the antibodies from a sheep with a particularly high anti-NGF titer. However, it

proved to be impossible to bring this animal from “dirty” Switzerland (not belonging to the European Union) to “clean” Germany. To overcome these difficulties I seriously considered the possibility of bringing the sheep across an unmanned part of the border from Switzerland to “clean” France and from there without any problems to “clean” Germany. I knew the open borders in the surroundings of Basel quite well, but I had mixed feelings about the possibility of being arrested as a newly appointed Max Planck director and bringing not only myself but also the Max Planck Society into trouble. We therefore decided to boost the sheep for a last time and to collect several liters of serum, a stock that lasted for a long time. However, we had decided from the very beginning that we would also produce monoclonal antibodies against mouse NGF. This method was still in its infancy after Milstein and Köhler had published the principles of this revolutionary method in the late 1970s. The available myeloma cell lines were not yet optimal (“partial producers”). After some initial difficulties we accomplished several successful fusions and obtained many positive clones. For the enzyme immuno-assay we selected a clone exclusively on the basis of its high affinity to NGF. It was sheer luck that it did not cross-react with any other neurotrophin. Amazingly, this clone (27/21) is still in use and is up to date in commercial kits offered for the immunological determination of NGF.

The sensitivity of this assay enabled NGF levels to be determined in target tissues of sympathetic neurons with not only dense but also sparse innervation. In general there was a positive correlation between the density of sympathetic innervation and NGF levels. For instance, the levels were high in the densely innervated iris and the heart atria but low in the more sparsely innervated heart ventricles. By far the highest concentrations of NGF were found in sympathetic ganglia (determined in superior cervical and stellate ganglia) that do not produce NGF themselves but accumulate it through retrograde axonal transport. This assay also provided the possibility to directly demonstrate the retrograde axonal transport of endogenous NGF. After crushing the sciatic nerve we observed a very rapid 10 to 15-fold increase in NGF distally to the location of the crush. Proximally to the crush site the NGF levels were reduced, very soon reaching the lower detection limit. It was important to restrict the duration of the analysis to a short time period, that is, fewer than 10 hours after the nerve was crushed to avoid interference with the local synthesis of NGF after nerve lesion (see below). In complementary experiments we investigated the consequences of the interference with the retrograde axonal transport of NGF by destroying the adrenergic nerve terminals with 6-HODA or blockade by colchicine. Within 12 to 15 hours the NGF levels in sympathetically innervated tissues increased two- to fourfold whereas the levels in sympathetic ganglia (superior cervical and stellate ganglion) decayed with a half-time of 4 to 5 hours to reach minimal levels of 4% to 5%. The increase in NGF levels in sympathetically innervated tissues after blockade of the retrograde axonal transport with

6-HODA is also determined by the NGF uptake by NGF-responsive sensory neurons that are not destroyed by 6-HODA. Under normal physiological conditions, these two populations of neurons compete for NGF. Accordingly, after administration of 6-HODA the levels of NGF increased in the corresponding sensory ganglia and the levels of their NGF-regulated neuronal peptides, such as substance P, increased after administration of 6-HODA.

### *Quantification and Cellular Localization of NGF mRNA*

After having exploited the new sensitive NGF enzyme immuno-assay to resolve many open questions, we felt that it was essential to complement these results with a reliable method for NGF mRNA quantification. In the early 1980s it was virtually impossible to convince molecular geneticists to work on a neurobiological question. They were afraid of ruining their reputation by becoming involved in such "dirty systems." The only way out was to acquire the necessary skills ourselves and to adapt the available methods to the requirements of neurobiology. In my lab Rolf Heumann took over this task. After joining my research group, Rolf had already made an important contribution by unambiguously demonstrating that the regulatory effects of NGF at the transcriptional level were mediated by second messenger mechanism(s) rather than by a direct transfer of NGF to the nucleus. This possibility was hotly disputed at the time because of the artifactual redistribution of J<sup>125</sup> NGF after producing cell fractions of PC12 cells that had taken up J<sup>125</sup> NGF.

First Rolf became acquainted with the still very laborious process of synthesizing oligonucleotides, which were necessary for the production of adequate probes to quantify NGF mRNA in Northern blots. He developed a very sensitive assay many years before the arrival of PCR that permitted the quantification of mRNA in small tissue samples. As was found when determining the NGF protein levels, the NGF mRNA was highest in those peripheral tissues with the densest sympathetic innervation. By contrast, in sympathetic ganglia, which had by far the highest NGF protein levels, NGF mRNA was at best at the detection limit. The sensitive assays for NGF protein and NGF mRNA also enabled us to expand our analysis to the central nervous system. This was of particular interest in the context of the support of cholinergic neurons by NGF and the relatively high levels of NGF mRNA in the projection fields of cholinergic neurons of the basal forebrain nuclei (see below).

The next logical step for a more refined analysis was the development of an in situ hybridization procedure that enabled us to determine the cellular localization of the really very rare NGF mRNA. Christine Bandtlow, a graduate student, established a procedure using S<sup>35</sup> RNA probes. With the rapid progress in the automatic synthesis of oligonucleotide probes, she complemented her results with S<sup>35</sup> labeled oligonucleotides. In densely innervated sympathetic tissues, the NGF mRNA was not only localized in smooth muscle

and fibroblast cells, but at higher relative density in epithelial cells. In the iris, for instance, the densest labeling was in the cuboidal epithelial layer on the posterior side of the iris. In the skin, particularly in the whisker pad, the surface epithelium, and the epithelium of the hair follicles were much more densely labeled than the underlying stromal cells. These astonishing results were confirmed by Northern Blot hybridization in separated samples of epithelium and underlying stromal cells. Interestingly, in the whisker pad the rapid increase in NGF mRNA in the epithelium correlated with the in-growing sensory axons, suggesting the possibility of a causal relationship, that is, that the in-growing axons initiate the synthesis of NGF. However, this interpretation, at least as a general concept, was challenged by observations made in chick embryos. When the neural tube was removed at a very early developmental stage, a chick embryo was produced that had no innervation of the skin. Nevertheless, NGF mRNA developed in the skin independently of any innervation.

With these tools in hand we were also able to analyze in greater detail the regulatory mechanisms of NGF synthesis coming into play after nerve lesion. These experiments were predominantly performed in the rat sciatic nerve. In newborn animals intermediate levels of NGF mRNA are expressed by Schwann cells and cells of the epineurium. In adult rats virtually no NGF mRNA was detectable. However, after nerve lesion there was a very rapid increase in NGF mRNA, which was of short duration, followed by a slower more protracted increase. These changes were only visible in the segments distal to the lesion. Proximally these changes were restricted to the domain of the lesion site. Interestingly, if segments of the sciatic nerve were cultivated, the time course of the NGF mRNA changes differed distinctly. We observed only the initial rapid increase, whereas the more protracted changes were not detectable. We reasoned that the difference between organ culture and in vivo experiments might be due to the absence of immigrating macrophages in organ cultures. Indeed, we then added activated macrophages to the organ cultures and the in vivo situation could virtually completely be restored. At this stage of the experiments, Dan Lindholm, who had a background in rheumatology, joined my laboratory. Based on his knowledge we investigated which molecules, produced by activated macrophages, were responsible for the protracted increase in NGF mRNA. Tumor necrosis factor and platelet derive growth factor produced a very modest increase in NGF mRNA. However, interleukin-1 $\beta$  resulted in a dramatic 15-fold increase in NGF mRNA. Similar effects could also be achieved by medium conditioned by activated macrophages that could be blocked by anti-interleukin-1 $\beta$  antibodies. It is instructive to recall that at that time only antibodies against human interleukin-1 $\beta$  were available, which did not cross-react with rat interleukin. We thus had to use a "mixed system," namely organ cultures of rat sciatic nerves and human-activated macrophages obtained from patients undergoing peritoneal dialysis.

*A Memorable Evening with the Father of NGF*

I had already worked in the field of NGF for several years before I met Viktor Hamburger personally. It was in the very early 1980s when I gave a seminar in St. Louis, Missouri. After the seminar Viktor took me out for dinner and immediately told me that he had deliberately not invited anybody else. The reason was that he wanted to speak German. I was baffled, because I knew his sad story. I would have expected him to avoid speaking German as far as possible, and there would have been more than reasons enough. In the course of our conversation it became clear that he had very strong emotional links to his home country, Germany. This did not make him any less grateful to the United States for providing him shelter after he was expelled from Germany and giving him the opportunity to pursue a successful scientific career. We shared memories of the Black Forest, in particular the Feldberg and the Notschrei, places that Viktor had visited in all seasons, including wintertime with old-fashioned skiing equipment. I had enjoyed the same places decades later, skiing in the winter on well-prepared cross-country tracks. His glowing eyes showed how much he loved this country. Later on I gradually discovered that he originated from a family with strong national feelings, German patriots, serving in the German army during World War I. To be expelled from your home country by foreign intruders is a bitter and damaging experience, but to be chased away like a scabby dog by the countrymen you identify yourself with is a tragedy of unimaginable proportions. In the following years I met Viktor more frequently, including on the occasion when I gave the Viktor Hamburger Lecture in St. Louis. I became aware of very personal predilections, for example, that it was a mistake to bring him *Himbeergeist* (raspberry brandy) as a souvenir from the Black Forest, as he definitely preferred *Zwetschgenwasser* (plum brandy). The memories of my contacts with Viktor Hamburger would be incomplete if I did not mention his sad exclusion from the Nobel Prize awarded to Rita Levi-Montalcini and Stanley Cohen. Viktor was bitter and depressed not so much for not being awarded the Nobel Prize but on account of unnecessary, offending remarks by one of the laureates.

*Purification of Choline Acetyltransferase (ChAT);  
Production of Poly- and Monoclonal Antibodies*

In our initial investigations on the possible functions of NGF in the CNS we injected NGF or anti-NGF antibodies into the immediate vicinity of the substantia nigra and the locus coeruleus. Against our expectation this did not result in any changes of TH activity. However, after injection of J<sup>125</sup> NGF into the projection field of the locus coeruleus, we observed a retrograde labeling of neurons in the basal forebrain that we suspected to be cholinergic. Moreover, the injection of NGF into the lateral cerebral ventricles resulted

in a marked increase in choline acetyltransferase (ChAT) activity in the basal forebrain. The precise identification of the sites of ChAT synthesis required the production of specific antibodies. This was accomplished by a graduate student, Felix Eckenstein, under the guidance of Yves-Alain Barde. As for the purification of BDNF we used pig brain also for the purification of ChAT. The ChAT activity was not particularly high, but it was compensated by the large quantities of pig brain we could obtain immediately after the animals were killed. The purification procedure included several precipitation steps, adsorption to hydrophobic columns, and a final HPLC column step that resulted in a more than 1 million-fold purification. After SDS gel electrophoresis all the ChAT activity was localized in a single band with an apparent molecular weight of 68,000. The production of monoclonal antibodies proved to be difficult and laborious. Our efforts resulted in one single useful positive clone that was not suitable for immunohistochemistry. However, it could be used for the affinity purification of ChAT from other species and to produce polyclonal antisera that made the immunohistochemical localization of ChAT possible.

The availability of reliable anti-ChAT antibodies had been awaited by the scientific community for a long time. In an initial set of experiments, partially in collaboration with Mike Sofroniew and Claudio Cuello (Oxford University, U.K.) we identified a series of cholinergic neurons in the forebrain, in particular in the nucleus tractus diagonalis (Broca), medial septum, medial forebrain bundle, caudate-putamen, and portions of the globus pallidus. Interestingly, in layer II–VI of the entire cerebral cortex there were predominantly bipolar spindle-shaped ChAT positive cells. The cell bodies and proximal dendrites could be visualized. However, the quality of the antibodies and our immunohistochemical techniques were not good enough to visualize the distal dendritic arborization and, above all, the axonal projections from basal forebrain cholinergic neurons to the hippocampus and the cerebral cortex. Their presence could be deduced from retrograde tracing and axonal transection experiments. The latter led to a drastic reduction of ChAT in the projection fields. This was particularly illuminating for “undercut” experiments of the visual cortex which led to a strong reduction, but not to a complete disappearance of ChAT, providing the counterpart to the immunohistochemical localization of ChAT in the spindle-shaped interneurons of the cerebral cortex. Some basic important questions could be resolved in this way, but a lot of other questions were still waiting to be elucidated, particularly in the context of the pharmacological manipulation of central and peripheral cholinergic neurons. The various ChAT antibodies were in great demand, and Felix Eckenstein became a much-courted postdoc.

For us the anti-ChAT antibodies remained a useful tool for future experiments in which, in addition to NGF, we analyzed the response of ChAT levels to other neurotrophic molecules. The purification procedure for ChAT was also the basis for the determination of its partial amino acid sequence.

This information was then handed over to the laboratory of Jacques Mallet in Paris who cloned ChAT and determined its genomic organization. My own lab could not pursue this project to this level. We had to set priorities and had more than enough to do elucidating the physiological functions of the members of the neurotrophin family and CNTF, including the production of corresponding KO animals.

#### *Retrograde Trans-Synaptic Effects of NGF on Preganglionic Cholinergic Neurons*

In previous experiments performed in Basel, we observed that treatment of newborn rats with NGF resulted in an increase in ChAT activity in the superior cervical ganglion. This increase did not result from a direct effect of NGF on the preganglionic cholinergic neurons. Neither J<sup>125</sup>-NGF nor NGF-HRP injected into the projection field of postganglionic adrenergic neurons was transferred to the preganglionic cholinergic nerve terminals. As a positive control we injected labeled tetanus toxin that was, as expected, transferred trans-synaptically.

Theo Schäfer, under the guidance of Martin Schwab, determined the morphometric changes occurring in the superior cervical ganglion of the rat and the corresponding preganglionic cervical trunk. Under physiological conditions the number of preganglionic cholinergic axons drops from 13,000 at birth to 7,000 at postnatal day 10, reflecting the physiological neuronal cell death in the superior cervical ganglion during this time period. NGF treatment did not only prevent this loss but even increased the number of axons to over 30,000 after 10 days of treatment with NGF. The retrograde labeling of the preganglionic cholinergic neurons in the spinal cord showed a similar distribution in controls and NGF-treated animals, that is, from C6 to T6. However, the number of neurons was 1.5 times higher in the NGF-treated animals, providing evidence for an augmented survival of the preganglionic cholinergic neurons.

#### *Projects Not Discussed in Detail*

At an early stage after our move to Munich, Motoharu Hayashi, under the guidance of David Edgar, investigated the rate of development of substance P, somatostatin, and vascular intestinal protein. They produced specific antisera against fragments of these peptides in rabbits, developed immunoassays, and then determined the rate of development of these peptides in the paravertebral sympathetic ganglia and spinal sensory neurons of chick embryos. These peptide changes occurred independently of each other. Similar, rather complex changes in the expression of these peptides occurred when large quantities of NGF were administered daily to the allantois of chick embryos.

In another set of experiments we compared the changes in NGF and p75<sup>NTR</sup> mRNA in the sciatic nerve during development and after lesion. These experiments were performed in cooperation with the laboratory of Eric Shooter.

In cooperation with Ruppert Timpl, David Edgar and I identified the heparin-binding domain of laminin as responsible for the potentiating effect of laminin on NGF-mediated neurite outgrowth and the potentiation of the survival effect of NGF on chick sympathetic neurons.

Although the “Campenot multi-chamber system” proved to be disappointing for the detailed analysis of the kinetics of the retrograde axonal transport, it led to initial experiments demonstrating that central myelin, produced by oligodendrocytes, inhibits the regeneration of axons after their lesion. In experiments subsequently carried out at the Brain Research Institute in Zürich, Martin Schwab purified one of the most essential inhibitory molecules, Nogo, and demonstrated in highly sophisticated experiments that monoclonal antibodies directed against specific domains of Nogo could at least partially restore the regeneration of transected axons in the spinal cord.

### *Compulsory Retirement Determines Research Strategies*

In the Max Planck Society the compulsory retirement of the directors at the age of 68 is an iron rule. It is expected that all the staff positions of scientists are available for a prospective successor. This policy ensures a high degree of flexibility in the selection of new, innovative directions of research.

For many years my research strategies were determined by my approaching retirement. On the one hand I wanted to finish as much as possible ongoing research projects requiring expertise in advanced neuroanatomy and immunohistochemistry in the context of the analysis of already produced KO mice. Postdocs fulfilling these qualifications expected to become acquainted with contemporary methods of molecular genetics, such as the overexpression of genes under the control of a tissue- or cell-specific promoter, or to learn all the necessary techniques to produce KO mice. In this way a relatively broad spectrum of projects was initiated, thought to provide the basis for future independent research groups outside our institute after my retirement. In this way we made the first interesting observations that, contrary to all expectations, calbindin-28 KO mice did not show increased sensitivity to the excitotoxic effects of glutamate. However, in view of the high concentrations of calbindin-28 in Purkinje cells the involvement of these neurons was thought to be responsible for the slight impairments of equilibrium and coordination. Indeed, later on Michael Meyer, Jaroslaw Barski, and Matti Airaksinen demonstrated by the selective targeting of the calbindin-28 gene in Purkinje cells that this was in fact the case.

In my own research I concentrated more and more on the modulatory role of neurotrophins, in particular BDNF, in activity-dependent neuronal

plasticity. By contrast with the very generous support for an active Max Planck director, the level of support was very modest after retirement, and the topics to be pursued had to be selected very carefully, and the work, unavoidably, proceeded at a reduced pace.

### *Modulatory Role of BDNF in Activity-Dependent Neuronal Plasticity*

Activity-dependent regulation of BDNF synthesis in the CNS was shown to occur very rapidly, that is, within minutes, suggesting its regulation as an immediate early gene. Under physiological conditions, BDNF in the CNS is exclusively expressed in neurons. The pattern of expression changed depending on the stimulation parameters used. The regulatory mechanisms also included physiological stimuli such as light input that elicited characteristic changes in BDNF expression in the neurons of the visual cortex. These observations led me to propose at an early stage of BDNF research that BDNF might be involved in activity-dependent neuronal plasticity. This hypothesis was strongly supported when Martin Korte demonstrated that in acute hippocampal slices of BDNF KO mice, long-term potentiation in the CA-3/CA-1 system was strongly reduced. Interestingly, this reduction was the same in homo- and heterozygous BDNF KO mice, demonstrating that this modulatory role of BDNF depends on a minimal critical level of BDNF. Very soon the relationship between BDNF and activity-dependent neuronal plasticity attracted the interest of many other laboratories, and it is impossible to give appropriate credit to all the contributions that were made in rapid sequence.

In view of my approaching retirement, with a drastic reduction of my experimental possibilities, I had to concentrate on specific details, such as the mechanism and site of secretion of neurotrophins at the light and EM level. Although fragmentary, these investigations led to surprising results: contrary to our expectations, the activity-dependent secretion of neurotrophins (initial experiments performed with NGF because suitable antibodies against BDNF were not yet available) did not depend on extracellular  $Ca^{++}$  but exclusively on intact intracellular  $Ca^{++}$  stores and the  $Ca^{++}$  released therefrom. The levels of endogenous neurotrophins were so low that for the majority of the experiments a chemical- or virus-mediated transduction was necessary. The necessity for overexpression led us to question whether the results obtained were representative of the physiological situation *in vivo*. Not unexpectedly, given the different methods used, controversial results were reported and many questions are still unresolved, such as the site of the synthesis of BDNF; it is not known whether its synthesis is confined to the perikaryon or whether BDNF mRNA is selectively transported to distal parts of the neurons, in particular dendrites. Controversial observations were also reported on the nature of the compartment in which BDNF protein is transported and secreted from the different parts of the neuron. More recently the question arose as to whether neurotrophins, in particular NGF and BDNF,

are secreted as precursors or mature proteins. This aspect became even more important when it was demonstrated that the precursor molecules were bound with higher affinity to p75<sup>NTR</sup> receptors and enhanced the evolving cascade of signal transduction.

Although the analysis of all the detailed functions of neurotrophins is justified in its own right, I feel that the increasing evidence for a modulatory role of BDNF in activity-dependent neuronal plasticity, long-term potentiation and long-term depression, opens up more general conceptual aspects. It is accepted that our memory is based on activity-dependent changes in synaptic strength. The mechanisms brought into play by the Hebbian activation pattern provide only limited possibilities for variability. The intensity of the activation of these rather ubiquitous mechanisms such as the activation of NMDA receptors, activation of CaM kinase II and IV, and calcium activated cAMP leaves little room for locally restricted modulations. If we put this limited armamentarium in context with the numberless engrams that are stored in the human brain (several languages, faces, names, broad spectrum of general knowledge, and the ever-increasing flood of new scientific data), it is comforting to know that mechanisms exist that can modulate these basic mechanisms with local restriction and variability. Neurotrophins, in particular BDNF, fulfill the requirements for such a function. I would like to emphasize that I do not consider this function to be unique. However, in spite of the numerous unresolved questions, knowledge on BDNF is relatively far advanced by comparison with that of other potential modulatory molecules. It is conceivable that many other molecules, including numerous neuropeptides, have similar functions. A direct link between the function of BDNF and memory has recently been reported. In the United States a regional cohort of predominantly European origin showed a relatively high percentage (2%) of a single nucleotide polymorphism that led to an exchange of the amino acid valine by methionine. This exchange was accompanied by subtle deficits in declarative memory and was also reflected in distinct changes in functional magnetic resonance imaging (fMRI). In animal experiments the same mutation in the precursor domain of BDNF led to changes in its sorting and a reduction of the activity-dependent secretion of BDNF. Moreover, there is increasing evidence for a possible relationship between the expression of BDNF and the therapeutic effect of antidepressants. When antidepressants were analyzed in animal models, there was a close correlation between the appearance of the antidepressive actions and the increase of BDNF levels in different regions of the CNS, in particular in the hippocampus and the amygdala. These antidepressants were introduced on account of their blocking action on the uptake of biogenic amines. Because this uptake blockade is an instantaneous effect, one would also expect a short-term therapeutic response. However, the therapeutic effect does not become apparent until several weeks after beginning of the administration of the antidepressant.

Although I am no longer actively contributing to the field of neurotrophic factors, it gives me great pleasure and satisfaction to see how this field developed in so many unexpected directions. When we embarked on these challenging, high-risk projects our goals were much more modest, namely to find neurotrophic factors for populations of neurons for which molecules of trophic support had not yet been identified.

## Conclusion

When I was invited to write an autobiographical contribution I was very hesitant about doing so. Now, having come to the end I ask myself whether I have written it in an appropriate form. The simple description of valuable contributions made by my laboratory did not seem to be particularly interesting as such. Today the findings that were of high current interest at the time of their detection are considered self-evident, and at best they became textbook knowledge. As far as possible I have included aspects that have not been published in our original papers. They include fortunate combinations of circumstances but also the pitfalls we encountered, including barely explainable bad luck as for example, in the case of the screening of genomic libraries, when no clone coding for BDNF was present. I have also tried to shed light on the experimental situation—its possibilities and limitations—from a contemporary point of view. The biological problems were not infrequently clearly identified and the correct questions were asked. However, the experimental tools necessary to obtain a direct answer were simply not available. In this situation I felt it would be instructive to describe the approaches taken to obtain at least a partial answer.

Failures are just as much part of the everyday scientific life as the few moments of really exciting new insights. In between there are the long periods of hard work necessary to complete the many details of a project and to carry out all the necessary controls.

Sharing moments of success and overcoming difficulties with friends and motivated colleagues, based on absolute confidence, is one of the most positive aspects of doing research. However, it would be dishonest to exclude frictions and even painful personal experiences. They are part of our scientific life, even if our memory has the features to eliminate them and to present, retrospectively, a picture that is too rosy and does not correspond to the realities.

When I look back and try to evaluate what my coworkers and I contributed to science and what science meant to us, I realize that it is important to remember that the wheel is reinvented again and again. Although we contributed to the improvement of wheels or in rare cases even invented an initial primitive wheel ourselves, we cannot expect that these contributions will be acknowledged forever. Further progress is based on the very latest successful steps. What we think are earthshaking new insights now will

become very soon textbook knowledge. I am happy that I had the opportunity to participate in a field of research that developed in such an extraordinary manner. Although for several years I have now been moving from the function of an active player to that of an interested spectator, I am still anxious to grasp as much as possible of the exciting new developments. However, at the same time I am also conscious that even the most important pillars of our science will disappear together with our planet. What remains is my gratitude that I have lived in a period of evolution brought about by a fortunate combination of numerous variables that made such interesting events possible including our own existence and that I was privileged to become a scientist.

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