Review

Contributions to the field of neurotransmitters by Japanese scientists, and reflections on my own research

By Masanori Otsuka[†]

(Contributed by Masanori Otsuka, m.j.a.)

Abstract: Part I describes important contributions made by some Japanese pioneers in the field of neurotransmitters: (their achievements in parentheses) J. Takamine (isolation and crystallization of adrenaline); K. Shimidzu (early hint for acetylcholine as a neurotransmitter); F. Kanematsu (donation of the Kanematsu Memorial Institute in Sydney); T. Hayashi (discovery of the excitatory action of glutamate and the inhibitory action of GABA); and I. Sano (discovery of a high concentration of dopamine in striatum, its reduction in a patient with Parkinson's disease and the treatment with DOPA). In Part II, I present some of my reflections on my research on neurotransmitters. The work of my colleagues and myself has made some significant contributions to the establishment of neurotransmitter roles played by GABA and substance P, the first amino acid and the first peptide neurotransmitters, respectively. By the early 1960s, 3 substances, i.e., acetylcholine, noradrenaline, and adrenaline, had been established as neurotransmitters. Now the number of neurotransmitters is believed to be as many as 50 or even more mainly due to the inclusion of several amino acids and a large number of peptide transmitters.

Keywords: Neurotransmitter, GABA, substance P, enkephalin, Japanese pioneers

Introduction

Research on neurotransmitters occupies an important field in neuroscience, and many Japanese scientists have made important contributions to that field. When the Editorial Board of the Proceedings of the Japan Academy (PJA) decided to invite all members of the Academy to contribute archival reviews on their own research, I was hesitant to accept the invitation for various reasons. On the other hand, I thought that it might be a good opportunity to review some of the important contributions made by Japanese scientists. After some thought, therefore, I decided to accept to write an archival review, firstly on some important contributions to the field of neurotransmitters made by Japanese scientists (Part I), and secondly on my own research (Part II). Indeed, my research on neurotransmitters represents an extension of the path paved by many international scientists including the Japanese pioneers described here.

Abbreviations: CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione; CNS: central nervous system; CSF: cerebrospinal fluid; DAEA: [D-Ala^2]-methionine-enkephalinamide; D-APV: D(−)-2-amino-5-phosphonovaleric acid; DOPA: 3,4-dihydroxyphenylalanine; EPSP: excitatory postsynaptic potential; GABA: γ-aminobutyric acid; HPLC: high-performance liquid chromatography; IMG: inferior mesenteric ganglion; IPSP: inhibitory postsynaptic potential; MSR: monosynaptic reflex; NKA: neurokinin A; NKB: neurokinin B; PI: principal investigator; PJA: Proceedings of the Japan Academy; PNAS: Proceedings of the National Academy of Sciences, USA; RIA: radioimmunoassay; SP: substance P; VRP: ventral root potential.

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Part I. Some Japanese pioneers

1. Chemical transmission and neurotransmitters. In the 1920s and 1930s, O. Loewi, H.H. Dale and their collaborators established the concept of chemical transmission, according to which, neural signals, either excitatory or inhibitory, are transmitted at a majority of synapses by means of chemical messengers, i.e. neurotransmitters or chemical trans-

Fig. 1. Jokichi Takamine (1854-1922) at the age of 15 (arrow). From Ref. 2.

mitters. By the early 1960s, 3 substances, i.e., acetylcholine, noradrenaline, and adrenaline, had been established as neurotransmitters. Subsequent studies from the 1960s to 1980s, however, provided evidence that several amino acids and a large number of peptides serve as neurotransmitters, and as a result the number of neurotransmitters is now thought to be as many as 50 or even more, including ATP and NO.[1)]

2. J. Takamine and adrenaline. Jokichi Takamine was born in 1854 in Takaoka in the middle part of Japan. This was more than ten years before the modern revolution of Japan (the Meiji Restoration), which opened the country to the world after its almost complete closure that had lasted for more than 2 centuries. Figure 1 shows Takamine at the age of 15 in a pre-modern costume. He studied applied chemistry in the newly founded University of Engineering in Tokyo and graduated therefrom in 1879.[3)] The University of Engineering soon became the Faculty of Engineering of the Imperial University of Tokyo, the first university in Japan that was founded in 1877. It was a period of rapid modernization of Japan.

In 1900, Takamine, together with his assistant Keizo Uenaka, was trying to isolate the active principle from the extract of mammalian adrenal glands in a private laboratory in New York, that was affiliated to a pharmaceutical company, Parke Davies. Six years before, G. Oliver and E.A. Schäfer had discovered that the extract of mammalian adrenal glands exerts a powerful hypertensive action on blood pressure.[1)] Based on this observation, trials to isolate the active principle were made by J.J. Abel, O. von Fürth and Takamine in a competitive setting.[3)]

Uenaka (1876-1960) had been trained by Prof. Nagayoshi Nagai in the Imperial University of Tokyo and just joined Takamine in 1900. In August of this year, Uenaka succeeded in crystallizing the active principle of adrenal glands by adding a large excess of ammonia to a highly concentrated extract.[3),4)] Its powerful vasoconstrictive action was immediately confirmed. The crystallized principle was named Adrenalin and its molecular formula was determined by Takamine and Uenaka as $C_{10}H_{15}NO_3$. The latter was soon slightly corrected by their colleague T.B. Aldrich as $C_9H_{13}NO_3$. In the early 1901, Parke Davies & Co. distributed Adrenalin widely in the United States for its clinical use.[3)] Takamine patented his discovery in 1900 and reported it at a meeting of the British Physiological Society in 1901.[5)]

Abel, on the other hand, had reported a few years earlier the isolation of the active principle from the extract of adrenal glands, named it epinephrin, and reported the molecular formula as $C_{17}H_{15}NO_4$. It turned out that epinephrin contained one benzoyl residue as could be suggested from its molecular formula. Furthermore, the activity of Abel's product was probably small.[3),6)] Abel reminisced later that Takamine had visited his lab in the autumn of 1900, and thus a discussion was raised about the possibility that Takamine might have obtained a hint for the isolation of adrenaline then.[6),7)] Recently, however, the experimental record of Uenaka (Memorandum) was found, which shows that his success of crystallization was from July the 21st to August the 4th whereas the visit of Takamine to Abel's lab was after September.[3),6)]

Dale wrote in his paper published in 1906: In accordance with physiological custom the name "adrenaline" is used throughout this paper to denote the active principle of the supra-renal gland $\cdots$.[8)] Since Adrenalin was a patented name of Parke Davies & Co., whereas Dale belonged to another pharmaceutical company, Wellcome, a pressure from some executives of Wellcome & Co. was exerted on Dale not to use the name adrenaline. Dale resented that interference and it was one of the reasons of his leaving Wellcome & Co.[1),4)] In their paper of 1910, G. Barger and Dale used the term adrenine instead of adrenaline.[9)]

M.H. Lewandowsky (1899) and J.N. Langley (1901), extending the observation of Oliver and Schäfer, found that the effects of the extract of

adrenal glands were similar to those of sympathetic nerve stimulation.[10),11)] After adrenaline became available, Langley examined the action of adrenaline on various visceral organs and reached the generalization that the effect of adrenaline is the same as the effect of exciting the sympathetic nerves. T.R. Elliott, a medical student working in Langley's department, extended Langley's observations and presented the results at a meeting of the Physiological Society, and concluded his presentation with the often-quoted statement that: "Adrenalin might then be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery".[12)] This is considered as the origin of the concept of chemical transmission.

3. K. Shimidzu and an early hint for acetylcholine as a neurotransmitter. By his classical experiment performed in 1920, Otto Loewi in Graz, Austria, showed that the stimulation of the vagus nerve, which exerts an inhibitory effect on the heart of the frog, caused a release of a substance mediating an inhibitory action on the heart, and called this substance "Vagusstoff" (vagal substance). Later study of Loewi and E. Navratil (1926) demonstrated that Vagusstoff was acetylcholine, which represents the establishment of the first neurotransmitter.[1)]

In the meantime, W.R. Hess in Zürich reported at the XIth International Physiological Congress in 1923 that acetylcholine caused a contraction of frog skeletal muscle and that an acetylcholine-like substance was released into the perfusate of the muscle in response to motor nerve stimulation.[13)] Kenmatsu Shimidzu (Fig. 2A) from Japan was working in the department of Prof. Hess at that time and examined this problem. Stimulation of a motor nerve innervating a skeletal muscle of the frog resulted in a liberation into the perfusate of a substance, which produced a contraction of frog intestine, and an inhibition of frog heart contraction, and both these effects were blocked by atropine. Shimidzu therefore suggested that the released substance was similar to acetylcholine.[14)] This suggestion of Hess and Shimidzu was later confirmed by Dale, W. Feldberg and M. Vogt at the mammalian neuromuscular junction in 1936.[15)]

The early hint by Hess and Shimidzu for acetylcholine as the neuromuscular transmitter was cited in the paper of Dale *et al.*,[15)] and therefore I used to mention Shimidzu's name and his achievements in my lecture to medical students at the University

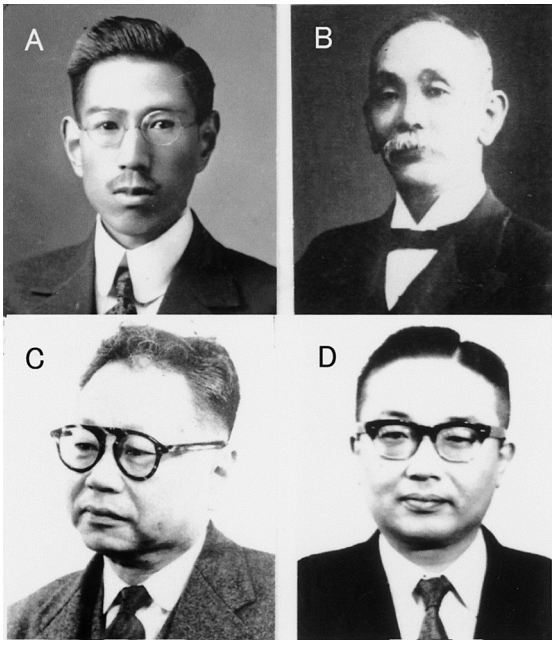

Fig. 2. Four Japanese pioneers. (A) Kenmatsu Shimidzu (1893-1992); (B) Fusajiro Kanematsu (1845-1913); (C) Takashi Hayashi (1897-1969); and (D) Isamu Sano (1924-1975). (B) and (D), from Refs. 18 and 110.

of Tokyo, but we did not know where Shimidzu was. My mentor, Prof. Setsuro Ebashi, wrote an essay in the journal of the Japanese Academic Community Club, and at the end of the article he added a note: "To our regret we don't know where Dr. Shimidzu is".[16)] Very soon he received a letter from Shimidzu, who wrote that he had graduated from the University of Tokyo, Faculty of Medicine in 1918, studied physiology and pharmacology in Europe from 1923 to 1925, came back to Japan and had been working in a private clinic in Kanazawa as an ophthalmologist. Naturally he was very pleased.

4. F. Kanematsu and the Kanematsu Memorial Institute in Sydney. Fusajiro Kanematsu (Fig. 2B) was not a scientist but a successful merchant, who founded a company Kanematsu dealing in the trade of wool and rice between Australia and Japan. In 1929 to commemorate the 17th anniversary of the death of F. Kanematsu and to promote the friendship between Australia and Japan, Kanematsu & Co. made a donation to the Sydney National Hospital, by which the Kanematsu Memorial Institute of Pathology was founded in 1933.[17),18)] It is well known that in the early 1940s the three giants in neurophysiology, J.C. Eccles, B. Katz and S.W. Kuffler, had worked in this institute and pub-

lished a series of brilliant papers on neuromuscular transmission, which consolidated the postulate of Dale *et al.* that acetylcholine is the transmitter at the neuromuscular junction of the vertebrates.[15]

During that period World War II broke out. In one of their papers it is written in a footnote that: "Dr. Eccles' paper was posted from Australia on December 17, 1941. Owing to conditions prevailing in the South Pacific he has not been able to examine proofs. Drs. Katz and Kuffler, who have papers in this issue, were also unable to read their proofs".[19] Since Australia was fighting against Japan it was proposed to eliminate the name of Kanematsu from the Institute. However the Director of the Institute, Sir Norman Paul, resisted to that move, postulating that the donation was based on the goodwill of Kanematsu & Co. and this should be appreciated. Because of this action, Sir Norman Paul risked his position.[17]

Later when I met Prof. Kuffler (see Part II), I asked him how it had been possible that a miraculous gathering of three eminent scientists could occur, like a triple collision of comets. I don't remember the exact wording of Dr. Kuffler, but his reply meant that many young scientists could have a possibility of encountering such a luck. I think he wanted to say that he was a simple young man when he arrived in Sydney. The comment of Dr. Kuffler shows his usual humbleness and encouragement to young people.

5. T. Hayashi and the discovery of the excitatory action of glutamate and the inhibitory action of GABA. Takashi Hayashi (Fig. 2C) was Professor of Physiology in Keio University, School of Medicine. He was trained by Prof. Genichi Kato and then went to Soviet Union to study in the laboratory of Prof. I.P. Pavlov from 1932 to 1933. His main interest was in "chemical physiology of excitation".[20] In 1943, he applied a concentrated solution of Na-glutamate to the motor cortex of dogs and found that it elicited a convulsion. Based on this observation he suggested that glutamate may play some physiological role.[21),22] This was a remarkably early discovery of the excitatory action of glutamate. The study was published in Japanese during World War II and in English in 1954.[22] The first well known study dealing with the excitatory action of L-glutamate on the crustacean neuro-muscular system was published by J. Robbins in 1958.[23] One of the reasons why Hayashi was interested in glutamate was its abundant presence in the brain.

Hayashi also studied the effect of GABA on dog motor cortex and found its inhibitory action. The result was reported at the XXth International Physiological Congress in Brussels in 1956.[24] In the same year A. Bazemore *et al.* reported their study describing the inhibitory action of GABA on crustacean stretch receptor.[25] These studies represent the first finding of the inhibitory action of GABA.

The neurotransmitter roles of L-glutamate and GABA were elucidated much later from the 1960s to 1980s.

6. I. Sano, dopamine and Parkinson's disease. In 1959, Isamu Sano (Fig. 2D) and his colleagues examined the distribution of dopamine in the human brain and found that dopamine, but not noradrenaline, was specifically concentrated in the striatum.[26] This finding was made simultaneously with and independently from the similar finding of A. Carlsson in the dog brain,[27] and suggested that dopamine has not only a role as a precursor of noradrenaline but has a role of its own in the CNS. Since the extrapyramidal system, comprising the striatum, was known to be involved in Parkinson's disease, Sano measured the dopamine contents in the striatum of the brain of an autopsied patient with Parkinson's disease in August 1959. In this single patient Sano found a definite decrease of dopamine in the striatum and the finding was reported in a neuropathology meeting in Tokyo in February 1960.[28] Furthermore, Sano injected a precursor of dopamine, DOPA, intravenously into 5 patients with Parkinson's disease, and noted that it had some beneficial effect. The effect was not dramatic as he wrote in a review published in a Japanese journal (1960),[29] presumably because he used D, L-DOPA instead of L-DOPA, the latter being unavailable for him then.[28] Sano did not further pursue this treatment with racemic DOPA.

The finding by Sano of the decrease of dopamine in the striatum of a Parkinson's disease patient and the effectiveness of DOPA injection in patients with Parkinson's disease was only preliminary and was published in a review in the October 1960 issue of a Japanese journal.[29] But the findings were quite early and appear to precede the reports by Hornykiewicz *et al.*[30),31]

Part II. My research on neurotransmitters

1. To Prof. Kuffler's laboratory. In 1964

I had a great luck to be accepted as a postdoc in the Laboratory of Neurophysiology, Harvard Medical School, presided by Prof. Stephen Kuffler. In retrospect I feel how fortunate I was, because I later heard that, due to the fame of Prof. Kuffler, every day a letter of application for a postdoc position arrived in his office so that the probability of acceptance was extremely low. Indeed the group of Kuffler, belonging to the Dept. of Pharmacology, was very small comprising only about ten researchers.

In early 1963, my mentor, Ebashi, Professor of Pharmacology, University of Tokyo, Faculty of Medicine, arranged an interview for me with a Rockefeller Foundation officer, Dr. L. Gregg for a possible fellowship. As a result I was given an opportunity to go abroad with a Rockefeller Foundation fellowship. I wrote a letter to Prof. Kuffler expressing my eager hope to study in his laboratory. Very soon I received his reply advising me that, since there was no vacancy in his laboratory to accept me, I might possibly seek the possibility of working with someone in the Dept. of Pharmacology, Harvard Medical School. Then Prof. Ebashi proceeded in a difficult negotiation to persuade Prof. Kuffler, who wrote suddenly in the summer of 1963 to advise me to come to Boston. It was extremely fortunate for me and I am still puzzled why he changed his mind.

In September 1964, I arrived at Boston Airport accompanied by my wife and two small children. We were met there by Dr. David Potter, who was already a famous neuroscientist for his discovery together with Dr. E.J. Furshpan of the first electrical synapse. Dave brought us to an apartment near the Medical School, where, to our surprise, the refrigerator was full of food and the beds were covered with fresh sheets, pillows etc.

2. Abdominal ganglia of the lobster. The next morning, leaving my family in confusion, I went to the lab and met Steve (Prof. Kuffler), who told me that I should work with Dave on the GABA project. It was soon after the publication of a series of the now classical papers authored by Edward Kravitz, Kuffler, Potter and their colleagues in J. Neurophysiology,[32] showing that inhibitory axons of lobsters contained a high concentration of GABA, more than 100 times higher than that in excitatory axons.[33] This finding suggested strongly that GABA was the inhibitory transmitter in the lobster peripheral nervous system.

Dave told me that we were going to work on cell bodies in the lobster abdominal ganglia. So I went to the cold room, picked up a live lobster and dissected out a chain of abdominal ganglia which were connected in situ to the abdominal part of the lobster. When I removed the connective tissue sheath covering the ganglion, I could see vaguely something like cell bodies. I impaled a microelectrode into one of those, and thus obtained the resting potential. In the electrophysiological setup of Steve's group there was a switch which connected the microelectrode either to a preamplifier or a stimulator. By the use of this arrangement I tried to give a stimulus through the microelectrode. To my surprise I saw a faint movement of the tail evoked by the stimulus. I reported the observation to Dave, who was greatly excited by my report and walked down the corridor and told the members of the laboratory: "Masanori developed a wonderful preparation!". People came one after another to visit my setup, gave a stimulus and confirmed the movement. I was astonished by the familial atmosphere of Steve's lab. In retrospect it seems to me that Dave was trying to encourage a middle-aged newcomer who was under great tension after arriving at the high standard laboratory. After repeating similar experiments almost every day, it became clear that every lobster abdominal ganglion had a more or less constant cellular architecture (Fig. 3). Dave and I identified more than 10 excitatory cell bodies each innervating a specific muscle or muscles.

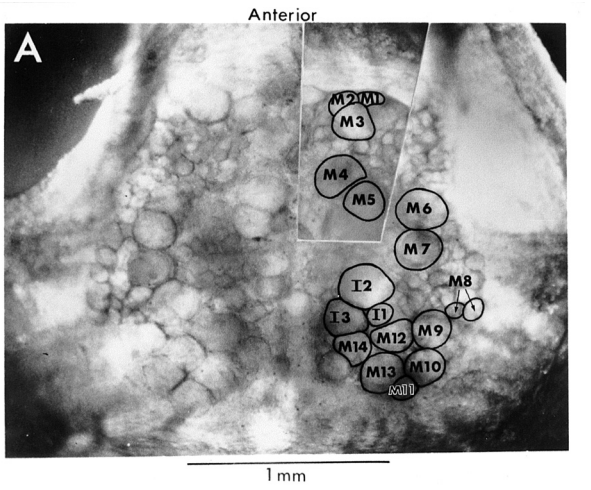

Fig. 3. Architecture of the 2nd abdominal ganglion of the lobster. M_{1-14} are excitatory cell bodies and I_{1-3} are inhibitory cell bodies. From Ref. 35.

One day I was recording intracellularly from one of the abdominal muscles, i.e. superficial flexor mus-

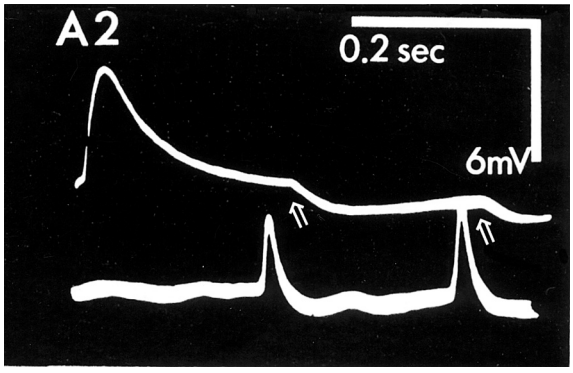

Fig. 4. Simultaneous recordings from I_1 cell body and superficial flexor muscle. Arrows indicate the coincidence between the action potential in the cell body (lower trace) and IPSP in the muscle (arrows). From Ref. 35.

cle, and noticed that the muscle fibers displayed frequent spontaneous IPSPs. This presented a nice opportunity for finding an inhibitory cell body. I searched for a cell body producing these IPSPs in the muscle by recording from one cell body to the next in ganglia. I was very pleased when I found a small-sized cell body (I_1 in Fig. 3) displaying action potentials which showed one-to-one coincidence with IPSPs in the muscle (Fig. 4). I reported it to Dave, and again I received many visitors who confirmed the coincidence between action potentials and IPSPs. This was the first inhibitory cell body we found.

Neighboring this inhibitory cell body (I_1), there were two large cell bodies which did not evoke muscular contraction upon stimulation (I_2 and I_3 in Fig. 3). Ed Kravitz and Dave proposed to isolate these silent cell bodies and measure their GABA contents, and as a result we found that these silent cells contained a high concentration of GABA. Therefore Dave and I stimulated intracellularly these cell bodies and looked for IPSPs in various muscles in the abdomen. It soon turned out that these two large inhibitory cell bodies innervate flexor and extensor muscles respectively.

Dave and I accumulated daily new information on the physiological and chemical architecture of the abdominal ganglia. It gradually became clear that many, or possibly all, cell bodies have constant sizes and locations in the ganglia. Excitatory cell bodies always contained a low concentration of GABA, whereas inhibitory cell bodies contained a high concentration of GABA, which was in good parallel with the finding of Ed, Steve and Dave on excitatory and inhibitory axons as published in 1963.[32]

We decided that I would present these data at the Federation of American Societies for Experimental Biology (FASEB) meeting at Atlantic City in the spring of 1965. During those years in Steve's group it was customary to organize a rehearsal before the presentation at any scientific meeting. Furthermore Dave patiently corrected my rehearsal many times even after arriving at Atlantic City. As a result my presentation was received with great enthusiasm by the audience with three rounds of big applause.[34] After I came back to Boston, my wife received a letter from Steve saying: "Well, you should know that Masanori had a very great success and I don't remember any other brief talk with such an enthusiastic reception". Again Steve was trying to encourage his new postdoc in his typical manner. I still cherish this letter now.

After the meeting I continued perfecting the map of the ganglion by identifying as many cells as possible. One day in the corridor Steve asked me what I was doing, and when I replied to him that I was trying to complete the mapping, he said, if I did that, it would take a lifetime. I was surprised and stopped pursuing the mapping.

I wrote a lengthy manuscript on the lobster abdominal ganglion. Dave praised my writing but rewrote it completely.[35] Steve read it thoroughly and gave us many useful comments. Furthermore Dave Hubel and Torsten Wiesel kindly read our manuscript and gave their comments. I felt honored because I thought that it was rare for them to be interested in works from other unrelated field. Probably they might have been interested in the constancy of the cellular architecture of the lobster ganglion.

3. Demonstration of the release of GABA from the lobster neuromuscular junction. I felt a great sense of relief because I thought that now I could go back home with an accomplishment after my stay in this high standard laboratory. Therefore I wanted the challenge of a risky but important project. Although the work of Ed, Dave, Steve *et al.* in 1963 strongly suggested that GABA was an inhibitory neurotransmitter in the crustacean nervous system, an important piece of evidence was still missing for the final acceptance of GABA as a transmitter, namely the demonstration of the release of GABA from inhibitory axon upon stimulation. This represented an important final item among the criteria for the identification of transmitter.

Before starting the experiment I thought that

many people must have previously tried similar experiments with the same purpose without success. So I decided to take advantage of our recent findings. I converted radioactive L-glutamate to radioactive GABA by the use of glutamic decarboxylase and filled a microelectrode with the hot GABA. I then inserted the microelectrode into an inhibitory cell body (I_1) innervating the superficial flexor muscle. After some time, presuming that the radioactive GABA was transported to the nerve endings of the inhibitory neuron, I isolated the nerve-muscle preparation, perfused it with artificial sea water and counted the radioactivity of the perfusates with a liquid scintillation counter. Upon stimulation of the nerve bundle containing an inhibitory axon innervating the muscle, I found no change in the radioactivity.

Then I soaked a nerve-superficial flexor muscle preparation in an artificial sea water containing the radioactive GABA. After some time presuming that the radioactive GABA was taken up by nerve terminals of the inhibitory neuron, I perfused the preparation with cold sea water and measured the radioactivity in the perfusates. In some experiments, there were small but definite increases in the radioactivity in the perfusates upon nerve stimulation. I showed the results to my colleagues, but no one showed any enthusiasm.

After a few months of working in vain, I was discouraged. One day I met Steve in the corridor, and he asked me about the progress of my experiments. When I told him that I could not get an increase in GABA release upon stimulation, he said: "Maybe GABA is not the transmitter". It was clear that he was joking. But I was slightly upset because when I was desperate, I often said to myself: "This is Steve's project, and I know that his ideas are always right". Steve prepared, instead, an opener muscle of a walking leg of a lobster for a release experiment. While I was impressed by the beauty of the preparation, the experimental result was again negative. I consulted my colleague Dick Orkand working next door as to what I should do now. He said that, since I had invested too much time it was too late for me to change the project.

One day Ed Kravitz told me that he and Leslie Iversen, who had come recently from Dr. J. Axelrod's lab to join us, succeeded in developing a new method to measure GABA, and I should simply isolate a nerve-muscle preparation of the lobster, perfuse it, stimulate the inhibitory axon, and give them the perfusates. The new GABA assay consisted of separating GABA from a large amount of salts in the perfusates, the molar ratio being around $1{:}10^8$, by the use of ion-exchange resin columns, and then measuring GABA by the usual enzymatic method.

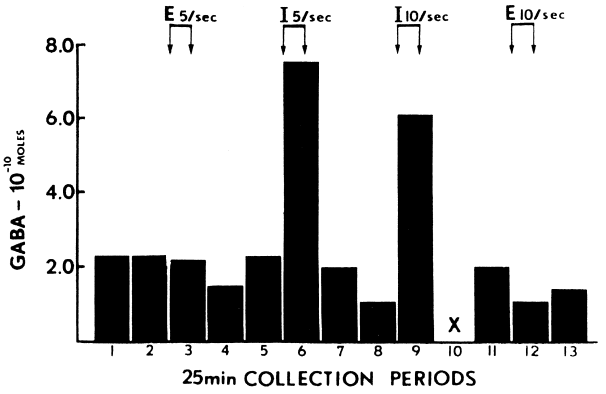

Fig. 5. Release of GABA from an inhibitory axon innervating the opener muscle of a lobster claw upon stimulation. E and I: stimulation of single excitatory and inhibitory axons. X: failure of assay. From Ref. 36.

This experiment based on this simple principle clearly showed that GABA release from the superficial flexor muscle was increased upon stimulation of the nerve bundle containing an inhibitory axon. Ed dissected the nerve bundle separating the single inhibitory axon innervating the muscle, demonstrating his great skill in dissection. As a result it was shown that the GABA release was increased upon stimulation of the inhibitory axon. We asked Zach Hall, then a Ph. D. student, to join us, so that Ed, Les, Zach and I tried to increase the number of experiments and to make the results as convincing as possible. Ed proposed to try the opener muscle of big claw of the lobster and he himself skillfully dissected single excitatory and single inhibitory axons innervating the muscle. The GABA release was increased upon stimulation of the inhibitory, but not the excitatory, axon (Fig.5). People worked hard until past midnight and I was astonished by the stamina shown by my colleagues, whereas I was exhausted. There was very little time left for us because Les, Zach and I were to leave the laboratory in a few months.

Shortly before my departure, it was clear that we had conclusive evidence for GABA release and that we were going to write a paper to be published in PNAS, and we wanted Steve to join us as a coauthor, since the GABA project was started by him in the

1950s, he participated in some of the experiments, and the paper would represent the final goal of the project. Ed urged me to go to Steve to persuade him, but he added that there was very little hope. I did go and asked Steve, but he firmly refused our proposal.

After the departure of Zach to California and mine to Japan, Ed and Les completed the final experiments and wrote a manuscript to be introduced by Steve to PNAS.[36] In addition, Les presented a paper to the British Physiological Society in November 1966,[37] Ed and Dave attended a symposium in Stockholm on "Structure and Function of Inhibitory Neuronal Mechanisms" in September 1966 and presented our results on the mapping of the lobster ganglion and GABA release from the lobster neuromuscular junction upon stimulation of the inhibitory axon.[38),39] After these presentations Dr. J. Dudel summarized the session and commented as follows: "As you all know there are only a few instances of synaptic transmission in which the transmitter is fully identified according to the criteria cited above $\cdots$. After this session we can add another substance to the list of approved transmitter, namely GABA as an inhibitory transmitter in the crustacean neuromuscular junction".[40] Indeed this was the moment when the third neurotransmitter, following acetylcholine and catecholamines, was approved.

After coming back to Japan, I received a letter from Steve saying: "Your success was complete". I knew by then that it was his typical kindness, but I felt a great relief from the tension of staying in this extremely high standard laboratory. My stay in Harvard was from 1964 to 1966 soon after the end of the presidency of J.F. Kennedy, whose influence to induce excitement and optimism, it seemed to me, still prevailed the American scientific community.

4. To Tokyo Medical and Dental University. Early in 1966 I was appointed as Professor of Pharmacology at Tokyo Medical and Dental University, Faculty of Medicine, and the dean, Prof. Yasuji Katsuki asked me strictly to return to Tokyo by June, forcing me to leave Ed and Les in the final stage of the experiments. Before leaving I learned from Ed the enzymatic assay of GABA developed by W.B. Jacoby and E.M. Scott.[41] So after my return to Tokyo I tried to increase the sensitivity of the GABA assay by combining the enzymatic cycling of O. Lowry.[42] Thus we could increase the sensitivity of the enzymatic GABA assay by two to three orders of magnitude.[43]

The previous experiments of Kunihiko Obata and Masao Ito gave strong evidence that cerebellar Purkinje cells send their axons to vestibular nucleus and exert a GABA-mediated inhibition on dorsal Deiters cells.[44] Obata had moved from Ito's lab to join us as an Assistant Prof. He isolated Deiters cells from the cat brain stem for the high sensitivity GABA assay. We found that isolated dorsal, but not ventral, Deiters cells contained a high concentration of GABA, which, however, was markedly reduced after the removal of the cerebellar vermis.[43] This suggested that GABA was concentrated in axon terminals of Purkinje neurons making synapses with dorsal Deiters cells, which of course was in accordance with the original hypothesis of Obata and Ito.[44] Our finding suggesting the presence of GABA in axon terminals attached to dorsal Deiters cells was further confirmed by Y. Okada with direct measurement of GABA contents of presynaptic terminals attached to dorsal Deiters cells.[45]

5. Research on substance P. Around 1970 I felt that the role of GABA as an inhibitory transmitter was established both at the crustacean neuromuscular junction and in the mammalian CNS. However there had been still little information about excitatory transmitters in the mammalian CNS. I reflected that at the start of their GABA project, Ed, Steve and Dave had isolated single excitatory and inhibitory axons and compared the contents of physiologically active substances in both kinds of axons.[32]

A similar arrangement had been known to exist in the mammalian CNS, namely, the ventral and dorsal roots of spinal nerves. Loewi and H. Hellauer in 1938 had shown that ventral roots of the dog and cat consisting of motor nerve fibers contained a high concentration of acetylcholine, i.e. their transmitter, whereas the dorsal roots consisting of sensory fibers did not.[46] Extending this finding, Hellauer and Umrath compared the vasodilator activity of the extracts of bovine dorsal and ventral roots.[47] This attempt was based on the statement of Dale in his Nothnagel Lecture that the discovery and identification of a chemical transmitter of axon-reflex vasodilatation would furnish a hint as to the nature of the transmission process at a central synapse.[48] This suggested, in other words, that a sensory neurotransmitter might be a vasodilator substance. As expected from this suggestion, Hellauer and Umrath

found that the vasodilator activity was higher in the dorsal, than ventral, root extract.[47] Since the discovery of substance P (SP) by U.S. von Euler and J.H. Gaddum in 1931,[49] this substance had been known to be one of the endogenous vasodilator substances. In 1953, several groups of investigators found that SP-like activities, including a vasodilator action, is much higher in dorsal roots than in ventral roots of mammalian spinal nerves.[50),51] Based on this finding, Fred Lembeck proposed a hypothesis that SP may be a sensory transmitter of primary sensory fibers.[50]

This hypothesis, however, had not been accepted during the following two decades. There were two main obstacles: first the chemical nature of SP had not been elucidated (except for its peptide nature) in spite of many attempts to purify SP, and second, electrophoretic application of crude SP preparation to central neurons of the cat brainstem revealed neither excitatory nor inhibitory action.[52] In 1969, M. Vogt stated as follows: Among polypeptides occurring in the brain "substance P", discovered many years ago by von Euler & Gaddum (1931), is thought by some to act as a transmitter substance; there is, however, to date, no good evidence for this view and the suspicion that it may act as a local vasodilator is perhaps a little more likely.[53] Such a view represented the general climate surrounding the hypothesis of Lembeck that SP may be a sensory transmitter.

Around 1970 I became interested in SP because the situation of SP resembled that of GABA in the crustacean peripheral nervous system. Therefore I wanted to examine whether or not SP had any action on central neurons. However, after reading the paper of Galindo *et al.*,[52] I thought that if we used the same methodology, i.e., electrophoretic application of crude SP to central neurons, we would certainly get a similar result. At that time Dr. Makoto Katori working next door gave me a Symposium Record containing his article. In that record Dr. Terumi Nakajima also contributed an article in which he stated that SP in mammals corresponded to physalaemin in non-mammals including amphibians.[54] Although the structure of SP was still unknown it was believed that SP and physalaemin are similar peptides belonging to a group called tachykinins. I called Dr. Nakajima who informed me that Dr. Y. Nobuhara of Dainihon Pharmaceutical Co. had already synthesized physalaemin, so we might be able to get some from him. At my request Dr. Nobuhara kindly gave us synthesized physalaemin and several shorter analogs.

Shiro Konishi, who had recently joined us as a graduate student, and I dissected an isolated spinal cord of a bullfrog, recorded from a ventral root extracellularly and applied physalaemin by perfusion. Thus we used a synthetic peptide and applied it in a way so that we were sure that the peptide reached central neurons at an appropriate concentration. The results showed that a submicromolar concentration of physalaemin produced a depolarization of the ventral root. Since a large part of the depolarization remained after the treatment with tetrodotoxin, which blocked synaptic transmission, we concluded that physalaemin exerted a depolarizing, i.e. excitatory, action on frog spinal motoneurons.[55),56]

At that time M.M. Chang, S.E. Leeman and H.D. Niall[57] reported the structure of SP, 40 years after its discovery in 1931. The structure of SP, in particular its C-terminus, was very similar to that of physalaemin and also to those of some C-terminal analogs of physalaemin, which showed a similar depolarizing action on frog spinal motoneurons. These results strongly supported Lembeck's hypothesis that SP, or some similar substance, is a neurotransmitter of sensory neurons.

Tomoyuki Takahashi joined us then as a graduate student. Konishi, Takahashi and I went to the slaughter-house and collected spinal cords attached with ventral, dorsal roots and cauda equina from hemisected cows. The slaughter-house permitted us to collect the spinal cords, mostly hemisected, from dead cows by ourselves. It was long before the outbreak of BSE (mad cow disease) in the UK (1986) and I feel now fortunate that we could avoid the danger we had never thought of at that time. We dissected ventral and dorsal roots separately and brought them back to the lab to extract them according to the procedure used by Leeman and her colleagues so that the extracts would contain SP, which had been isolated by Chang and Leeman from bovine hypothalamus, if it existed. After the fractionation of the extracts using cation exchange chromatography, we applied the fractions which possibly contained SP to the isolated bullfrog's spinal cord. Only the fraction from the dorsal, but not ventral, roots produced a depolarization of the bullfrog's ventral root. This depolarizing action was abolished after treatment with chymotrypsin, suggesting the

peptide nature of the active principle. Since these results were exactly as expected, we wrote a short paper and submitted it to the PJA, in which Prof. Masahiro Okada, Member of the Japan Academy, introduced our paper.[58]

In the summer of 1972 I was invited to give a plenary lecture at the International Congress on Pharmacology in San Francisco. The main topic suggested by the program committee was GABA, but toward the end of my lecture I presented some new results about physalaemin and the peptide, presumably SP, extracted from bovine dorsal roots. Many people in the audience were very interested in our results on the peptides. Dr. M. Vogt suggested that I should go to Boston to meet Dr. Leeman and ask her for a sample of the newly synthesized SP. After the congress I went to Boston, visited the Neurobiology Department of Harvard (my home lab had now changed its name), where my colleagues kindly arranged a seminar for me with Dr. Susan Leeman as chairperson. It turned out that Dr. Leeman had been working in the Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, just next door to the Dept. of Neurobiology. Susan and I had a fruitful discussion. She gave me a sample of the precious synthetic SP preparation, and we planned a future possible collaboration.

On my way back home I visited Carl Rovainen at Washington University in St. Louis, who had been working next door as a Ph. D. student in Steve's lab in the 1960s. Carl introduced me to Dr. Garland Marshall who had been Carl's friend since they had studied together at California Institute of Technology. I told Marshall that we would need synthetic SP, and he suggested me to synthesize it by ourselves, saying that it was not so difficult.

When I came back to Tokyo, Takahashi compared the motoneuron depolarizing peptide obtained from bovine dorsal roots (we called it tentatively "dorsal root peptide") with Leeman's synthetic SP using high voltage electrophoresis, and easily confirmed that they were identical. Konishi and I applied the synthetic SP to the isolated frog's spinal cord, and confirmed that the action of the synthetic SP was very similar to that of physalaemin as well as to that of the dorsal root peptide. Thus the results were exactly as we had expected.[59),60)]

In the meantime, I received a brief message from Dr. Marshall before his leaving from Tokyo Airport. He wrote that he had attended a meeting in Kyoto and there met Dr. Haruaki Yajima of Kyoto University, who had synthesized SP, so that we should contact him. Dr. Yajima gave us generously a large amount of SP, which greatly facilitated our study.

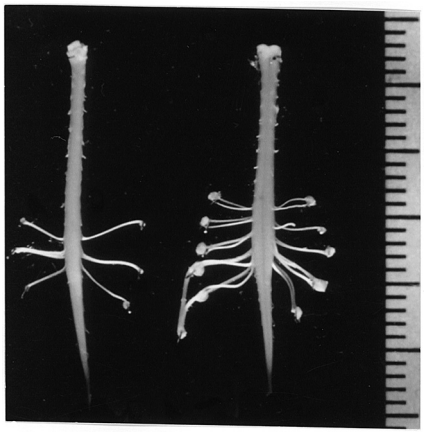

Fig. 6. Isolated spinal cords of newborn rats before (right) and after (left) hemisection. One division in the scale is 1 mm.

Until this point we had been using the isolated spinal cord of the bullfrog, which could be kept functional in oxygenated Ringer's solution. Since we had been aiming at a sensory transmitter in mammals, we should have used a mammalian spinal cord for bioassay. It was believed that the isolated spinal cord of mammals could not survive in the artificial physiological solution. Why can they not survive? The first reason we could think of was the lack of oxygen. We thought that we might be able to supply enough oxygen by diffusion if we used a mammalian spinal cord of small size. Therefore we tried to isolate the spinal cord of a newborn mouse. Konishi was extremely skillful at dissection but even for him it was too difficult at that time because of the small size. One day I went to the animal house to pick up baby mice for experiments, and by chance saw a baby rat which looked huge to me. So I brought back baby rats instead of baby mice; the dissection was easy and we could keep the isolated spinal cord functional and have stable electrophysiological recordings for more than 10 hours (Fig. 6).[61)] Application of synthetic SP at submicromolar concentration range produced a definite depolarization of the ventral root and excitation of spinal neurons as recorded from the ventral root.[62)] A large part of the depolarization was blocked by tetrodotoxin, indicating that the depolarization of motoneurons was mainly due to a transsynaptic action with a small

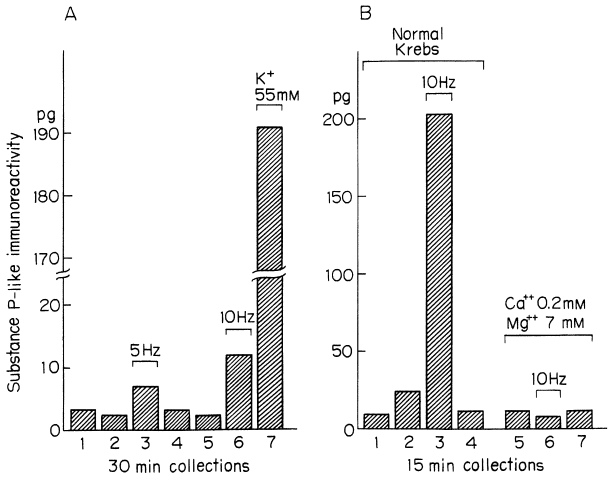

Fig. 7. Release of SP from isolated spinal cords of newborn rats. Stimulation periods and frequencies are indicated in the figure. The duration of each stimulating pulse was 0.01 ms in (A) and 0.3 ms in (B). from Ref. 66.

part being due to a direct action on motoneurons.[63] I should have paid more attention on this observation but did not.

In this connection Takahashi examined the distribution of SP in the cat spinal cord by bioassay and found that SP was most concentrated in the dorsal part of the dorsal horn. The section of the incoming dorsal roots resulted in an almost complete disappearance of SP in this area.[64] These results were confirmed and extended by the immunohistochemical study by Hökfelt and his colleagues.[65] The electrophysiological, neurochemical and immunohistochemical studies altogether suggested that SP was concentrated in axon terminals of incoming primary sensory fibers synapsing on dorsal horn neurons, and SP, if released, would act on these dorsal horn neurons. But at this stage my knowledge about the physiology of the spinal cord was rather poor.

Konishi and I perfused the isolated spinal cord of newborn rats with artificial CSF, stimulated dorsal roots, and collected the perfusates for radioimmunoassay (RIA) of SP. Together with Prof. Noboru Yanaihara, we had raised the antibody against SP, and we sent our samples to Yanaihara's lab in Shizuoka for RIA. As expected, the SP contents in the perfusates during stimulation showed definitely higher values than those during the resting period. The amount of SP released during the stimulation was much larger when we used long duration stimuli (Fig. 7),[66] which are effective for mobilizing thin fibers,[67] than when we used short stimuli.

A few years later Hiroyuki Akagi, an expert of peptide chemistry who joined us as a graduate student, examined the chemical nature of the immunoreactive SP released during the stimulation with high K^+ by the use of HPLC combined with the RIA for SP. The result confirmed that the released SP was indeed the undecapeptide SP identified by Leeman and her colleagues.[68]

The antibody for SP we had been using was C-terminus specific. Since we learned that my old friend Leslie Iversen had recently developed a RIA for SP using a N-terminus specific SP antibody, I thought that if we could send him the lyophilized perfusates of isolated newborn rat spinal cords during stimulation, and obtain the same values of the immunoreactivity using the C-terminus specific and N-terminus specific antibodies, we might be able to conclude that the released SP immunoreactivity would be entirely due to the undecapeptide SP identified by Chang and Leeman. When we sent our samples to Les, after some time in early 1982 I received a letter from him that he had obtained a puzzling result in that the C-terminus specific antibody gave about two times larger values than the N-terminus specific antibody for samples during stimulation. In retrospect, the result was consistent with the idea that the released peptides consisted of SP and neurokinin A (NKA). At that time I did not pay much attention to the results Les informed us, but NKA was discovered in the next year by Kimura *et al.*[69]

6. A stumbling block. One difficulty of working on a possible new neurotransmitter is that the intention of the group becomes obvious for other people so that the group is forced to defend a hypothesis which is not yet proven. When one is invited to talk at a meeting, the audience always asks about the actual status of the new transmitter. I think that one should submit the hypothesis as early as possible to a most rigorous test and if the result is contrary to what one hopes, one should abandon the hypothesis without hesitation.

In 1975, we became interested in a drug called baclofen (Lioresal). In the isolated spinal cord of the newborn rat, baclofen at a low concentration blocked reversibly the monosynaptic and polysynaptic reflexes. Furthermore the drug clearly depressed the depolarization of the ventral root induced by SP.[70] We were excited by these findings, because they seemed to suggest the possibility that SP might be the transmitter mediating the well studied monosyn-

aptic reflex (MSR) in the spinal cord, a hypothesis we had kept in mind for preceding years. In retrospect there were some observations forewarning us that some caution was required. Takahashi examined the hypotensive action of SP on the blood pressure in a dog and found that the SP action was not influenced by baclofen at all. Furthermore, the depolarizing action of glutamate on the ventral root of the newborn rat was also moderately reduced by baclofen. In spite of these facts I continued to be attracted for some time by a slight possibility that SP might be the MSR transmitter.

It was somewhat unfortunate that I had been invited during this period to several international meetings, including some important ones such as the 40th Cold Spring Harbor Symposium in 1975.[71] Although I carefully avoided to suggest explicitly the possibility of SP's being the MSR transmitter, people got an impression at my presentation that we were postulating that, and I did not have definite evidence to deny the hypothesis.

A decisive observation which eliminated the possibility came in 1981. Nagy and colleagues showed that neonatal treatment with a drug called capsaicin in rats resulted in an almost complete disappearance of unmyelinated fibers as well as of SP in dorsal roots of adult rats, whereas myelinated fibers were left intact.[72] Therefore, myelinated group Ia fibers producing the MSR did not appear to contain SP. This had been already suggested in the immunohistochemical paper of Hökfelt *et al.* in 1975.[65] Baclofen turned out to be a $GABA_B$ receptor agonist, and to block spinal reflexes and the SP action on spinal neurons by pre- and/or postsynaptic mechanisms.

7. Tachykinin receptor antagonists. In 1981 the International Congress of Pharmacology was held in Tokyo. After the meeting we organized a satellite symposium on SP in Kyoto. There Dr. Sune Rosell of Karolinska Institute reported that he and his colleagues had succeeded in synthesizing peptides which exerted an antagonistic action against SP.[73] This was the start of the long and steady progress in the development of antagonists against SP and related peptides.

In addition, in the early 1980s there were two important discoveries made by other groups in the field of SP. Firstly, there had been a suggestion of the existence of peptides related to SP in the mammalian CNS. In 1983 Kimura *et al.* reported their discovery of two SP-related peptides in the mammalian spinal cord, neurokinin α and β.[69] These peptides were later named as neurokinin A (NKA) and neurokinin B (NKB) at an International Nomenclature Meeting.[74] Two other groups also reported their independent discoveries of NKA and NKB with different namings.[75),76]

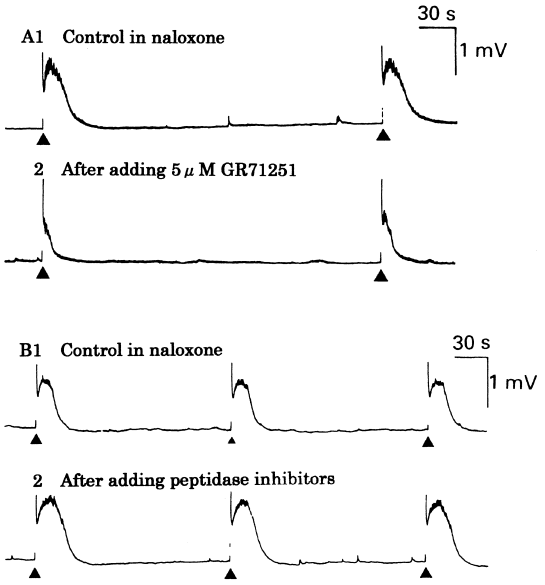

Fig. 8. Effects of an NK_1 receptor antagonist (A) and a mixture of peptidase inhibitors (B) on the slow VRP in the isolated spinal cords of newborn rats. From Ref. 82.

Secondly, there had been steady progress in elucidating the nature of receptors to SP and related peptides, which were classified as NK_1, NK_2 and NK_3 corresponding to SP, NKA and NKB[74] (the reality of the correspondence is not quite so simple). Nakanishi and his colleagues cloned the three types of tachykinins receptors as G-protein coupled receptors.[77]

From 1982 to 1994 we had been for a long time studying the nature of the slow ventral root depolarization (VRP, P stands for potential) induced by stimulation of a dorsal root or a saphenous nerve containing mainly small diameter sensory fibers. The purpose was to establish the neurotransmitter roles of SP and NKA in an experimental system where a stable recording of a response, which from many lines of evidence was suggested to involve tachykinins, was possible. The slow VRP lasts about 30 seconds, corresponding presumably to the time course of tachykininergic slow EPSP in dorsal horn neurons. The slow VRP was markedly curtailed by treatment with capsaicin, which is known to cause depletion

of SP and NKA from sensory C-fibers. The slow VRP was markedly and reversibly depressed by a series of synthetic SP antagonists (NK_1 receptor antagonists) (Fig. 8A).[67), 78)–81)] At an early stage, there had been some criticism concerning the neurotoxicity and specificity of NK_1 antagonists. However there had been continual improvement in the nature of SP antagonists, and the fact that C-fiber-evoked slow VRP lasting about 30 sec was markedly depressed by these SP antagonists provided persuasive, though indirect, evidence that tachykininergic slow EPSPs were involved. In this connection, if we had examined the effects of SP antagonists in the dorsal horn where SP-operated synapses are abundant, using microelectrode and electrophoretic drug application, besides the difficulty of obtaining stable recordings, evidence would have been still indirect because of the complicated anatomy of neural circuits in the dorsal horn.

After experiments with NK_1 receptor antagonists, we examined the effects of the mixture of peptidase inhibitors which inhibited the degradation of SP and NKA by the homogenate of the newborn rat spinal cord. The slow VRP was clearly prolonged by treatment with the mixture of peptidase inhibitors (Fig. 8B).[82)] Although we could not specify the peptidase involved, the results strongly suggested that a peptide-operated slow EPSP is involved in the slow VRP. In these experiments with NK_1 receptor antagonists and peptidase inhibitors, I had in mind the classical work of Eccles, Katz and Kuffler (1942) showing the effects of curare and eserine on end-plate potential.[19)] Although our results were much more crude than the classical work a long time ago, I still was pleased to see our results in good parallel with those of Eccles *et al.*

8. Slow EPSP recorded in spinal motoneurons. The saphenous nerve-evoked slow VRP represents a polysynaptic response induced by primary afferents, in which tachykininergic slow EPSP in dorsal horn neurons are presumably involved. We wanted, however, to study more directly the synaptic potential which may be mediated by tachykinins. Ulfhake *et al.* have shown that certain descending fibers originating from medullary raphe nuclei contain serotonin, thyrotropin-releasing hormone, and SP and that some of these fibers form synapses with spinal motoneurons.[83)] Furthermore there was evidence of the existence on motoneurons of NK_1 receptors responding to NKA and SP.[63)]

Takashi Kurihara, a graduate student in my lab, and colleagues, therefore, examined the nature of the synaptic response induced by stimulation of descending fibers and recorded from motoneurons in the isolated spinal cord of the newborn rat. When we stimulated the cervical spinal cord electrically, and recorded extracellularly from a lumbar ventral root, fast depolarizing potentials followed by a slow depolarization was recorded. A large part of the response was eliminated by the mixture of excitatory amino acid receptor antagonists, i.e., D-APV and CNQX, and a serotonin antagonist, ketanserin, but a small part of the depolarization remained. This depolarization lasting about 1 min could be also recorded intracellularly from motoneurons. The slow depolarization was potentiated by a peptidase inhibitor, thiorphan, and depressed by a tachykinin NK_1 receptor antagonist GR71251. Furthermore, when we treated the isolated spinal cord with a serotonin neurotoxin, 5,7-dihydroxytryptamine, for several hours, the slow depolarization became smaller and was no longer potentiated by the peptidase inhibitor or depressed by the NK_1 receptor antagonist. These results strongly suggested that SP and NKA released from descending serotonergic fibers produce a slow EPSP in motoneurons.[84)]

I had wished to pursue this study because there was a chance to study a monosynaptic slow EPSP caused by SP and NKA, which may be released from descending serotonergic fibers, and this might serve as a prototype of tachykininergic slow EPSP in other areas in the CNS. But I could not finish this line of study.

9. SP in sympathetic ganglia. The presence of SP in sympathetic ganglia of dogs was early demonstrated by Pernow.[85)] This was confirmed by immunohistochemical studies that further showed that principal cells in the inferior mesenteric ganglion (IMG) of the guinea pig are surrounded by dense networks of varicose SP-positive fibers.[86)] Furthermore in 1979, Steve and the Drs. Jan had accomplished beautiful work showing that an LHRH-like peptide was probably a transmitter producing EPSP of long time course in the frog sympathetic ganglia.[87)]

Konishi, A. Tsunoo and myself, therefore, studied the possible neurotransmitter role of SP in the IMG of the guinea pig. We had rapidly accumulated the results fulfilling the criteria for transmitter identification: brief-pulse bath application of SP to ganglion cells induced a depolarization that mim-

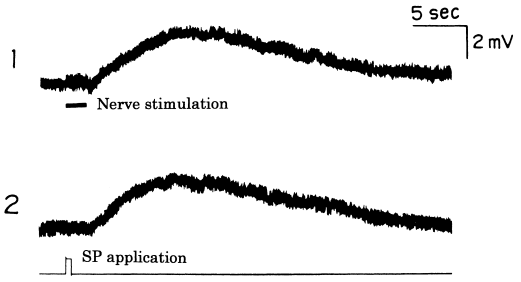

Fig. 9. Slow EPSP (1) and SP-induced depolarization (2) recorded intracellularly from a cell of an IMG of the guinea pig. From Ref. 90.

icked the slow EPSP induced by preganglionic nerve stimulation (Fig. 9); both the slow EPSP and the depolarization induced by SP was blocked by NK_1 receptor antagonists; the ganglia were shown to contain a high concentration of SP, which was markedly reduced by section of the preganglionic nerves; application of high K^+ solution to the ganglia induced the release of SP immunoreactivity in a Ca^{2+}-dependent manner.[88)–91)] In parallel with our study, immunohistochemical and electronmicroscopic studies of Dalsgaard, Matthews, Cuello *et al.* showed that SP-containing fibers in the IMG are collateral branches of primary sensory neurons and form axodendritic synapses upon ganglionic neurons.[92), 93)] In addition, electrophysiological study by Dun *et al.* gave an independent support for the neurotransmitter role of SP in the IMG.[94)]

In the early 1990s, Dr. Fei-Yue Zhao came from the People's Republic of China to visit our laboratory for 2 years as a postdoc and had accomplished a nice piece of work on the prevertebral ganglia of the guinea pig. He and his colleagues had shown that there were two kinds of ganglion cells, one showing a slow EPSP in response to preganglionic nerve stimulation and another a shortening of after-hyperpolarization following action potentials, both effects being of excitatory nature. The two kinds of postsynaptic responses were mimicked by the application of SP or NKA, blocked by an NK_1 receptor antagonist, GR71251, and prolonged by the mixture of peptidase inhibitors.[95)] A large part of Zhao's work was later confirmed by Jobling *et al.*[96)]

Thus the prevertebral ganglia of the guinea pig provided us with an excellent opportunity for examining the possible neurotransmitter role of SP and NKA, and indeed we have here the best pieces of evidence that SP and NKA are released from axon collaterals of primary afferent neurons that innervate principal ganglion cells and that these tachykinin peptides serve as neurotransmitters for slow EPSP and another type of excitation (see above).

10. Enkephalins in sympathetic ganglia. The immunohistochemical study of Schultzberg *et al.* had shown the existence of enkephalin-like immunoreactivity in nerve terminals surrounding nerve cells in the IMG of the guinea pig.[97)] These enkephalin-containing nerve terminals originate in sympathetic preganglionic nuclei of the spinal cord.[98)] Konishi, Tsunoo and myself examined the possible neurotransmitter role of enkephalins in the guinea pig IMG.[99), 100)] Bath-application of Met-, Leu-enkephalins or a metabolically stable Met-enkephalin analog (DAEA) produced an inhibition of cholinergic fast EPSP, but did not affect the response of the ganglion cells to exogenously applied acetylcholine, thus suggesting the presynaptic mechanism of inhibition by enkephalins. Stimulation of lumbar splanchnic nerves also produced an inhibition of cholinergic fast EPSP. In order to examine the mechanism of the neural inhibition, we performed a quantal analysis of the neurally evoked cholinergic EPSP under normal condition, and under conditioning stimulation of the preganglionic splanchnic nerves. The quantal analysis clearly showed the presynaptic mechanism of the inhibition. Furthermore the neurally evoked inhibition was blocked by an opioid receptor antagonist, naloxone. Thus the results altogether strongly suggested that enkephalins serve as neurotransmitters of presynaptic inhibition of cholinergic EPSP in this ganglion. I think that this is probably the most convincing piece of evidence for the transmitter role of enkephalins. We have also obtained evidence that enkephalins serve as transmitters to inhibit presynaptically tachykininergic slow EPSP in the IMG of the guinea pig.

Later in the 1990s, Hidenori Suzuki and colleagues attempted an electrophysiological and biochemical study to examine the possible enkephalinergic mechanism in the isolated spinal cord of the newborn rat.[101)] Although the results suggested the existence of enkephalinergic inhibition in the spinal cord, we could not complete the evidence before my retirement.

Clinical applications of tachykinin antagonists

One of the ultimate purposes of the basic study on SP is its clinical applications. At present many

pharmaceutical companies are actively working in order to develop clinically useful drugs starting from NK_1, NK_2 and NK_3 receptor antagonists. The first drug on the market was an NK_1 receptor antagonist, aprepitant (Emend) of Merck, which is used as an anti-emetic in combination with a serotonin 5-HT_3 receptor antagonist and dexamethasone.[102] Since 2002 its sale has been doubling every year so that it may be widely used in the near future.

Several years ago Kramer *et al.* of Merck published a very promising result of a clinical study on MK-869 (same substance as aprepitant) against depression and anxiety.[103] Recently this drug as an antidepressant was abandoned. However several pharmaceutical companies are continuing the study for the development of antidepressant and anxiolytic drugs starting from tachykinin antagonists, and some of the studies are in Phase II or III.

In experimental animals it is well established that NK_1 receptor antagonists exert an analgesic action. But in humans the results have so far been disappointing. Since many tachykinin antagonists will become available on the market in the near future it may be possible that some tachykinin antagonists might possibly be found to be effective as analgesics in some particular type of pain. The studies on the clinical use of tachykinin antagonists appear to have just started.

SP may be regarded as a prototype of peptide neurotransmitters, which as a whole may represent the largest group of neurotransmitters. Each member may play a relatively minor role in human physiological functions. But this situation may provide us with an excellent opportunity for modulating delicately some of the functions in humans. Needless to say, this may also apply to opioid receptor-related drugs such as morphine.

Conclusions

I have described the scientific accomplishments of some Japanese pioneers in the field of neurotransmitters, and also my reflections on the work I have done as an extension of the tradition of these Japanese pioneers as well as many international scientists.

My work in collaboration with my colleagues contributed to the establishment of GABA as the first amino acid neurotransmitter and SP as the first peptide neurotransmitter. GABA is the most prevalent inhibitory transmitter in the CNS, and SP is the representative in the largest group of neurotransmitters comprising probably a large number of peptides. While I could not make any unexpected discovery, I feel fortunate that the basic ideas of my work were right. I have made some mistakes but they were trivial and faded away with time. In this respect, I remember a most memorable line of Steve when he was introducing Sir John Eccles before his lecture: "He has often been wrong, but always about important things."[104] I attended this lecture given at Harvard, and as I remember, Steve finished his introduction by saying: "Let's listen to what he is going to tell us today." Prof. Eccles was a little taken aback and said: "I have never been introduced in such a way." But the friendly atmosphere between the mentor (J.C.E.) and his pupil (S.W.K.) was not at all lost on that occasion.

In order to avoid serious mistakes I have tried to examine the hypothesis with two or more tests from different disciplines, e.g., electrophysiological and neurochemical. Since the 1980s SP became gradually recognized as a neurotransmitter and some papers cited our work as presenting persuasive evidence for its transmitter role.[105, 106] Nowadays we may say that SP is widely recognized as an important neurotransmitter.[1, 107]

When I was approaching the age of becoming a PI, my mentor Ebashi said to me and other pupils: "Never try to organize a big group for your project. Work with your hands." By the late 1970s I followed Ebashi's advice. But afterward I had 5 or more collaborators, and I tended to organize a group, because I was in a hurry to consolidate the hypothesis of SP's being a transmitter. In a sense this may have been regrettable because I had wished to follow the puritanism of, e.g., Stephen Kuffler. But in another sense it was worthwhile to have had many excellent colleagues and I am now grateful to them for the happy time I have had with them. I had also wished to, but could not, complete some work, e.g. thoroughly elucidating, at the level obtained for acetylcholine at the frog neuromuscular junction, the role of SP as a transmitter of slow EPSP in spinal motoneurons, or prevertebral ganglia, and the role of enkephalins as transmitters of presynaptic inhibition in the same ganglia. These works may hopefully be accomplished by future investigators.

In his letter to the members of the Japan Academy, Prof. Yamakawa, Editor-in-Chief of PJA, requested archival reviews that would serve as a mes-

sage to the next generation. If I were asked to give some advice to a young scientist, I would remind him/her of the title of a book by A.L.Hodgkin, "Chance and Design".[108] Stephen Kuffler is an excellent example. He did not miss the chance of his meeting J.C. Eccles at the tennis court and subsequently B. Katz. He had mistakenly thought that he could not compete with these senior scientists on the intellectual plane, and therefore decided to take advantage of his manual skills which led him to develop the first isolated nerve-muscle junction.[109] Kuffler later paved the path from neuron to brain. Otto Loewi's experiment in 1920 based on his dream is another good example of this philosophy. He opened up the field of chemistry and pharmacology of the brain.

Acknowledgements

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Profile

Masanori Otsuka was born in 1929 and graduated from the Faculty of Medicine, University of Tokyo in 1953. He started his research career in 1954 in the Department of Pharmacology, University of Tokyo as a graduate student and studied muscle chemistry under the guidance of Dr. S. Ebashi. In 1955, he went to France as a Fellow of the French Government, and studied cardiac electrophysiology at the Laboratoire de Physiologie générale, Sorbonne, Paris. In 1956 he was appointed Assistant in Physiology, University of Berne, and continued to study cardiac electrophysiology under the guidance of Dr. S. Weidmann. In 1958 he was appointed Assistant Professor in Pharmacology, University of Tokyo. In 1964 he became a Rockefeller Foundation Fellow and went to the Neurophysiology Laboratory, Dept. of Pharmacology, Harvard Medical School, and participated in research on GABA under the guidance of Prof. S.W. Kuffler. In 1966 he was appointed Professor of Pharmacology, Faculty of Medicine, Tokyo Medical and Dental University, where he remained until 1994. At present he is Prof. Emeritus of Tokyo Medical and Dental University and an honorary member of the Japanese Pharmacological Society, the Japanese Neuroscience Society and the Japanese Association for the Study of Pain. He was awarded Yamaji Prize in Science, Erwin von Bälz Prize, Takeda Prize in Medicine, and Japan Academy Prize. He was elected to a member of the Japan Academy in 1995.