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Shigetada Nakanishi

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Visiting Associate, Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health (1971–1974)
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In his early studies, Shigetada Nakanishi elucidated the characteristic precursor architectures of various neuropeptides and vasoactive peptides by introducing recombinant DNA technology. Subsequently, he established a novel functional cloning strategy for membrane receptors and ion channels by combining electrophysiology and Xenopus oocyte expression. He determined the molecular structure and elucidated the regulatory mechanisms of several peptide receptors as well as several G protein-coupled metabotropic-type and NMDA-type glutamate receptors. He also developed new techniques for manipulating specific components of neuronal circuitry in olfactory bulb, basal ganglia, and cerebellum, thereby illuminating aspects of the function of these networks.

Shigetada Nakanishi

Family and Childhood

I was born in a rural area called Ogaki near Nagoya on January 7, 1942, just one month after the outbreak of the World War II in Asia. Ogaki is an old castle town with a population of about 100,000 people. My ancestors up to my parents lived in Ogaki as a Samurai family for nearly 400 years. During the war, almost all areas of Ogaki were burnt by incendiary bombs. My house was relatively large for a Japanese house, having a stable, a storehouse, gardens, and a pond. Fortunately it escaped any damage from the war.

Because my sister is 8 years older than me, I received all of the attention from my parents, just like an only child. My family was relatively wealthy before the war, but inflation after the war severely hurt my family. My father was a librarian in the City of Ogaki and was too poor to adapt to the tremendous changes in the economic situation during the postwar period. My mother was a daughter of a priest near Ogaki and was forced to manage this difficult situation for my family. My parents maintained their livelihood by selling off parts of our big house one after another. However, I knew nothing about the severe situation of my family and continued to receive all of my parents' affection during my childhood. They believed that education is the most important thing that they could give me for my future life, and they were devoted to supporting my educational career as much as they could.

During my childhood, after compulsory education of primary school and junior high school, only half of the students moved on to senior high school. Furthermore, only half of these students went on to attend universities. I went to public schools in Ogaki from primary school through senior high school. I was good in mathematics, but my most favorite subjects were chemistry and history. My parents took great care in supporting my school life and hoped that I would go to a high-ranking university in Japan. My parents, however, were very careful not to overburden me, and I enjoyed my school life during primary school and junior high school. After the war, baseball and swimming were very popular in Japan. After school, I played baseball on the school campus until sunset and enjoyed swimming in the river during summer.

Because I grew up in a rural town, I had no chance to think of my life at an international level, something which would later become necessary for my research life. When Japan opened our country during the Meiji Restoration, the Meiji government sent some elite graduates to Europe with government support to bring back the latest knowledge and technology to Japan.

My grandfather was one of those engineers and was engaged in designing and constructing tunnels and bridges at the earlier time of development of the Japanese railroad after his return from England. In addition, during my childhood, several relatives of my family were university faculty members and spent several years as postdoctoral fellows in the United States. Because they often let me know about their lives abroad, I felt that the foreign countries were close to me. Particularly, one of my relatives, Koji Nakanishi, was an associate professor at the Department of Chemistry, Nagoya University after his return from Harvard University and took me to his University during my childhood. When I saw him enjoying research and discussions in the laboratory, I longed for a research life.

With respect to the choice of my major when I entered university, I wondered whether I should choose chemistry (my favorite subject) or medicine. Because my mother had a hard time after the war, she hoped that I would take a medical course and live a stable life as a clinical physician. I also thought that involvement in the cure of patients would be valuable as a life work, and I thus finally decided to major in medicine. I chose the medical school of Kyoto University, because Kyoto University is located in the quiet old capital city in Japan and the school was known to be liberal.

Medical School

In 1960, I entered the medical school of Kyoto University, in which the freshmen class consisted of 55 students. We became friendly very soon after entrance into the University. Among my classmates, Tasuku Honjo has been my best friend for more than 40 years, not only in academic life but also in family relationships. We took the same course of study in biochemistry and molecular biology, although his major concern has been molecular mechanisms of the immune system. It was not too difficult to pass the medical courses in those days. At the time I belonged to a rugby club at our medical school and played games with teams from other medical schools almost every weekend during the rugby season. During winter, I also enjoyed skiing in the Japan Alps. I played a lot of mah-jongg games with classmates, which was a most popular pastime for university students.

The medical course consisted of 2 years of premedicine, 2 years of basic medicine, and 2 years of clinical medicine. During the basic medical course, I was excited by the superb biochemistry lectures of Osamu Hayaishi who came to our school at the age of 38 after being a laboratory chief at NIH. Several student-initiated journal courses also took place among our medical students. I participated in some of these courses such as modern physiology and oncology and was impressed with some of the new developments in these fields. I had interest in the subjects of internal medicine and pediatric medicine within the clinical medical course and during the poly-clinical course. I felt that clinical practice in these fields would be valuable as a life

work for my future. During the clinical course, however, the etiology of many diseases was poorly understood and, in every case, was explained by either hereditary inheritance; disorders of the immune, nervous, or endocrine systems; nutritional deficits; and so on. I was deeply disappointed with the lectures on clinical medicine.

I very much enjoyed the life of a medical student and safely passed the graduate examination in 1966. I then took a one-year internship course at Kyoto University Hospital. However, noninvasive imaging technologies such as computed tomography and magnetic resonance imaging were not available yet, and the tool of biochemical and pathological examination was not well developed. Therefore, although I was greatly interested in dealing with patients, I could have no self-confidence that I had examined and treated my patients substantively. Because the curriculum of the internship course was not well organized in those days, I made time to work in some laboratories of basic medicine and was involved in helping out their research work. Through this activity, I became gradually more interested in research on basic medicine and began to think that I should go into basic medicine to explore the mechanisms of diseases rather than to practice in clinical work.

My mother strongly opposed my decision to go into basic medicine, because she believed that I was not the type of a scientist who must work all day long conducting research, and because she hoped that I would return to my hometown as a clinician in the future. My father supported my decision under the condition that it was made under sincere and sufficient consideration. When my parents later saw that I had been heartily enjoying basic science and had been accredited for making some scientific achievements from the scientific world, they were honestly delighted that I had made a correct decision.

Graduate Course

For my graduate studies after the one-year internship, I entered the Department of Medical Chemistry in 1967, which was organized by Osamu Hayaishi and Shosaku Numa. Numa was supposed to come back from Germany one year later. I chose Numa's laboratory and received training from an Associate Professor Masamichi Tachibana in the first year of my graduate course. The department was large, consisting of more than 50 scientists including staff, graduate students, and researchers from several companies. In this department, a journal club (seminar) took place at lunchtime every day and a progress report meeting was held on every Saturday. In the journal club, the laboratory members introduced a wide variety of topics covering many different biological fields. The members were highly talented and made very strict and effective discussions in the journal club and the progress report meetings. The policy of training by both professors was that science is an international activity, and it is essential to make scientific achievements in Japan that are widely recognized at the international level.

In the first year of the graduate course, Tachibana was indeed a good mentor. He dedicated his time to training a young scientist, which highly influenced my scientific career. He taught me several important principles of biochemical research. For example, because biochemical approaches are based on chemical analysis, it is essential to investigate the biological system as quantitatively as possible. It is also important to establish an easy and reproducible methodology to develop a new field and to perform research as logically as possible on the basis of a step-by-step progress. The subject in which Tachibana was engaged was whether the same type or different types of carbamyl phosphate synthetase are responsible for production of carbamyl phosphate, which is the first metabolite for the urea cycle and for pyrimidine biosynthesis. I simply followed the procedure by Tachibana and fortunately disclosed that two separate types of the enzyme exist in the respective metabolic pathways and are regulated differently by the end products of the two pathways. I was deeply impressed with my results, in which a metabolic mechanism was explained at molecular levels and felt that biochemical approaches were a good fit for my sensitivity and thinking about research work.

In the next year, Shosaku Numa returned from Germany and continued on his life work concerning acetyl-CoA carboxylase, which is a rate-limiting enzyme in the metabolic pathway of fatty acid synthesis. He assigned me to work on protein synthesis and degradation of this enzyme by its purification and antibody production. However, acetyl-CoA carboxylase is cold sensitive and too unstable to be purified. This enzyme was known to require an allosteric activator (citrate) to be maximally activated in the enzyme assay at room temperature. I hit on the idea that citrate may stabilize acetyl-CoA carboxylase at room temperature, which could make it easy to purify under this condition. Indeed, this was the case, and I succeeded in purifying the enzyme to homogeneity and generating an effective antibody against it. However, conflicts at the university became severe in 1969, and we had to completely stop experiments for almost one year because our campus was closed with barricades. When I restarted experiments, I found that an essentially similar study had been reported in *Journal of Biological Chemistry*. In spite of these setbacks, I continued working toward gaining a Ph.D. degree with this work (Nakanishi and Numa, 1970). Because most of the data from my experiments were already reported in the published paper, it was very tough for me, and I learned that I would never do overlapping works in the future.

The cessation of experimental work during the University's closure was a big frustration, but this also gave me an unexpected opportunity to seriously consider what direction I should take in the future on the basis of my 2 years of research experience. I realized that I liked biochemical approaches but also that the biochemical approaches, particularly enzymology, had come to a very mature stage and could rarely discover new principles about

biological mechanisms. The big and unexplored field of bioscience was how genetic information is encoded by genomes and how this information is expressed and regulated in the biological system. I thought that this was now the direction of my research career. However, there was no way to approach this direction in mammals of my interest. In contrast, some specific genes in E. coli were enriched by the transducing mechanisms of λ phage and investigation of their expression and regulation was extensively conducted in combination with biochemical techniques. This strategy, called "genetic biochemistry," highly attracted me although this research direction, at the time, was not directly related to the mammalian genomes of my interest. Among several laboratories working on genetic biochemistry, I was particularly impressed with the study of Ira Pastan at the Laboratory of Molecular Biology, National Cancer Institute (NCI), National Institutes of Health (NIH). His group worked on gene regulation of the lactose operon in E. coli using a λ transduced lactose operon, and they reported a number of important findings concerning the mechanisms of the cyclic adenosine monophosphate (cAMP)-mediated induction of the lactose operon expression and its relation to catabolite repression. Although I had no personal connection with Ira Pastan, I wrote a letter to him to express my wish to work in his laboratory as a postdoctoral fellow. Because of a recommendation letter by Hayaishi and Numa, and a kind contact by Hayaishi's NIH friend, Ira accepted me as the first Japanese postdoctoral fellow in his laboratory.

NIH

I started my research at NIH from 1971 immediately after the graduate course of Kyoto University. Because I had no experience in dealing with deoxyribonucleic acid (DNA) and because many accumulated studies were reported for both λ phage and E. coli genomes, I had to struggle to understand the background of this research field. Two leading geneticists, Max Gottesman and Sankar Adhya, in the same laboratory were very helpful and let me effectively work on the E. coli galactose operon with an in vitro transcription system. Using hundreds of mutant genes isolated by Max and Sankar, I purified galactose repressor and investigated the regulatory mechanisms of the operator and promoter of the galactose operon gene. This collaboration was fruitful, and I was able to publish several papers as first author together with Sankar, Max, and Ira in that order (Nakanishi et al., 1972). Through this, I recognized again how important and effective the use of a specifically enriched gene is to analyze gene function. I also kept my great interest in the study of mammalian genes and intently attended NIH seminars whenever topics on mammalian genes were presented.

In those days, Paul Berg and his colleagues reported the establishment of recombinant DNA technology by incorporating the SV40 genes into the λ

phage (Jackson et al., 1972). From my ongoing study, I immediately realized that this was the best strategy to enrich and amplify a specific mammalian gene and would lead to a revolution of mammalian molecular biology. I therefore decided to use this technology in the near future and prepared to move to Yale University after my 3-year postdoc at NIH. At Yale University, I planned to work on how the mature SV40 messenger ribonucleic acid (mRNA) was generated from the apparently large heterogenous nuclear ribonucleic acid (RNA) reported from several laboratories. However, when I was ready to get a permanent visa for the United States to work at Yale University, I received a call from Shosaku Numa who offered me a position as associate professor in Kyoto University. I thus finally decided to return to Japan and to work at Kyoto University in 1974.

POMC and Enkephalin Precursors

In the middle of the 1970s, molecular biology of the mammalian system in Japan was far behind that of the United States and Europe. We therefore needed to prepare every material and technique necessary for molecular biology with our own hands. I decided to change the subject of my study from the very competitive field of the SV40 virus to the not-yet well-characterized field of the mammalian system. During my stay at NIH, I intently attended seminars related to mammalian molecular biology and learned that many leading laboratories had been energetically working on the possibility of using insulin and growth hormone for therapeutic purposes. In contrast, only a minor population of molecular biologists was interested in and working on other endocrine systems. I thought that the endocrine system was an attractive target for molecular biological techniques in mammals for several reasons. First, a high amount of mRNA for a peptide hormone is synthesized in a specific endocrine tissue. Thus there is an easy route to characterizing a peptide hormone mRNA. Second, the regulation of peptide hormone production had been extensively studied with respect to regulatory extracellular signaling factors such as steroid hormones, peptide-releasing factors, and so on. Third, and most interestingly, accumulated but indirect evidence indicated that a small peptide hormone is initially synthesized as a large precursor in vivo, which is in turn specifically cleaved to produce a biologically active hormone. Accordingly, I believed that investigating peptide hormone biosynthesis would be fruitful as a target of mammalian molecular biology and a good project for applying recombinant DNA technology in the near future.

Shosaku Numa agreed with my proposal, and I initiated a molecular study of adrenocorticotropic hormone (ACTH) using the in vitro translation system. ACTH is a 39 amino-acid peptide and was assumed to be derived from a large precursor. My rationale for using the in vitro translation system was that this system is devoid of any cleavage enzymes responsible for

the generation of a biologically active ACTH in vivo and could thus allow us to identify an ACTH precursor molecule synthesized from the pituitary mRNA as a template. However, none of the biochemists or molecular biologists in Japan used the in vitro translation system at that time. I thus had to establish every step of in vitro translation from the initial stages, including searching for a source of wheat germ, preparation of wheat germ extracts and oligo-dT cellulose and all the other tools necessary for in vitro protein synthesis. Hiroo Imura at Kobe University had effective antibodies against several different parts of the ACTH sequence. We started collaborative work with Imura and spent 1½ years establishing all necessary procedures for in vitro translation of an ACTH precursor. We finally succeeded in proving that an ACTH precursor is about 7 times larger than the natural ACTH peptide in 1976 (Nakanishi et al., 1976).

In 1997, Mains and Eipper reported an excellent study, indicating that ACTH and an endogenous opioid peptide β-endorphin are derived from a common precursor (Mains et al., 1977). However, neither the structural organization of ACTH/β-endorphin nor more than half of this precursor sequence was clarified yet. The obvious question was what structure is present in the precursor molecule other than ACTH and β-endorphin. The best way to answer this question was to conduct molecular cloning of the ACTH complementary deoxyribonucleic acid (cDNA), followed by its sequence determination. In those days, purification of a target mRNA to homogeneity was a prerequisite for cloning of the corresponding cDNA. ACTH was generally believed to be synthesized in the anterior lobe of the pituitary. However, when a Ph.D. student, Shunzo Taii, quantified ACTH mRNA levels in the anterior, intermediate, and posterior lobes of the pituitary, he found the surprising result that the ACTH mRNA represents about 30% of the intermediate lobe mRNA. Three Ph.D. students, Taii, Toru Kita, and Akira Inoue, tried to purify the ACTH mRNA by isolating about 40 pituitaries from a slaughterhouse every day and finally achieved purification of the ACTH mRNA to homogeneity. The 1970s was a period for developing recombinant DNA technology, and none of the Japanese scientists was engaged in recombinant DNA research on mammals. Our friend, Robert Schimke at Stanford University, was invited to the annual meeting of the Japanese Biochemical Society in 1977. He told us to be ready to clone an ovalubmin cDNA and was kindly willing to help me with molecular cloning of the ACTH cDNA.

On February in 1978, I brought 1μg of a homogenous ACTH mRNA to Stanford University and started collaborative work with Schimke and Stanley Cohen. However, I found that the trial of molecular cloning by Schimke's group was not going well. I thus had to check every process of molecular cloning, one-by-one, with my own hands, such as the optimal conditions for reverse transcription, double-stranded cDNA synthesis and insertion of a cDNA mixture into a vector DNA, selection of an appropriate

cloning vector, optimal hybridization conditions, and so on. Although this was a tough time, Jack Nunberg in Schimke's laboratory and Annie Chang in Cohen's laboratory were very helpful and collaborative and gave me much valuable advice. Furthermore, I received very useful information about many unpublished protocols for molecular cloning during my stay at Stanford University. I am confident that, had I not worked at Stanford, I would not have succeeded in cloning the ACTH cDNA. I worked very hard, day and night, for 4 months and finally succeeded in isolating more than 10 clones constituting the ACTH cDNA. I am sure that this 4-month period is the time in which I most intensely concentrated on research work during my entire research life.

Stanley recommended extending my stay at Stanford to determine the sequence of the cloned ACTH cDNA. However, I was so tired by these 4 months of hard work that I returned to Japan, and this time I brought back cloned ACTH cDNAs with me. DNA sequencing was just at the beginning, but, fortunately, Mitsuru Takanami and his group at the Institute for Chemical Research at Kyoto University had established DNA sequencing techniques in Japan. Under the guidance of Takanami's group, we determined the sequence of the ACTH cDNA and deduced the whole amino acid sequence of the ACTH precursor (Nakanishi et al., 1979). The deduced amino acid sequence revealed that the ACTH sequence is followed by the β-lipotropin sequence that contains β-melanocyte-stimulating hormone (β-MSH) and β-endorphin. The biologically active peptides are all flanked by paired basic amino acids in which proteolytic processing takes place. The most exciting finding was the presence of an additional new MSH sequence at the amino-terminal portion of the precursor. We termed this sequence "γ-MSH." This precursor consists of the repetitive MSH core sequences followed by the β-endorphin sequence. Our study not only proved that recombinant DNA technology is very powerful for elucidating peptide precursors but also provided the first evidence that the peptide precursor possesses a characteristic structure consisting of repetitive core peptide sequences. In vitro cDNA synthesis or cDNA cloning may cause possible sequence errors of the cloned cDNA. (In most cases, however, the reported errors turned out to be errors in DNA sequencing rather than in cDNA cloning.) Some endocrinologists called the predicted sequence "Nakanishi's structure" rather than the sequence of the ACTH precursor. The correctness of the ACTH precursor sequence was confirmed by a partial sequence determination of the precursor protein as well as through determination of the ACTH precursor in other mammalian species and its genomic sequence (Nakanishi et al., 1980). We simply named this precursor ACTH-β-lipotropin precursor, but it is now designated with a more attractive name, proopiomelanocortin (POMC).

The next question we were interested in was whether a polypeptide structure is common in precursor molecules for other biologically active peptides.

Because endogenous Leu-enkephalin and several extending peptides containing either Met-enkephalin or Leu-enkephalin were not contained in the POMC sequence, the existence of additional enkephalin precursors was predicted. However, endogenous enkephalins are widely distributed in various brain regions and some peripheral tissues. It was thus impossible to purify the enkephalin mRNA from a particular tissue or brain region. We therefore attempted to develop a different approach to clone the enkephalin precursors.

According to mRNA–cDNA hybridization analysis, the total number of mRNA copies present in a particular tissue was calculated to be around 5×10^5 copies. It was therefore expected that at least several cDNA clones of interest exist in a cDNA library consisting of randomly synthesized 5×10^5 cDNA clonal mixture. The question was then how to identify a cDNA clone of interest from such a cDNA library. A Ph.D. student, Masaharu Noda, found an interesting report of a model experiment of the globin cDNA by Itakura's group (Wallace et al., 1981). They reported that an oligonucleotide sequence of about 20 nucleotide residues complementary to an mRNA sequence effectively and specifically hybridized with its coding cDNA sequence. At the meeting, I met Tadaaki Hirose who was a main contributor in chemical synthesis of oligonucleotides at Itakura's laboratory and who had just returned to Keio University. We decided to start collaborative work on molecular screening with the use of synthetic oligonucleotide probes.

Another important issue for screening a cDNA library arose. Because the location of a peptide sequence in a precursor molecule is not known, it is desirable to construct a cDNA library containing so far as possible a full-length cDNA mixture. When I stayed at Stanford University, Hiroto Okayama, a former Ph.D. student of Hayaishi's laboratory, worked in Paul Berg's laboratory and aimed at designing a plasmid vector that contained a full-length cDNA. He finally developed such a vector, the so-called Okayama-Berg vector (Okayama and Berg, 1982) and kindly let me know his unpublished protocol when he visited Japan. Masaharu Noda and Hitoshi Kakidani constructed cDNA libraries and attempted to isolate cDNA clones for enkephalin precursors by hybridization with a mixture of synthetic oligonucleotides deduced from the amino acid sequences of the extending enkephalin sequences. Using this cloning strategy, we succeeded in cloning preproenkephalin and preprodynorphin (Kakidani et al., 1982; Noda et al., 1982). Preproenkephalin contains six copies of Met-enkephalin and one copy of Leu-enkephalin, whereas preprodynorphin contains three copies of Leu-enkephalin. The two enkephalin precursors and POMC all contain a cysteine-rich region preceding the peptide core sequences and followed by a signal peptide. These studies revealed the roots of all endogenous enkephalin peptides and led to the important realization that peptides acting in coordination are derived from common precursors by a specific proteolytic cleavage.

Vasoactive Peptides and Neuropeptides

Numa and I discussed almost everyday how to proceed on our projects and enjoyed the progress of our projects in the new field of the neuroendocrine system. Based on my achievements in these works, I was promoted to professor at a new laboratory of the same university in 1981. In those days, we had to leave the ongoing subject to the former laboratory and had to start a new project. Also, we could not expect any financial support from the University to open a new laboratory and had to struggle in getting research grants from the government and private foundations. In those days, there was no postdoctoral fellowship system in Japan. Hence we had to solicit undergraduate students to work together and to train them from the beginning of bench work. At the new laboratory, I therefore decided to extend my peptide project on a different biological system.

The renin-angiotensin system and possibly the kallikrein-kinin system are involved in the regulation of blood pressure (see Nakanishi et al., 1985). In the former system, the vasoconstrictive angiotensin II is generated from angiotensinogen followed by enzymic processing with angiotensin-converting enzyme. The vasodilative bradykinin is also initially synthesized as kininogen and liberated by kallikrein. Because kinase II, which degrades bradykinin, is the same enzyme as angiotensin-converting enzyme, the kallikrein-kinin system was also implicated in blood pressure regulation. However, because in most patients with essential hypertension, renin, the rate-limiting enzyme, is not high in blood plasma, little attention was paid to the influence of the renin-angiotensin system on blood pressure regulation and the pathogenesis of essential hypertension. Despite this fact, many references I read indicated that angiotensin-converting enzyme inhibitors effectively lowered hypertension in humans and could possibly be widely used as drugs for treating hypertensive patients (Ondetti and Cushman, 1982). However, the renin-angiotensin system was poorly understood at the molecular level.

Kininogen also had an interesting molecular aspect in terms of the generation of this precursor molecule. There are two kininogens, termed "high-molecular-weight" (HMW) and "low-molecular-weight" (LMW) kininogen, in which not only is the bradykinin moiety and its following 12-amino acid sequence identical, there is also complete divergence between the two kininogens in their downstream sequences. This structural relationship of the two kininogens strongly suggested involvement of alternative splicing mechanisms as revealed in SV40 and some mammalian mRNAs a few years ago. New staff researchers in my laboratory, Hiroaki Ohkubo and Naomi Kitamura, started and succeeded in molecular cloning of angiotensinogen and two kininogens by screening cDNA libraries in combination with hybridization with synthetic oligonucleotide probes. Angiotensin is located at the amino-terminus of angiotensinogen, followed by a large carboxyl-terminal sequence

(Ohkubo et al., 1983). The two kininogens are identical throughout their large amino-terminal portions up to 12 amino acid residues distal to the bradykinin moiety, and they then diverge at their carboxyl-terminal sequences (Kitamura et al., 1983). In fact, genomic cloning indicated that the two kininogens are encoded by a single gene and generated by alternative RNA splicing in combination with different polyadenylation events. The large carboxyl-terminal domain of angiotensinogen and the common amino-terminal domain of kininogens both share this sequence similarity with proteinase inhibitors (Tanaka et al., 1984). In fact, kininogen turned out to be the same as the cysteine proteinase inhibitor. The angiotensinogen and kininogens possess no repetitive peptide core sequence and thus seem to evolve in a different manner from the polypeptide precursors. Rather, the multifunctional domains characteristic of these precursors implicated evolutionary significance in the process of blood pressure regulation and inflammatory reactions.

We also started one more project concerning the precursor of the neuropeptide substance P. Substance P is one of the best-characterized neuropeptides and acts as a pain-generating peptide in the sensory nervous system (see Nakanishi, 1986). This peptide belongs to the tachykinin peptide family but was thought to be the only tachykinin peptide in mammals. However, some nonmammalian tachykinins such as amphibian kassinin were reported to be pharmacologically more potent than substance P in some mammalian assays (Erspamer, 1981). I considered the possibility that one or more additional mammalian tachykinins, like opioid precursors, may exist in the substance P precursor. Using the same cloning strategy described above, a Ph.D. student, Hiroyuki Nawa, identified two types of cDNA clones encoding the substance P precursor (termed "preprotachykinin-A," PPT-A) (Nawa et al.,1983). One (α-PPT-A) contains a single substance P sequence in the precursor, whereas the other (β-PPT-A) possesses an additional tachykinin sequence. This sequence is strikingly similar to that of kassinin and was thus termed "substance K." Its genomic sequence and S1-nuclease mapping analysis indicated that substance K is precisely specified by one of the exons of the PPT-A gene (Nawa et al., 1984b). More interestingly, the expression of α- and β-PPT-A mRNAs is regulated in a tissue-specific manner by including or excluding a substance K–coding sequence by alternative RNA splicing events. The PPT-A gene provided the first example in which alternative RNA splicing is independent of different promoters or polyadenylation and is directly involved in determining the production of a specific peptide. Substance K (also termed "neurokinin A" or "neuromedin L") and a third mammalian tachykinin, neuromedin K/neurokinin B, were independently identified as naturally occurring tachykinin peptides from mammalian tissues. We also cloned the neuromedin K precursor (preprotachykinin-B, PPT-B) and indicated that three mammalian tachykinins are derived from the two genes (Kotani et al., 1986).

About one year before our publication of alternative splicing mechanisms of kininogens and PPT-A, Susan Amara, Ron Evans, and Michael Rosenfeld reported that a novel calcitonin-gene-related peptide (CGRP) is contained in the calcitonin precursor and its expression is regulated by tissue-specific alternative splicing in combination with different polyadenylation (Amara et al., 1982). Furthermore, a number of leading laboratories reported on their extensive studies of alternative splicing and polyadenylation mechanisms. The study of renin-angiotensin and kallikrein-kinin systems in blood pressure regulation and hypertension was also very interesting and important. We actually showed that transgenic mice carrying the renin and angiotensinogen transgenes became hypertensive and that the angiotensinogen mRNA was markedly upregulated by inflammatory reaction. However, knockout techniques were not yet available and if I were to pursue this project, I would need to shift from molecular studies to more pharmacological and physiological studies. I felt that this was not my field, and I was therefore perplexed about what direction to take next in our research.

Peptide Receptors

When we identified substance K as a new mammalian tachykinin, we chemically synthesized substance K and subjected it to parallel pharmacological analyses with substance P. This study clearly indicated that the two peptides possess common pharmacological activities but markedly differ in their potencies and kinetics (Nawa et al., 1984a). This finding strongly suggested the existence of different tachykinin receptors. The substance P receptor is coupled to intracellular G proteins, but the molecular entity of G protein-coupled receptors (GPCRs) per se remained to be clarified in those days. Membrane receptors and ion channels are composed of hydrophobic amino acid clusters embedded in plasma membranes and hydrophilic amino acid clusters exposed to the extracellular and intracellular milieus. Furthermore, membrane proteins generally represent a minor population of the cellular components. These features of membrane receptors and ion channels hamper protein purification by biochemical approaches. However, when chemical properties of membrane proteins are regarded at the mRNA level, there is no difference in the chemical constituents between soluble proteins and membrane proteins. Therefore, if we can make up a good strategy for identifying cDNA clones for a receptor or an ion channel, we would be able to isolate such clones from a cDNA library.

In 1984, I had two staff researchers and about 10 graduate students. And as a result of the above achievements, I was able to obtain government funding to maintain my laboratory. I therefore decided to move on to a more challenging project by developing a new tool. Eric Barnard and his colleagues had reported that when tissue mRNA such as brain mRNA is injected into Xenopus laevis oocytes, the functional receptor or ion channel is expressed

in the oocyte plasma membrane (Barnard et al., 1982). Consequently, the voltage-gated ion channels and ligand-gated ion channels are characterized by the recording of an electrophysiological response of Xenopus oocytes after changing membrane potentials and after applying an appropriate receptor-specific ligand, respectively. Furthermore, when a GPCR is coupled to intracellular Ca^{2+} signaling, the activation of this receptor increases intracellular Ca^{2+}, which in turn stimulates Ca^{2+}-dependent Cl^- channels in Xenopus oocytes. Therefore, the expression of such GPCR is characterized by an electrophysiological recording of oocyte Cl^- currents after ligand application. Because the tachykinin receptors are coupled to intracellular Ca^{2+} signaling, we extended Barnard's finding to develop a new functional cloning strategy for tachykinin receptors.

Our cloning strategy was as follows (Masu et al., 1987). We first synthesized a cDNA mixture from the brain mRNA. We then constructed a cDNA library by inserting the cDNA mixture immediately downstream into an appropriate promoter in the vector DNA. We then extracted a clonal cDNA mixture from the cDNA library and subjected it to in vitro transcription by a specific RNA polymerase. Then the mRNA synthesized in vitro was injected into Xenopus oocytes, which were then tested for expression of a receptor or ion channel by electrophysiological recording after ligand application or membrane polarization. When a positive response was observed, we serially fractionated the cDNA library until a single functional cDNA clone could be identified. This strategy does not require any protein purification and is a straightforward way to circumvent many of the problems involved in the cloning of membrane receptors and ion channels.

Because our group had no experience with electrophysiological research, I shared my ideas with Motoy Kuno of the Department of Physiology and asked for his assistance and collaboration in experiments, as well as for the training of our graduate students. This collaboration was not only very fruitful but also enjoyable because I learned some of the logic and thinking patterns of electrophysiologists, which are sometimes different from those of our molecular biologists. Two Ph.D. students, Kazuhisa Nakayama and Yasuo Masu, started experiments but encountered several unexpected problems. These included the difficulty of synthesizing in vitro a full-length cDNA mixture covering the functional protein sequences, construction of an appropriate vector that allowed the incorporation of a full-length cDNA mixture, and the variability of electrophysiological responses of individual oocytes. To solve these problems, it took a longer time than I predicted, but they finally established this technique and reported the molecular cloning of a functional substance K receptor cDNA (Masu et al., 1987). This cloning was the first cloning of peptide receptors and provided compelling evidence that the peptide receptor belongs to the family of GPCRs. Independently, David Julius and Richard Axel reported the same cloning technique for the serotonin receptor in 1988 (Julius et al., 1988). Before publication of our

work, however, Lefkowitz's group and Numa's group had reported their success in molecular cloning of the adrenergic receptor and the muscarinic receptor on the basis of protein purification of these receptors (Dixon et al., 1986; Kubo et al., 1986).

As we intended, our cloning strategy became a widely applicable strategy for molecular cloning of membrane receptors and ion channels. This methodology was used for cloning of the endothelin receptor and the neurotensin receptor in our laboratory (Arai et al., 1990; Tanaka et al., 1990) and the PAF receptor, the histamine receptor, and the bradykinin receptor in other laboratories. Because our cloning strategy was applicable for ion channels by membrane polarization, we examined such electrophysiological responses in oocytes injected with several tissue mRNAs. A Ph.D. student, Toru Takumi, found that a kidney mRNA induced very potent voltage-dependent currents in its expression in Xenopus oocytes (Takumi et al., 1988). Because the size of this mRNA was unusually smaller than those of conventional voltage-dependent ion channels, we cloned and determined the sequence of the corresponding functional cDNA. The cloned cDNA encodes a novel small protein with a single transmembrane domain but induces selective permeation of K^+ by membrane depolarization. Because the structure of this protein is peculiar, it remained elusive whether it acts as a discrete K^+ channel per se or modulates the channel activity of endogenous oocyte K^+ channels. Later, this protein turned out to be a potently activating accessory subunit of K^+ channels and its mutations cause arrhythmia in humans (Abbott and Goldstein, 1998).

A Ph.D. student, Fumihiro Yokota, elucidated the molecular entity of the substance P receptor by cross-hybridization in combination with the Xenopus oocyte expression system (Yokota et al., 1989). Another Ph.D. student, Ryuichi Shigemoto at the laboratory of Noboru Mizuno in the Department of Morphological Brain Science, came to my laboratory to learn molecular biology and cloned neuromedin K receptor (Shigemoto et al., 1990). He is a very talented scientist, and we enjoyed working on many collaborative projects after he returned to his own laboratory. On the basis of molecular cloning of the three tachykinin receptors, we demonstrated the agonist and antagonist selectivities, and the intracellular signaling mechanisms of the three tachykinin receptors by use of heterologously expressing cells. We also indicated the distribution of individual tachykinin receptor mRNAs in various brain regions and peripheral tissues as well as the interesting negative regulation of the substance P receptor by steroid hormones. Our studies thus led to a comprehensive demonstration of the mammalian system from its biosynthesis to receptor interaction and intracellular signaling mechanisms (Nakanishi, 1991).

Yokota investigated the ligand binding mechanism of tachykinin receptors by expressing a series of chimeric receptors between substance P and substance K receptors in heterologously transfected cells. This analysis showed

that the extracellular domain and the transmembrane regions are involved in selective peptide binding of the tachykinin receptors (Yokota et al., 1992). However, Lefkowitz's group characterized the detailed ligand binding mechanisms of the adrenergic receptor by analyzing binding of a small-molecule ligand to this receptor. In contrast, the peptide binding of the tachykinin receptor was too complicated to be solved at the molecular level. We also hoped to examine mechanisms underlying the different response profiles observed between substance P and substance K receptors by detailed analysis in heterologously transfected cells. However, this study failed to reproduce such differences in the heterologous expression system. This finding suggested that the different response patterns of the two receptors result from endogenous intracellular signaling mechanisms following receptor activation. More important, the tachykinin system lacks explicit evidence indicating the physiological and pathophysiological role of tachykinin peptides and their receptors in the biological system. My standpoint toward molecular biology is that molecular biology is very powerful for logically exploring mechanisms of the biological system but is not so effective for unravelling a novel physiological or pathophysiological role of the biological system. I therefore gradually moved toward investigating a more attractive system for our molecular research approach.

Cloning of Glutamate Receptors

Glutamate is the main excitatory neurotransmitter in the nervous system. Numerous lines of evidence demonstrated that glutamatergic transmission plays an essential role in memory and learning, neural development, and neuronal cell degeneration (see Nakanishi, 1992; Nakanishi and Masu, 1994). Exploring regulatory and integrative mechanisms of glutamatergic transmission was thus not only one of the central subjects of neuroscience but also an intriguing target of application for our functional cloning strategy. Our colleagues wished to extend the project to molecular elucidation of glutamate receptors and to uncover regulatory and integrative synaptic mechanisms underlying glutamate-mediated brain functions at the molecular level.

Previous electrophysiological and pharmacological studies categorized glutamate receptors into two groups, termed "ionotropic receptors" and "metabotropic receptors" (mGluRs) (see Nakanishi, 1992). Ionotropic receptors are glutamate-gated cation channels and are subdivided into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors and N-methyl-D-aspartate (NMDA) receptors. AMPA receptors are responsible for the rapid excitation of neuronal cells, whereas NMDA receptors are important for neural plasticity and neural development as well as neurotoxicity. mGluRs are GPCRs and are involved in the modulation of glutamatergic transmission. In 1989, Stephan Heinemann and his colleagues reported the

molecular cloning of an AMPA receptor using the functional oocyte cloning strategy (Hollmann et al., 1989). Since then, AMPA/kainate receptors have been shown to comprise diverse members of the receptor family.

A Ph.D. student, Masayuki Masu, initiated an attempt to clone mGluR as an extension of the GPCR family. This cloning took a longer time than predicted because the mGluR mRNA encoded an unusually large extracellular domain and was unexpectedly large as compared with mRNAs for other conventional GPCRs. In 1991, he finally succeeded in cloning mGluR1, which was coupled to the IP_3/Ca^{2+} signaling cascade (Masu et al., 1991).

The NMDA receptor is not only essential for memory, learning, and neurotoxicity but also is distinguished from AMPA/kainate receptors by its unique properties, including high Ca^{2+} permeability, a voltage-dependent channel block by Mg^{2+} and the action of a number of selective agonists and antagonists (see Nakanishi, 1992). A number of laboratories attempted but failed to identify NMDA receptors through molecular cloning with the use of conventional protein purification or with the oocyte expression system. Koki Moriyoshi was trained in recombinant DNA techniques during his medical course at my laboratory and came to my laboratory as a Ph.D. student in 1991.

I thought that there were two possible explanations for the difficulty of cloning the NMDA receptor by the oocyte expression system. One was that the NMDA receptor mRNA represents a minor population of the brain mRNA. If that were the case, we needed to increase the number of cDNA clones of the cDNA library to identify a rare NMDA receptor cDNA clone. The other possible explanation was that the current activity of a single functional NMDA receptor subunit may be too low in oocytes to identify its clone in the large population of a cDNA library. If this were the case, it would be better to decrease the number of cDNA clones from a cDNA library. I recommended that Moriyoshi reduced the number of the clonal mixture from the standard 500,000 clones to 1000 clones of a brain cDNA library. This was the case for the situation of the NMDA receptor and allowed him to isolate a number of functional receptor cDNA clones less than 3 months after the start of this project (Moriyoshi et al., 1991). The cloned functional subunit shares a common structural characteristic with ionotropic receptors and shows all the physiological and pharmacological properties reported for the NMDA receptor. We submitted this study to *Nature* by summarizing the sequence and electrophysiological and pharmacological properties of the cloned MNDA receptor subunit (NR1) and its wide distribution in various brain regions. Two weeks later, we received a galley proof of our manuscript by fax. This was our first and only experience thus far to correct a faxed proof of a *Nature* paper.

The NMDA currents of the cloned NR1 receptor subunit were much smaller than the ones that were induced with the whole brain mRNA in Xenopus oocytes. This finding strongly indicated that additional subunits of

the NMDA receptors existed and that the full activity of the NMDA receptors would be expressed by a heteromeric assembly. The groups of Masayoshi Mishina and Peter Seeburg as well as a Ph.D. student, Takahiro Ishii, in my laboratory demonstrated the presence of four additional NMDA receptor subunits (NR2A-NR2D) by PCR and cross-hybridization techniques (see Ishii et al., 1993). The individual NR2 subunits, in contrast to NR1, evoked no electrophysiological response after the agonist application. However, combined expression of NR1 with the NR2 subunits markedly potentiated responses to NMDA or glutamate. Thus, NR1 serves as a fundamental subunit necessary for the NMDA receptor-channel complex, and it forms a heteromeric assembly with different kinds of NR2 subunits. The combination of NR1 with different subunits shows functional variability in electrophysiological and pharmacological properties. The affinity for agonists, the effectiveness of antagonists, the kinetics of responses, the sensitivity to Mg^{2+} block, and the stimulatory effect of glycine are all different, depending on the subunit composition.

Upon mutational analysis of the AMPA receptor, it was shown that substitution of glutamine for asparagine at the channel pore segment allowed the AMPA receptor to highly permeate Ca^{2+}. Because asparagine is present in the corresponding pore segment of the cloned NR1 subunit, we proposed that this asparagine is a key residue for allowing the high Ca^{2+} permeability of the NMDA receptor. A fellow from the Kyowa Hakko Kogyo Company, Kazuhiro Sakurada, as well as Mishina's group and Seeburg's group showed that asparagine is essential for Ca^{2+} permeability and inhibition by Mg^{++}, Zn^{++}, and an NMDA receptor antagonist, MK801 (see Sakurada et al., 1993).

The NR1 mRNA is expressed ubiquitously in almost all neuronal cells throughout the brain. In contrast, the mRNAs for different NR2 subunits show overlapping but different expression patterns in the brain. Thus, the anatomical and functional differences among the NR2 subunits provide the molecular basis for the generation of heterogeneity in the physiological and pharmacological properties of the NMDA receptors in different neuronal cells and brain regions (Nakanishi, 1992).

Several Ph.D. students, Yasuto Tanabe, Takaaki Abe, and Naoyuki Okamoto, expanded cloning of the mGluR family by cross-hybridization and PCR techniques. They revealed the existence of seven different subtypes (mGluR1 through mGluR7) (Abe et al., 1992; Nakanishi, 1994; Okamoto et al., 1994; Tanabe et al., 1992), whereas Duvoisin reported one more subtype (mGluR8) of the mGluR family (Duvoisin et al., 1995). A fellow from the Eizai Pharmaceutical Company, Ichiro Aramori, and a Ph.D. student, Yasunori Hayashi, participated in investigating the properties of individual mGluRs by means of DNA transfection and stable expression in Chinese hamster ovary cells (Aramori and Nakanishi, 1992; Hayashi et al., 1993). Eight different mGluR subtypes were classified into three groups according

to their sequence similarity, agonist and antagonist selectivity, and intracellular signaling mechanisms. Group 1 mGluRs (mGluR1 and mGluR5) react most potently with quisqualate and stimulate IP_3 formation and intracellular Ca^{2+} mobilization. The other six mGluR subtypes inhibit the forskolin-stimulated accumulation of intracellular cAMP, but group 2 mGluRs (mGluR2 and mGluR3) and group 3 mGluRs (mGluR4, mGluR6-mGluR8) are different in their agonist and antagonist specificity. Interestingly, glutamate was believed to be an excitatory neurotransmitter. However, the linkage of group 2 and group 3 mGluRs to the inhibitory cAMP cascade suggested suppression rather than excitation of neurotransmission, because in many cases the receptors linked to the cAMP inhibition are involved in suppression of neurotransmission. Later on we pursued the physiological role and detailed mechanisms of mGluR-mediated synaptic modulation in some neural networks.

In situ hybridization and immunohistochemistry revealed that different mGluR subtypes show overlapping but different distribution patterns in the brain. All but mGluR6 of group 3 mGluRs are widely localized at the presynaptic sites of many neuronal cells, again indicating that these subtypes serve as inhibitory autoreceptors at the presynaptic sites. In contrast, mGluR1 and mGluR2 are more restrictedly distributed in specific neuronal cells, and the functions of these subtypes were investigated in detail as described later.

Glutamatergic Transmission in the Retina

Thanks to the molecular cloning of diverse types of ionotropic receptors, many laboratories were engaged in precise characterization of the properties and regulatory mechanisms of these receptors at the molecular level. In contrast, the physiological and functional role of mGluRs in brain function largely remained to be investigated. The receptors of the GPCR family are usually involved in the regulation of neurotransmission via intracellular signaling mechanisms. Furthermore, a single molecular entity of an individual mGluR would allow us to delineate its regulatory role in glutamatergic transmission more easily than would a heteromeric assembly of ionotropic receptors. We therefore focused on the role and mechanisms of mGluRs in the neural network.

At the time we reported the first cloning of mGluR, two groups, Nawy and Jahr and Schiells and Falk, reported a very interesting finding. A putative mGluR that responds to 2-amino-4-phosponobutyrate (L-AP4), a glutamate analogue, most likely mediates synaptic transmission from photoreceptors to ON bipolar cells in the retina through coupling to the inhibitory signaling cascade (Nawy and Jahr, 1990; Shiells and Falk, 1990). In the retina, glutamate is used as a common neurotransmitter from photoreceptors to ON and OFF bipolar cells, but this common transmitter induces opposite responses, namely, depolarization of OFF bipolar cells and hyperpolarization of ON

bipolar cells, thus resulting in the discrimination of light and dark signals at the level of bipolar cells (see Nakanishi, 1995). The excitation of OFF bipolar cells by glutamate was easily explained by the involvement of the ionotropic receptor, but hyperpolarization of ON bipolar cells by glutamate could not simply be accounted for by the usual glutamate receptor. A Ph.D. student, Yoshiaki Nakajima, thus initiated screening of a retinal cDNA library and identified mGluR6, which in fact responded effectively to L-AP4 (Nakajima et al., 1993).

Several Ph.D. students, Akinori Nomura, Masayuki Masu, and Hideki Iwakabe, then characterized the role of mGluR6 in glutamatergic transmission from photoreceptors to ON bipolar cells by immunohistochemistry, immunoelectron microscopy, and intracellular signaling mechanisms. This study explicitly demonstrated that mGluR6 is confined to the postsynaptic site of ON bipolar cells (Nomura et al., 1994). We then took advantage of the restricted expression of mGluR6 in retinal ON bipolar cells and generated mGluR6 knock-out mice by gene targeting. This investigation provided compelling evidence that mGluR6 is essential for evoking ON responses in ON bipolar cells (Masu et al., 1995). Interestingly, the mGluR6-deficient knock-out mice retained the ability to respond to visual input. Because the OFF response remained unchanged in mGluR6 knock-out mice, this finding indicated that not only an ON response but also an OFF response is essential as a signal for visual transmission. The mGluR6 deficiency, however, impaired the detection of visual contrast (Iwakabe et al., 1997). Furthermore, Dryja's group recently reported that in human patients mGluR6 mutations induce an abnormal ON response electroretinogram and cause night blindness (Dryja et al., 2005), indicating the indispensable role played by mGluR6 as well in human visual discrimination.

These studies explored the mechanisms underlying light and dark discrimination at the level of bipolar cells (Nakanishi, 1995). When exposed to light, photoreceptor cells hyperpolarize, and the AMPA receptor and mGluR6 remain inactive. The inactive AMPA receptor shuts off the OFF pathway. Conversely, the inactive mGluR6 is uncoupled to the inhibitory intracellular signaling and keeps cation channels active, thus resulting in the excitation of ON bipolar cells. In contrast, when the light is shut off, glutamate activates the AMPA receptor and mGluR6, and the activated mGluR6 stimulates the inhibitory signal cascade and in turn shuts off the ON pathway. Therefore, the two types of glutamate receptors, namely mGluR6 in ON bipolar cells and the AMPA receptor in OFF bipolar cells, are effectively used for discrimination of light and dark signals at the level of bipolar cells.

Accessory Olfactory Bulb Neurotransmission

The distinctive roles of the two types of glutamate receptors seemed to be rather unusual because they are often expressed in the same neurons.

We therefore investigated the cooperative function of ionotropic and metabotropic receptors in several networks. Abundant mGluR2, as analyzed by in situ hybridization and immunoelectron microscopy, is located at the dendrites of granule cells in the accessory olfactory bulb (Hayashi et al., 1993). Consequently, the AMPA receptor and mGluR2 are located at the postsynaptic site of granule cells. In the accessory olfactory bulb, mitral cells are excited by input from the vomeronasal nerve, that is, pheromonal receptor neurons. Mitral cells are known to form typical dendrodendritic synapses with inhibitory granule cells, and these synapses undergo reciprocal regulation, namely excitation of granule cells by glutamate from the mitral cell and inhibition of mitral cells by γ-aminobutyric acid (GABA) from the granule cell. The question, however, then arose as to how the odorant stimulus is transmitted to the central olfactory pathway when the granule cell-mediated GABAergic inhibition is working on mitral cells (see Nakanishi, 1995).

The development of selective and potent agonists for mGluR subtypes is indispensable for mGluR research. Ohfune and coworkers in Suntory Institute for Bioorganic Research chemically synthesized eight isomers of the conformationally restricted glutamate analogue, 2-(carboxycyclo-propyl) glycine (CCG) (see Shinozaki et al., 1989). Shinozaki and Ohfune reported that two of the L-isomers of CCGs, L-CCG-I and L-CCG-II, potently activate the IP3-coupled mGluR. A Ph.D. student, Yasunori Hayashi, in collaboration with Ohfune, investigated the agonist potencies and selectivity of eight CCG isomers by examining their effects on the signal transduction of the representative mGluR subtypes and identified L-CCG-1 as a potent agonist for group2 mGluRs. He further revealed that DCG-IV, which was initially abandoned as a weak NMDA receptor antagonist, is a more potent and selective agonist for mGluR2 and mGluR3. Thus, DCG-IV is very useful for distinguishing the functions of different subtypes of mGluRs (Hayashi et al., 1993).

Hayashi in collaboration with Tomoyuki Takahashi of the Department of Physiology examined whether the activation of mGluR2 by DCG-IV can regulate granule cell-mediated GABA transmission in the accessory olfactory bulb. In slice preparations, he made whole-cell recordings of a mitral cell after electrical stimulation of a granule cell. This analysis clearly showed that the activation of mGluR2 abolishes GABA-mediated inhibitory postsynaptic currents in mitral cells. On the basis of this and other findings, we proposed an interesting mechanism for olfactory transmission (Hayashi et al., 1993). In the accessory olfactory bulb, excitation of mitral cells releases glutamate and in turn stimulates granule cells through the AMPA receptor. This stimulation of the granule cell is thought to cause self-inhibition and lateral inhibition through the inhibitory GABA transmitter. Our observation, however, also indicated that the simultaneous activation of mGluR2 relieves GABA-mediated self-inhibition. Importantly, this inhibition is restricted to synapses with the excited mitral cell and would maintain lateral inhibition with neighboring mitral cells. This mechanism evidently enhances

the signal-to-noise ratio of strong odorant stimuli from background stimuli and would contribute to the discrimination of different olfactory stimuli. Our study demonstrates the importance of the cooperative function of ionotropic and metabotropic receptors in synaptic integration.

Behaviorally, the accessory olfactory bulb is known to be important for pheromonal memory formation (Brennan et al., 1990). When a female mouse mates, she forms a memory of male pheromones during mating and maintains pregnancy during exposure to familiar pheromones. However, when the pregnant female is exposed to unfamiliar pheromones, development of embryos is prevented and pregnancy is blocked. This pheromonal memory was initially discovered by Bruce and is thus named the "Bruce effect."

Keverne and his colleagues extensively studied the mechanisms of the Bruce effect (Brennan et al., 1990). They showed that coital stimulation persistently enhances norepinephrine in the accessory olfactory bulb and in turn reduces GABA transmission from granule cells to mitral cells. This coitus-stimulated, norepinephrine-mediated excitation of mitral cells results in the formation of a memory specific to the pheromones exposed during mating. I was deeply impressed with the review article of this mechanism written by Brennan et al. (1990). My interest was the following; because norepinephrine and DCG-IV commonly reduce GABA transmission, I wondered whether DCG-IV infusion into the accessory olfactory bulb may also create a specific pheromonal memory without mating. Kaba is a rare Japanese name. Thus, I originally thought that he was a foreign scientist, but I noticed that he was a Japanese scientist working at the Kochi Medical School. I then discussed the possibility of doing collaborative work with Hideto Kaba and immediately sent Hayashi to his laboratory. Our study demonstrated that the persistent activation of mGluR2 by DCG-IV is capable of inducing an olfactory recognition memory without mating (Kaba et al., 1994). Thus, mGluR2 in the accessory olfactory bulb plays an important role in neural plasticity responsible for olfactory memory formation.

Cerebellar Neurotransmission

The cooperative roles of the AMPA receptor and mGluR were also revealed in synaptic transmission of Golgi cells in the cerebellar network (Watanabe and Nakanishi, 2003). The principal network of the cerebellar cortex consists of mossy fibers, granule cells, parallel fibers, and Purkinje cells (see Nakanishi, 2005). In addition, Purkinje cells receive excitatory input from climbing fibers. This circuit serves as the single output system of the cerebellar cortex. Coincident excitation by parallel fibers and climbing fibers results in long-term depression (LTD) at parallel fiber-Purkinje cell synapses and attenuates the inhibitory output of Purkinje cells to deep cerebellar nuclei. Such LTD is a cellular model system that has been implicated in a portion of the engram for some forms of motor learning. Besides this main

network, Golgi cells receive excitatory parallel fiber input and in turn suppress granule cell excitation through an inhibitory GABA transmitter. This feedback inhibition is thought to be essential for filtering mossy fiber input before distributing input to Purkinje cells. However, this feedback inhibition is so strong that well-defined control of this feedback inhibition is necessary. Otherwise, this strong feedback inhibition would block information transmission of mossy fiber input to Purkinje cells.

To study the mechanisms of Golgi cell synaptic modulation, we focused on mGluR2, which is abundantly distributed at the postsynaptic sites of Golgi cells. A Ph.D. student, Dai Watanabe, performed whole-cell recordings of green fluorescent protein (GFP)-positive Golgi cells after electrical stimulation of parallel fibers in cerebellar slices of wild-type and mGluR2 knockout mice. A series of electrophysiological, pharmacological and biochemical experiments indicated that mGluR2 is coupled to G protein-coupled, inwardly rectifying K^+ channels (GIRK) and suppresses Golgi cell excitability through the activation of GIRK-mediated K^+ permeation. Our study also revealed that when Golgi cells receive glutamatergic input from parallel fibers, the AMPA receptor first excites Golgi cells and mGluR2 then suppresses Golgi cell excitability in a stimulus strength-dependent manner. As a result, postsynaptic mGluR2 is capable of sensing the strength of presynaptic granule cell input and relieving Golgi cell-mediated feedback inhibition in a stimulus strength-dependent manner. Therefore, the cooperative function of ionotropic and metabotropic receptors plays an important role in spatiotemporal processing of incoming input in the cerebellar network (Nakanishi, 2005).

We also investigated the specialized functions and intracellular signaling mechanisms of an individual subtype of the mGluR family in some specific neuronal cells and neural networks. In collaboration with my group, Hitoshi Ohishi of the Department of Morphological Brain Science showed that mGluR2 is located not only at the postsynaptic site but also at the presynaptic site of cerebellar Golgi cells. Because Golgi cell axon terminals are not directly connected with the glutamatergic synapses of mossy fibers, we proposed that glutamate spillover released from the mossy fiber terminals could activate the presynaptic mGluR2 of Golgi cells, which in turn heterosynaptically inhibits GABA-mediated inhibition of Golgi cells onto granule cells (Ohishi et al., 1994). This inhibitory mechanism of mGluR2 was supported by electrophysiological experiments reported by Mitchell and Silver (2000). Therefore, mGluR2 plays an important role in the modulation of Golgi cell transmission presynaptically and postsynaptically.

With respect to mGluR1 in Purkinje cells, Shigemoto raised antibodies against two distinct extracellular sequences of mGluR1 expressed as bacterial fusion proteins and indicated that these antibodies inhibit the mGluR1-stimulated IP_3 formation in transfected CHO cells. Shigemoto and Tomoo Hirano of the Department of Physiology then revealed that both of the

mGluR1 antibodies completely block induction of LTD at the parallel fiber-Purkinje cell synapses in cultured Purkinje cells (Shigemoto et al., 1994). Shigemoto further extended the physiological importance of Purkinje cell mGluR1 in collaboration with Peter Sillevis-Smith of the Department of Neuro-Oncology at the University Hospital Rotterdam. We showed that an mGluR1 autoantibody is generated in patients with Hodgkin's disease and causes paraneoplastic cerebellar ataxia (Sillevis-Smitt et al., 2000). This antibody abrogated mGluR1-induced IP_3 formation and caused severe ataxia in mice as well after injection into the subarachinoid space. This was the first report of an autoantibody associated with a neural antigen in the central nervous system.

Because many Ca^{2+} signaling molecules are highly expressed in Purkinje cells, a Ph.D. student, Jun Kitano, investigated the functional linkage of mGluR1 with Ca^{2+} signaling molecules in Purkinje cells (Kitano et al., 2003). He showed that mGluR1 forms a protein assembly with the $Ca_v2.1$ subunit of P-type Ca^{2+} channels and inhibits $Ca_v2.1$-mediated increases in agonist-dependent and agonist-independent manners. Furthermore, the simultaneous activation of mGluR1 and $Ca_v2.1$ channels enhances the $Ca_v2.1$-mediated Ca^{2+} increase, suggesting that the physical coupling of mGluR1 with $Ca_v2.1$ ensures efficient spatiotemporal regulation of intracellular Ca^{2+} during glutamatergic transmission in Purkinje cells.

Upon single-cell analysis of Ca^{2+} responses in heterologously transfected cells, Sigeki Kawabata and Masamichi Okada from Yamanouchi Pharmaceutical Company in collaboration with our group found a clear difference in intracellular Ca^{2+} responses between mGluR1 and mGluR5 (Kawabata et al., 1996). Glutamate induces a single-peaked nonoscillatory Ca^{2+} increase in mGluR1-expressing cells but elicits Ca^{2+} oscillations in mGluR5-expressing cells. Chimeric analysis indicated that this different pattern results from a single amino acid substitution, aspartate or threonine, located at the corresponding G protein-interacting domains of mGluR1 and mGluR5, respectively. Interestingly, this threonine of mGluR5, as analyzed by pharmacology and peptide mapping, is phosphorylated by protein kinase C (PKC). Our data suggested a novel mechanism by which phosphorylation and dephosphorylation of mGluR5 can generate Ca^{2+} oscillations, whereas the absence of the corresponding PKC phosphorylation leads to glutamate-induced non-oscillatory Ca^{2+} responses in mGluR1. A Ph.D. student, Kiyoshi Nakahara, further showed that glutamate evokes oscillatory Ca^{2+} responses via the PKC phosphorylation/dephosphorylation mechanism in cultured astrocytes expressing mGluR5 but not mGluR1 (Nakahara et al., 1997). The physiological significance of mGluR5-induced Ca^{2+} oscillations remains to be clarified. However, because the frequency of Ca^{2+} oscillations is dependent on the glutamate concentrations applied, I still believe that this frequency modulation mode of Ca^{2+} signaling would be important as a distinct intracellular signal, characteristic of mGluR5.

3D Structure of the mGluR Glutamate-Binding Domain

The eight mGluRs are considerably larger than the conventional GPCRs and show a common structural architecture with an extremely large extracellular amino-terminal domain that precedes seven transmembrane segments. This structural characteristic turned out to be common in GPCR subfamilies such as pheromone receptors, $GABA_B$ receptors and the calcium sensor. A Ph.D. student, Katsu Takahashi, performed chimeric analysis between mGluR1 and mGluR2 and provided compelling evidence that the large extracellular domain of mGluR is a site for glutamate binding (Takahashi et al., 1993).

Hisato Jingami from the Biomolecular Engineering Research Institute (BELI), my former Ph.D. student, was interested in determining the 3D structure of the extracellular glutamate-binding domain of mGluR1. In collaborative work, the Banyu Company chemically synthesized ^{3}H-labeled quisqualate, a potent and selective agonist for mGluR1. To quantitate agonist binding to the extracellular glutamate-binding domain that is free from the transmembrane and G protein-interacting domains of mGluR1, the Banyu Company kindly gave us a ^{3}H-labeled quisqualate. Using a ^{3}H-labeled quisqualate-binding assay, we succeeded in purifying a soluble extracellular domain of mGluR1 to homogeneity from extracts of a buculovirus expression system.

The 3D structure of mGluR1 was then elucidated by Jingami, Kosuke Morikawa, and their colleagues in BELI with the use of X-ray crystallography (Jingami et al., 2003; Kunishima et al., 2000). The glutamate-binding domain of mGluR1 forms a dimeric structure through a packed α-helical interface between the two homomers. The glutamate-free structure consists of an open-open conformation and an open-closed conformation at the glutamate-binding site. Glutamate binding exclusively shifts this structure to an open-closed conformation, whereas a glutamate antagonist such as α-methyl-carboxyphenylglycine (MCPG) fixes this structure into an open–open conformation. The active and inactive forms of mGluR thus exist in equilibrium between these two conformations. This conformational change triggers activation of intracellular G proteins, but the activation mechanism, as is the case for other GPCRs, still remains elusive.

Immunotoxin-Mediated Cell Ablation

Using multidisciplinary approaches including electrophysiology, pharmacology, knock-out/transgenic techniques, and so on., several Ph.D. students further revealed that different glutamate receptor subunits or subtypes show specialized functions in different brain regions. These include the key role of mGluR2 in induction of LTD at the mossy fiber-pyramidal cell synapses in the hippocampal CA3 region (Yokoi et al., 1996), the necessity of

the heteromeric assembly of NR2A and NR2C NMDA receptor subunits at cerebellar mossy fiber-granule cell synapses for movement coordination (Kadotani et al., 1996), and the critical role of mGluR7 in fear responses and conditioned taste aversion (Masugi et al., 1999). However, such conventional approaches alone were not sufficient for investigating synaptic mechanisms of a specific network. In particular, it is difficult to target a specific neural network by conventional approaches.

After I left NIH, Ira Pastan shifted his research interest to oncogenic mechanisms and cancer therapy from the molecular biology of E. coli gene regulation. His group nicely elaborated a new immunotherapy technique termed "immunotoxin-mediated cell targeting" (IMCT). This technology was developed to treat human adult T cell leukemia, which expresses a high amount of interleukin-2 (IL-2) receptor. In this technique, the recombinant immunotoxin composed of human IL-2 receptor α-subunit monoclonal antibody fused to Pseudomonas extoxin is generated and injected to selectively kill T cell leukemia. Ira often visited Kyoto to enjoy the atmosphere of old temples and gardens in Kyoto. Once when he visited my laboratory, he emphasized that IMCT is selective for eliminating target cells by apoptosis and has no deteriorating effects on other cell types. He recommended applying the IMCT technique to neuroscience. We discussed doing collaborative work, and a Ph.D. student, Dai Watanabe, decided to apply this technique to investigate synaptic mechanisms of the cerebellar network by focusing on Golgi cells in the cerebellum (Watanabe et al., 1998).

In the cerebellar circuitry, Golgi cells mediate feedback inhibition onto granule cells. However, Golgi cells represent less than 1% of the cerebellar cell population, and the physiological and functional role of Golgi cells in the cerebellar circuitry largely remains to be determined. With the IMCT technology, we generated transgenic mice that express the human IL-2 receptor under the control of a neuron-specific promoter. We then injected the immunotoxin that is specific to the human IL-2 receptor and not cross-reactive with the endogenous mouse IL-2 receptor. The immunotoxin binds to the membrane human IL-2 receptor in transgenic mice and this complex is internalized and kills target cells, thereby leading to the elimination of a specific neuronal cell type.

We constructed a transgene that contained the mGluR2 promoter, followed by human IL-2 receptor fused to GFP. In the cerebellum of transgenic mice, the human IL-2 receptor was specifically expressed in Golgi cells. Immunotoxin injection ablated Golgi cells in transgenic mice but not in wild-type mice, and this cell ablation had no influence on any other cerebellar cell types. Golgi cell-ablated transgenic mice showed severe acute motor disorders after immunotoxin injection but, interestingly, the severe motor disorders gradually recovered in the chronic phase. However, these mice still failed to perform more complex motor tasks such as staying on a rapidly rotating rotarod.

We addressed how synaptic transmission is perturbed in the acute phase and how this perturbation is at least partly compensated in the chronic phase. We performed a series of electrophysiological and optical recordings of cerebellar slices of wild-type and Golgi cell-eliminated mice with the assistance of Keisuke Toyama of the Department of Physiology, Kyoto Prefectural University, and Masanobu Kano of the Department of Physiology, Kanazawa University. These studies allowed us to explore the novel synaptic mechanisms of the mossy fiber-granule cell-Golgi cell synapses. Under normal circumstances, GABA inhibition and NMDA receptors play an important role in the synaptic integration responsible for complex motor coordination. When Golgi cells are eliminated, GABA is depleted, and this depletion results in overexcitation of NMDA receptors and causes severe acute motor disorders. In the chronic phase, NMDA receptors are adaptively attenuated, thus relieving the overexcitation of granule cells. Importantly, AMPA receptors remain unchanged before and after Golgi cell elimination, and these AMPA receptors allow animals to perform simple motor movements. However, NMDA receptors are attenuated, thus causing functional deficits in directing more complex movement coordination. Our studies demonstrate that excitatory and inhibitory neurotransmissions concertedly act at the mossy fiber-granule cell-Golgi cell synapses, and this cooperative action is important not only for integrative brain functioning but also for the compensation of brain dysfunction.

We extended the IMCT technique to other neural networks. The basal ganglia control motor balance and reward-based learning. In the basal ganglia, cortical information reaches medium-sized spiny neurons in the striatum and is transmitted to substantia nigra pars reticulata (SNr)/ventral tegmental area (VTA) through two parallel routes termed the direct and indirect pathways. The two pathways exert opposite effects on SNr/VTA and control the dynamic balance of the basal ganglia-thalamocortical circuitry. Dopamine from substantia nigra pars compacta (SNc) is crucial for exciting and inhibiting the direct and indirect pathways, respectively. As a consequence, dopamine depletion in Parkinson's disease causes severe motor imbalance. Acetylcholine is locally released from striatal cholinergic interneurons, which represent 1% to 2% of the striatal cell population. Because acetylcholine agonists and antagonists cause global effects on many other brain regions, previous pharmacological studies failed to indicate the physiological and behavioral role of local acetylcholine in the striatum.

Satoshi Kaneko spent 5 years as a neurologist and came to my laboratory as a graduate student. He was interested in the regulatory mechanism of the basal ganglia and looked at striatal functions of our knock-out and transgenic mice. One day, I recalled Kaneko's finding that one specific striatal cell type showed a high intensity of GFP/IL-2 receptor fluorescence in the IMCT transgenic mice. I pointed out the possibility that this cell type could correspond to striatal cholinergic interneurons and may be a good

target for the IMCT technique to characterize the local cholinergic action in the basal ganglia function. In fact, this turned out to be the case, and the immunotoxin injection selectively eliminated cholinergic neurons in the basal ganglia network. Unilateral ablation of cholinergic neurons caused an acute abnormal turning behavior and then showed a gradual recovery thereafter. This recovery, however, was incomplete, and mice continued to display abnormal turning behavior when there was an excess of either stimulation or inhibition produced by dopamine. His series of experiments led to the important conclusion that the reduction of basal ganglia acetylcholine results in dopamine having the predominant effect. Then D1 and D2 dopamine receptors are adaptively down-regulated, thus relieving the dopamine overaction and compensating acetylcholine-depleted synaptic perturbation. However, this compensation is still defective in responding to excessive stimulation and inhibition by dopamine. Therefore, we can conclude that the acetylcholine–dopamine interaction is concertedly and adaptively regulated to control the basal ganglia function.

Two more Ph.D. students, Takatoshi Hikida and Yasushi Kitabatake, came to our laboratory and worked on another important function of the striatum (Hikida et al., 2003). The nucleus accumbens (NAc), the ventral part of the striatum, is a key neural substrate in the addiction to abusive drugs such as cocaine and morphine. They showed that ablation of the NAc cholinergic neurons enhances the acute and long-lasting behavioral changes of abusive drugs, such as cocaine-induced hyperlocomotion and conditioned place preference to cocaine and morphine. Importantly, acetylcholinesterase inhibitors suppress cocaine- and morphine-induced behavioral changes, and this suppression is abolished by ablation of the NAc cholinergic neurons. These studies demonstrate that acetylcholine synthesized locally in the NAc plays a key role in the acute and chronic actions of cocaine and morphine.

Another project focusing on retinal circuitry was performed using IMCT by a Ph.D. student, Kazumichi Yoshida et al. (2001). In retinal circuitry, ON-OFF ganglion cells respond maximally when a stimulus moves in a preferred direction, but little or no response is evoked when the stimulus moves in the opposite null direction. This mechanism, called "direction selectivity," represents primitive pattern recognition of visual information. Among the diverse types of amacrine cells in the retina, one type of amacrine cell, called the "starburst cell," forms an asymmetric connection with ON or OFF bipolar cells and ON-OFF direction-selective ganglion cells. The starburst cell was implicated in the mechanism of direction selectivity of ON-OFF ganglion cells. However, the role of the starburst cell in direction selectivity was controversial.

Consistent with our previous observation that mGluR2 is specifically expressed in starburst cells within the retina, the IL-2 receptor/GFP fusion protein was found to be highly expressed in starburst cells of the IMCT

transgenic mice. Yoshida intravitreally microinjected immunotoxin into adult mice and showed that starburst cells are specifically ablated after immunotoxin injection. We then started collaborative work with Masao Tachibana of the Psychology Department, University of Tokyo. Upon measuring responses to light movement in eight directions, we showed that ON-OFF ganglion cells in starburst cell-eliminated transgenic mice respond to all directions of light movement, indicating that starburst cell elimination abolishes the direction selectivity of ON-OFF ganglion cells. Furthermore, in these transgenic mice, optokinetic nystagmus, which was observed before immunotoxin injection, became negligible after starburst cell elimination. Our study thus provided compelling evidence that starburst cells play a key role not only in originating retinal direction selectivity but also in deriving optokinetic eye movement.

Osaka Bioscience Institute: Reversible Neurotransmission Block

We are obliged to retire from Kyoto University at the age of 63. I was fortunate to have an offer to become the Director of Osaka Bioscience Institute (OBI). OBI is located in a quiet and beautiful suburb of the city of Osaka and is financially supported by the city. The aim of this Institute is to achieve research of internationally acclaimed quality in the basic fields of bioscience and medicine. My position as Director is to be responsible for the administration of OBI. But this Institute is small, consisting of only five laboratories. I can thus continue research by organizing my own group of about 10 members of postdoctoral fellows and graduate students from affiliate universities such as Kyoto University and Osaka University.

In one of our projects at OBI, we are focusing on the synaptic mechanisms underlying cerebellum-dependent motor learning. The conditioned eye-blink response is a typical cerebellum-dependent motor learning. When a conditioned sensory stimulus such as a tone is paired with an unconditioned stimulus, the trained animals exhibit a tone-dependent eye-blink response in the absence of unconditioned stimulation. Information about the conditioned stimulus is transmitted through the pons, mossy fibers, granule cells, and parallel fibers, whereas information about the unconditioned stimulus is transmitted through the inferior olive and climbing fibers. Importantly, this information converges on Purkinje cells and deep cerebellar nuclei, namely, the interpositus nucleus. However, the relative importance of these two sites and the underlying mechanism of cerebellum-dependent eye-blink motor learning have remained largely unclear.

The IMCT technology is very useful for delineating the synaptic mechanisms of a specific neural network. However, this technique irreversibly ablates synaptic transmission in the target network. We therefore developed a novel technique termed “reversible neurotransmission blocking”

(RNB) that allows us to reversibly block synaptic transmission in a specific neural network. Mutsuya Yamamoto came from the Mitsubishi Pharma Company to learn mammalian molecular biology and together with a Ph.D. student, Norio Wada, developed the RNB technique during my stay at Kyoto University (Yamamoto et al., 2003). With the RNB technology, we generated two lines of transgenic mice. One line of transgenic mice selectively expressed tetracycline-activating transcription factor in granule cells under the control of the $GABA_A$ α 6 promoter. In the second line, the fusion protein of tetanus toxin and GFP was controlled by the tetracycline-activating transcription factor. Tetanus toxin is a bacterial toxin that selectively cleaves the synaptic vesicle VMMP2 and blocks transmitter release from the synaptic vesicle. When these two lines were mated, tetanus toxin was selectively expressed in granule cells, dependent on the administration and omission of a tetracycline derivative, DOX. This procedure selectively and reversibly blocked granule cell transmission to Purkinje cells. As a consequence, the conditioned stimulus is not transmitted to Purkinje cells, but this information is still conveyed to the interpositus nucleus. Therefore, a reversible blockade of granule cell transmission can delineate the role of the two information pathways in conditioned eye-blink responses.

Norio Wada performed electrophysiology and behavioral experiments on conditioned eye blink motor learning (Wada et al., 2007). We confirmed the reversibility of granule cell transmission to Purkinje cells by extracellular recording of Purkinje cells in awake animals. This blockage, however, had no influence on climbing fiber transmission. We therefore succeeded in reversely manipulating the blocking of granule cell transmission to Purkinje cells without any impairment of responses to the climbing fiber input. We then tested the conditioned eye-blink responses in this model animal. In RNB mice, the conditioned eye-blink response disappeared during the administration of DOX and was recovered by the omission of DOX. Importantly, when granule cell transmission was recovered by the withdrawal of DOX, the normal conditioned response was immediately induced at the beginning of the second conditioning session of the pretrained RNB mice. This finding explicitly demonstrated that although the conditioned response is not expressed during DOX treatment, this memory is acquired and stored in RNB mice during DOX treatment. We further confirmed that memory acquisition and storage in DOX-withdrawn RNB mice was completely abolished by a bilateral lesion of the interpositus nucleus.

These results demonstrate that the information in conditioned signals to Purkinje cells is necessary for the expression of conditioned responses, but the memory is acquired and stored despite the absence of conditioned signals to Purkinje cells. The most plausible interpretation of our study is that the blockage of granule cell transmission relieves tonic Purkinje cell inhibition, and that the interpositus nucleus induces latent neural plasticity in response to paired conditioned and unconditioned signals. This neural

plasticity should be critical for memory acquisition and storage and would allow prompt expression of conditioned responses once the expression process is restored by the recovery of granule cell transmission.

Concluding Remarks

When I look back upon my research life of more than 40 years, starting from the age of 25 years old, I feel that I have performed my research work on the basis of consistent thinking and attitudes toward my research projects. First, I have maintained a great interest in the mechanisms underlying intercellular communication from the standpoint of investigating molecular constituents involving a targeted biological system. This attitude probably reflects my fondness for chemical analysis, which has certainly stimulated my imagination to explore molecular mechanisms underlying intercellular communication in various parts of biological systems. For those who have this inclination, recombinant DNA technology provides a superb approach for predicting the possible functions of a molecule of interest from its deduced structural characteristics, and for verifying its functions through various techniques related to recombinant DNA technology. I was fortunate in being able to enjoy working during a revolutionary period in the development of recombinant DNA technology.

Second and needless to say, different approaches such as molecular biology and electrophysiology have greatly contributed to establishing the principal concepts of biological systems. However, because common mechanisms often underlie apparently distinct biological phenomena, it is usually very difficult to find a novel principle using a well-established methodology. I have therefore aimed at developing new and logical approaches as much as possible and also directed our research projects toward the boundary between different fields. In this context, I like Sydney Brenner's witty remark, saying, "Progress in science depends on new techniques, new discoveries, and new ideas, possibly in that order." In many cases, the introduction of a new tool did not work as well as expected, but in some cases it led to a new and expected finding. More exciting, new approaches sometimes led to an unexpected and more interesting finding. To establish a new tool, it is essential to organize a collaborative team with members from different fields. In Kyoto University, we fortunately were able to organize a nice collaborative triangle between molecular biologists (our group), electrophysiologists (Motoy Kuno, Haruki Ohmori, Tomoyuki Takahashi, Tomoo Hirano, Toshiya Manabe, etc.) and morphologists (Ryuichi Shigemoto, Noboru Mizuno, etc.) This triad was not only fruitful for directing new research projects through collaborative studies but I also greatly enjoyed many exciting discussions about the different viewpoints of our colleagues in different fields.

Finally, as I mentioned, our research advances were mostly achieved by a number of Ph.D. students working with me. They had to gain the basic

knowledge and techniques of bioscience and medical science at the beginning and to learn how to design and proceed in experimental research from the beginning. My graduate students were so talented and self-motivated that they came to organize their own research projects and to explore exciting mechanisms during the latter stages of their Ph.D. course. Consequently, I enjoyed not only looking at the exciting progress of their research works, but also recognizing the remarkable development in their abilities as research scientists. My 40-year research life has been a very pleasant time during which I have been able to experience the creativity of science and young talented scientists.

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