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Excitatory Synaptic Connections to Layer 5 Neurons from Layer 2/3 in Visual Cortex

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ABSTRACT

In order to elucidate the organization of visual information processing in visual cortex, the relation between signal type and receptor type in excitatory synaptic transmission to layer 5 neuron from layer 2/3 was studied using slice preparation of cat visual cortex. The effects of application of agonists and antagonists for glutamate receptor on EPSP evoked in layer 5 neurons by electrical stimulation of layer 2/3 were investigated since the type of synaptic potential depends on the combination of transmitter and receptor. The results showed that two types of receptors for glutamate were responsible for the generation of two types of EPSPs separately.

Key Words : Cat Visual Cortex, Layer 5 Neurons, Excitatory Synaptic Transmission, NMDA Receptors

1. Introduction

In the layer 5 of visual cortex, there exist pyramidal cells which project to superior colliculus. Visual information from retina to superior colliculus is transmitted not only directly through optic nerve but also indirectly through visual cortex and influences the function of superior colliculus neurons¹⁾. It is based on studies of degenerating fibers²⁾ and results from intracellular injecting experiments of HRP³⁾ that the prime input to layer 5 of visual cortex is from layer 2/3, however there have not been the electrophysiological reports on the input from layer 2/3 to layer 5 neurons except the case of rat⁴⁾. Besides, recently the organization of information processing in visual cortex have been investigated from viewpoints of transmitters and receptors in addition to signal types in synapses. Therefore studies on neural network of visual cortex have proceeded in the approach from transmitters of excitatory^{5, 6, 7)} or inhibitory amino acids⁸⁾. At present, glutamate of excitatory amino acid comes to be considered as convincing candidate for excitatory transmitter in visual cortex^{5, 6, 7)}. Receptors for the

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excitatory amino acids are divided into two groups; one is non-NMDA type receptor which have specific sensitivity to kainate and quisqualate, and another is NMDA type receptor which is specifically sensitive to an N-methyl-D-aspartate (NMDA)⁹. Especially, NMDA type receptor is suggested to be involved in neural plasticity¹⁰. In this study, to obtain the electrophysiological and pharmacological informations about the projection from layer 2/3 to layer 5 in cat against the anatomical informations on it, we investigated the relationship among the receptors of excitatory amino acid, the excitatory inputs and the excitatory synapses and examined the organizations of excitatory transmission of the neural circuit.

2. Method

2.1 Preparation and Maintenance of Tissue Slice

Experiments were performed using kitten (2-3 monthes old) deeply anesthetized by intraperitoneal injection of pentobarbital sodium (40mg/kg). After decapitation and quick removing of whole brain from skull, a block of tissue in primary visual cortex was cut off by vibrating cutter and cooled in the petri dish of cold oxygenated (4°C) standard medium. Then the tissue was mounted on microslicer and 500 μ m thick slices were made parallel to frontal plane. Slices were then transfered into a bottle with oxygenated standard medium of room temprature and stayed in it for at least 1 hour before recording to make slice recover from trauma of slicing procedure. Before transfer of the slice to recording chamber, Gennari's band was observed under binocular microscope to identify layer 4. Then the tissue was submerged in recording chamber where oxygenated standard medium of 34°C was continuously perfused with the flow rate of 2-3 ml/min. The composition of the standard medium is NaCl, 124mM; KCl, 5mM; NaH₂PO₄, 1.25mM; MgSO₄, 1mM; CaCl₂, 3mM; NaHCO₃, 25mM; Glucose, 10mM.

2.2 Electrical Stimulation and Recording

As shown Fig. 1, bipolar stimulating electrodes were inserted to the site 250 μ m inward from surface of the cortex for electrical stimulation of layer 2/3. These electrodes were made of insulated platinumiridium wire exposed only at the tip (tip diameter, 30-50 μ m; the interval between each electrode, 50-75 μ m; exposed part, 30-50 μ m). Another bipolar stimulating electrodes consisting of insulated acupuncture needles were placed in white matter. Constant current of square pulses 0.1msec in duration was applied to the slice with intesity of several tens to several hundreds microampere. Glass micropipettes filled with 4M potassium acetate (resistance; 50-80M Ω) were inserted into layer 5 locating at two thirds of cortex thickness to record synaptic potentials or synaptic current. Those signals were amplified with DC amplifier for microelectrode or voltage clamp amplifier (Axoclamp-2A, Axon Instruments Inc.), observed on digital memory scope display, stored on digital audiotape recorder (PC-108M, SONY) and plotted on X-Y recorder.

2.3 Agonists and Antagonists Applications

Micro-application of glutamate or NMDA to soma of layer 5 neuron was made iontophoretically from multibarreled microelectrode attached to 3 dimensional hydraulic micromanipulator (Fig. 1, C). The degree of access to cell soma was monitored on 3-D coordinates values of the drug electrode tip to the recording electrode tip and the intensity of responses to iontophoretically applied glutamate. Excitatory amino acids antagonists (Kyn; Kynurenic acid, APV; 2-amino-5-phosphonovalerate, CNQX; 6-cyano-7-nitroquinoxaline-2, 3-dione) were applied to perfusate to investigate the actions on EPSPs (excitatory post synaptic potential) evoked by layer 2/3 electrical stimulation and on potentials by

Figure 1. Schema of the experiment. a, bipolar stimulating electrodes of platinum-iridium. b, recording glass micropipette. c, multibarreled glass electrode. d, insulated acupuncture needle electrodes for stimulation of white matter.

inotophoresis of glutamate and NMDA. GABA (γ -Aminobutyric acid) antagonist picrotoxin (PTX) was added to perfusion fluid to block GABAergic inhibition in voltage clamp experiment.

3. Result

3.1. EPSPs Evoked by Electrical Stimulation of Layer 2/3

Stable intracellular recordings were obtained in 35 cells of layer 5 with resting membrane potentials between -60 and -70 mV. Electrical stimulations of layer 2/3 induced EPSPs and the following IPSPs (inhibitory post synaptic potential). Those EPSPs had latencies of 2.5-5.0 msec (mean = 3.4 msec), amplitudes of 8-15 mV and durations of 10-100 msec. They consisted of fast and slow components which showed a rapidly rising phase ($1.5-4$ V/sec) and a slowly rising phase (below 1 V/sec) respectively. Fig. 2A shows that response patterns varied with the changes of stimulus intensities. Both components had nearly same threshold (25μ A) and started to respond from low intensity. With increased intensities, the amplitude of fast component exceeded that of slow one and the slope in early part of the falling phase became so steep that each components could be definitely recognized. In some cells, it was observed that the shape of fast EPSP turned sharp with further increment of stimulus intensities (Fig. 2A, 35μ A). Since this sharpening of fast EPSP became more clear with the membrane depolarizations (Fig. 2B), IPSP proved to be involved in this phenomenon. The latencies in these fast EPSPs were not fluctuated with different stimulus intensities and therefore afferent inputs for fast EPSPs are considered to be monosynaptically transmitted. The axon collaterals of pyramidal cells in layer 5 are extending to layer 2/3, hence antidromic potentials to the stimulation of layer 2/3 were expected to be recorded, however those responses were never observed.

3.2. Effects of Applications of Excitatory Amino Acid Agonists and Antagonists

Figure 2. A, EPSPs evoked in layer 5 neuron by different intensities of electrical stimulation of layer 2/3. B, Check of IPSP by depolarization of the membrane potential from -60 mV (resting membrane potential) to -47 mV.

3.2.1 Existence of NMDA Receptor and the Participation on EPSP

EPSPs induced by layer 2/3 stimulations almost disappeared by bath application of kynurenic acid (5 mM) which was nonspecific antagonist of excitatory amino acid (Fig. 3A), and therefore it was suggested that excitatory amino acids were involved in the excitatory synaptic transmission from layer 2/3 to layer 5. To examine whether layer 5 cells have receptors of excitatory amino acid, glutamate and NMDA were iontophoretically applied to the vicinity of the cell body or proximal dendrites in layer 5 cells. Applications of them produced strong excitations (Fig. 3B GLU.100 nA, NMDA 100 nA) although the response to NMDA was slower than that to glutamate and the excitations by NMDA were completely blocked by bath application of APV (Fig. 3B NMDA + APV (50 μ M)). Even after synaptic transmission were blocked in perfusing fluid of low Ca^{++} (0.1 mM) and high Mg^{++} (5 mM) (Fig. 3B lower row right), the iontophoresis of NMDA elicited excitations though the excitability reduced than in normal medium (Mg^{++} concentration 1 mM) (Fig. 3B lower row center).

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Figure 3. Effects of agonists and antagonists of excitatory amino acids.

Therefore it is suggested that this cell have NMDA receptors located at proximal dendrite or soma.

From those, EPSP elicited by layer 2/3 stimulation was considered probably to have the properties of NMDA receptors. It is well known that NMDA receptors are subjected to blockade by Mg^{++} in normal medium and inhibited by APV application. In Mg^{++} free solution, the EPSP augmented (Fig. 3C) and addition of APV to normal medium gave rise to the amplitude reduction in falling phase and 30% reduction in duration (Fig. 3D). These properties also were examined in the medium contained non-NMDA antagonist CNQX where non-NMDA receptor mediated EPSP was blocked. In Fig. 4, the effects of CNQX, Mg^{++} and APV on EPSPs are shown. Threshold for action potential was $120 \mu A$ in normal medium and CNQX bath application ($10 \mu M$) eliminated action potential though slowly rising EPSP remained (Fig. 4b, $120 \mu A$). This CNQX resistant EPSP was augmented by remove of Mg^{++}

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Figure 4. Effects of Mg^{++} removing and APV bath application on CNQX resistant EPSP.

from bathing medium (Fig. 4c) but completely blocked by bath application of APV ($50 \mu M$) (Fig. 4d). Then the fast and slow EPSP recovered after return to normal medium (with normal Mg^{++} concentration and without CNQX and APV) (Fig. 4e). Accordingly, this CNQX resistant component would be mediated through NMDA receptor. Another example is shown in Fig. 5 where GABA antagonist PTX was added in the medium to block GABAergic inhibition since IPSPs appear to mask a part of EPSPs as shown in Fig. 2B. Here the threshold of action potential was $240 \mu A$ in normal medium and under presence of PTX ($50 \mu M$) the CNQX resistant components were observed from stimulus intensity of $160 \mu A$. The CNQX resistant components rise very slowly though slope turn steep with the increase in the intensity. Then the application of APV ($50 \mu M$) eliminated the CNQX resistant component in stimulus intensity of 200, 240 and $260 \mu A$. From these results it is shown that EPSPs induced by stimulation of layer 2/3 have the same properties as NMDA receptors show and therefore NMDA receptors also would be involved in the excitatory transmission from layer 2/3 to layer 5 neuron even in normal medium.

3.2.2 Direct Participation of NMDA Receptor

As mentioned above, it was shown that layer 5 neurons possessed NMDA receptors and considered that the receptor was involved in excitatory transmission from layer 2/3 to layer 5. This gives rise to question whether NMDA receptor directly participates in the transmission. In order to examine this point, EPSCs correspondent to EPSPs evoked by layer 2/3 stimulation were recorded and the

dependency of EPSCs on membrane potentials was examined in the voltage clamp experiments after recording of intracellular potentials in the same cell as Fig. 5. If layer 5 neurons receive inputs from layer 2/3 directly through NMDA receptors locating on layer 5 cells, CNQX resistant EPSCs should show the increment of the amplitude with membrane depolarizations. It is shown in Fig. 6 that membrane potential levels were shifted at interval of 10mV from -100mV to -30mV while recording post synaptic current and applying antagonists to bath. In normal medium and with stimulus intensity of $160 \mu\text{A}$, inward current component at resting membrane potential of 70mV increased with hyperpolarization, reversed at -60mV and outward current component increased with depolarization. These inward and outward current components had fast falling phases. With CNQX and PTX application, these components disappeared in both hyperpolarization and depolarization levels. Therefore this component corresponds to the EPSP component of fast rising phase. As the amplitude of new slow inward EPSC appeared with CNQX and PTX application was small in stimulus intensity of $160 \mu\text{A}$, the intensity was increased to $240 \mu\text{A}$ (Fig. 6, CNQX $10 \mu\text{M}$ + PTX $50 \mu\text{M}$). Even in this intensity fast component was not present due to block of non-NMDA component by CNQX, whereas the EPSC component of slowly falling phase appeared in resting membrane potential despite normal Mg^{++} concentration. This new slow component corresponds to EPSP in the same condition of Fig. 5. Though the amplitude of this EPSC was still small in resting level, it increased with membrane potential depolarization showing the nature of response mediated through NMDA receptor. Furthermore, this slow inward component was completely blocked by application of APV ($50 \mu\text{M}$).

4. Discussion

Since the fast EPSP component was completely blocked by CNQX of the specific antagonist of non-NMDA type receptors while it was not blocked by APV of the specific antagonist of NMDA type receptors, it can be said that non-NMDA receptors are involved in the transmission of this component. We could not directly show that layer 5 neurons have non-NMDA receptors by micro-application of non-NMDA type receptor agonists such as kainate and quisqualate to the soma proximity, however as it is highly possible that the EPSP is generated by monosynaptic pathway from layer 2/3 as mentioned in the previous section, it is strongly suggested that this component is mediated through non-NMDA type receptors on layer 5 cell. It seems likely due to direct binding of glutamate to this receptor that the time course of excitatory response to electrophoretic application of glutamate was faster than in the case of NMDA application. Despite blocking of synaptic transmission with medium of low Ca^{++} and high Mg^{++} , layer 5 neurons showed excitatory responses to electrophoretic micro-application of NMDA to soma proximity. This means that NMDA receptors are located on proximal part of the soma. Accordingly first, in order to examine whether the excitatory inputs from layer 2/3 are received via NMDA receptors, effects of Mg^{++} , APV and CNQX application on the EPSPs were investigated. The slow EPSP component evoked by layer 2/3 stimulation was augmented in Mg^{++} free medium, eliminated by APV application but resisted CNQX. These results show that NMDA receptors were involved in slow EPSP generation.

The excitatory response mediated directly through NMDA receptor have the property that the amplitude increases in the depolarizing level of membrane potential because Mg^{++} which occludes NMDA receptor is released from the receptor at depolarizing level. Thus the voltage dependency of the inward slow current persisted under CNQX and PTX application was investigated. Its amplitude increased

Figure 5. Effects of CNQX, PTX and APV on EPSCs

Figure 6. Effects of CNQX, PTX and APV on EPSCs and dependency of EPSCs on membrane potentials. Arrows indicate stimulus onset. inward current downward.

with the shift of membrane potential to depolarizing level and showed the same property as the response mediated through NMDA receptors (Fig. 6 middle column and Fig. 7). From these results, the slow EPSP component corresponding to this EPSC component is considered to be transmitted directly through NMDA receptors on layer 5 neuron.

The latencies of EPSPs were 3.4msec on average but these were for the fast EPSPs. As the slow component is masked by fast EPSP and IPSP, it was recorded while blocking those components by CNQX and PTX. Since in the cell of Fig. 5 the latencies of fast and slow components were 3.6msec and 4.6msec respectively and did not show any latency fluctuation to the changes of stimulus intensity, it is strongly suggested that both components in this cell are the responses induced by monosynaptic input.

Since NMDA receptors are blocked by Mg^{++} in normal medium, it has been considered that the excitatory transmission is difficult to occur in standard medium. However in this study the results were obtained that layer 5 cells showed high excitability to the local application of NMDA even under such a high Mg^{++} concentration that synaptic transmission is blocked and that the component mediated through NMDA receptors existed in such EPSPs that enabled to generate action potential. These results suggest that NMDA receptors play some physiological roles under normal condition.

5. Conclusion

We studied electrophysiological and pharmacological properties of EPSP evoked in layer 5 neurons by electrical stimulation of layer 2/3 in vitro slices of kitten (2-3M old) visual cortex. The EPSP showed the following properties.

- (1) The mean latency of the EPSP was 3.4msec and latencies to different stimulus intensities remained same.
- (2) It consisted of two components; the fast one showed a rapidly rising phase and the slow one a

Figure 7. Dependency of EPSC on membrane potentials (○, EPSC corresponding to fast EPSP; ●, EPSC to slow EPSP). Inward current minus.

slowly rising phase. The fast component was abolished by bath application of CNQX, a selective antagonist of non-NMDA receptors, but was unaffected by bath application of APV ($50\ \mu\text{M}$), a specific antagonist of NMDA receptors. In contrast, the slow component was not affected by CNQX, but depressed by APV. In Mg^{++} free medium only the slow component was augmented in size and prolonged in duration but this was blocked by APV.

(3) In a low Ca^{++} -high Mg^{++} bathing medium which blocked synaptic transmission, the layer 5 neurons were strongly depolarized by iontophoresis of NMDA. This effect was blocked by APV.

(4) Using a single electrode voltage-clamp method, the slow EPSC was recorded in the medium containing CNQX, the amplitude of which increased with a depolarization of the membrane potential.

These results suggest that the fast EPSP is generated by monosynaptic pathway via non-NMDA receptors on layer 5 neurons and that NMDA receptors on layer 5 cells are responsible for the slow EPSP.

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