Electrochemical Monitoring of Glutamate Release at Multiple Positions in a Rat Hippocampal Slice

Nahoko KASAI,*† Yasuhiko JIMBO,* and Keiichi TORIMITSU*,**

*NTT Basic Research Laboratories, NTT Corporation, 3-1, Morinosato Wakamiya, Atsugi, Kanagawa 243-0198, Japan

**CREST, JST, 4-1-8-12F, Honcho, Kawaguchi, Saitama 332-0012, Japan

The continuous monitoring of the distribution of glutamate (Glu), a neurotransmitter released at synaptic terminals, is important in terms of understanding the signal transfer mechanism in the brain. In this study, we monitored the concentration of Glu released at multiple positions in a hippocampal slice continuously, and obtained an approximate Glu distribution by using our electrochemical glutamate sensor array. After confirming our sensor's high sensitivity to Glu, we placed a slice on the array, and measured the currents at selected electrodes in the array. When we stimulated a specific position in the slice electrically, the glutamate concentration increased in different areas after several tens of seconds. The presence of glutamate receptor blockers suppressed these increases. This suggests that the electrical signal was transferred along with neurons through synapses and stimulated the Glu release. Our multichannel glutamate sensor should be a powerful tool to determining the distribution of real-time glutamate non-invasively for the studies using biological samples.

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Introduction

Glutamate (Glu) is one of the excitatory amino acid neurotransmitters released at synaptic terminals. Recently, numerous attempts have been made to measure the glutamate concentration in the brain in order to reveal the role of Glu.¹⁻⁴ However, the objective of these studies was to achieve real-time monitoring with high-spatial resolution, and little attention has been directed toward the distribution and dynamics of Glu. In the hippocampus in particular, the simultaneous monitoring of the Glu distribution along the signal transfer route could provide us with further information about the signal transfer mechanism and the mechanism of memory and learning in the brain.

Our group has succeeded in monitoring the concentration of Glu released at multiple positions in a hippocampal slice by using our novel electrochemical sensor.⁵ This multichannel glutamate sensor is capable of the simultaneous monitoring of real-time glutamate concentrations non-invasively. Our previous result suggested that it is a powerful tool for understanding the role of glutamate in the brain.

In this study, we observed changes in the Glu distribution with time when the hippocampal slice was electrically stimulated. The hippocampus has a distinctive structure and its signaling route is well known. As shown in Fig. 1, the active potential generated by the electrical stimulation of Area I in the hippocampus, the so-called "dentate gyrus", is confirmed to be transferred, thus generating the potential of the neurons in Area II, "CA3", and further to Area III, "CA1". However, there have been no successful observations as regards the neurotransmitter.

Therefore, the glutamate distribution could provide a new clue to understanding how the signal is transferred in terms of neurotransmitter release.

Experimental

Electrode array

We fabricated our multichannel electrochemical Glu sensor using an indium-tin-oxide (ITO) planar electrode array (Fig. 2).⁶ The method we employed, which includes the immobilization of enzymes (HRP and glutamate oxidase), has been explained elsewhere.⁵

Hippocampal slices

We cultured hippocampal slices (300- μ m-thick) obtained

Fig. 1 Illustration of a rat hippocampal slice. The electric stimulation was given at the white arrow in Area I.

† To whom correspondence should be addressed.
E-mail: nahoko@will.brl.ntt.co.jp

Fig. 2 Electrode array used in this study.

from Wistar rats (8-day post-natal) on pretreated porous polycarbonate membranes (thickness: 7 μm , pore size: 0.8 μm) for 7 days under 5% CO_2 water-saturated conditions at 34°C in Dulbecco's modified Eagle's medium (Gibco) containing heat-inactivated fetal bovine serum, heat-inactivated horse serum, insulin, penicillin, and streptomycin. We pretreated the membrane surface using laminin and poly-D-lysine before cultivation.⁴

Procedure

We positioned a membrane with a cultured slice sample on the Glu sensor array on an inverted microscope using a Nylon mesh. HEPES buffer consisting of 148 mM Na^+ , 2.8 mM K^+ , 2 mM Ca^{2+} and 2 mM Mg^{2+} (pH 7.4, saturated with a mixed gas of 95% O_2 /5% CO_2) was allowed to flow at 0.7 ml/min during the measurement and the currents at the electrodes of the array were obtained at -100 mV vs. Ag/AgCl by using a multipotentiostat (HA1010mM8, Hokuto Denko). The electrical stimulation (30 V, 20 Hz, 600 ms) was provided using a tungsten stereo-type electrode (tip size: 1–2 μm , tip distance: 125 μm). Then a mixed solution of glutamate receptor blockers ((+)-MK-801 hydrogen maleate, MK801 (RBI), DL-2-amino-5-phosphonopentanoic acid, AP-5 (Sigma), and 6-cyano-7-nitroquinoxaline-2,3-dione, CNQX (RBI)), was added to the flowing solution and the flow was maintained for 30 min before the next stimulation was provided. We selected from four to seven of the 64 electrodes for the measurement. We chose the electrodes with the best performance as regards sensitivity and selectivity, and with suitable positions in the slice that represented each region in the hippocampus.

Results and Discussion

First, we confirmed the electrodes' characteristics. Figure 3 shows calibration curves for four electrodes from the array, obtained from the steady-state current responses that occurred when glutamate was added to the solution. As shown in the figure, the responses increased almost linearly with increasing Glu concentration. This electrode array was able to detect the presence of sub-micromolar Glu concentrations. We also examined the selectivity of the electrodes and ascertained that the electrodes' responses were more than 100 times more sensitive to Glu than to ascorbic acid (AA), an electrochemically active substance in the hippocampus (data not shown). Here, we considered the ambient AA concentration in the extracellular fluid in hippocampal slices and the increased amount that results from an epileptic seizure.^{7,8} The selected electrodes also had other characteristics, such as high sensitivity

Fig. 3 Calibration curves of the 4 selected sensors to glutamate.

to Glu (about 1 pA to 1 μM Glu) and a fast response speed (about 5 s time constant). The difference between the electrodes is probably due to the non-uniform distribution of the effective enzymes and/or mediators in the modified membrane on each electrode, and/or to the diffusion rate of the substrate, Glu, which is also related to the partial difference in the modified membrane thickness. We confirmed that the electrodes had the same responses to glutamate both before and after exposure to the slice (data not shown).

We then monitored the reduction currents at the selected electrodes in this array when a slice was placed on the array, and obtained the glutamate concentrations from each reduction current using the calibration curve of each electrode. Figure 4 shows images of the approximate distribution of the Glu concentrations. The colored squares in the photograph indicate the electrode positions used in the measurement and the glutamate concentration at each position. We enlarged the electrode by 9 times to emphasize its color (The actual electrode size can be observed in the photograph in Fig. 2). Figure 4 also shows the change in the Glu distribution after stimulation. Electrical stimulation was provided at the time indicated at the position shown by x in the photograph. In HEPES buffer without the blockers (Fig. 4(A)), we observed several increases in the reduction current, indicating the increase in glutamate concentration especially in Area II over a long time scale of tens of seconds after the stimulation. However, the increase in most areas of the hippocampus was suppressed when we added the glutamate receptor blocker to the solution (Fig. 4(B)).

This would suggest that the electrical signal caused by the stimulation was transferred along with neurons through synapses as shown in Fig. 1, and at the same time the Glu released in the same area as the signaling route. This suggests that the synaptic transmission would involve the glutamate release at the synaptic terminal.

The glutamate receptor blocker we used in this study was a mixture of three different blockers that are believed to block most of the different types of glutamate receptor. Therefore, the signal transfer caused by the glutamate release would be inhibited when the blocker was added as observed in Fig. 4(B).

However, the glutamate release we observed occurred several tens of seconds after the stimulation, which is much slower than the electrical signal transfer rate of millisecond order. In an

Fig. 4 Change in the glutamate distribution. Glutamate concentration was indicated as colors. The electrical stimulation was provided at the white x in each photograph. Time in this photograph indicates the time after stimulation. Electrode size was enlarged to emphasize its color.

electrophysiological study using a voltage sensitive dye, the electrical signaling transmission was monitored at a millisecond order.⁹ In that study, the electrical stimulation spread inside the CA1 area (Area III) in a hippocampal slice in tens of milliseconds. The study also found that long-term depolarization, namely the increase in postsynaptic potential, occurred in tens of minutes. These results optically demonstrated the transition of the electrical signal inside the hippocampus.

Although the response speed of the electrode was slow, of the order of seconds, we were able to observe the successive release of glutamate from the slices. The slow Glu release that we observed suggests that glutamate would be related to the long-term transition. We can therefore assume that there may be many different Glu release pathways. We need to undertake a further investigation of glutamate release mechanisms.

Conclusion

We were able to monitor real-time glutamate release at multiple positions in a network of neurons in a rat-hippocampal slice by using our multichannel Glu monitoring system consisting of an enzyme-immobilized electrode array. Although a great deal of effort has been devoted to the visualization of the distribution and localization of receptor subunits using approaches such as the *in-situ* hybridization of mRNA,^{10,11} there have been very few attempts to visualize the distribution of Glu itself. Unlike the invasive methods that involve immersing the samples in a fluorescent dye, our method using electrodes is noninvasive, thus enabling us to undertake long or repetitive measurements in biological samples. Although our approach is rather primitive and requires further improvement in such areas as spatial resolution, this method should prove useful to those interested in obtaining a Glu distribution map by non-invasive means. Furthermore, this method could be a valuable tool for

pharmacological and biochemical analysis, and for the study of such subjects as memory and learning, and neural development.

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