Review

Tetrodotoxin

—A brief history—

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Abstract: Tetrodotoxin (TTX), contained in puffer, has become an extremely popular chemical tool in the physiological and pharmacological laboratories since our discovery of its channel blocking action in the early 1960s. This brief review describes the history of discovery of TTX action on sodium channels, and represents a story primarily of my own work. TTX inhibits voltage-gated sodium channels in a highly potent and selective manner without effects on any other receptor and ion channel systems. TTX blocks the sodium channel only from outside of the nerve membrane, and is due to binding to the selectivity filter resulting in prevention of sodium ion flow. It does not impairs the channel gating mechanism. More recently, the TTX-resistant sodium channels have been discovered in the nervous system and received much attention because of their role in pain sensation. TTX is now known to be produced not by puffer but by bacteria, and reaches various species of animals via food chain.

Keywords: tetrodotoxin, saxitoxin, sodium channels, sodium currents, puffer, selectivity filter

Introduction

Tetrodotoxin (TTX) is a major toxic component contained in puffer of the Family Tetraodontidae. Despite the toxicity, or perhaps because of it, puffer has long been regarded as one of the most delicious fish in Japan, and 30–50 cases of intoxications occurred every year.[1)] The present article is not intended to be a comprehensive review of TTX. It is a story primarily of my own study of TTX. Readers who are interested in the relevant or more complete information are encouraged to refer to papers quoted in this article. There has been a long history of the study of TTX, especially by Japanese pharmacologists, but it was not until the discovery of the selective and potent blocking action of TTX on voltage-gated sodium channels[2),3)] that the toxin received the world-wide attention in the fields of physiology and pharmacology.

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My encounter with TTX

In the late 1950s, I was working on the mechanism of action of insecticides on the nervous system in the Faculty of Agriculture, University of Tokyo. My colleague Dr. Norimoto Urakawa, who was studying the effects of a toxin called maltoxin on the muscle, asked me to collaborate with him using the intracellular microelectrode technique I was using. This technique was fairly new at that time having been developed by Nastuk and Hodgkin.[4)] It turned out that maltoxin was a neuromuscular agent blocking the endplate acetylcholine (ACh) receptor of the frog.[5)] In the course of experiments, we thought that TTX might have a similar effect based on the information available at that time as a nerve-muscle blocking toxin. Therefore, we did experiments on TTX using the nerve-sartorius muscle preparations isolated from the frog. However, TTX was totally different from maltoxin, blocking the muscle action potential evoked by membrane depolarization. TTX did not change the resting potential, the membrane conductance, and the delayed rectification which is indicative of potassium channel activation (Fig. 1). Thus, we proposed a hypothesis that TTX selec-

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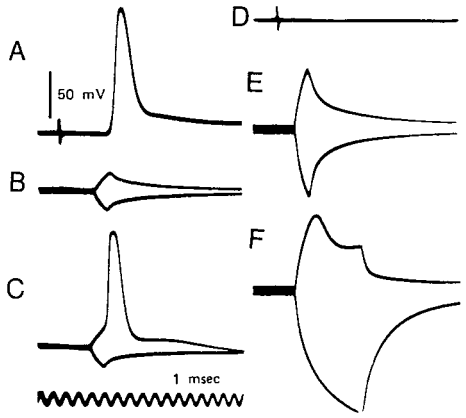

Fig. 1. Tetrodotoxin (TTX) block of muscle action potential without effect on delayed rectification. Intracellular microelectrode recording from a frog nerve-sartorius muscle preparation. (A) Normal muscle action potential evoked by nerve stimulation. (B) Responses to direct subthreshold depolarization and hyperpolarization in normal muscle. (C) Action potential generated by direct suprathreshold depolarization and hyperpolarization in normal muscle. (D) After application of 300 nM TTX, nerve stimulation failed to evoke muscle action potential. (E) In TTX, direct depolarization and hyperpolarization failed to evoke muscle action potential. (F) In TTX, stronger direct depolarization and hyperpolarization still failed to evoke muscle action potential revealing the presence of delayed rectification, indicative of potassium channel activation.[2)]

tively inhibited the activation of sodium channels. However, voltage clamp experiments were required to demonstrate this hypothesis. I reported the TTX study at an annual meeting of the Japanese Pharmacological Society in 1960. There were not many pharmacologists who were working on ion channels at that time, yet a few who understood the area raised sharp questions evoking intense discussions and dragging the allocated time to well over 30 minutes. Shortly after that time we published this paper in the American Journal of Physiology.[2)]

I was also greatly inspired by a pair of enormous review articles written by Abraham Shanes[6),7)] who not only summarized the progress but also proposed the future direction of research in the field of cellular neuropharmacology. It was indeed my dream to explain the mechanism of action of drugs and chemicals in terms of interactions with ion channels and to promote the field of cellular neuropharmacology. On the day of my departure for the US in 1961, Dr. Urakawa came to the airport to see me off and slipped a small vial containing TTX into my pocket. We were hoping that some day we would be able to demonstrate our hypothesis of the selective TTX block of sodium channels by voltage clamp experiments which were extremely difficult to perform at that time. This chance finally arrived in the late 1962 when I was a faculty at Duke University Medical Center, albeit I had only one month or so to work on TTX before returning to Japan for immigrant visa. I collaborated with Dr. John W. Moore, an expert in voltage clamp technologies, and William Scott, a medical student at that time. We had to use lobster giant axons (~80 μm in diameter) because squid was not available in North Carolina. The only method for voltage clamping of such "smaller" giant axons was to apply the sucrose gap technique.[8),9)] This technique was not only difficult but also far from perfect from the technical point of view, and numerous data had to be discarded because of imperfect ionic current records. Experiments were continued literally day and night during the Christmas and New Year holidays, and we were jubilant at proving that our original hypothesis was correct indeed. I took the barely dried films containing current records (no computer at that time) back to Japan for analysis. When I submitted the manuscript to the Journal of General Physiology, I received the very first request for a TTX sample which was jotted down at the end of manuscript review with his signature. This was indeed a dawn of cellular neurophysiology and neuropharmacology.[3)]

TTX has since not only received world-wide attentions as a useful chemical tool in the laboratory, but equally importantly also laid the foundation to pursue the mechanism of action of drugs and chemicals in terms of interactions with ion channels.[10)] In fact, before that time it was inconceivable to use a chemical or toxin to study the function of ion channels. One distinguished neurophysiologist even announced publicly that "I am proud of being a physiologist in not using dirty chemicals; I use ions."

I thought time was ripe for further promotion of cellular neuropharmacology field. Along this line, Dr. C. Paul Bianchi (then at the University of Pennsylvania) and I planned to start a new journal tentatively called "Cellular Pharmacology", and sent out many letters to physiologists and pharmacologists in the early 1970s asking whether they would be interested in contributing their papers to

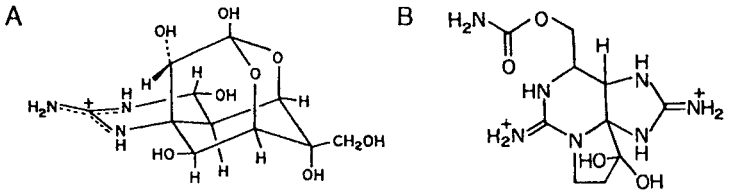

Fig. 2. Structures of tetrodotoxin (A)[15] and saxitoxin (B).[16]

such a journal. We received overwhelmingly positive responses. At that time, however, we both received an invitation to join the Specific Field Editors of the Journal of Pharmacology and Experimental Therapeutics (JPET) to create a new section called "Cellular Pharmacology". We decided to accept the invitation in order to promote this field. This section had lasted for 25 years until JPET underwent re-organization in 1999. During my tenure as a field editor, my partners who were handling the cellular and molecular pharmacology section changed to Drs. George Weiss, Ronald Rubin and Edson Albuquerque.

Chemistry, sources, distribution and origin of TTX

Several excellent reviews have been published concerning the chemistry, sources, distribution and origin of TTX. Older literature before the mid-1960s was comprehensively reviewed by Kao.[11] The chemical structure of TTX was firmly established by two Japanese groups and one American group (Fig. 2).[12–14] The total synthesis of TTX that required elaborate 26 steps was accomplished by Kishi *et al.*[17,18] Yotsu-Yamashita[19] has published an excellent review of the chemistry of TTX. Animals that contain TTX are not limited to certain species of puffer. A wide variety of marine and terrestrial animals are now known to have TTX, including, but not limited to, puffer, salamanders, frogs, horseshoe crabs, xanthid crabs, blue-ringed octopus, and starfish.[20] In the puffer, TTX is concentrated in the overy and liver, but other organs including skin, intestine, and muscle contain TTX in some species of puffer. The reason for such a wide distribution is that TTX is not produced by puffer but produced by certain species of bacteria including *Vibrio* sp. and reaches the animals through the food chain.[21–23] Reflecting the bacterial origin of TTX, if puffer is cultured in an environment in which the invasion of TTX-bearing bacteria is prevented, it would be possible to produce puffer without TTX. This has been demonstrated to be the case.[24] TTX is very toxic to mammals with an LD_{50} in the order of 10 μg/kg. Thus, the animals having TTX in the body may be resistant to TTX toxicity. This has indeed proven to be the case.[25]

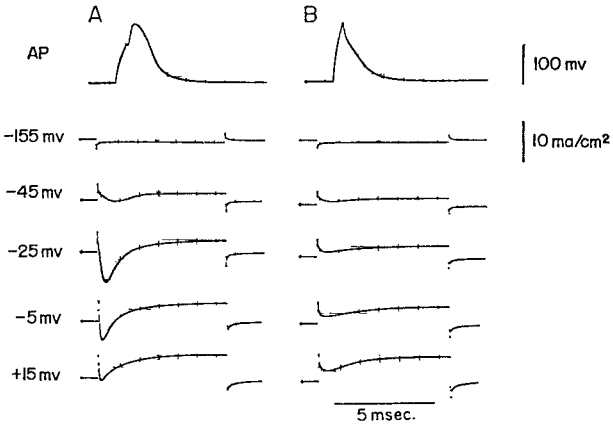

Fig. 3. Tetrodotoxin (94 nM) blocks action potentials and sodium channel currents without effect of potassium channel currents. Lobster giant axon under sucrose-gap voltage clamp. (A) Before and (B) during application of TTX. Holding potential was −120 mV including hyperpolarization caused by sucrose-gap conditions. Downward transient currents represent inward sodium currents, and upward steady-state currents represent outward potassium currents. Because of sucrose-gap hyperpolarization, a large depolarizing current was needed to evoke an action potential in A, and a large depolarizing current did not produce the action potential in the presence of TTX in B.[3]

Mechanism of action of TTX on sodium channels

Voltage clamp demonstration of selective TTX block of sodium channels. An example of sucrose-gap current clamp and voltage clamp experiments using lobster giant axons is illustrated in Fig. 3.[3] The action potential was blocked by 3×10^{-8} g/ml (94 nM) TTX, and voltage clamp

experiments clearly showed that sodium currents were blocked while potassium currents were kept intact. The selective block of sodium channels was unique as local anesthetics were shown to inhibit both sodium and potassium channels.[26)–28)]

Measurements of sodium channel density. At Duke University in 1966, we were fortunate enough to have Dr. Trevor Shaw as a visiting professor from Cambridge University in the UK. He brought with him an astonishing idea to count the number of sodium channels using TTX. Initially, I did not believe it possible, yet we did experiments using bioassays of TTX. The idea was to measure the amount of TTX absorbed to nerve membranes, and together with the measurements of membrane surface area and extracellular space, we could calculate the density of sodium channels. We assumed one-to-one stoichiometry for TTX binding to sodium channels; this was later demonstrated to be the case.[29)] Using the nerve bundles isolated from lobster walking legs, we came up a sodium channel density of 13 μm^2 of the nerve membrane as a maximal value.[30)] This was an unexpectedly small density, as it indicated that two sodium channels of a few ångstroms in diameter were separated by a distance of ~3000 ångstroms. More accurate measurements of sodium channel density were made much later by several other groups who used the tritiated form of saxitoxin (STX) which is originally derived from dinoflagellates[31)] and which blocks sodium channels in the same manner as TTX.[32)] It turned out that our original measurement using bioassays was underestimate. Many nerve tissues have 100–300 sodium channels per μm^2 of the membrane (Table 1). Counting of sodium channel density is one of the remarkable examples of using TTX and STX as tools.

Site of action of TTX on sodium channels

In contrast to the potent blocking action of externally applied TTX on sodium channels, TTX was devoid of such blocking action when perfused internally through squid giant axons.[28)] The IC_{50} values of TTX applied externally are generally in the range of 1–10 nM, but when applied internally no such block was observed even at 1 μM. This is in sharp contrast with local anesthetics which act on either side of the nerve membrane.[28)] In fact, local anesthetic which is applied externally in clinical situation penetrates the nerve membrane in the uncharged molecular form, is dissociated into the charged cation form in the axoplasm, and the cation form blocks sodium and potassium channels from inside the channels (Fig. 4).[40),41)] A variety of compounds are now known to block the sodium channels, albeit not as potent as and not as selective as TTX. In most such cases, block occurs from inside the sodium channels as exemplified by pancuronium which blocks the channels from inside when the channels open.[43),44)]

TTX molecule has a guanidinium group which can fit the external orifice of sodium channels but the rest of the molecule is too large to penetrate the channels. This results in plugging the sodium channels from outside preventing the flow of sodium ions even though the gating mechanism operates normally upon depolarizing stimulation. This was shown by the measurement of gating currents which were not affected by TTX.[45),46)]

TTX-binding proteins have been obtained and

Table 1. Densities of sodium channels as measured by bioassay of TTX and by binding of [^{3}H] STX binding

Method Reference	Preparation	Density/μm^2	Reference
TTX binding bioassay	Lobster walking leg nerve	<13	30
[^{3}H] STX binding	Lobster walking leg nerve	90	33
[^{3}H] STX binding	Garfish olfactory nerve	35	33
[^{3}H] STX binding	Squid giant axon	290	34
[^{3}H] STX binding	Rabbit vagus nerve	110	35
[^{3}H] STX binding	Mouse neuroblastoma cell	78	36
[^{3}H] STX binding	Frog sartorius muscle	380	37
[^{3}H] STX binding	Rat diaphragm muscle	209	38
[^{3}H] STX binding	Rat soleus muscle	371	39
[^{3}H] STX binding	Rabbit sciatic node	12000	35

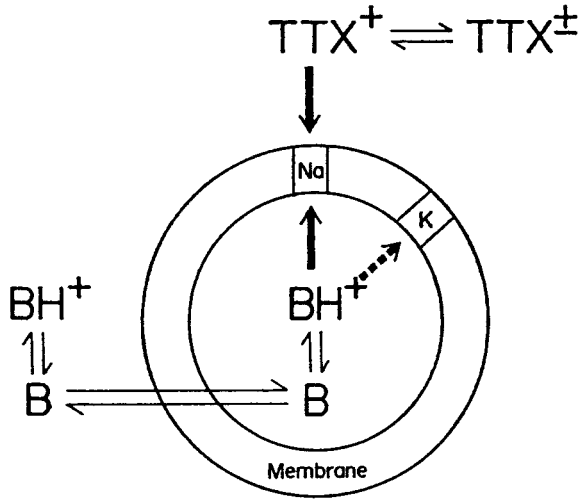

Fig. 4. Tetrodotoxin (TTX) blocks sodium channels from outside the nerve membrane in the cationic form, whereas local anesthetic molecules penetrate the nerve membrane in the uncharged form (B) and block both sodium and potassium channels from inside the nerve membrane in the cationic form.[42]

identified (reviewed by Catterall).[47] Agnew *et al.*[48] obtained a protein of ~270 kDa, and subsequently Hartshorne and Catterall[49] and Hartshorne *et al.*[50] identified a complex of α (260 kDa), β1 (36 kDa), and β2 (33 kDa) subunits. The TTX-binding component of sodium channel was also purified from eel electroplax as a 270 kDa single protein.[51] These and other studies led to the isolation of cDNAs encoding the entire polypeptide using electroplax mRNA.[52]

Single sodium channel block by TTX. One-to-one stoichiometry of TTX block of sodium channels by plugging them at the external orifice implies that characteristics of single sodium channels are not affected by TTX and that the number of observations of open sodium channels decreases dose dependently with increasing concentration of TTX. This has been demonstrated as shown in Fig. 5.[53),54]

Molecular binding site of TTX in sodium channels. Neurotoxin binding sites on sodium channels can be classified into six categories.[55] Examples of toxins that bind to each site are: site 1, TTX, STX, μ-conotoxin; site 2, batrachotoxin, grayanotoxins, veratridine; site 3, α-scorpion tox-

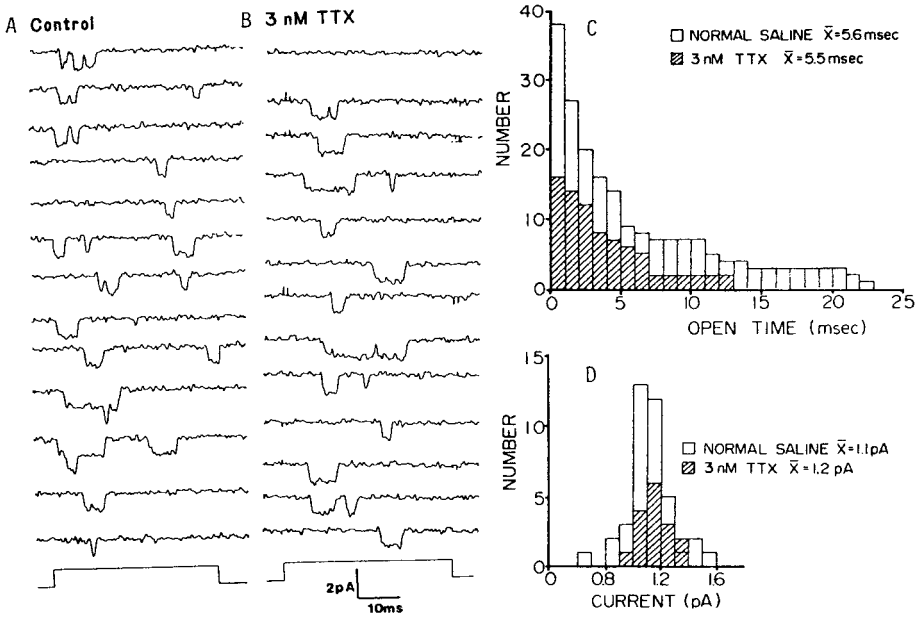

Fig. 5. Tetrodotoxin (TTX) block of single sodium channel currents. (A) Single channel currents recorded from an outside-out membrane patch isolated from a neuroblastoma cell (N1E-115) in response to depolarization from a holding potential of −90 mV to −30 mV as shown at the bottom. (B) After application of 3 nM TTX to the external membrane surface. (C) Open time distributions before and after exposure to TTX. (D) Amplitude histograms before and after TTX. Temperature 10 °C. TTX did not change the open channel characteristics but decreased the number of open channels to approximately half as 3 nM TTX was close to its IC_{50}.[53),54]

ins, sea anemone toxins; site 4, β-scorpion toxins; site 5, brevetoxins and site 6, δ-conotoxin. The amino acid residues that form site 1 in the α subunit are located in the pore loop and to form the ion selectivity filter.[55)–58)]

Sodium channels in the nervous system comprise the pore-forming α subunit and β_1 through β_4 subunits.[55),59),60)] The α subunit is sufficient for functioning, and the β subunits modify the kinetics and voltage dependence. The α subunit is composed of four homologous domains (I–IV), and each domain contains six transmembrane α helices (S1–S6). There also is an additional pore loop connecting the S5 with the S6 segments.[55),59)] Negatively charged amino acids located between transmembrane segments 5 and 6 in all four domains are postulated to form the selectivity filter where TTX and STX bind.[47)]

Subtypes of sodium channels

Not all sodium channels are sensitive to the blocking action of TTX. TTX-resistant (TTX-R) sodium channels with micromolar IC_{50}s had been known in denervated skeletal muscle and cardiac muscle. However, TTX-R sodium channels are also present in the nervous system. The first analysis of TTX-R and TTX-sensitive (TTX-S) sodium channels of rat dorsal root ganglion (DRG) neurons was made by Kostyuk *et al.*[61)] However, that paper did not receive much attention for unknown reasons, and only a few papers by other investigators on this topic were published in a decade that followed.

We decided to analyze TTX-S and TTX-R sodium channels of rat DRG neurons in detail.[62)] TTX-R currents were much slower than TTX-S currents in their activation and inactivation kinetics. The IC_{50} values for blocking TTX-S and TTX-R currents, respectively, were 0.3 nM and 100 μM for TTX, and 0.5 nM and 10 μM for STX (Fig. 6). The voltage dependences of both activation and inactivation of TTX-R channels were shifted in the depolarizing direction by 11 mV and 30 mV, respectively, compared with those of TTX-S channels. This is important as shifts affect the sensitivity to various drugs. For example, the action potential from TTX-S sodium channels were much more sensitive to the blocking action of phenytoin and carbamazepine than that from TTX-R sodium channels, and this differential sensitivity could be largely explained in terms of the difference in voltage dependence of TTX-S and TTX-R sodium channels.[63)]

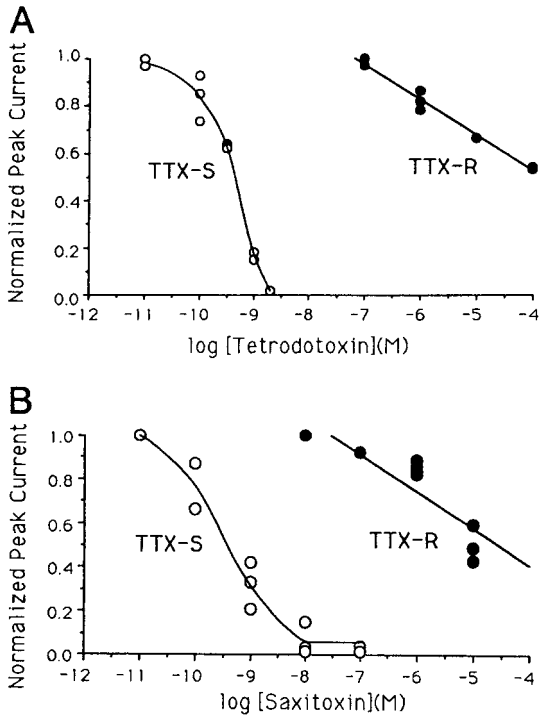

Fig. 6. Dose-response relationships for TTX and STX block. Dorsal root ganglion cells expressing TTX-S (n = 3) or TTX-R (n = 3) currents were exposed to increasing concentrations of TTX or STX and pulsed once per minute to +10 mV to determine peak current amplitude. Steady-state peak current amplitudes reached at each concentration were normalized to control toxin-free amplitudes and plotted against toxin concentration. (A) TTX dose-response curve, with IC_{50} values of 0.3 nM (TTX-S) and 100 μM (TTX-R). (B) STX dose-response curve, with IC_{50} values of 0.5 nM (TTX-S) and 10 μM (TTX-R).[62)]

Since the publication of our paper,[62)] TTX insensitivity of DRG neurons has received much attention, because TTX-R sodium channels are present in C fibers that convey pain sensations to the brain. If a chemical that blocks TTX-R sodium channels without any effect on TTX-S sodium channels is discovered, it could become a useful pain killer without serious side effects. It should be noted that pain is one of the most crucial biomedical issues these days.

A number of recent studies using molecular approaches have disclosed at least nine subtypes of sodium channels, $Na_v1.1$-$Na_v1.9$.[59),64)] Heart muscle contains $Na_v1.5$ (TTX-R), DRG contains $Na_v1.8$ (TTX-R), and peripheral nervous system contains

$Na_v1.9$ (TTX-R). Skeletal muscle contains $Na_v1.4$ (TTX-S). $Na_v1.1$, 1.2, 1.3, 1.6 and 1.7, all of which are TTX-S sodium channels, are found in the nervous system.

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Profile

Toshio Narahashi, John Evans Professor of Pharmacology at Northwestern University Feinberg School of Medicine in Chicago, received his DVM in 1948 and Ph. D. (Sc. D.) in 1960 from the University of Tokyo. After spending about 10 years as a faculty member, he came to the United States first to the University of Chicago in 1961, and then to Duke University Medical Center (1965) where he rose from Assistant Professor to Associate Professor in 1967, and then to Professor in 1969. In 1977, he was recruited to Northwestern University as Professor and Chairman of the Department of Pharmacology. He stepped down from the Chairmanship in 1994, and has since continued his research and teaching activities as Professor. His research involves pharmacology and toxicology of receptors and ion channels of excitable cells using voltage clamp and patch clamp techniques. During his tenure at the University of Tokyo, he devoted himself in the study of physiological mechanism of action of insecticides, and is credited in discovering the sodium channel modulation caused by DDT and pyrethroids as the major mechanism that kills insects. He also undertook a study suggesting the selective tetrodotoxin (TTX) inhibition of sodium channels (1960). This hypothesis was clearly demonstrated in his voltage clamp experiments conducted at Duke University (1964). TTX has since become an extremely important chemical tool in the laboratory. Equally important is the fact that his TTX study has opened the door leading to cellular and molecular pharmacology which now flourishes as one of the most crucial biomedical science fields. More recently, he has been working on the mechanism of action of alcohol, anesthetics, and Alzheimer's drugs on neuroreceptors and channels. On the basis of these pioneering studies, he has received numerous awards such as the Merit Award (1991) and the Distinguished Toxicology Scholar Award (2008) from the Society of Toxicology, the Otto Krayer Award (2000) from the American Society of Pharmacology and Experimental Therapeutics, and Burdick & Jackson International Award (1989) from the American Chemical Society, to mention a few. He has trained some 140 postdoctorals and graduate students in his laboratory, many of whom held prestigious academic positions in the U.S., Japan and other countries.