Calcium Signaling Pathways Involved in Long-Term Potentiation at Excitatory Synapses on Parvalbumin Positive Fast-Spiking GABAergic Neurons in the Mouse Visual Cortex

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Abstract
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Objective: Recently, we reported the presence of metabotropic glutamate receptor type 5 (mGluR-5) dependent long-term potentiation (LTP) at excitatory synapses on fast-spiking GABAergic (FS-GABA) cells in the visual cortex. In this study, we report a Ca2+ signaling pathway involved in this LTP type.

Materials and Methods: Brain slices from GAD67-GFP knock-in mice were used. Using whole-cell patch-clamp recording followed by immunohistochemical staining on parvalbumin-positive (PV+) FS-GABA cells, we studied the Ca2+ signaling pathway involved in excitatory LTP of certain visual cortical interneuron subtypes.

Results: U-73122- a phospholipase c (PLC) inhibitor (10 μM), inositol triphosphate (IP3) inhibitors such as 2-APB (3 μM) and heparin (10 IU/ml), and CPA- the internally stored Ca2+ release inhibitor- (5 μM) blocked the mGluR5 signaling pathway to induce LTP at excitatory synapses on PV+ fast-spiking cells in the visual cortex. However, application of the vehicles alone had no effect.

Conclusion: Our results indicate that mGluR-5 at FS-GABA neurons activate PLC and IP3 production. This leads to Ca2+ release, promotes LTP induction, and its maintenance is supported by internal Ca2+ stores. Considering the key role of PV+ FS inhibitory neurons in the visual cortex circuits, we suggest that the metabotropic glutamate receptor-dependent LTP of excitatory synapses to FS cells plays a crucial role in the visual cortex plasticity.

Keywords: GABA, Long-Term Potentiation, Visual Cortex, Metabotropic Glutamate Receptor, Calcium Signaling

Introduction
The transmission and plasticity at interneuron excitatory synapses are pathway- and synapse- specific (1-3). Inhibitory interneurons are a heterogeneous cell group forming complex local circuit networks with principal neurons (4). GABAergic neurons are classified functionally into at least two subgroups, fast-spiking (FS) and non-FS neurons, and may correspond approximately to basket and non-basket cells morphologically and to parvalbumin -positive (PV+) and non-PV cells neurochemically (5, 6).

It is difficult to record excitatory synaptic responses from GABAergic neurons in the visual cortex because GABAergic neurons only comprise 15-25% of all rodent neocortex neurons and their soma sizes are generally smaller than those of excitatory neurons (5, 7-9). To overcome this problem we used transgenic mice (GAD67-GFP knock-in mice), in which expression of green fluorescence protein (GFP) is regulated by the glutamic acid decarboxylase 67 (GAD67) promoter (10). Under a fluorescence microscope, the GABAergic neurons in the cortical slices from these transgenic mice were then easily identifiable.

Depending on the subgroups of postsynaptic GABAergic neurons, excitatory synapses on GABAergic neurons may have different types of LTP; furthermore, the mechanisms underlying LTP may differ. We have shown that LTP is induced reliably in excitatory synapses on FS-GABA neurons (11). This LTP is postsynaptic in origin and is Ca2+-dependent. Induction and maintenance of this...
LTP is facilitated by the sub-type 5 of metabotropic glutamate receptors (mGluR5) (11). Furthermore, it should be noted that the Ca²⁺ signals selectively activated by each subtype of group I mGluRs, as well as the Ca²⁺ signals specifically involved in LTP induction at excitatory synapses on interneurons remain undetermined at this time.

A well-established pathway of mGluR1/5 Ca²⁺ signaling is a G-protein-dependent intracellular Ca²⁺ release via phospholipase C (PLC) and inositol-1, 4, 5-trisphosphate (IP3) activation (12, 13). This Ca²⁺ signal regulates the induction of many forms of LTP (14-16). Moreover, it has been reported that group I mGluRs can be linked to a member of the canonical subfamily of transient receptor potentials (17), Ca²⁺ release from ryanodine-sensitive stores (18) and Ca²⁺ transients associated with outwardly rectifying currents (19).

Interestingly, in addition to the well-established G protein-dependent signaling cascade, mGluR1/5 also signals via Src family of tyrosine kinases (20). However, the role of Ca²⁺ signaling pathways in specific synaptic plasticity at certain synapses remains unexplored. Thus, our goals were to firstly determine synaptic plasticity at certain synapses re-

**Materials and Methods**

**Slice preparation**

GAD67-GFP knock-in mice, aged from 16 to 19 postnatal days (P16-P19), were anesthetized with Isoflurane (Abbott, Abbott Park, IL), then decapitated. The brains were rapidly removed and placed in cold oxygenated artificial cerebrospinal fluid (ACSF). Coronal slices of the visual cortex (300 μm thick) were obtained using a tissue slicer (Vibratome 3000, The Vibratome Co., St. Louis, MO). All experimental procedures were carried out in accordance with the guidelines of the Animal Experimental Committee of RIKEN Brain Science Institute. All slices were placed in an incubating chamber of oxygenated ACSF at 31°C for at least one hour before recording. The pH 7.4 ACSF had the following composition (in mM): NaCl, 124; KCl, 3.0; CaCl₂, 2.0; MgCl₂, 1.0; NaH₂PO₄, 1.25; NaHCO₃, 26.0; and glucose, 10.0. It was diffused continuously with 95% O₂-5% CO₂. ACSF flow rate was about 2.5 ml/min. The slice chamber recording temperature was 29-31°C.

**Whole-cell recordings**

Whole-cell recordings were made from GABAergic neurons in layer II/III of the visual cortex under infrared differential interference contrast optics. GABAergic neurons in cortical slices of GAD67-GFP knock-in mice were visualized with an epifluorescence microscope (BX51WI, Olympus, Tokyo, Japan). We identified fast-spiking by injecting depolarizing currents of 50 to 400 pA for 500 ms, as described in the results section. In most cases, excitatory postsynaptic potentials (EPSPs) evoked by test stimulation of layer IV at 0.05 Hz were recorded in the current-clamp mode; they were recorded with a multi-clamp amplifier (700B, Molecular Devices, Palo Alto, CA), filtered at 2-5 kHz, digitized at 10 kHz, and uploaded to a Pentium 4 personal computer with a digitizer computer interface (PCI-MIO-16E-4, National Instruments, Austin, TX). The analysis was made using Igor 4.01 program.

Recording electrodes were pulled from borosilicate capillary glasses with filaments (0.86 mm inner diameter, 1.5 mm outer diameter). Electrode resistance was 5-7 MΩ. The internal solution was adjusted to pH 7.2 with KOH and its composition was as follows (in mM): K-gluconate, 130; KCl, 10; HEPES, 10; EGTA, 0.2; MgATP, 3; and Na₃GTP, 0.5. The solution osmolarity was 270-285 mOsm. To identify the morphology of recorded neurons, biocytin (0.2%) was injected into neurons through recording pipettes in part of the experiments. In experiments involving postsynaptic-cell drug exposure, drugs were administered to the internal solution of the recording electrodes, and the baseline EPSPs were recorded for at least 10 min after rupture. Similarly, given drugs and dimethyl sulfoxide (DMSO) (0.1% in ACSF) were added to the internal solution as vehicle and are mentioned in the results section.

**LTP induction**

EPSPs of GABAergic neurons in layer II/III of visual cortical slices were elicited by a test stimulation at 0.05 Hz through a concentric bipolar stimulating electrode positioned in layer IV. After recording baseline responses for 10 minutes, a theta burst stimulation (TBS) paired with postsynaptic depolarization at 0 mV for 30 seconds was applied to layer IV. TBS consisted of 3 trains at 0.1 Hz, each train of 10 bursts at 5 Hz, and each burst of 4 pulses at 100 Hz. Thereafter, responses to test stimulation at 0.05 Hz were recorded again for at least 30 minutes.

**Drugs**

Drugs were applied either through the perfusion...
medium or the internal solution of recording pipettes. When drugs were applied through the internal solution alone or the vehicle (DMSO solution) alone were made in slices from the same mice used for test recordings. In case of the bath application, interleaved control recordings were made in slices without drug from the same animals. To block signaling pathways following the activation of mGluRs-5, we used 10 μM of the PLC inhibitor 1-[6-[(17β)-3-methoxysteriodien-17-yl]amino]hexyl]-1H-pyrole-2,5-dione (U-73122) and 5 μM cyclopiazonic acid (CPA, Sigma Aldrich, St. Louis, MO) as a Ca2+ pump blocker to deplete Ca2+ in intracellular Ca2+ stores. As IP3 receptor inhibitor, we used either 3 μM 2-aminoethyl diphenyl borate (2-APB, Sigma Aldrich) or 10 IU/ml heparin (Sigma Aldrich). In case of nimodipine administration, CPA, 2-APB, and DMSO (0.1%) were used as vehicle.

Analysis of morphology of recorded neurons

After electrophysiological recordings, slices containing biocytin-loaded neurons were processed in the similar way as described in previous study (21). In brief, the slices were fixed by immersion in 10% paraformaldehyde (Sigma Aldrich) and 10% sucrose (w/v) in 0.1 M phosphate buffer solution (PBS) overnight at 4°C. Each slice was then washed in 0.1 M PBS 5-7 times for 10 minutes, and incubated in 0.2% Triton X-100 (Sigma Aldrich) for one night. After being incubated with the mouse monoclonal antibody against PV (isotype: IgG1, Chemicon, Temecula, CA; 1:500, diluted in the same solution as above) for 2 days at 4°C, the slices were washed in 0.1 M PBS 5 times each for 10 minutes, then incubated in the solution mentioned above for one day at 4°C with the secondary antibody, anti-mouse IgG1 conjugated with Alexa Fluor 647 (1:100, Molecular Probes, Eugene, OR). Finally, each slice was washed in 0.1 M PBS 5-7 times for 10 minutes.

Statistical analysis

In the present study, values are given as Mean±SEM, unless otherwise mentioned. For statistical analysis, when the numbers showed normal distribution, values before and after TBS obtained from the same cell and values between two groups of cells were compared with paired and unpaired t-tests respectively. The statistical evaluation of normal distribution was made using the Kolmogorov-Smirnov test.

Results

Morphology and immunohistochemical analysis of Fast-spiking GABAergic cells

As previously reported (5, 6), these cells were morphologically basket cells (Fig 1A), and immunohistochemically PV+ (Fig 1C). Twenty-seven of the 29 immunohistochemically stained FS-GABA neurons were positive for anti-PV antibody (Fig 1).

Phospholipase C (PLC) inhibitor can block mGluR5 signaling pathways to induce LTP at excitatory synapses on PV+ fast-spiking cells in the visual cortex

To address the question of which intracellular signaling pathways are involved in mGluR5-mediated LTP of excitatory synapses on FS-GABA neurons in the visual cortex, we initially applied an inhibitor for PLC, U-73122 (10 μM) (23). We found that this inhibitor blocked LTP of EPSPs of FS-GABA neurons, but not that of pyramidal cells (Fig 2). The mean ratios of EPSP slope 20-25 minutes after TBS to those before TBS were 1.01 ± 0.15 with U-73122 (n=7) and 1.48 ± 0.10 without U-73122 (n=9) in FS-GABA neurons. The difference between these two values was significant (p<0.05). In pyramidal cells on the other hand, this inhibitor was not effective. The values with and without U-73122 were 1.61 ± 0.16 (n=7) and 1.66 ± 0.21 (n=7), respectively. The difference was not significant (p>0.05, unpaired t test).
Fig 1: Parvalbumin (PV) immunoreactivity of FS-GABA neurons. A. GFP-positive neurons in a visual cortical slice obtained from a GAD67-GFP knock-in mouse at P17. B. Biocytin image of an electrophysiologically confirmed fast-spiking GABA neuron. C. Immunohistochemical PV image of the neurons shown in A. D. Superposed image of A-C. Scale bar in A indicates 30 μm and applies also to B-D.

Fig 2: U-73122 (10 ± M), an inhibitor of PLC, can block LTP induction in FS GABAergic cells but not in pyramidal cells. Time courses of the mean EPSP slopes of 9 fast-spiking (FS) controls, 7 FS and 7 pyramidal (PY) neurons in presence of U-73122. The EPSP slope was normalized to the value before TBS. Vertical bars indicate Means ± SEMs. Insets, superimposed traces of EPSPs, before (a) and 20-25 minutes after the application of TBS (b). Each trace is an average of 15 consecutive EPSPs.
IP3 linked with PLC is involved in mGlu5-dependent LTP at excitatory synapses on PV+ fast-spiking cells in the visual cortex

We used IP3 inhibitors, 2-APB (24) and heparin (25), to address the question of whether IP3 produced by the activation of PLC is involved in LTP of excitatory synapses on FS-GABA neurons. We injected these inhibitors into postsynaptic neurons through recording pipettes, and found that both inhibitors blocked LTP (Fig 3A). In case of 2-APB, DMSO was added to the internal solution as vehicle. As shown in Fig 3A, DMSO had no effect on LTP. The mean ratio of EPSP slope 20-25 minutes after the application to that before the application of TBS was 1.48 ± 0.12 (n=8) with DMSO; this value was not significantly different from that of the control at 1.51 ± 0.10 (n=10). With 2-APB, the mean ratio of EPSP slope was 0.87 ± 0.06 (n=7), which was significantly (p<0.01) smaller than that with DMSO alone.

The intracellular injection of heparin also blocked the induction of LTP (Fig 3A). The mean ratio of EPSP slope after TBS application to that before the application of heparin was 0.95 ± 0.11 (n=7), which was significantly (p<0.01) smaller than that with the vehicle alone (1.48 ± 0.12, n=8) (no heparin).

Source of Ca2+ for LTP induction in fast-spiking cells is Ca2+ released from internal stores

We applied CPA, an inhibitor of Ca2+ release from internal stores to the bath solution (26). The bath application of CPA at 5 μM blocked LTP of EPSPs of FS-GABA neurons, but the application of the vehicle alone had no effect. The mean ratios of EPSP slopes after TBS to those before TBS were 1.05 ± 0.09 with CPA (n=9) and 1.52 ± 0.11 with vehicle (n=9). The difference between these two values was significant (p<0.01). However, there was no significant difference between control and vehicle alone (Fig 3B). These results indicate that mGluR5-dependent LTP is induced through the pathway of PLC, IP3, and Ca2+ release from internal stores.

Discussion

In our recent study, we reported normal properties of cortical GABAergic neurons of GAD67-GFP knock-in mice (11). Based on that report, in the present work, we focused on the PV+ fast-spiking cells to uncover the signaling mechanism of mGluR-5 dependent LTP. Here, we showed that mGluR-5 at FS-GABA neurons activates PLC and IP3 production. Also, Ca2+ for LTP induction and maintenance came from internal stores. However, we cannot exclude the possibility that voltage-gated Ca2+ channels other than L and T types, such as N and/or P/Q types, were involved. This is because blockers for these channels abolish synaptic transmission itself and thus could not be tested in the present study.

Previous immunohistochemical studies indeed reported that the intense expression of mGluR5 was seen in telencephalic regions including the visual cortex of the rat (27, 28). In a study using the in situ hybridization technique, it was reported that mGluR5 signals were intensely labeled in some GABAergic neurons such as PV+ cells in the neocortex (29). On the other hand, in hippocampal cells, mGluR1 and 5 coexist. Concurrent activation of mGluR1 and mGluR5 by bath-applied agonists produces mixed Ca2+ entries through different pathways (30, 31), even though these receptors may differentially regulate hippocampal cell function (32). A major finding in our study, which dis-
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criminates it from previous works such as Topolnik et al. (19), is that we could specifically show the signaling pathway for mGluR5 subtype in certain interneurons. Also, visual cortex structure is completely different from hippocampus; for example, the synaptic plasticity properties in visual cortex are layer dependent. The transmission and plasticity at interneuron excitatory synapses are pathway- and synapse- specific (1-3); furthermore, in the present study, we report a signaling mechanism in visual cortical neuron synapses which was absent in other areas of the brain such as the hippocampus. Finally, in the rat hippocampus, Topolnik et al. (19) reported that the activation of mGluR1/5 and subsequent Ca$^{2+}$ signaling pathways played a role in LTP of excitatory synapses at interneurons. In the mouse visual cortex, we further identified that mGluR5, but not mGluR1, played a crucial role in the activation of Ca$^{2+}$ signaling pathways such as PLC, IP3 and that this Ca$^{2+}$ was released from internal stores (Fig 4).

Our results are consistent with previous reports (12, 13) indicating that G-protein-dependent intracellular Ca$^{2+}$ is released via phospholipase C (PLC) and inositol-1, 4, 5-trisphosphate (IP3) activation in mGluR-5 subtypes. Based on immunohistochemical studies showing little expression of mGluR1 in the visual cortex (27, 28), we can simply exclude signaling mechanisms other than PLC or IP3 Ca$^{2+}$ release from internal stores, as well as repudiate reports of their presence in the hippocampus (19, 20). Furthermore, new studies are needed to elucidate the role of mGluR1 receptors in synaptic plasticity of the visual cortex interneurons. In addition, the necessity or feasibility of using the dual patch method to record unitary EPSP in FS cells in order to exclude the role of synapses (other than their excitatory input to the FS-GABA neurons) remains to be examined.

![Fig 4: Schematic diagram showing signaling pathways to the induction of mGluR5-dependent LTP of excitatory synapses on FS-GABA neurons. Inhibitors are indicated by T bars.](image)
Conclusion
In conclusion, based on crucial role of PV+ FS-GABA cells in critical period of cortical circuit development (33) our result suggesting mGluR5-dependent LTP in excitatory synapses on these cells might have an important role in cortical neuronal development.

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