in the facilitation of synaptic transmission through these mechanisms. Recently, it has been suggested that Munc13-1, one of the non-PKC C_1 domain-containing receptors, localized to active zones in the presynaptic terminal, may also be involved in the regulation of synaptic efficacy by BPEs [54–56]. When the DAG/BPE-binding site of Munc13-1 is genetically deficient or non-functional, the effects of BPEs are largely impaired [56, 57]. Although the present study did not prove whether BPEs potentiate MF synaptic transmission through Munc13-1, it does not conflict with the notion that the PKC-dependent and Munc13-1-dependent pathways synergistically modulate the exocytosis [4, 5].

The ΔSpH unsilencing

We found that the ΔSpH, which was negligibly small at sampling 1, become obvious at sampling 2 after the PDAc in some LMFBs. These synapses are possibly presynaptically silent or weak in the release ability before PDAc [34]. Alternatively, the transmitter release might be rapidly depressed during repetitive stimulation. These possibilities should be clarified in future studies, e.g., the simultaneous recordings of EPSC and SpH fluorescence. Recent studies using cultured networks of developing hippocampal neurons have described the presence of presynaptically silent synapses, which become transmittable through mechanisms dependent on cAMP-protein kinase A (PKA) or BDNF-Cdc signaling cascades [58–62]. At the CA3-CA1 synapses of the mouse hippocampus, the slow presynaptic component of long-term potentiation (LTP), which is detected by the change of SpH fluorescence, is mediated by the PKA and the L-type Ca^{2+} channels [39]. It is possible that some boutons are unsilenced in the ΔSpH. Since MF-LTP is dependent on the AC-PKA cascade [63, 64], it is probably accompanied with the increased ΔSpH. The unsilencing of ΔSpH might be related to the accumulation and organization of the large arrays of vesicular and non-vesicular molecules required for exocytosis as they are during synaptogenesis [65, 66]. PKC is one of the protein kinases involved in these processes. For example, PKC phosphorylates GAP-43, one of major proteins of the presynaptic and growth cone membrane, during synaptic potentiation [67]. Another PKC substrate is myristoylated alanine-rich C kinase substrate (MARCKS), which is involved in both synaptic maturation and the synaptic plasticity [68, 69].

In the hippocampus of a living animal the signals are usually a train of impulses of variable frequencies [70], sometimes at around 10 Hz (a theta rhythm) [71, 72]. When DAG/BPEs facilitate the fusion probability by increasing the Ca^{2+} influx and the Ca^{2+} sensitivity of exocytosis [10], they up-regulate the transmission efficacy for impulses arriving early in a train, but down-regulate it for impulses arriving later because of the depletion of RRP. This effect could explain the reduction of the paired-pulse ratio. Even if the RRP were to be increased, the potentiation should be transient if it is not replenished. On the other hand, the staurosporine-sensitive enhancement of exocytosis followed a more prolonged time course. Therefore, the DAG/BPE-dependent signaling cascade is suggested to be involved in the facilitation of the vesicle replenishment through a staurosporine-sensitive mechanism and to maintain the fidelity of transmission at a high level during a train of repetitive firings of the presynaptic neuron.

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Appendix

Reduction of the paired-pulse ratio

According to the quantal hypothesis [36, 73], a postsynaptic response (E) is related to the following relationship.

$$E = Npq. \tag{1}$$

where q is the postsynaptic response by a single quantum (quantal size). The meanings of N and p are definition-dependent. If p is regarded as the probability of vesicle fusion to the plasma membrane, then N refers to the number of vesicles drawn from the next action potential, the readily releasable pool (RRP) or the release-ready pool [20–22]. On the other hand, if p is regarded as the release probability of a release site, N should be the number of release sites, the morphological correlates of which are the number of active zones of a presynaptic terminal. When a presynaptic axon is stimulated by two pulses of a short interval, the vesicle fusion probability by the second action potential (p') is generally increased by some mechanisms dependent on the residual Ca^{2+} [74–78]. On the analogy of the Eq. 1, the second postsynaptic response (E') will be expressed as,

$$E' = \{N(1-p) + R\}p'q'. \tag{2}$$

Here, R is the number of vesicles replenished to the RRP during the interval between the first and the second action potentials, and q' is the quantal size of the second response.

The paired-pulse ratio (r) is thus the function of p with the relationship,

$$r = E'/E = (1 - p + R/N)(p'/p)(q'/q). \quad (3)$$

The value R/N is dependent on the vesicle recycling mechanisms. The value p'/p is dependent on the underlying mechanisms of facilitation. The value q'/q is mainly influenced by the postsynaptic factors, such as the desensitization of transmitter receptors. It is also influenced by the synaptic geometry, such as the narrowness of the synaptic cleft, the transmitter clearance activities, such as the glutamate uptake by the astrocytes as well as the presynaptic factors, such as the transmitter density in the vesicle [48, 49]. Although these values appear to be less variable than p, their effects have to be taken into consideration. Since r is negatively related to p, the enhancement of the vesicle fusion probability at the first action potential (p) is expected to be accompanied with the reduction of r.

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Technical note

Cannula-aided penetration: A simple method to insert structurally weak electrodes into brain through the dura mater

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ABSTRACT

We developed a simple and inexpensive method to insert structurally weak electrodes into the brain through the thickened dura mater in chronic animal experiments. It uses a commonly available intravenous (IV) needle and a cannula to secure a small puncture in the dura mater, through which an electrode is advanced into the underlying cerebral cortex. In addition to its simplicity and cost-effectiveness, this method provides greater degree of freedom regarding the shape and the placement of electrodes compared to the conventional guide tube systems.

1. Introduction

Recent development of neuron recording techniques has led to the use of diverse kinds of electrodes. Examples of such electrodes include silicon-based multichannel electrodes (Csicsvari et al., 2003; Olsson et al., 2003), glass pipettes for iontophoresis (Shima and Tanji, 1993), carbon fiber electrodes for voltammetric measurement of neurotransmitters (Walker et al., 2000), wire-bundle electrodes such as tetrodes (Recce and O'Keefe, 1989; Gray et al., 1995; Jog et al., 2002; Takahashi et al., 2003), dodecatrodes (Takahashi and Sakurai, 2005), etc. The use of these electrodes is expected to bring important findings in neurophysiological and neuropharmacological studies which have not been possible with conventional metal electrodes. Application of these electrodes in awake, behaving animals is, however, limited because these electrodes are generally fragile and prone to break easily when they pass through the dura mater. Therefore, researchers have either made incisions in the dura, or used guide tubes to insert fragile electrodes. However, these conventional methods contain adverse consequences: making incisions in the dura requires anesthesia which is unsuitable for behavioral experiments, and causes a risk of infection and bleeding. Use of guide tubes is another frequently employed method. Some manufacturers provide guide tube systems that consist of guide tube holders and microdrives for electrodes. However, they are precision-machined and accordingly expensive. Moreover, the length and narrow lumen of a guide tube often precludes the use of electrodes with short and/or tapered shanks, such as glass pipettes for iontophoresis.

To overcome these problems, we developed a simple, inexpensive method to assist the insertion of structurally weak electrodes into the brain of chronic experimental animal models. Using this method, we succeeded in making stable recordings by such electrodes through the thickened dura mater, which would otherwise have been impossible.

2. Materials and methods

2.1. Construction of the cannula assembly

Fig. 1 illustrates the structure and use of the device to puncture the dura. The device consists of a common, commercially available intravenous (IV) needle, a cannula (Terumo Inc., Japan) and a Teflon coated tungsten wire (#796000, A-M Systems, Inc., USA, insulated diameter = 0.114 mm) to record neuronal activity. Table 1 shows the sizes of the IV needles and cannulas used in this study. The choice of the cannula varies depending on the diameter of the electrode to pass through it. The cannula is cut at

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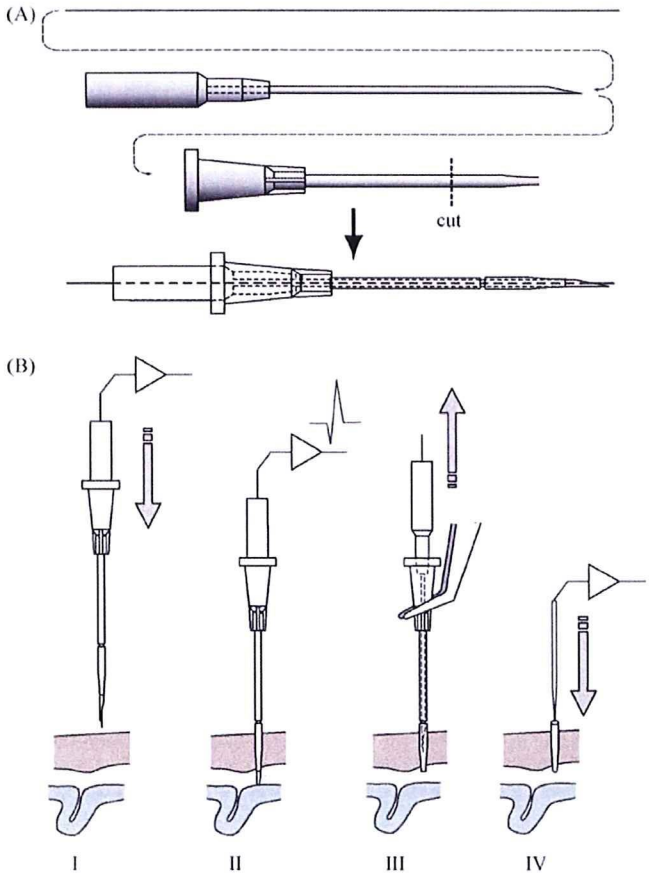

Fig. 1. Appearance of the dura puncture device and its usage. (A) Materials and the construction of the device. A Teflon insulated tungsten wire (top) which serves as the probing electrode is passed through the lumen of the intravenous needle (second row from the top) and secured by epoxy glue. Then, the cannula is cut at several millimeters from the tip (third row). Finally, the needle and the tungsten wire are passed through the cannula (bottom row). (B) Usage of the dura puncture device. The device is advanced into the dura (I) until action potentials are recorded through the probing electrode (II). Then, while holding the flange of the cannula by a pair of tweezers, the inner needle is removed (III). Finally, the recording electrode is advanced through the cannula which secures the opening of the dura (IV).

5–10 mm from its tip. The cut section must be long enough so that its top end appears over the dura. If it is too short, its top will not appear over the dura, which makes it difficult to insert the recording electrode. Moreover, if the top end is not visible, removal of the cannula after the recording is also difficult.

The IV needle holds the Teflon coated tungsten wire in its lumen. For thicker IV needles (#20 and above), the wire could be substituted with FHC's metal microelectrode (product code UESLGCSDN1-E, FHC Inc., USA, insulated diameter = 0.3 mm). Either the wire or the electrode is passed through the needle from its tip with the back-end first, fixed by epoxy glue and serves as a probing electrode during the puncture. We use this probing electrode to monitor the appearance of action potential while advancing the whole assembly to know when the dura is penetrated.

Table 1
The sizes of IV needles and cannulas used in the present study. Units are in mm. O.D.: outer diameter; I.D.: inner diameter.

	Manufacturer's size #			
	18	20	22	24
Inner needle				
O.D.	0.9	0.7	0.55	0.4
I.D.	0.66	0.48	0.37	0.22
Cannula				
O.D.	1.3	1.1	0.85	0.67
I.D.	0.95	0.8	0.6	0.47

2.2. Puncture

Fig. 2A shows the experimental set-up. The puncturing device was attached to a manipulator (SM-12, Narishige Inc., Japan) via a retainer, positioned at a desired location by an X–Y manipulator attached to a stereotaxic frame, and driven into the dura. While advancing the device, we looked for the appearance of action potentials through the tungsten wire to detect its entry into the cortex. When an action potential appeared, we stopped advancing the device. Then, while holding the flange of the cannula with a pair of tweezers, we withdrew the needle. Care was taken not to move the cannula during this process. This left the cut section of the cannula in place (Fig. 2B). Then, the needle and its manipulator were replaced with a hydraulic microdrive (MO-81, Narishige Inc, Japan) which held the recording electrode. The recording electrode was aligned with the cannula's cut section in the dura by the X–Y manipulator, through which we advanced it into the cortex.

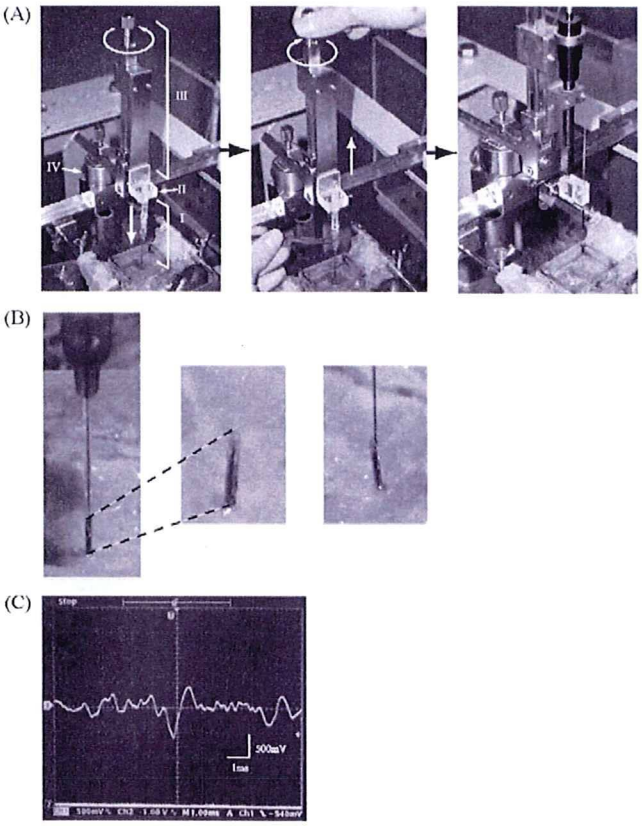

Fig. 2. Puncture of the dura by the needle. (A) Left: the puncture device (I) is attached to the manipulator (III) via a retainer (II), and positioned at the desired location by an X–Y manipulator (III). The retainer has a hole in it to which the device snug-fits. Middle: upon puncture, the inner needle is withdrawn while holding the cannula. Right: the device and its manipulator is replaced with an hydraulic microdrive. (B) Left: the dura puncture device is advanced into the dura. The cut section of the cannula is painted by a marker for clarity. Middle: after the needle is withdrawn, the cannula's cut section is left in place. Right: a thin metal electrode is inserted through the cannula. (C). An action potential recorded though the probing electrode during the puncture.

At the end of the recording, the cannula was removed with a pair of tweezers. The opening in the dura quickly closed after the removal of the cannula. Bleeding, though it occurred infrequently, was stopped easily by placing sterile cotton balls on the opening. After the recording, we gave antibiotics to the animal. The whole procedure was done in an awake, unanesthetized animal.

2.3. Animal

For chronic animal preparation, we selected a female Japanese macaque (macaca fuscata, b.w. = 5.8 kg) which had been used for single neuron recording for over a year. The animal was cared for in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals, and Guidelines for Institutional Animal Care and Use published by the Tohoku University. It had a chamber over the skull opening (approximately 20 mm × 30 mm) which allowed the access to the medial portion of the cerebral hemisphere. The dura which covered the opening had grown to 7–9 mm in thickness as estimated from the distance over which a metal electrode was advanced from its surface until reaching the cortex. Before the testing of this method, no special effort had been made to reduce the thickness of the dura in order to make the penetrations easier.

3. Results

3.1. Penetration of the dura by the puncture device

We tested IV needles of four different sizes (#24, #22, #20 and #18). Larger IV needles made later passage of recording electrodes easier. On the other hand, they increased the risk of bleeding. Moreover, they caused considerable dimpling of the dura during penetration. Therefore, we mainly used #22 in the present study. Fig. 2C shows the action potential recorded by the probing electrode. When an FHC electrode was used as the probing electrode, action potentials were clearly identified upon penetration. On the other hand, a Teflon coated tungsten wire did not record clear single neuron activity. Still, the sound of multi-unit activity was audible from a speaker connected to the amplifier. This helped us ascertain when the IV needle penetrated the dura mater. Leak of the cerebrospinal fluid through the cannula was negligible with cannulas thinner than #20 and longer than 10 mm.

3.2. Recording

To test the feasibility of this method, we used the electrodes shown in Fig. 3. They include (1) parylene-coated tungsten electrodes (diameter = 0.15 mm); (2) glass capillary carbon fiber electrodes (diameter < 0.3 mm within 3 mm from the tip) constructed by the procedure described previously (Sawaguchi et al., 1986; Shima and Tanji, 1993); (3) silicon-based, multi-recording site electrodes made from 0.1 mm thick silicon wafer; and (4) glass-tungsten composite electrodes which had microcircuits printed on glass wafers that measured 0.15 mm × 0.2 mm × 40 mm, embedded in tungsten needle (diameter = 0.35 mm).

These electrodes successfully reached the cerebral cortex through the opening in the dura secured by the cannula. In contrast, none of them could reach the cortex when driven directly into the dura. The parylene-coated tungsten electrodes tended to bend before penetrating the dura. Even in a few cases in which they reached the cortex, abrasion with the hardened dura mater damaged the insulation, which compromised the quality of recording. The other three types of electrodes never reached the cortex without using a cannula.

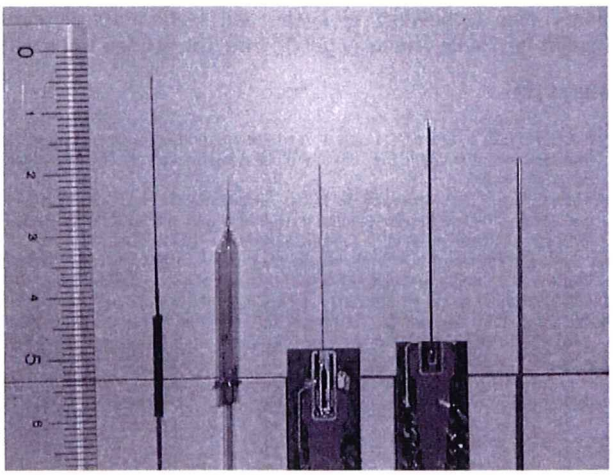

Fig. 3. The four recording electrodes used in this study. From left to right, a parylene-coated tungsten electrode, a glass pipette carbon fiber electrode, a silicon electrode, a glass-tungsten composite electrode, and a stainless steel pipe (outer diameter = 0.6 mm) for size comparison.

4. Discussion

In this paper, we presented a simple yet versatile method to pass structurally weak electrodes through the hardened dura mater of animals. The method requires no costly materials. It consists of commonly available, inexpensive IV needles and Teflon coated tungsten wires. Further, it allows the use of a wide variety of electrode shapes due to the short and relatively wide lumen of the cannula. Finally, the cannula takes little space over the dura, thus makes it easy to place multiple electrodes in proximity, if necessary. With this method, we have succeeded in recording neurons with electrodes which otherwise failed to reach the cerebral cortex.

In previous studies, researchers made incisions in the dura (Shima and Tanji, 1993) or used a guide tube to insert fragile electrodes into the cortex. However, surgical incisions require anesthesia, which results in longer preparation time in daily experiments. Moreover, incisions cause the risk of bleeding and infection. Other methods, such as guide tubes (Malpeli et al., 1992) or needles (Chen et al., 2001) to penetrate the dura, have disadvantages. The length of the guide tubes often restricts the choice of electrodes. The needle, once withdrawn, leaves no clear clue on the dura as to where the puncture is, because such a small hole quickly closes and/or is hidden by bleeding after the needle is withdrawn. Moreover, neither a guide tube nor a needle has the means to record action potentials to ascertain when the dura is penetrated.

In contrast, the use of the probing electrode in our method helps us know when the dura is penetrated during the puncture, thus reducing the chance to cause unnecessary damage to the cortex. Moreover, the cannula left in place clearly shows where the opening is, thus helps to align the recording electrode with it. And finally, this method requires relatively short time to secure the access to the cortex. One shortcoming is that this method restricts the selection of the chamber. Because the whole procedure needs to be done under direct vision, use of a large chamber is recommended to use this method.

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Science and Technology of Japan, and Exploratory Research Program for Young Scientists (ERYS) from the Tohoku University.

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Development of Double-sided Si Neural Probe with Microfluidic Channels Using Wafer Direct Bonding Technique

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Abstract— **We have proposed the intelligent Si neural probe system which can realize high density and multifunctional recording of neuronal behaviors. In this device, LSI chips such as amplifiers, A/D converters, and multiplexers are integrated on the intelligent Si neural probe. In this paper, we report the development of a novel Si neural probe with microfluidic channels which is the key part of the intelligent Si neural probe system. The Si neural probe has microfluidic channels fabricated using a wafer bonding technique to deliver drugs into the brain when neuronal action potentials are recorded. Furthermore, our Si neural probe has recording sites on both front- and back-side of Si to realize high density recording. We fabricated the carefully-designed Si neural probe, and evaluated characteristics of microfluidic channels. From the liquid ejection test, we confirmed that there was no void at bonding interfaces. We observed the liner relationship between the flow rate and the pressure drop, and the relationship was identical to that from the calculation, which indicated that the microfluidic channel was successfully formed. In addition, we fabricated the Si neural probe for *in vivo* neural recording. Both front- and back-side recording sites of the fabricated Si neural probe had impedance values of 1.5 MΩ and 1.2 MΩ at 1 kHz, respectively, which indicated that both recording sites had equivalent characteristics. The neuronal action potentials in motor area of Japanese macaque's brain were successfully recorded by using the fabricated Si neural probe.**

Keywords- intelligent Si neural probe system; wafer bonding technique; simultaneous multi-site recording

I. INTRODUCTION

With the progressing of ageing society, the number of patient with brain diseases such as paralysis, epilepsy, and Parkinson's disease has been increasing remarkably. However, medical cures for these diseases have not been established yet. For medical care or rehabilitation of brain diseases, not only patients but also their families have been under heavy strain. In order to improve these situations, neurophysiology has attracted much attention all over the world. In neurophysiology, recording of neuronal signals from the brain plays an important role in advanced studies of neuronal circuit dynamics and relationships between actions of the body and activities of the brain. In the past few decades, various types of probes made of Si have been developed to record neuronal signals from the brain of animals, and lots of useful results were obtained [1-2]. We have proposed an intelligent Si neural probe system, and have been developing key parts of the system [3]. Fig. 1 shows the configuration of the intelligent Si neural probe system.

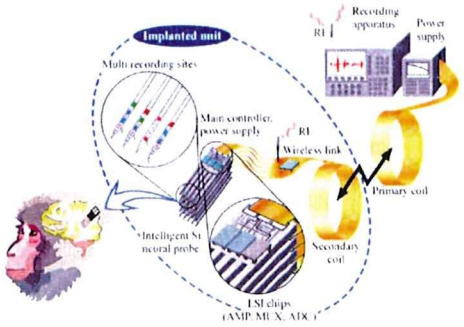

Figure 1. Configuration of the intelligent Si neural probe system.

In this system, a large number of recording sites are located on the tips of the intelligent Si neural probe for simultaneous multi-site recording of neuronal action potentials. Furthermore, electric circuits such as amplifiers (AMPs), A/D converters (ADCs), and multiplexers (MUXs) are integrated on the Si neural probe. Therefore, weak neuronal signals recorded from the brain are amplified with keeping low noise levels.

In the present paper, we propose a novel Si neural probe with microfluidic channels and fabricated the Si neural probe for *in vivo* neural recording that are the key components of the intelligent Si neural probe system. We designed the Si neural probes carefully, and we evaluated characteristics of both microfluidic channels and recording sites. Subsequently, using the fabricated Si neural probe, we performed an *in vivo* experiment of recording neuronal action potentials on motor area in brain of Japanese macaque.

II. SI NEURAL PROBE WITH MICROFLUIDIC CHANNELS

A. Proposal of Si neural probe with microfluidic channels

Si probes have been widely used because Si probes can be fabricated by using advanced CMOS processes which produce several important advantages such as mass production, high

repeatability, and high accurate processes, and the Si is also highly biocompatible material. However, number of neurons that can be observed by conventional Si probes is limited, and neuronal action potentials can only be recorded from neurons. Additionally, considering neuron damages by a Si probe, further improvement of Si probe has been demanded. To solve these problems, we propose a novel Si neural probe with microfluidic channels which has recording sites on both front- and back-side of Si. By integrating microfluidic channel into the microelectrode, it becomes possible to deliver drugs into the neural tissue, and to understand the drug's effect to neurons. Moreover, by placing recording sites on both sides, high density 3-D recording of neuronal action potentials can be realized.

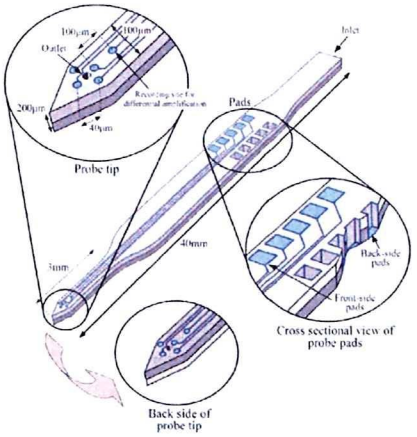

Figure 2. Structure of Si neural probe with microfluidic channels.

The overall structure of the Si neural probe with microfluidic channels is shown in Fig. 2. This probe has 8 recording sites on both front- and back-side for neuronal cell sorting. This probe has a length of 40 mm. The width and thickness were 100 μm and 200 μm, respectively. Recording sites are made of Au with circular pattern. Diameter of circular pattern is 12.5 μm and the distance between two recording sites is 40 μm in center to center. Wirings are also made of Au and isolated by SiO_2 films. Behind 200 μm from the tip of the probe, the recording site for differential amplification is placed. The microfluidic channel has the length of 40 mm, the width of 50 μm, and the depth of 10 μm. The fluidic outlet at the probe tip has diameter of 10 μm, and the fluidic inlet is 50 × 10 μm^2 large. At the middle of the microelectrode, bonding pads for connecting to external recording apparatus were formed. Back-side pads leaded from back-side recording sites are formed by Si etching and connected to recording apparatus by wire bonding through Si hole.

B. Fabrication of Si neural probe with microfluidic channels

Our probe was fabricated by combining standard photolithography with bulk micromachining process. Two 100μm-thick Si wafers were used as a substrate of the probe. The fabrication sequence of the probe is illustrated in Fig. 3. First, microfluidic channel with the depth of 10 μm was formed on the surface of the Si wafer using deep reactive ion etching (DRIE) with SF_6 and C_4F_8 gases. The etched Si wafer and other Si wafer were rinsed by standard clean 1 (SC-1), and both surfaces of 2 wafers were covered with thin oxidized layer. Then, these wafers were stacked and bonded by hydrogen bonds. By annealing these wafers, hydrogen bonds changed to covalent Si-O bonds with the reaction below:

$$SiOH - OH_2 - OH_2 - OHSi \leftrightarrow Si - O - Si + 3H_2O. \quad (1)$$

Next, Au/Cr wirings were formed on the front surface of the wafer by sputtering and wet etching with an iodine etchant and a ceric ammonium nitrate solution. The thickness of Au and Cr were 250 nm and 100 nm, respectively. Then, SiO_2 with a thickness of 1.5 μm was deposited using plasma enhanced chemical vapor deposition (PECVD) for insulation of wirings. Back-side surface of Si wafer was also processed with the same sequence. After that, recording sites and bonding pads were formed by wet etching with buffered HF solution (HF/NH_4F 1/10), followed by formation of the shape of the probe and back-side pads using DRIE. As a result, the Si neural probe was obtained. As shown in Fig. 4, the Si neural probe with microfluidic channels was fabricated successfully.

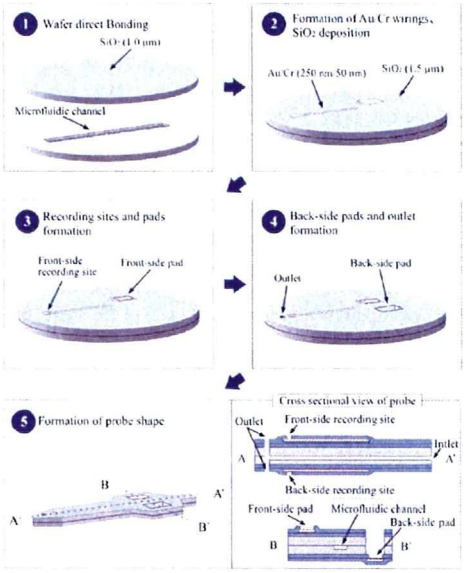

Figure 3. Fabrication sequence of Si neural probe with microfluidic channels.

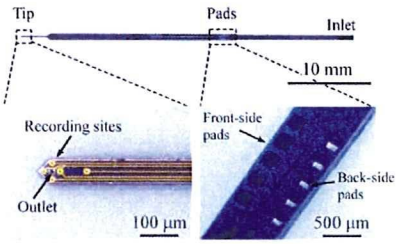

Figure 4. Fabricated Si neural probe with microfluidic channels.

C. *Evaluation of microfluidic channels of fabricated Si neural probe*

At first, we confirmed the strength of the fluidic channel. In the experiment, we injected red ink into the fluidic channel with flow pressure of 1000 mmHg. Fig. 5 shows the ejection of red ink from the fluidic outlet. From this experiment, it was confirmed that the microfluidic channel had enough strength to inject fluidic medicine.

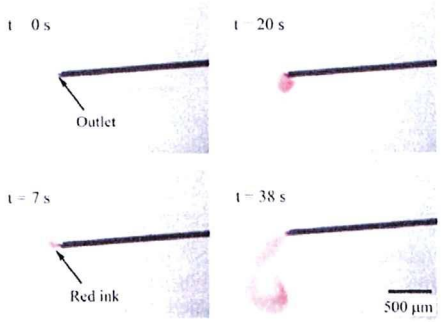

Figure 5. Photographs of ejected red ink from the fluidic outlet.

We also evaluated relationships between the flow rate and the pressure drop through microfluidic channel. We changed a flow rate from 0.1 to 0.4 μl/min with the syringe pump. We measured the pressure drop across the channel with the pressure meter, and we compared measured values with theoretical calculations. The pressure drop (Δp) is defined as:

$$\Delta p = \frac{\mu V (\mathrm{Re} \times f) L}{2D^2} \qquad (2)$$

where μ is the viscosity of fluid (~8.9×10^{-4} kg/ms at 25 °C for water), Re × f is the product of the Reynolds number and the Darcy friction factor (~64 in laminar flow), L is the length of the channel (40 mm), and D is the hydraulic diameter of the channel as defined by

$$D = 4 \times (\text{Cross-sectional area / wetted perimeter}). \qquad (3)$$

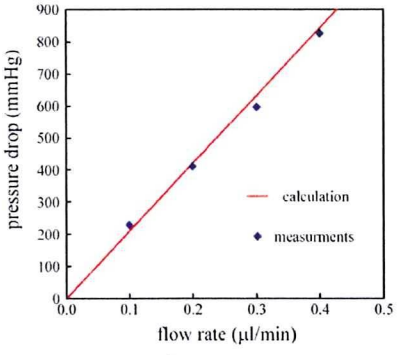

Figure 6. Relationship between the flow rate and the pressure drop.

As shown in Fig. 6, the measured values agreed well with the theoretical calculations, indicating that the microfluidic channel has the same shape as we designed.

III. Si Neural Probe for In Vivo Neural Recording

A. *Design and fabrication of Si neural probe for in vivo neural recording*

The overall structure of the Si neural probe for *in vivo* neural recording is shown in Fig. 7. This probe has single slim shank for *in vivo* recording and 9 recording sites on both front- and back-side tip. To analyze deeper region of the brain such as basal ganglia or to apply to large animals like primates, this probe has a length of 40 mm. The width and thickness were 135 μm and 100 μm, respectively. Recording sites are made of Au. Diameter of circular pattern is 15 μm and the distance between two recording site is 100 μm in center to center. Wirings are also made of Au and isolated by SiO_2 films. Behind 1255 μm from the tip of the probe, the recording site for differential amplification is placed. At the end of the probe, bonding pads for connecting to external recording apparatus were formed. Back-side pads are connected to recording apparatus by wire bonding through Si hole.

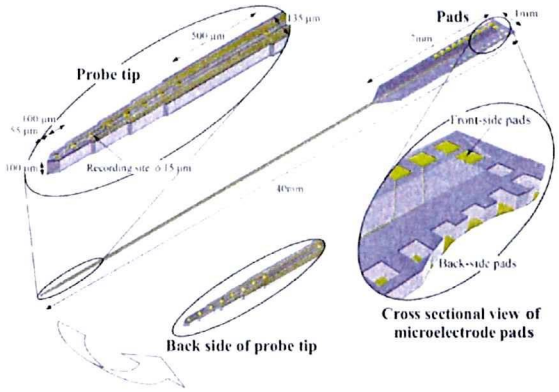

Figure 7. Structure of Si neural probe for *in vivo* neural recording.

Fabrication sequence of this probe is almost the same as that of the Si neural probe with microfluidic channels. In fabrication of this probe, microfluidic channels were not formed. As shown in Fig. 8, the Si neural probe for *in vivo* neural recording was fabricated successfully.

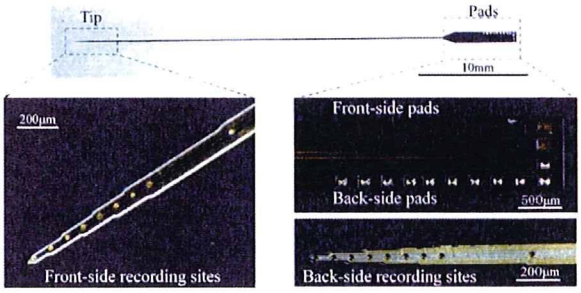

Figure 8. Fabricated Si neural probe for *in vivo* neural recording.

B. *Characterization of recording sites of the Si neural probe for in vivo neural recording*

In order to characterize the interface between recording site and electrolyte, we measured electrical impedance of the Si neural probe before animal experiments. Measurements were performed with the 10 mV AC signal and the frequency ranging from 100 Hz to 10 MHz in 0.9 % saline solution. An Ag/AgCl electrode and a Pt electrode were employed as a reference electrode and counter electrode, respectively. Fig. 9 shows the impedance characteristics. While front-side recording site had the impedance value of 1.5 MΩ at 1 kHz, back-side recording site had the impedance value of 1.2 MΩ at 1 kHz. This result indicated that both sites had almost the same electric characteristics.

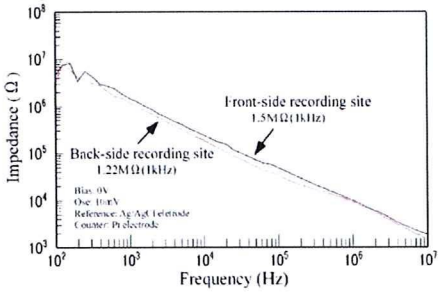

Figure 9. Impedance spectra of fabricated Si neural probe for *in vivo* neural recording.

C. *Neuronal action potential recording using Si neural probe*

We performed an *in vivo* experiment of neuronal action potentials recording using the Si neural probe. A female Japanese macaque (b.w. = 5.5 kg) was used. The animal was cared in accordance with the Guiding Principles for the Care and Use of Laboratory Animals of the National Institutes of Health and the Guidelines for Institutional Animal Care and Use published by our institute. The probe was inserted into the left supplementary motor area (SMA) through the dura matter by a hydraulic microdrive (Narishige MO-81; Tokyo, Japan) as shown in Fig. 10. Then, the neuronal activity was amplified (×5000), band-pass filtered (0.3-3.2 kHz), sampled at 20 kHz, and stored in a computer disk for offline inspection. Individual neuronal action potentials were discriminated by a spike-sorting software (MSD; Alpha Omega Engineering, Nazareth, Israel) based on template matching, as shown in Fig. 11.

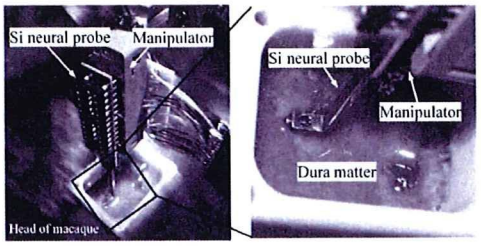

Figure 10. Photographs of Si neural probe inserted into brain of Japanese macaque.

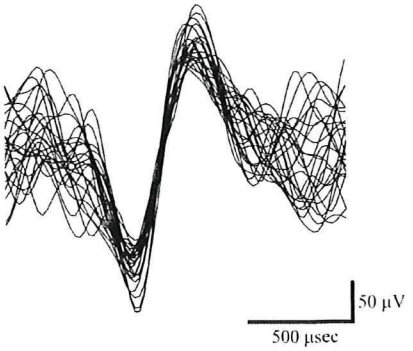

Figure 11. Recorded neuronal spike potentials.

IV. Conclusions

We fabricated the novel Si neural probe with both microfluidic channels and recording sites on front- and back-side of Si. The probe was fabricated successfully using wafer direct bonding techniques. From fluidic experiments, it became clear that there was no void at bonding interfaces and the bonding strength of the fabricated probe withstood the flow pressure larger than 1000 mmHg. Additionally, we confirmed the measured fluidic characteristics of the microfluidic channel agreed well with the theoretical calculations. Both front- and back-side recording sites of the fabricated microelectrode had impedances of 1.5 MΩ and 1.2 MΩ at 1 kHz, respectively, which indicated both recording sites had equivalent characteristics. Using our probe, we successfully recorded neuronal action potentials from motor area of brain of Japanese macaque.

Acknowledgment

This work was partially supported by Grant-in-Aid for Scientific Research on Priority Areas-< Integrative Brain Research >-from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grant-in-Aid for JSPS Fellows. This device was fabricated in Micro/Nano-Machining Research and Education Center, Tohoku University, Japan.

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A Simple Device Allowing Silicon Microelectrode Insertion for Chronic Neural Recording in Primates

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Abstract—**Micro-machined silicon microelectrodes are useful for obtaining high-density, high-spatial resolution sampling of neuronal activity within the brain, and hold promise for revealing the spatiotemporal dynamics of local circuits. However, the fragile nature of silicon electrodes precludes their application in chronic recordings for a long period of time in which electrodes are repeatedly passed through the hardened dura matter. Here, we describe a newly developed holder designed to make a micro-perforation through the dura matter in which a silicon electrode can easily be inserted.**

Keywords - silicon microelectrode, neuronal activity, chronic recording, dura matter, holder, micro-manipurator

I. INTRODUCTION

The single-neuron recording technique using microelectrodes in behaving animals, especially in monkeys performing behavioral tasks, [1] has contributed much to revealing the higher functions in the brain (e.g., [2][3]). In particular, by three-dimensional mapping the characteristics of neuronal activities over wide areas in the cerebral cortex (e.g., [4][5]), the functions of those cortical areas have been able to be discussed in the single-neuronal level.

For neuronal mappings in this sort of chronic neurophysiological studies, microelectrodes have to be inserted into the cortex repeatedly and single-neuronal activities have to be recorded one by one in each layer of the cortex by moving the electrode by a micromanipulator within daily experimental sessions for a period as long as several months or more. So as to avoid the risks caused by the long experimental period, such as infection, the dura matter is left intact. And, at the initial surgery, the skull around the cortical area of interest is removed and a recording chamber, which keeps the surface of the dura over the recording site clean throughout the recording period, is attached to the skull around the recording site. Usage of the recording chamber also enables the experimental procedure in daily recording session quite easy. However, during such a long period of recording session, the dura matter within the recording chamber inevitably thickens, which is the reason why only mechanically-strong metal electrodes have long been used in this sort of chronic neurophysiological studies.

In recent years, increasing numbers of studies are recording the activities of multiple neurons simultaneously, using multiple electrodes inserted in multiple sites within a area or areas of interest to reveal the dynamics of the underlying neuronal networks (e.g., [6][7]). However, the relationships between the dynamics and the structure of the network, specifically the laminar structure of the cortex are less well understood and would be revealed through simultaneous recording.

In order to record neuronal activities from different laminae of the cortex simultaneously, silicon microelectrodes have a great advantage. This is because one silicon microelectrode can have multiple recording sites, spaced at accurate intervals along its shaft using silicon-based micromachining (e.g., [8][9]).

At present, however, because of the fragile nature of silicon, the main application of these silicon microelectrodes *in vivo* is for acute recordings after cutting the dura matter [10]. Thus far, silicon microelectrodes have not been applied to chronic neurophysiological recordings for neuronal mapping in behaving monkeys because the dura matter becomes thickened during the long recording period.

Here, we describe a new technique that allows the use of fragile silicon electrodes for chronic neurophysiological recordings or mappings in monkeys. For this purpose, we developed a novel holder for a silicon electrode combined with a punch-out pipe. This pipe makes a micro-perforation through the dura matter, and the silicon electrode is inserted through this perforation to the cortex. Using this technique, we succeeded in the simultaneous recording of multiple single-neuron activities from the cerebral cortex of a monkey.

II. MATERIALS AND METHODS

A. The Silicon Electrode Holder

Schematic views for the new silicon electrode holder are shown in Fig. 1(a). This holder is attached to a micro-manipulator which is widely used in chronic neuronal recordings of monkeys (MO-95, Narishige, Tokyo, Japan). "The micro-manipulator attachment", combined with stopper A (the details for stoppers A and B are described below), serves

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this purpose. The socket for a silicon electrode and the connector to the head amplifier were attached to "the silicon electrode holder". "The position adjuster" is used to set the tip of the silicon electrode and the punch-out pipe (described below) coaxially.

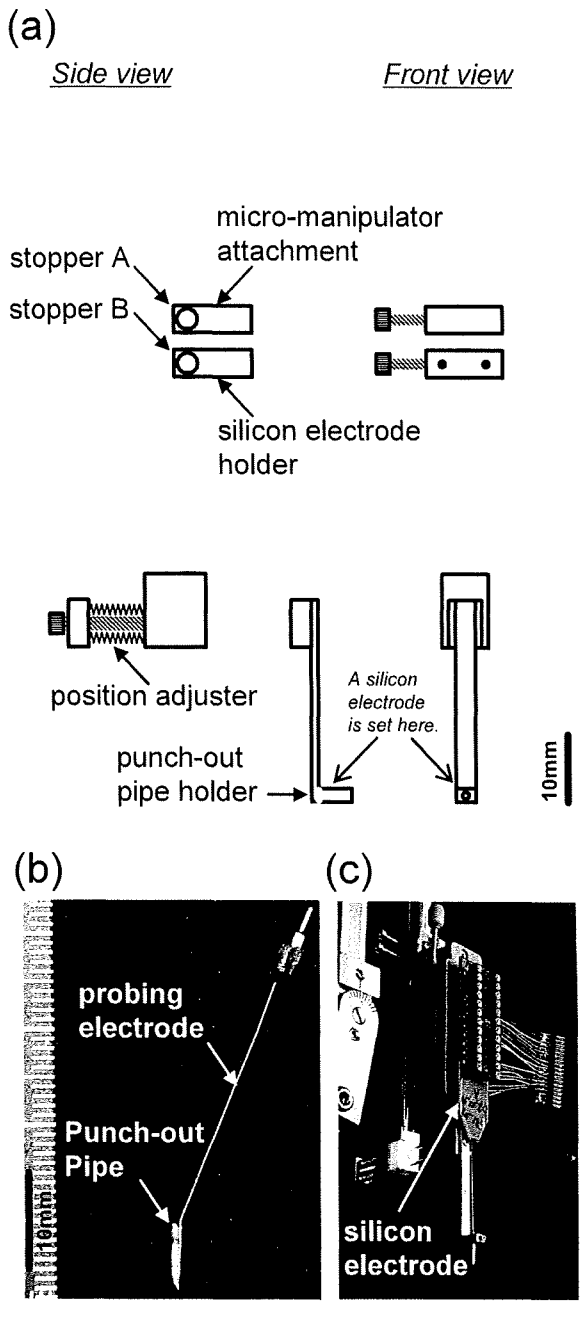

Figure 1. The silicon electrode holder. (a) Front and side views of the holder. (b) The punch-out pipe with a probing electrode. (c) The overall structure of the recording system using the new holder

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B. Fabrication of the Punch-out Pipe

The punch-out pipe is essential for making a micro perforation through the hardened dura matter. Through this perforation, a fragile silicon electrode is inserted into the cerebral cortex. For this purpose, the punch-out pipe has to penetrate the dura matter. However, the punch-out pipe must not be inserted into the cortex deeply, to allow the recording of neuronal signals from several points within the laminar structure of the cortex simultaneously by using the silicon electrode that has several recording sites along its shaft. Therefore, one has to know whether the punch-out pipe has penetrated the dura matter.

In order to detect the penetration, a conventional metal microelectrode (polyimide-coated tungsten electrode, 1-2 MΩ at 1kHz, Maruho-Hatsujo Kogyo, Kyoto, Japan) was attached to the punch-out pipe as a probing electrode. The punch-out pipe can be fabricated easily by cutting an indwelling needle covered by a thin plastic tube or a cannula (18G×1・1/4, OD 1.3mm, ID 0.95mm, TERUMO, Tokyo, Japan). The metal electrode was positioned between the metal needle and the plastic tube (Fig. 1(b)).

The punch-out pipe with a probing electrode, the holder for silicon electrode and a silicon electrode were assembled as shown in Fig.1(c). This was attached to the micro-manipulator for chronic neurophysiology as described above.

C. Silicon Electrode

Recordings were made with single-shank silicon electrodes which had 16 recording sites (our newly developed electrodes [11], or NeuroNexus, Ann Arbor, MI, or Yamagata Electronic Corp., Yamagata, Japan).

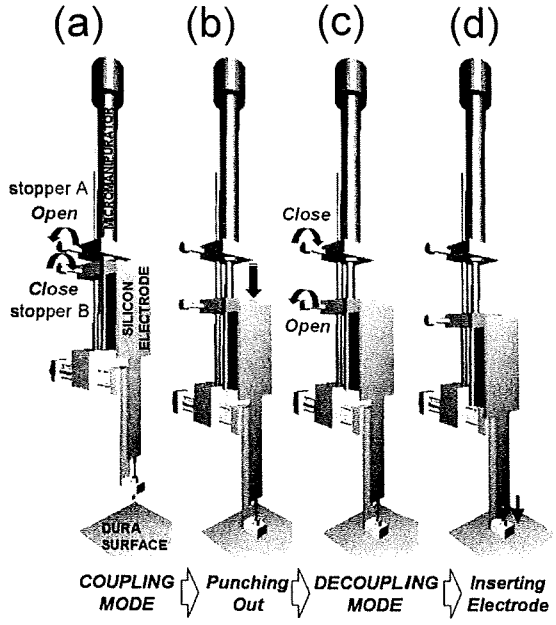

Figure 2. Schematic outline of the procedure of insertion of a silicon electrode using the new holder.

D. How to Insert a Silicon Electrode under the Dura Matter

First (Fig. 2(a)), stopper A should be opened, while stopper B is closed (COUPLING MODE). In this mode, the silicon-electrode holder and punch-out pipe holder are movable integrally. At this stage, the tip of the silicon electrode was set within the punch-out pipe.

Then, the silicon-electrode holder and punch-out pipe holder are lowered together until the punch-out pipe penetrates the dura matter, driven by the micro-manipulator (Fig. 2(b)). Penetration of the dura matter is detected by the probing electrode attached to the pipe. As in ordinary electrophysiological recordings of extracellular activities, the electric signals from the probing electrode were monitored not only with an oscilloscope but also with a speaker. This sound monitoring, in particular, let the experimenters know if the electrode has reached the cortex very clearly.

After the punch-out pipe penetrates the dura matter, stopper A is closed and stopper B is opened (Fig. 2(c), DECOUPLING MODE). Unlike before, only the silicon-electrode holder is movable, while the punch-out pipe holds the position in this mode.

Finally, only the silicon-electrode holder holding the silicon electrode is driven down through the punch-out pipe to the cortex (Fig. 2(d)).

III. Results

The novel silicon electrode holder was used to insert an electrode in a Japanese monkey (*Macaca fuscata*, 7.5 kg) that had been used for chronic neural recordings before this experiment. The animal was cared for in accordance with the Guiding Principles for the Care and Use of Laboratory Animals of the National Institutes of Health and the Guidelines for Animal Care and Use of our institution. For additional detailed descriptions of the surgical procedures, see [12]. Briefly, an acrylic recording chamber had already been attached to the monkey's skull under aseptic conditions. During surgery, the animal was anesthetized with ketamine hydrochloride (10 mg/kg intramuscularly) and pentobarbital sodium (30 mg/kg intramuscularly). The dura was not removed and left intact. Following surgery, cortical sulci were identified using a magnetic resonance imaging scanner (OPART 3D-System; Toshiba, Tokyo, Japan). Fig. 3(a) shows the punch-out pipe, attached to the holder, inserted into the dura matter. Neuronal activities were recorded using a Cheetah data acquisition system (Neuralynx, Tucson, AZ).

Fig. 3(b) shows an example of a single neuron recorded from a silicon electrode inserted into the frontal cortex of a monkey using our new technique. We found that this neuron was recorded stably throughout the recording session. Fig.3(c) indicates that spike activities were recorded from almost all of the recording sites on the shaft of a silicon electrode, demonstrating the possibility of neuronal recording using a fragile silicon electrode inserted through the hardened dura matter of a monkey subjected to repeated experiments. We moved the silicon electrode by the micro-manipulator until neuronal activities stopped being recorded from the recording site near the tip, which indicates the electrode penetrated the entire cortical layers. This implies that neuronal activities were recorded from each layer.

We have not evaluated the influence of the penetration for the tissue histologically, because the monkey is been served for a behavioral task and the physiological experiment during its execution. Though a small damage must be inevitable for each penetration in our experimental procedure, the penetrated hole in the dura shut up after drawing the pipe out of the dura, and any severe incidences of damage have not been observed.

IV. Discussion

In this paper, we developed a novel holder for fragile silicon electrodes. In order for the silicon electrode to pass through the hardened dura matter in chronic neurophysiological recordings, a punch-out pipe was set to the holder. This holder enables us to manipulate the silicon electrode and punch-out pipe simultaneously, and then to pass the silicon electrode solely through the pipe once it was positioned properly. By using this simple device, we succeeded in recording spike activities of the cortical neurons through each recording site of a silicon electrode that had penetrated through the hardened dura matter of a monkey.

Silicon electrodes were usually implanted for chronic neurophysiological studies (e.g. [13]). In those studies, once the electrodes were implanted aseptically, they were completely surrounded by dental acrylic cement or similar sort of materials as soon as possible. This type of experiment has advantages in observing neuronal activities at certain sites for a long period of time. However, mapping neuronal activities of a wide area in the cortex has a complementary role to former studies using silicon electrodes in neuroscience. Our simple but new method would have expanded the range of application of silicon electrodes to a traditional type of chronic neurophysiology or neuronal mappings.

Our simple device presented in this paper would be applicable to other fragile probes. For example, a confocal imaging probe is a powerful candidate, which enables us to examine the spatiotemporal patterns of various biological substances [14]. Recently, a microprobe for confocal imaging was applied in a freely moving mouse, and activities of many individual neurons were measured simultaneously [15]. Such a microprobe could be passed through the hardened dura matter of chronic monkeys by using our novel device. By combining various measurement techniques, fine structures of spatiotemporal dynamics of the brain will be revealed.

Acknowledgment

The authors would like to thank Mr. Y. Takahashi and Mr. M. Kurama (Physiology) for technical assistance. We also would like to thank M. Watanabe and F. Hayasaka of Yamagata Electronic Corp. for providing us the silicon electrodes.

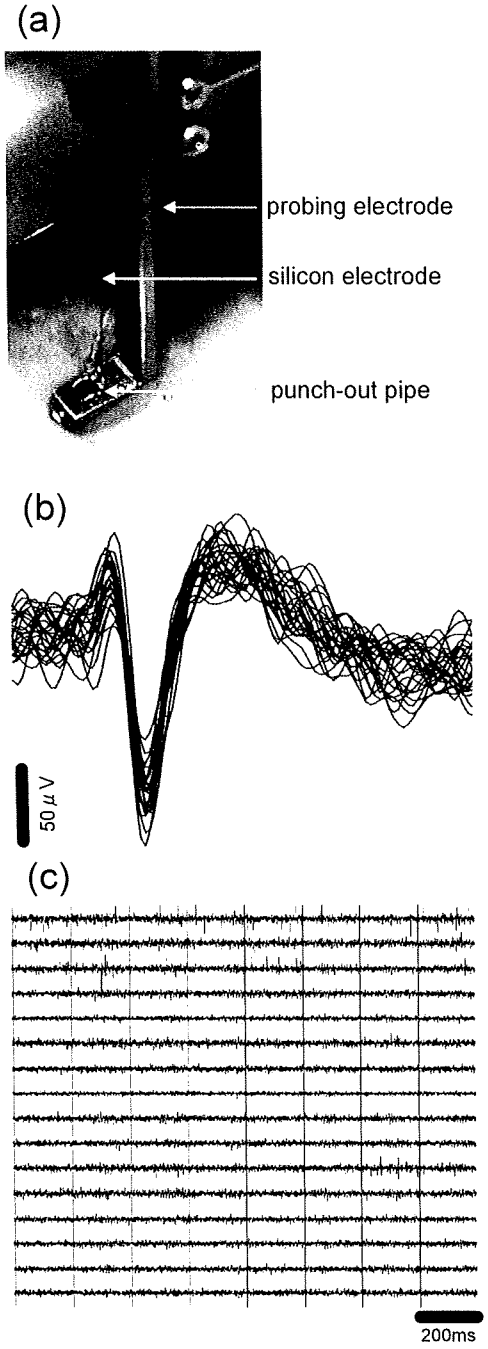

Figure 3. The recording of neuronal activities from a chronic monkey. (a) This picture shows a silicon electrode which was inserted into the cerebral cortex of the monkey through the punch-out pipe. (b) An example of single-neuronal activity. (c) Spike activities of neurons were recorded from almost all of the recording sites on the silicon electrode.

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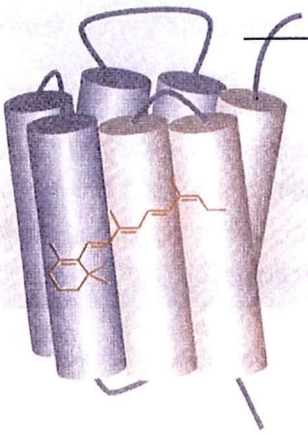
●光信号を電気情報に換えるタンパク質●

チャネルロドプシン
――オプトジェネティクスへの招待――



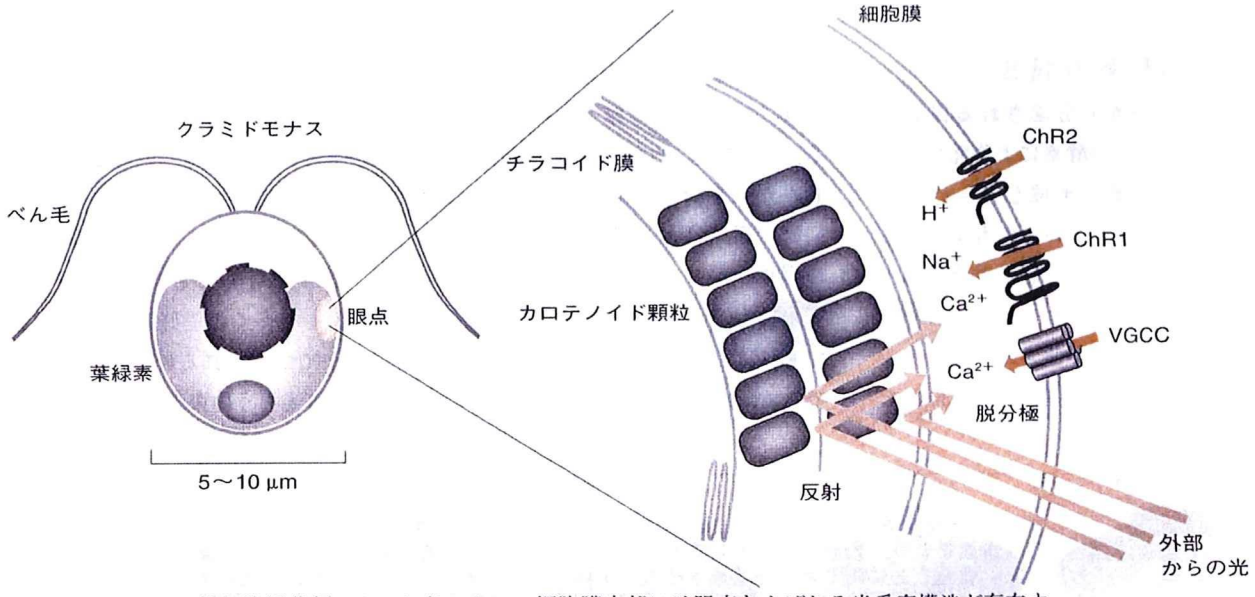
杉山友香　石塚　徹
王　紅霞　八尾　寛

チャネルロドプシンは，光受容体機能とイオンチャネル機能を合わせ備えたユニークなタンパク質である．光エネルギーを電気的情報に変換する一風変わった性質は，生物学的な興味を引きつけるに留まらず，これまで計測が難しかったニューロンネットワークを解明したり，視覚機能を回復したりするための新たなバイオツールとしての可能性も拡がりつつある．

オプトジェネティクスの黎明

ヒトを含むさまざまな動物において，脳の機能は，神経細胞（ニューロン）のネットワークの活動に依存している．したがって，ニューロンネットワークにおける信号の流れを解読することに，脳研究の主要な目的がある．スペインの神経解剖学者のラモニ・カハールがこのアイデアを提案してから，約100年経過したにもかかわらず，ニューロンネットワークは脳研究においてブラックボックスとして扱われてきた．ネットワークを構成するニューロンが，多様な形と機能をもち，複雑なつながりをつくっており，従来の研究手法が使えないことがその理由である．

しかし，近年になり，大きなブレークスルーがあった．



単細胞緑藻類のクラミドモナスの細胞膜内部には眼点とよばれる光受容構造が存在する．眼点では色素顆粒とそれを支える内膜が層構造を形成している．外部から入った光はカロテノイド顆粒に反射し，細胞膜上にあるチャネルロドプシンへと届く．チャネルロドプシンを透過した陽イオンにより細胞内が脱分極し，電位依存性カルシウムチャネル（VGCC）が開き，膜電位変化を2本のべん毛へと伝えると考えられている．

図1　クラミドモナス眼点と光受容構造

それは，緑色蛍光タンパク質（GFP）による細胞やタンパク質の可視化に始まっている．GFPやその改変体を利用して，pHやカルシウム濃度，膜電位の変動に応じて蛍光強度が変化するレポータータンパク質が開発されてきた．GFPの遺伝子や機能タンパク質の遺伝子をもとにこれらのレポータータンパク質の設計図にあたる遺伝子をつくることができる．この遺伝子をニューロンに組込んで発現させ，蛍光の波長や強度を光学的に計測することによって，そのニューロンの機能を知ることができる．すなわち，**遺伝子工学と光学計測技術の組合わせ技術（オプトジェネティクス）**の出現である．数年前までは，オプトジェネティクスは，ネットワーク機能の計測（アウトプット）の目的に限定されていた．ネットワークを解読するには，インプットの技術が不可欠なのだが，これを実現するタンパク質として，チャネルロドプシン（ChR）が注目されている．

チャネルロドプシンの発見

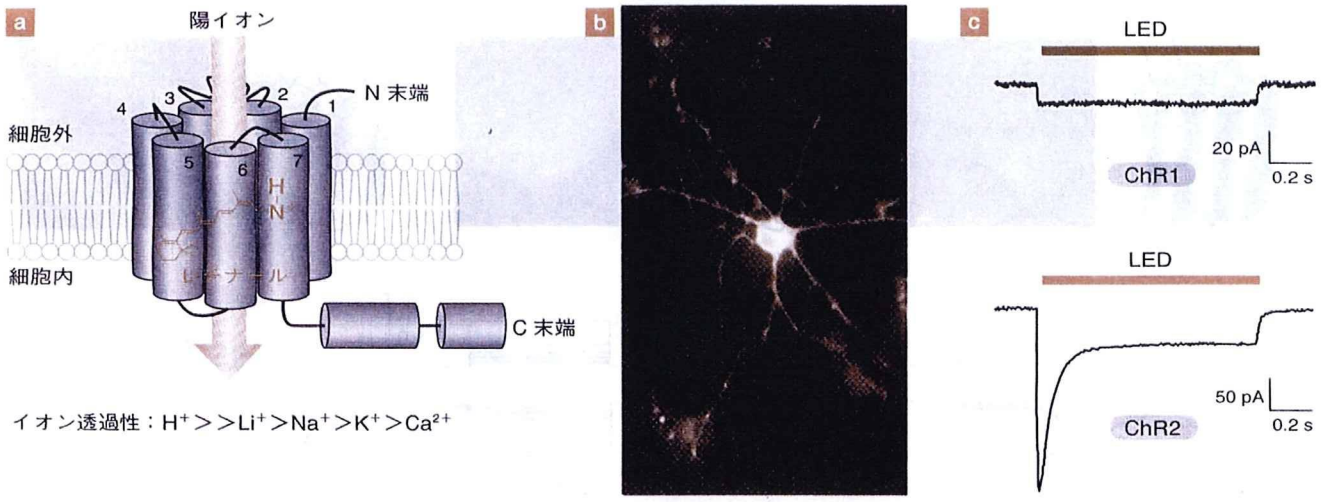
単細胞緑藻類の一種であるクラミドモナスは，淡水の池や沼などに生息している植物性プランクトンである．2本のべん毛と眼点とよばれる構造をもち，光合成のために必要な光に集まる走光性や，急激な光強度の変化で遊泳をやめる光驚動性を示す（図1）．

クラミドモナスに光を照射すると眼点上の形質膜で内向きの光電流が起こり，脱分極してべん毛にある電位依存性カルシウムチャネルが開き，べん毛運動に変換される．この光受容体は，動物と同じロドプシン類であるといわれていたが，長い間，そのタンパク質同定には至っていなかった．

しかし，2001年にかずさDNA研究所と米国デューク大学からクラミドモナスのゲノムデータベースが公開されると，複数のグループがほぼ同時に二つの古細菌型ロドプシンの関連遺伝子配列を同定した．なかでも，G. NagelとP. Hegemannのグループは，それ自体がイオンチャネルとして機能することを見いだし，それぞれをチャネルロドプシン1，2（ChR1，ChR2）と命名した．現在，これらの名前が広く用いられている．

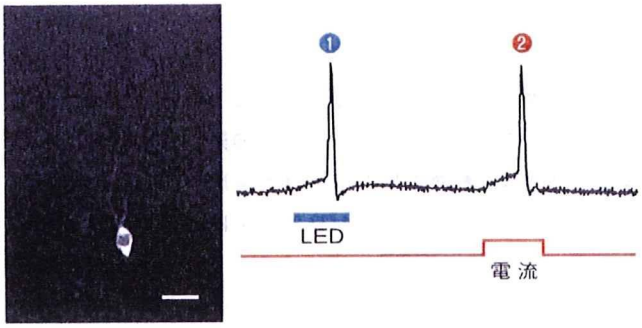
プランクトンの遺伝子をニューロンに組込む

チャネルロドプシンはおよそ700個のアミノ酸からなるチャネルオプシンタンパク質と発色団であるレチナール分子が結合したものである（図2）．このタンパク質分子は，ほかの古細菌型ロドプシン類と同様に7回膜貫通ヘリック



a）チャネルロドプシンはN末端側から300個程度のアミノ酸残基が7回膜貫通ヘリックス構造をとり，光感受性イオンチャネルを形成している．光に応じてチャネルが開くと，非選択的に陽イオンを透過させる．生体内でのイオン透過性は，Na^+に対して最も大きく，K^+やCa^{2+}は比較的小さい．b）ChR2の細胞内C末端に蛍光タンパク質を融合し，培養神経細胞に導入したもの．蛍光マーカーの発現により，ChR2が細胞膜全体に発現していることがわかる．c）7回膜貫通領域のチャネルロドプシンを発現させた培養細胞に青色LEDを1秒間照射して得られた光電流トレース．ChR2はピークと定常電流からなる，二相性の光電流を示す．ChR1光電流はチャネルロドプシン2に比べると非常に小さい．

図2　チャネルロドプシンの7回膜貫通構造と光電流トレース



ChR2-Veを発現している歯状回顆粒細胞（スケール10 mm）．①はLED照射，②は電流を流して誘発された活動電位記録．光照射のみで電気刺激と同様の活動電位が記録された．

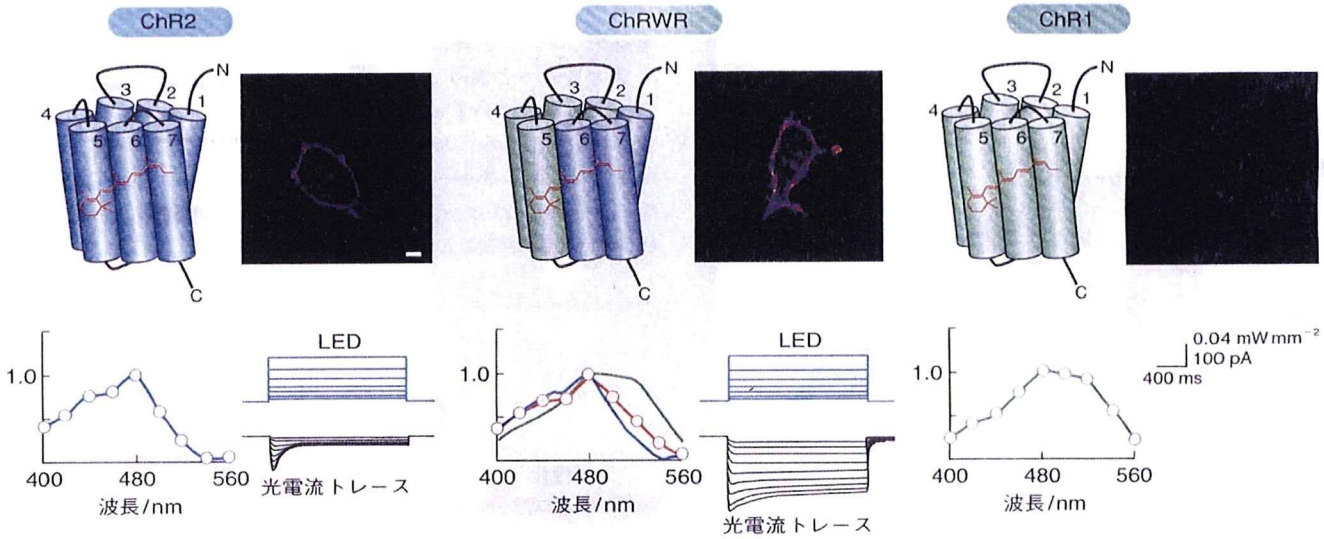
図3　チャネルロドプシン2を発現した，マウス海馬顆粒細胞と光刺激

ス構造をとり，光受容チャネルとして機能することが知られている（コラム1参照）．すなわち，チャネルロドプシン1は510 nm付近，チャネルロドプシン2は470 nm付近の光を吸収してポア（孔）が開き，H^+，Na^+，Ca^{2+}などの陽イオンを透過させる．これは，光で誘発される膜電流（光電流）をつくりだす．

チャネルロドプシンのこのような性質に注目して，ニューロンの光刺激に応用するオプトジェネティクスは，世界の三つのグループでほぼ同時にかつ独立に開始された．筆者らのグループ（石塚徹ら）は，チャネルロドプシン2のN末端1-315アミノ酸残基に蛍光標識としてGFP改変体のビーナス（Venus）を付加した融合タンパク質（ChR2-Ve）を作製し，シンドビスウイルスベクターを用いてマウス海馬の神経細胞にチャネルロドプシン2を遺伝子導入した（文献5）．約24時間後に脳を取出して海馬スライスを作製すると蛍光顕微鏡下にChR2-Veを発現している海馬ニューロンが確認される（図3左）．このチャネルロドプシン2を発現した細胞に青色LED照射を行うと，光パルス刺激に同期して活動電位が記録された（図3右）．チャネルロドプシン2を用いた光によるニューロンの活動制御は電極刺激や，ほかの光刺激法に比べても非常に精度が高く，細胞種特異的に数10 Hzでの連続刺激が可能である．

しかし，光刺激ツールとして眺めるとチャネルロドプシン2には，下記のようないくつかの改善すべき点がある．

1）チャネルロドプシン2に最も効果的な青色光は，光ファイバーや脳組織などの光媒体に吸収され，脳の深部に到達しにくい．また，緑色や赤色の光を吸収するチャネルロドプシンがあれば，ニューロンネットワー



チャネルロドプシン・ワイドレシーバー（ChRWR）はチャネルロドプシン1の第1～5ヘリックスとチャネルロドプシン2の第6～7ヘリックスからなるキメラタンパク質である．それぞれのC末端に蛍光マーカーを融合し，HEK293培養細胞で発現させると，ChRWRは特に発現が高いことがわかる．ChR2（青）と比べて500 nm付近での吸収が大きく，より長波長での刺激が可能となる．吸収スペクトルは最大吸収を1としたときの相対値を表している．LED光源の明るさを変化させて記録した光電流トレースでは，ChR2と比べて大きなピーク電流が見られ，脱感作が減少した分だけ，電流の総量が増加していることが見てとれる．ChR1の光電流は，相対的に小さいので省略した．

図4　チャネルロドプシン機能－構造連関研究の成果

クをマルチチャネルに駆動することができる．

2) チャネルロドプシン2の光電流が増強されれば，比較的微弱な光でもネットワークを駆動することができる．

3) 光を当て続けていると，チャネルロドプシン2の光電流は，急速に減少し，回復するのに数10秒の暗状態を要する（脱感作）．脱感作を弱めることにより，インプットの信頼性が高くなる．

光エネルギーを電気情報に変換するしくみ

チャネルロドプシンは，光受容体機能とイオンチャネル機能を合わせ備えたタンパク質として最初の報告であり，自然界においてきわめてユニークである．では，光エネルギーは，どのようにして電気情報に変換されるのだろうか？　この変換機能を担っているタンパク質構造を明らかにする研究は，生物学的に重要である．また，光を受容する構造やイオンを透過する構造などが解明されれば，これらの機能において改善されたチャネルロドプシンをデザインすることができる．

筆者らのグループ（王 紅霞ら）は，チャネルロドプシン1とチャネルロドプシン2の機能の違いに注目し，膜貫通ヘリックス構造それぞれの機能を解明した（文献6）．第1ヘリックスおよび第2ヘリックスは，タンパク質の形質膜導入に関係しており，チャネルロドプシン1由来の構造はこれを促進する．第5ヘリックスおよび第7ヘリックスは，吸収波長を決定しており，チャネルロドプシン1由来の構造は長波長吸収を促進する．第2ヘリックスおよび第6ヘリックスは，イオンの透過性に関係しており，チャネルロドプシン2由来の構造はイオンの流量を増大する．第2ヘリックスおよび第5ヘリックスは，脱感作に関係しており，チャネルロドプシン2由来の構造は脱感作を増大する．

さらに，このような機能がアミノ酸1残基に担われていることもわかった．たとえば，チャネルロドプシン2の第

コラム1　動物型と異なる古細菌型ロドプシンのレチナール光受容メカニズム

われわれの網膜に存在する光受容体など，高等動物に存在する動物型ロドプシンのもつレチナール発色団は基底状態で11シス形をとる．これは光吸収により異性化し全トランス形となり，レチナールがタンパク質から解離する．新たな11シス形レチナールがアポタンパクに結合することで，次の光受容が可能になる．動物型ロドプシンは，構造変化によりGタンパク質を介して細胞内分子カスケードを活性化し，細胞膜上にあるイオンチャネルの開閉に関わることが知られている．

ChRを含めた古細菌型ロドプシンのレチナールは基底状態で全トランス形をとり，第7ヘリックス内のリシン残基とシッフ塩基を介して結合している．青色光を吸収すると13シス形へ光異性化し，タンパク質構造変化のスイッチとなるが，タンパク質に結合したまま熱異性化により元の全トランス形へと戻り，そのまま次の光サイクルを始動する．チャネルロドプシンの機能にはレチナール分子との結合が必須であるが，ヒトやマウスなどの哺乳類では細胞の代謝によってレチナールが供給される．つまり外部から供給する必要がないため，光刺激ツールとして生体内など深部組織へ応用することが容易である．