Presynaptic Activity and Ca\(^{2+}\) Entry Are Required for the Maintenance of NMDA Receptor–Independent LTP at Visual Cortical Excitatory Synapses

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We have shown that some neural activity is required for the maintenance of long-term potentiation (LTP) at visual cortical inhibitory synapses. We tested whether this was also the case in N-methyl-D-aspartate (NMDA) receptor–independent LTP of excitatory connections in layer 2/3 cells of developing rat visual cortex. This LTP occurred after 2-Hz stimulation was applied for 15 min and always persisted for several hours while test stimulation was continued at 0.1 Hz. When test stimulation was stopped for 1 h after LTP induction, only one-third of the LTP instances disappeared, but most did disappear under a pharmacological suppression of spontaneous firing, indicating that LTP maintenance requires either evoked or spontaneous activities. LTP was totally abolished by a temporary blockade of action potentials with lidocaine or the removal of extracellular Ca\(^{2+}\) after LTP induction, but it persisted under a voltage clamp of postsynaptic cells or after a temporary blockade of postsynaptic activity with glutamate receptor antagonist kynurenate, suggesting that LTP maintenance requires presynaptic, but not postsynaptic, firing and Ca\(^{2+}\) entry. More than one-half of the LTP instances were abolished after a pharmacological blockade of P-type Ca\(^{2+}\) channels, whereas it persisted after either L-type or Ni\(^{2+}\)-sensitive Ca\(^{2+}\) channel blockades. These results show that the maintenance of NMDA receptor–independent excitatory LTP requires presynaptic firing and Ca\(^{2+}\) channel activation as inhibitory LTP; although the necessary level of firing and Ca\(^{2+}\) entry seems lower for the former than the latter and the Ca\(^{2+}\) channel types involved are only partly the same.

INTRODUCTION

A temporary increase in synaptic activity could lead to enduring alterations in the synaptic strength. These types of long-term synaptic modifications, long-term potentiation (LTP) and long-term depression, have been studied as a cellular basis of learning, memory, and activity-dependent maturations of visual circuits (Bliss and Collingridge 1993; Brown et al. 1990; Malenka and Nicoll 1999). It has been considered that modified synaptic strength is maintained by a mechanism independent of neural activity (Lisman 1994). However, in layer 5 cells of rat visual cortex, we have found that the maintenance of high-frequency stimulation-induced LTP at inhibitory synapses requires firing of presynaptic inhibitory cells at a low frequency (Komatsu and Yoshimura 2000). This maintenance is mediated by presynaptic Ca\(^{2+}\) entries through multiple (P, N, and L) types of high-threshold Ca\(^{2+}\) channels, which activate Ca\(^{2+}\)-dependent reactions different from those triggering transmitter release.

The aim of this study was to test whether neural activity is necessary to maintain other types of long-term modifications of synaptic transmission. This was examined in N-methyl-D-aspartate (NMDA) receptor–independent LTP of excitatory synaptic transmission in rat visual cortex. This LTP was found in kitten visual cortex (Komatsu et al. 1988), and recently, it was also shown in layer 2/3 pyramidal cells of rat visual cortex (Ohmura et al. 2003). The induction of LTP requires the activation of voltage-gated Ca\(^{2+}\) channels, which are likely T- or R-type Ca\(^{2+}\) channels, because LTP induction is blocked by a low dose of Ni\(^{2+}\) (Komatsu and Ikawaki 1992; Ohmura et al. 2003). This LTP occurred only during development in both rat and cat (Komatsu et al. 1988; Ohmura et al. 2003), and a high incidence of LTP was maintained until adulthood in visual cortex of dark-reared rats, as was the experience-dependent modification of visual responsiveness (Ohmura et al. 2003), suggesting that it could be a synaptic basis of experience-dependent development of visual cortical functions. The results in this study show that this excitatory LTP also requires the activity of presynaptic cells for maintenance. However, the activity requirement seems far less than that for inhibitory LTP, and the voltage-gated Ca\(^{2+}\) channels involved in LTP maintenance at these visual cortical excitatory and inhibitory synapses are only partly the same.

METH ODS

As described previously (Komatsu 1994; Ohmura et al. 2003), coronal slices (400 μm thick) of primary visual cortex were prepared from Sprague-Dawley rats at postnatal 20–30 days under deep anesthesia with isoflurane and maintained in an interface type chamber perfused with an artificial cerebrospinal fluid (ACSF; in mM): 126 NaCl, 3 KCl, 1.3 MgSO\(_4\), 2.4 CaCl\(_2\), 1.2 Na\(_2\)HPO\(_4\), 26 NaHCO\(_3\), and 10 glucose at 33°C. All experimental procedures were approved by the Animal Care Committee, Research Institute of Environmental Medicine, Nagoya University.

Excitatory synaptic field potentials evoked by layer 4 stimulation were recorded from layer 2/3 using glass microelectrodes filled with saline containing 2% pontamine sky blue, which was used to mark the recording sites (Fig. 1A) (Komatsu 1994). To test whether test stimulation produces antidromic spikes in layer 2/3 cells, intracellular responses were recorded with sharp electrodes filled with 2 M K-methanesulfate (40–60 MΩ). Excitatory postsynaptic potentials (EPSPs) and currents (EPSCs) evoked by layer 4 stimulation were recorded from layer 2/3 pyramidal cells using the blind patch whole cell recording method. Patch pipettes (4–6 MΩ) were filled with a

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solution containing (in mM) 140 K-glucuronate, 8 KCl, 2 NaCl, 0.2 EGTA, 10 HEPES, 3 MgATP, and 0.5 Na2GTP (pH 7.2 with KOH). Neurobiotin (0.3%) was included in the solution for later histological identification of recorded cells as pyramidal cells (Yoshimura et al. 2000). We selected cells with a high seal resistance (>1 GΩ), a series resistance <30 MΩ, and a stable resting membrane potential (<~55 mV) for analysis. When EPSP recording was employed, input resistance was monitored throughout the experiments by injecting hyperpolarizing current pulses. When EPSC recording was used, we held the membrane potential at ~70 mV and monitored series and input resistances by applying hyperpolarizing-voltage steps. We did not compensate for series resistance and conducted the experiments at room temperature (~25°C), because it was difficult to obtain stable recordings for longer period compared with the 33°C used in other experiments. We analyzed only monosynaptic EPSPs/EPSCs. Responses were considered monosynaptic when the onset latency was almost constant while the rising slope substantially changed at different stimulation intensities and during high-frequency stimulation (Komatsu et al. 1991).

Spontaneous EPSCs were recorded from visualized layer 2/3 pyramidal cells using patch pipettes filled with the internal solution containing Cs+ instead of K+ under infrared differential interference contrast (IR-DIC) optics (BX50WI, Olympus). To record spontaneous EPSCs isolated from inhibitory postsynaptic currents (IPSCs), cells were held at ~70 mV, which was the reversal potential of GABA_A receptor–mediated IPSCs in our experimental condition. Spontaneous action potentials were recorded from somata and layer 2/3 and 5 pyramidal cells and layer 4 star pyramidal cells using patch pipettes in the cell-attached mode under IR-DIC optics. In these cases, normal ACSF was used for the internal solution of the patch electrodes.

Two pairs of bipolar stimulating electrodes, separated from each other by ~0.7 mm, were placed in layer 4, and a surgical cut in layer 4–5 was made between the electrodes so that different groups of presynaptic fibers were activated (Fig. 1A). One of the stimulation electrodes was used to test the effect of conditioning stimulation and the other served as a control. Test stimulation was applied alternately to the electrodes at intervals of 5 s. As a conditioning stimulation to induce LTP, stimuli were applied at 2 Hz for 15 min. In the extracellular recording studies, the intensity of the test stimulation was adjusted to the value eliciting about one-half the maximal response, and the intensity employed as conditioning stimulation was twice that of the test stimulation. In some of the experiments using kynurenic acid, however, the intensity of test stimulation was adjusted to the value eliciting one-fifth of the maximal responses, while the intensity of conditioning stimulation was twice the intensity eliciting one-half the maximal responses. In the whole cell recording studies, very weak test stimuli were used to evoke EPSPs/EPSCs, without accompanying inhibitory postsynaptic responses (IPSPs/IPSCs). For this purpose, we selected cells in which the threshold intensity was lower for evoking EPSPs/EPSCs than it was for IPSPs/IPSCs, and for which the test stimulation intensity could be set to a value 10–30% higher than the threshold, at which no indication of superposition of IPSPs/IPSCs on EPSPs/EPSCs was detected. The intensity of the conditioning stimulation was adjusted to a value higher than that which evoked orthodromic action potentials. The laminar location of the stimulating and recording electrodes was histologically identified (Fig. 1A) (Komatsu 1994).

Data were expressed as means ± SE and Student’s t-test or Welch’s test was applied. The compounds used were obtained from the following sources: DL-2-amino-5-phosphonovaleric acid (APV) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) from Tocris (Bristol, UK); TTX, lidocaine, kynurenic acid, and bicuculline methiodide from Sigma (St. Louis, MO); ω-conotoxin GVIA and nifedipine from Research Biochemicals International (Natick, MA); ω-agatoxin IVA from Peptide Institute (Osaka, Japan); neurobiotin from Vector Laboratories (Burlingame, CA); and isoflurane from Abbott Laboratories (North Chicago, IL).
RESULTS

We analyzed postsynaptic responses recorded from layer 2/3 in response to stimulation of layer 4 in visual cortical slices prepared from developing rats at postnatal 20–30 days (Fig. 1A). At this age, a stimulation of layer 4 for 15 min at 2 Hz frequently induces NMDA receptor–independent LTP of field potentials, which is likely to be ascribable to LTP of EPSPs because this conditioning stimulation induced LTP of EPSPs recorded from layer 2/3 pyramidal cells in conditions where inhibition was locally blocked with pipettes containing bicuculline methiodide, a GABA_A receptor antagonist, placed near the whole cell recording patch electrodes (Ohmura et al. 2003). In this study, we employed mostly extracellular field potential recording because the analysis required a stable recording for a considerably long period. We first confirmed that 2-Hz conditioning stimulation produced LTP of EPSPs in the present experimental conditions, where field potentials were recorded with no pharmacological blockade of inhibition. For this purpose, we used a very weak test stimulation, which seemed to evoke monosynaptic EPSPs without accompanying IPSPs. Conditioning stimulation induced LTP of EPSPs (Fig. 1, B and C), specific to the conditioned pathway, with a time course and magnitude similar to that found in LTP of field potentials (Fig. 1, D and E). No significant difference ($P < 0.1$) was found in input resistance or resting membrane potential before (105 ± 8 MΩ, −71 ± 2 mV, $n = 7$) and 60 min after conditioning stimulation (103 ± 7 MΩ, −70 ± 2 mV). To avoid the inclusion of polysynaptic responses in the measurement of EPSPs, we measured the initial rising slope of EPSPs. In addition, we confirmed that test stimulation did not evoke monosynaptic IPSPs with a pharmacological blockade of EPSPs at the end of the recording experiments (Fig. 1B2), ensuring that enhancement occurred in the EPSPs themselves. Thus it is considered that changes in field potentials after conditioning stimulation are ascribed mainly to LTP of EPSPs.

In this study, analysis was conducted only in cases in which the magnitude of potentiation was a >20% increase from the baseline level at 30 min after conditioning stimulation. In such cases, LTP of field potentials (>20% increase) persisted >4 h as long as the test stimulation was continued at 0.1 Hz (Fig. 1, D and E). Figure 1F, comparing the magnitude of potentiation at 40–45 and 260–265 min after the termination of conditioning stimulation, summarizes the persistence of potentiation for the eight slices shown in Fig. 1E.

LTP maintenance requires neural activity

To test whether the maintenance of LTP requires neural activity, action potentials were blocked temporarily with a Na+ channel blocker. We used lidocaine, which can be washed out far more quickly than TTX. A bath application of lidocaine (5 mM) was started 45 min after the termination of conditioning stimulation and continued for 40 min (Fig. 2, A and B). This drug application completely blocked evoked responses (Fig. 2A1, c). After wash out, the responses of both test and control pathways returned to the baseline levels, indicating that LTP was abolished. This abolition of LTP was found in all nine slices tested (Fig. 2C), suggesting that the maintenance of LTP requires neural activity.

If this supposition is the case, neural activity evoked by test stimulation may be required for LTP maintenance. To test this possibility, the test stimulation for the test pathway was temporarily stopped. This cessation was started 1 h after conditioning stimulation and continued for 1 h. After resuming the test stimulation, LTP was abolished in one-third of the cases ($n = 4$; Fig. 3, A–C), while LTP persisted in the remaining cases ($n = 8$; Fig. 3, D–F). Thus neural activity induced by test stimulation was necessary to maintain LTP for the former cases.

Our previous study showed that spontaneous presynaptic spikes contributed to the maintenance of inhibitory LTP in visual cortex (Komatsu and Yoshimura 2000), suggesting the possibility that spontaneous neural activity contributed to LTP of field potentials, which persisted even after stimulation cessation. Thus we tested the possibility that excitatory cells innervating layer 2/3 cells fire spontaneously in our experimental conditions. Indeed, spontaneous spikes, albeit at a low frequency, were recorded from the soma of layer 2/3 and 5 pyramidal cells and layer 4 star pyramidal cells, which could send excitatory inputs to layer 2/3 pyramidal cells (Burkhalter 1989; Gilbert 1983; Lübke et al. 2000; Lund 1988), with patch electrodes in the cell-attached mode under IR-DIC optics (Fig. 4A). All cells elicited at least 1 spike/10 min. In layer 4 and 5 cells, the frequency varied widely, and the mean frequency was higher than that in layer 2/3 cells (Fig. 4B). However, this difference was not statistically significant ($P > 0.2$), and it could be mostly ascribed to the presence of exceptional cells showing high-frequency firing in layer 4 and 5.

If these spontaneously firing cells innervate layer 2/3 pyramidal cells, spontaneous EPSCs recorded from the latter cells would be sensitive to a pharmacological blockade of action potentials. We confirmed this with the whole cell recording method. To record EPSCs in isolation from IPSCs, cells were voltage clamped at −70 mV, which was the reversal potential of IPSCs in our experimental conditions. In normal ACSF, we...
found spontaneous EPSCs with various amplitudes (Fig. 5A1). A bath application of TTX (1 μM) abolished EPSCs except for those with small amplitudes, which were considered to be miniature EPSCs (Fig. 5, A2 and B), indicating that the larger EPSCs observed in normal ACSF were elicited by spontaneous firing of presynaptic cells. The addition of non-NMDA and NMDA receptor antagonists abolished the remaining responses (Fig. 5A3), although outward synaptic currents, which are considered to be IPSCs, were detected when the membrane potential was clamped at 0 mV (Fig. 5A4), ensuring that the synaptic currents recorded at ~70 mV were EPSCs.

We found this kind of reduction in the frequency (from 16 ± 3.2 to 5.3 ± 0.9 Hz) and amplitude of spontaneous EPSCs (from 15 ± 1.7 to 11 ± 1.2 pA) in five cells, but not in the remaining two cells (frequency, 12 ± 0.8–12 ± 0.6 Hz; amplitude, 17 ± 6.5–18 ± 7.7 pA), as shown in Fig. 5, C and D. The mean frequency of the EPSCs mediated by spontaneous presynaptic spikes, ~10 Hz (4–21 Hz) for the former cells and 7 Hz (0–21 Hz) for all of the recorded cells, was estimated by subtraction of the EPSC frequency in the presence of TTX from that in the control solution. Therefore it is likely that these spontaneous spikes contributed to LTP maintenance in cases in which LTP persisted even after the cessation of test stimulation.

This possibility was further tested under the pharmacological suppression of spontaneous firing. We found a reduction in the frequency (from 11.0 ± 3.7 to 2.8 ± 0.9 Hz, n = 5) and amplitude of spontaneous EPSCs (from 12.3 ± 3.0 to 8.1 ± 1.1 pA) in layer 2/3 pyramidal cells after a low dose (10 nM) of TTX was applied (Fig. 5, E–H). The reduction of the frequency was 8.2 ± 3.0 Hz. This value was almost the same (P > 0.8) as the mean frequency of the EPSCs mediated by spontaneous presynaptic spikes, which was estimated using 1 μM TTX. In the presence of 10 nM TTX, however, stimulation of layer 4 still evoked field potentials in layer 2/3 that were similar to those evoked under normal ACSF (Fig. 6). It is likely that, in our experimental conditions, TTX at this dose effectively suppresses persistent Na⁺ currents, which could contribute to spontaneous firing (Crill 1996), without accompanying substantial effects on rapidly inactivating Na⁺ currents, which are responsible for action potential generation.

In the presence of 10 nM TTX, LTP of field potentials was induced and maintained as long as test stimulation was continued (7 of 7 slices), just as in the control solution (Fig. 6, A–C). When test stimulation was stopped for 1 h, LTP was abolished in almost all of the cases (7 of 8 slices) after test stimulation was resumed (Fig. 6, D–F). It is unlikely that this LTP abolition was merely ascribed to the deterioration of plasticity mechanisms with time during the long recording period, because LTP abolished by test stimulation cessation was reestablished by applying conditioning stimulation again in all of the four slices tested (Fig. 7). These results indicate that neural activities evoked by test stimulation at 0.1 Hz are sufficient to maintain LTP even in the condition where spontaneous activity is low and that spontaneous spikes are often responsible for the maintenance of LTP when test stimulation is not given in normal ACSF.

![FIG. 3. Effects of test stimulation cessation on LTP maintenance. A1 and A2: example of LTP that was abolished after test stimulation cessation. A1: top (○) and bottom traces (□) represent responses for test and control pathways, respectively. Recorded time (a, b, and c) is indicated in A2. A2: amplitude of field potentials (FPs) plotted against time after start of conditioning stimulation. Test stimulation for test pathway was stopped for 60 min (interrupted line), starting 60 min after termination of conditioning stimulation. Squares and circles represent responses for test and control pathways, respectively. B: average time course for slices (n = 4), in which LTP was abolished after cessation of test stimulation. C: comparison of LTP magnitude before (55–60 min after termination of conditioning stimulation) and after cessation of test stimulation (175–180 min after termination of conditioning stimulation) in slices shown in B. D1–D3: similar to A–C, but for slices in which LTP persisted after cessation of test stimulation. D1–D2 and E: example case and average time course for 8 slices, respectively.](http://jn.physiology.org/)

![FIG. 4. Spontaneous firing of cortical cells: A: spontaneous spikes recorded from a layer 2/3 pyramidal cell (top), a layer 4 star pyramidal cell (middle), and a layer 5 pyramidal cell (bottom) using patch electrodes in the cell-attached mode. The layer 5 cell (bottom) elicited 4 spikes in a burst during the period shown here. B: scatter plot of the mean number of spikes per minute for layer 2/3 pyramidal cells (n = 12), layer 4 star pyramidal cells (n = 11), and layer 5 pyramidal cells (n = 19). Values were determined by the number of spikes during a 10-min recording period. Horizontal bars indicate mean values.](http://jn.physiology.org/)
LTP maintenance requires presynaptic, but not postsynaptic, activity and Ca\(^{2+}\) entry. The effect of neural activity on LTP maintenance might be mediated by Ca\(^{2+}\) entry into cells. This possibility was tested by removing Ca\(^{2+}\) from the extracellular medium for 60 min after LTP induction. This procedure eliminated evoked responses except for an early small component (Fig. 8A1, arrow in c), which may reflect presynaptic/antidromic spikes. After the normal level of Ca\(^{2+}\) concentration was resumed, the responses of both test and control pathways returned to the baseline levels (Fig. 8, A and B), just as in the case with lidocaine application. The same result was obtained in all of 12 slices tested (Fig. 8C), suggesting that Ca\(^{2+}\) entry associated with neural activities is required for the maintenance of LTP.

We attempted to determine whether pre- or postsynaptic activity and Ca\(^{2+}\) entry are responsible for LTP maintenance. Spontaneous and evoked synaptic transmission may produce postsynaptic depolarization and hence Ca\(^{2+}\) entry into postsynaptic cells through voltage-dependent Ca\(^{2+}\)-permeable channels such as NMDA receptors and voltage-gated Ca\(^{2+}\) channels. To prevent this entry, we recorded EPSCs from layer 2/3 pyramidal cells, which were kept under a whole cell voltage clamp at −70 mV during the entire test stimulation period (Fig. 6).

**FIG. 5.** Excitatory postsynaptic currents (EPSCs) elicited by spontaneous spikes of presynaptic cells. A: spontaneous postsynaptic currents recorded from a layer 2/3 pyramidal cell under whole cell voltage clamp. Traces were recorded at −70 mV in control (1), in the presence of 1 μM TTX (2), and 1 μM TTX, 40 μM DNQX, and 100 μM APV (3). 4: recorded at 0 mV in the presence of TTX, DNQX, and APV. B: frequency histogram of spontaneous EPSC amplitude in control (open) and in the presence of TTX (shaded), for the cell whose traces are shown in A. C: mean amplitude of spontaneous EPSCs in control and TTX solution. Each symbol represents a set of data obtained from individual pyramidal cells. D: similar to C, but for mean frequency of spontaneous EPSCs. In addition, frequency of EPSCs mediated by spontaneous presynaptic spikes, which was estimated by subtraction of EPSC frequency in the presence of TTX from that in the control solution, was plotted on the right (control-TTX). E–H: similar to A–D, but concentration of TTX applied was 10 nM.

**FIG. 6.** Test stimulation cessation abolishes LTP maintenance under reduced spontaneous activity with TTX. A1 and A2: example LTP that persisted as long as test stimulation was continued at 0.1 Hz in the presence of 10 nM TTX during the whole recording period. A1: top (c) and bottom traces (a) represent responses for test and control pathways, respectively. Recorded time (a, b, and c) is indicated in A2. A2: amplitude of field potentials (FPs) plotted against time after start of conditioning stimulation. Squares and circles represent responses for test and control pathways, respectively. B: average LTP time course for all of the tested slices (n = 7) in the presence of 10 nM TTX. LTP always persisted while test stimulation was continued. C: comparison of LTP magnitude 55–60 and 175–180 min after termination of conditioning stimulation in the slices shown in A and B. D–F: similar to A–C, but test stimulation for the test pathway was stopped for 60 min (interrupted line), starting 60 min after termination of conditioning stimulation. An example case is shown in D1–D2, and number of slices in E is 8. F: LTP magnitude was compared before (55–60 min after termination of conditioning stimulation) and after cessation of test stimulation (175–180 min after termination of conditioning stimulation).

LTP maintenance requires presynaptic, but not postsynaptic, activity and Ca\(^{2+}\) entry

The effect of neural activity on LTP maintenance might be mediated by Ca\(^{2+}\) entry into cells. This possibility was tested by removing Ca\(^{2+}\) from the extracellular medium for 60 min after LTP induction. This procedure eliminated evoked responses except for an early small component (Fig. 8A1, arrow in c), which may reflect presynaptic/antidromic spikes. After the normal level of Ca\(^{2+}\) concentration was resumed, the...
LTP is produced again after abolition due to test stimulation cessation. A1 and A2: example LTP that was produced again after it was once abolished by test stimulation cessation. TTX (10 nM) was present in artificial cerebrospinal fluid (ACSF) during the whole recording period. A1: top (□) and bottom traces (○) represent the responses for test and control pathways, respectively. Recorded time (a, b, c, and d) is indicated in A2. A2: amplitude of field potentials (FPs) plotted against time after start of conditioning stimulation. Squares and circles represent responses for test and control pathways, respectively. Test stimulation for the test pathway was stopped for 60 min (interrupted line), starting 60 min after termination of conditioning stimulation. B: average LTP time course for all of the tested slices (n = 4) in the presence of 10 nM TTX. LTP was always produced by the 2nd conditioning stimulation, which was applied after LTP abolition.

Fig. 8. Application of Ca\(^{2+}\)-free solution abolishes LTP maintenance. A1 and A2: example of LTP abolition after an application Ca\(^{2+}\)-free solution. A1: top (□) and bottom traces (○) represent responses for test and control pathways, respectively. Recorded time (a, b, c, and d) is indicated in A2. A2: amplitude of field potentials (FPs) plotted against time after start of conditioning stimulation. Arrows in c indicate presynaptic/antidromic spike components. A2: amplitude of field potentials (FPs) plotted against time after start of conditioning stimulation. Squares and circles represent responses for test and control pathways, respectively. Ca\(^{2+}\)-free solution was applied for 60 min (interrupted line), starting 45 min after termination of conditioning stimulation. Plot of response amplitude was blanked when responses disappeared. B: average time course for 12 slices, to which Ca\(^{2+}\)-free solution was applied. C: comparison of LTP magnitude before (40–45 min after termination of conditioning stimulation) and after application of the Ca\(^{2+}\)-free solution (220–225 min after termination of conditioning stimulation).
dromic action potentials were evoked in any of the tested cells (0/51) at this decreased intensity.

A bath application of 20 mM kynurenate blocked extracellular responses totally (Fig. 10B1, c). After washout, the responses for both test and control pathways almost returned to their respective levels before the application of the antagonist (Fig. 10, B–D). Similarly, LTP persisted after kynurenate application when test stimulation eliciting one-half the maximal responses was used instead as in the other experiments (Fig. 10E). These results suggest that postsynaptic activity and resultant Ca\(^{2+}\) entry are not necessary to maintain LTP.

However, even under the pharmacological blockade of excitatory synaptic transmission, layer 2/3 cells may fire spontaneously, allowing Ca\(^{2+}\) entry into these cells. When spontaneous firing was reduced by 10 nM TTX, test stimulation was necessary to maintain LTP in almost all of the cases tested (Fig. 6, D–F). In this reduced spontaneous activity, if postsynaptic responses and Ca\(^{2+}\) entry associated with test stimulation are necessary to maintain LTP, a pharmacological blockade of excitatory synaptic transmission should abolish LTP maintenance. Thus we tested the effect of 20 mM kynurenate on LTP maintenance in the presence of 10 nM TTX using the weak test stimulation. LTP was maintained in all of the tested cases (Fig. 9).

FIG. 9. LTP persists under a voltage clamp. A1 and A2: LTP of EPSCs persisted in a layer 2/3 pyramidal cell recorded with a patch electrode under a voltage clamp at −70 mV during the whole test stimulation period. Traces in A1 represent superimposed average EPSCs for test (c) and control pathways (C) before (a) and after (b) conditioning stimulation. Recorded time is indicated in A2. A2: amplitude of EPSCs plotted against time after start of conditioning stimulation. B: time course of EPSC LTP for an average of 5 tested cells.

Effects of Ca\(^{2+}\) channel blockers on LTP maintenance

Presynaptic Ca\(^{2+}\) entry involved in LTP maintenance might be mediated by voltage-gated Ca\(^{2+}\) channels, which are activated in association with action potentials. To test this possibility, we applied various subtype-specific Ca\(^{2+}\) channel blockers after LTP was induced. A low dose of \(\omega\)-agatoxin IVA (50 nM), which selectively blocks P-type Ca\(^{2+}\) channels at this dose (Mintz et al. 1992; Sather et al. 1993), did not affect the control pathway responses, indicating that P-type Ca\(^{2+}\) channels do not contribute to basal transmitter release (Fig. 12, A, B, D, and E). However, potentiated test pathway responses returned to the baseline level in more than one-half of the slices (n = 6, Fig. 12, A and C), while they were maintained, although the magnitude of LTP was reduced to some extent, in the rest of slices (n = 4; Fig. 12, D and F). Therefore P-type Ca\(^{2+}\) channels contribute to LTP maintenance at least in some cases.

FIG. 10. Complete blockade of postsynaptic responses by kynurenate does not affect LTP maintenance. A: postsynaptic responses evoked in a layer 2/3 pyramidal cell by strong layer 4 stimulation before (left) and after (right) application of 20 mM kynurenate. B1 and B2: example LTP that persisted after a bath application of kynurenate (20 mM). B1: top (c) and bottom traces (C) represent responses for test and control pathways, respectively. Recorded time (a, b, c, and d) is indicated in B2. B2: amplitude of field potentials (FPs) plotted against time after start of conditioning stimulation. Squares and circles represent responses for test and control pathways, respectively. Kynurenate was applied for 60 min (interrupted line), starting 45 min after termination of conditioning stimulation. Intensity of test stimulation was adjusted to that eliciting one-fifth of maximal responses. Plot of response amplitude was blanked when responses disappeared. C: effect of kynurenate on LTP maintenance is shown for an average of 12 slices. D: comparison of LTP magnitude before (40–45 min after termination of conditioning stimulation) and after application of kynurenate (220–225 min after termination of conditioning stimulation). E: similar to D, but intensity of test stimulation was adjusted to value eliciting one-half maximal responses.
L-type Ca$^{2+}$ application of fi

Wheeler et al. 1994), produced a very variable degree of

$\Delta V_{100}$ potentials was reduced to 68

a responses for test and control pathways, respectively. Recorded time (A, b, c, and d) is indicated in A2: A2: amplitude of field potentials (FPs) plotted against time after start of conditioning stimulation. Squares and circles represent responses for test and control pathways, respectively. Kynurenate was applied for 60 min (interrupted line), starting 45 min after termination of conditioning stimulation. Intensity of test stimulation was adjusted to that eliciting one-fifth of maximal responses. Plot of response amplitude was blanked when responses disappeared. B: average LTP time course for all of the tested slices ($n = 6$) in which experiments were conducted as shown in A1 and A2. C: comparison of LTP magnitude before ($40\text{--}45$ min after termination of conditioning stimulation) and after application of kynurenate ($220\text{--}225$ min after termination of conditioning stimulation) in slices shown in B.

A high dose of $\omega$-agatoxin IVA (1 $\mu$M), which also blocks Q-type Ca$^{2+}$ channels (Mintz et al. 1992; Sather et al. 1993; Wheeler et al. 1994), produced a very variable degree of reduction in the baseline responses. The amplitude of the field potentials was reduced to 68 $\pm$ 8% ($n = 15$) of the control when measured at the peak time in the control responses. An application of $\omega$-conotoxin GVIA (1 $\mu$M), a selective blocker for N-type Ca$^{2+}$ channels (Aosaki and Kasai 1989; Plummer et al. 1989), also produced a variable reduction in the baseline responses (58 $\pm$ 5% of the control level, $n = 16$). Thus we could not determine whether Q or N type Ca$^{2+}$ channels are involved in LTP maintenance.

A high dose of nifedipine (20 $\mu$M), a selective blocker for L-type Ca$^{2+}$ channels (Aosaki and Kasai 1989; Fox et al. 1987), affected neither baseline synaptic transmission nor LTP maintenance (Fig. 13A and B). The same results were obtained with 50 $\mu$M Ni$^{2+}$ (Fig. 13, C and D), which selectively blocks T- and R-type Ca$^{2+}$ channels at this dose (Fox et al. 1987; Narahashi et al. 1987; Zhang et al. 1993). These results indicate that P-type Ca$^{2+}$ channels, but not L-type or Ni$^{2+}$-sensitive Ca$^{2+}$ channels, contribute to LTP maintenance.

**DISCUSSION**

This study showed that NMDA receptor–independent LTP in rat visual cortex required neural activity for maintenance. After induction, LTP was always abolished by a temporary blockade of action potentials with lidocaine. In addition, almost all of the instances of LTP were abolished after a temporary cessation of test stimulation when spontaneous ac-


tivity was reduced by a low dose of TTX. However, LTP persisted when postsynaptic responses were recorded under a voltage clamp or they were temporarily blocked by the glutamate receptor antagonist kynurenate even under the reduced spontaneous activity. These observations strongly suggest that this LTP requires presynaptic, but not postsynaptic, activity for maintenance.

The application of Ca$^{2+}$-free solution terminated LTP, just as with the application of lidocaine, suggesting that LTP maintenance requires Ca$^{2+}$ entry associated with presynaptic spikes. Alternatively, LTP maintenance might merely require some level of intracellular Ca$^{2+}$ concentration in either pre- or postsynaptic cells rather than Ca$^{2+}$ entry associated with activity and that level was not maintained under the Ca$^{2+}$-free solution. However, in more than one-half of the tested cases, the blockade of P-type Ca$^{2+}$ channels abolished the maintenance of LTP without affecting the baseline synaptic transmission. These channels in postsynaptic cells could be rarely opened under either a voltage clamp at $-70$ mV or a glutamate receptor blockade together with reduced spontaneous activity. In such conditions, LTP always persisted when test stimulation was continued, suggesting that presynaptic Ca$^{2+}$ entry is nec-

![Fig. 11. Application of kynurenate fails to block LTP maintenance even under reduced spontaneous activities. A1 and A2: example LTP that persisted after bath application of kynurenate (20 mM) in the presence of 10 nM TTX during the whole recording period. A1: top (c) and bottom traces (c) represent responses for test and control pathways, respectively. Recorded time (a, b, c, and d) is indicated in A2. A2: amplitude of field potentials (FPs) plotted against time after start of conditioning stimulation. Squares and circles represent responses for test and control pathways, respectively. Kynurenate was applied for 60 min (interrupted line), starting 45 min after termination of conditioning stimulation. Intensity of test stimulation was adjusted to that eliciting one-fifth of maximal responses. Plot of response amplitude was blanked when responses disappeared. B: average LTP time course for all of the tested slices ($n = 6$) in which experiments were conducted as shown in A1 and A2. C: comparison of LTP magnitude before ($40\text{--}45$ min after termination of conditioning stimulation) and after application of kynurenate ($220\text{--}225$ min after termination of conditioning stimulation) in slices shown in B.](https://www.jn.org)

![Fig. 12. Effects of $\omega$-agatoxin IVA on LTP maintenance. A1 and A2: example of LTP that was abolished after application of $\omega$-agatoxin IVA (50 nM). A1: top (c) and bottom traces (c) represent responses for test and control pathways, respectively. Recorded time (a, b, and c) is indicated in A2. A2: amplitude of field potentials (FPs) plotted against time after start of conditioning stimulation. Toxin was applied for 60 min (interrupted line), starting 45 min after termination of conditioning stimulation. Squares and circles represent responses for test and control pathways, respectively. B: average time course for slices ($n = 6$) in which LTP was abolished after application of toxin. C: comparison of LTP magnitude before ($40\text{--}45$ min after termination of conditioning stimulation) and after application of $\omega$-agatoxin IVA (175–180 min after termination of conditioning stimulation) in slices shown in B. D–F: similar to A–C, but for slices ($n = 4$) in which LTP persisted.](https://www.jn.org)
A2: average time course of LTP for 9 slices. A2: average time course of LTP for 9 slices. A1: (c) and D: similar to A and B, but for effects of 50 μM Ni2+ on LTP maintenance. Example of traces obtained from a slice is shown in C1, and number of slices was 9 in C2. Data shown in A, B, C, and D both included 3 cases, in which application of nifedipine or Ni2+ was continued until end of recording experiment.

necessary for LTP maintenance, although postsynaptic Ca2+ might still have some role in maintenance too. Taken together, it is likely that LTP maintenance requires postsynaptic firing, subsequent Ca2+ entry mediated at least partly by voltage-gated Ca2+ channels, and the activation of Ca2+-dependent reactions different from those triggering transmitter release, as shown in visual cortical inhibitory LTP (Komatsu and Yoshimura 2000).

Ca2+ channels involved in LTP maintenance

Because presynaptic Ca2+ entry associated with action potentials is most likely mediated by voltage-gated Ca2+ channels, we attempted to test the effect of various Ca2+ channel blockers on LTP maintenance. It is known that nifedipine and ω-conotoxin GVIA, at the concentration used in this study, specifically and almost completely block L- and N-type Ca2+ channels, respectively (Aosaki and Kasai 1989; Fox et al. 1987; Plummer et al. 1989). The low dose of ω-agatoxin IVA used in this study selectively blocks P-type Ca2+ channels, and a high dose of this compound blocks both P- and Q-type Ca2+ channels without affecting other types of Ca2+ channels (Mintz et al. 1992; Sather et al. 1993; Wheeler et al. 1994). Because hippocampal mossy fiber LTP was produced in the presence of ω-agatoxin IVA even at the high dose (Castillo et al. 1994), it is unlikely that the effect of 50 nM ω-agatoxin IVA on LTP maintenance demonstrated in this study was ascribed to some side effects of the toxin. Compared with these blockers, Ni2+ blocks Ca2+ channels less selectively. We adopted the concentration at which Ni2+ substantially blocks T- and R-type Ca2+ channels but has a negligibly effect on P/Q-, N-, or L-type Ca2+ channels (Fox et al. 1987; Narahashi et al. 1987; Zhang et al. 1993). However, it is known that some T-type Ca2+ channels are blocked only weakly at the concentration used here (Lee et al. 1999).

Our results using these blockers showed that P-type, but not L-type or Ni2+-sensitive, Ca2+ channels were involved in LTP maintenance. However, we could not determine the involvement of N- and Q-type channels because the blockade of these channels reduced the basal synaptic transmission considerably and variably. This variability might be explained by the heterogeneity of the Ca2+ channels involved in transmitter release in connections mediating layer 4 stimulation-evoked responses in layer 2/3 cells. It is known that layer 2/3 pyramidal cells receive excitatory inputs from layer 2/3, 4, and 5 cells in addition to association and commissural afferents from other cortical areas (Burkhalter 1989; Gilbert 1983; Lund 1988). These different excitatory connections could utilize N- and Q-type Ca2+ channels with considerably different degrees of contribution to transmitter release.

We showed that the blockade of P-type Ca2+ channels abolished LTP maintenance in more than one-half of slices we tested. These channels were likely blocked almost totally by ω-agatoxin IVA at the dose used in this study (Mintz et al. 1992). Therefore other types of Ca2+ channels such as N- and Q-type Ca2+ channels might also contribute to LTP maintenance, even though we could not confirm that supposition. These channels could be located commonly at excitatory synapses or selectively at those in which LTP persisted after the blockade of P-type Ca2+ channels. If the former is the case, Ca2+ entry through Ca2+ channels other than P-type Ca2+ channels was sufficient to maintain LTP in those cases in which LTP persisted after P-type Ca2+ channel blockade. If the latter is the case, it is likely that different types of excitatory terminals have different types of Ca2+ channels contributing to LTP maintenance.

Comparison of two forms of LTP requiring activity for the maintenance

It may be considered that LTP is preserved by mechanisms independent of activity during the maintenance phase. In fact, it has been reported that LTP at hippocampal excitatory synapses persisted after the blockade of presynaptic spikes or Ca2+ channels (Castillo et al. 1994; Cormier et al. 1993; Malgrali and Tsien 1992; Manabe et al. 1992; Wheeler et al. 1994). A mechanism has been proposed for NMDA receptor–dependent LTP (Lisman 1994), which requires the activation of Ca2+/calmodulin-dependent protein kinase II for the induction (Malenka et al. 1989; Malinow et al. 1989; Silva et al. 1992). The activated state could be maintained by Ca2+-dependent autophosphorylation of the kinase, switching the molecule into an active state even at a low Ca2+ concentration (Miller and Kennedy 1986; Saitoh and Schwartz 1985). This type of LTP is reversed by low-frequency (1–5 Hz) stimulation continued for 10–15 min (Fujii et al. 1991; Staubli and Lynch 1990), indicating that it requires neural activity for its reversal.

However, we have found in visual cortex that two forms of LTP, LTP at inhibitory synapses of layer 5 cells and NMDA receptor–dependent LTP at excitatory synapses of layer 2/3 cells, require neural activity for maintenance and that their
reversal is attained by reducing neural activity. In regard to aspects other than maintenance, these two forms of LTP have properties that are both common and different. The frequency of conditioning stimulation that is effective in the induction of LTP is wider for inhibitory than excitatory LTP. Inhibitory LTP is induced by a brief high-frequency stimulation and a low-frequency stimulation continued for a longer period (Komatsu 1994), whereas excitatory LTP is induced by the latter but not the former type of stimulation (Komatsu et al. 1991; Ohmura et al. 2003). The induction of inhibitory and excitatory LTP required postsynaptic Ca\textsuperscript{2+} increase, although Ca\textsuperscript{2+} originated from the internal Ca\textsuperscript{2+} store in the former LTP, but from out side of cells in the latter LTP (Komatsu 1996; Komatsu and Iwakiri 1992; Ohmura et al. 2003). Both plastic changes occur mostly during development, suggesting their involvement in experience-dependent maturation of cortical functions (Komatsu 1994; Komatsu et al. 1988; Ohmura et al. 2003).

In regard to maintenance, the excitatory LTP studied in this experiment showed a close similarity to inhibitory LTP (Komatsu and Yoshimura 2000). Both LTPs required presynaptic, but not postsynaptic, Ca\textsuperscript{2+} entry associated with action potentials. In addition, the effect of presynaptic action potentials was, at least in part, mediated by voltage-gated Ca\textsuperscript{2+} channels. However, different subsets of Ca\textsuperscript{2+} channels contributed to the maintenance of the excitatory and inhibitory LTP. Maintenance of inhibitory LTP required the activation of L-, N-, and P-type Ca\textsuperscript{2+} channels but not of Ni\textsuperscript{2+}-sensitive (R- and T-type) Ca\textsuperscript{2+} channels (Komatsu and Yoshimura 2000). The contribution of Q-type Ca\textsuperscript{2+} channels could not be determined because IPSPs in layer 5 cells were completely blocked by the blockade of those channels. This study showed that P-type, but not L-type or Ni\textsuperscript{2+}-sensitive, Ca\textsuperscript{2+} channels were involved in excitatory LTP maintenance and kept open the possibility of N- or Q-type Ca\textsuperscript{2+} channel contribution. Thus it is concluded at present that P-type Ca\textsuperscript{2+} channels are commonly involved, but that L-type Ca\textsuperscript{2+} channels contribute only to inhibitory LTP. In addition to this difference, the blockade of any of the L-, N-, and P-type Ca\textsuperscript{2+} channels completely abolished inhibitory LTP in all of the cells tested, whereas blockade of P-type Ca\textsuperscript{2+} channels abolished excitatory LTP only in a part of cases. This suggests that either subtypes of Ca\textsuperscript{2+} channels involved in maintenance or Ca\textsuperscript{2+} increase levels required for maintenance are more variable at excitatory than inhibitory synapses.

It was strongly suggested that excitatory and inhibitory LTP differed in the level of presynaptic spike activity and Ca\textsuperscript{2+} elevation required for maintenance. Inhibitory LTP was maintained at a higher (4 mM), but not normal (2.4 mM), concentration of extracellular Ca\textsuperscript{2+} in the experimental condition in which excitatory synaptic transmission was pharmacologically blocked (Komatsu and Yoshimura 2000), whereas excitatory LTP was maintained at normal Ca\textsuperscript{2+} concentration. It is likely that the minimum frequency of presynaptic firing necessary to maintain LTP is higher for inhibitory than excitatory LTP. Inhibitory LTP was abolished in about two-thirds of cells after cessation of the test stimulation for 30 min (Komatsu and Yoshimura 2000), while excitatory LTP was abolished only in one-third of the cases after cessation of 60 min. The frequency of EPSCs elicited in layer 2/3 pyramidal cells by the spontaneous firing of presynaptic cells was almost the same as that of IPSCs in layer 5 pyramidal cells (both ~7 Hz) (Komatsu and Yoshimura 2000). Taken together, for maintenance, inhibitory LTP seems to need a considerably higher frequency and/or level of presynaptic intracellular Ca\textsuperscript{2+} elevation than excitatory LTP.

Functional significance of activity-dependent maintenance of LTP

The age and experience dependence of this excitatory LTP suggests that it could underlie the experience-dependent refinement of visual responsiveness during development (Komatsu and Iwakiri 1992; Komatsu et al. 1988; Ohmura et al. 2003) as inhibitory LTP (Komatsu 1994). The refinement of visual responsiveness proceeds gradually, and it is reversible during a critical period in early life (Frégnaç and Imbert 1984). Orientation selectivity is improved to a considerably high level in the middle of the critical period (Frégnaç and Imbert 1984), and a following deprivation of light, even for a few days, degrades the selectivity (Freeman et al. 1981). This supports the idea that neural activity is necessary to maintain visual responsiveness refined by visual experience during development. Thus the necessity of neural activity for the maintenance of excitatory LTP, shown in this study, is consistent with the hypothesis that this LTP is involved in the development of visual responsiveness. Synapses modified by a temporary neural activity may eventually lead to a persistent form independent of activity and contribute to the visual responsiveness of mature cortical cells if they are continued to be activated at least at a low frequency thereafter until the end of the critical period. This process might require RNA and protein synthesis, as is the case in the late phase of hippocampal NMDA receptor–dependent LTP (Frey et al. 1988; Nguyen et al. 1994).

In lateral geniculate nucleus, eye-specific laminar segregation of retinal ganglion cell axons is established activity-dependently during development (Shatz and Stryker 1988; Sretavan et al. 1988). During this developmental process, once segregated termination of ganglion cell axons returns to an overlapped state, when firing of retinal ganglion cells of both eyes was pharmacologically blocked (Chapman 2000). Therefore neural connections once refined by neural activity might often require some neural activity for their maintenance thereafter during development.

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