SUBTHRESHOLD CONTRIBUTION OF N-METHYL-D-ASPARTATE RECEPTORS TO LONG-TERM POTENTIATION INDUCED BY LOW-FREQUENCY PAIRING IN RAT HIPPOCAMPAL CA1 PYRAMIDAL CELLS

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Abstract—Long-term potentiation (LTP) is a use-dependent and persistent enhancement of synaptic strength. In the CA1 region of the hippocampus, LTP has Hebbian characteristics and requires precisely timed interaction between presynaptic firing and postsynaptic depolarization. Although depolarization is an absolute requirement for plasticity, it is still not clear whether the postsynaptic response during LTP induction should be subthreshold or suprathreshold for the generation of somatic action potential. Here, we use the whole-cell patch-clamp technique and different pairing protocols to examine systematically the postsynaptic induction requirements for LTP. We induce LTP by changes only in membrane potential while keeping the afferent stimulation constant and at minimal levels. This approach permits differentiation of two types of LTP: LTP induced with suprathreshold synaptic responses (LTP\textsubscript{AP}) and LTP induced with subthreshold excitatory postsynaptic current (EPSCs; LTPEPSC). We found that LTP\textsubscript{AP} (>40%) required paired depolarization (\(V_m\approx-40\) mV, for 40–60 s) with four to six (0.1 Hz) single synaptically initiated action potentials. LTPEPSC was of smaller magnitude (<30%) and required pairing of depolarization to −50 mV (60 s) with six subthreshold EPSCs. The N-methyl-D-aspartate receptor (NMDAR) antagonists aminophosphonovaleric acid and 7-chlorokynurenic acid consistently blocked LTPEPSC but were ineffective in preventing LTP\textsubscript{AP}. Robust, NMDAR-independent LTP is obtained by stronger postsynaptic depolarization that converts the EPSCs to suprathreshold somatic action potentials. Purely NMDAR-dependent LTP is obtained by pairing mild somatic depolarization with subthreshold afferent pulses to the postsynaptic cell. Our results indicate that the degree of postsynaptic depolarization in the presence of single afferent pulses determines the type and magnitude of LTP. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: action potential, depolarization, EPSC, glutamate, patch clamp, 7-chlorokynurenic acid.

Long-term potentiation (LTP) of synaptic transmission in the mammalian hippocampus is a model for studying the cellular and molecular mechanisms of learning and memory (Bliss and Lømo, 1973; Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). LTP is consistent with the theoretical assumption that the brain stores information in a form of use-dependent synaptic modification (Hebb, 1949; Brown et al., 1990). In the Schaffer collateral/commissural synapse onto CA1 pyramidal cells, LTP and its counterpart long-term depression (LTD) are Hebbian and require N-methyl-D-aspartate receptor (NMDAR) activation and elevation of postsynaptic calcium (Lynch et al., 1983; Malenka et al., 1988; Brown et al., 1990; Cummings et al., 1996).

LTP induction requires two events: synapse activation accompanied by transmitter release and depolarization of the postsynaptic membrane (Kelso et al., 1986; Malinow and Miller, 1986; Bliss and Collingridge, 1993). The requirement for sufficient membrane depolarization reflects the fact that for synaptic modifications to take place cooperative interaction between multiple afferents must be present (McNaughton et al., 1978; Lee, 1983; Debanne et al., 1996). This type of depolarization-mediated ‘cooperativity’ has been demonstrated in the hippocampus by increasing the number and rate of discharging presynaptic neurons or by directly depolarizing the postsynaptic cell (McNaughton et al., 1978; Lee, 1983; Kelso et al., 1986; Malinow and Miller, 1986; Gustafsson et al., 1987; Debanne et al., 1996). However, the degree of depolarization during induction alters the postsynaptic action potential threshold, which in turn may influence the specific mechanism of synaptic strengthening (Scharfman and Harvey, 1985; Magee and Johnston, 1997; Markram et al., 1997; Thomas et al., 1998; Pike et al., 1999; Sabatini et al., 2002). On the other hand, it has been suggested that the degree of postsynaptic depolarization during induction may determine the direction of synaptic change: mild depolarization leads to LTD, while LTP is induced with stronger depolarization (Kerr and Abraham, 1995; Goda and Stevens, 1996; Feldman et al., 1998; Ngezahayo et al., 2000; Shouval et al., 2002). Thus, the induction requirements for plasticity in CA1 pyramidal cells remain unclear.

The ‘pairing protocols’ for LTP induction allow better control over the postsynaptic voltage during coincidental...
presynaptic and postsynaptic activity. A 'pairing protocol' usually couples a constant rate of afferent stimulation with a steady depolarization, provided by the somatic intracellular or patch electrode (Kelso et al., 1986; Gustafsson et al., 1987; Stricker et al., 1996). Mechanistically, the depolarization provided by the voltage-clamp reflects the direct measurement of the 'cooperativity' requirement. However, this measurement is usually confounded by the depolarization produced by afferent stimulation, which may differ in duration, frequency or intensity. Reducing the rate and number of presynaptic pulses during induction allows better separation of synaptically evoked depolarization from that provided by voltage-clamp. Low-frequency pairing also permits the differentiation and direct examination of two different types of input specific LTP: LTP induced by pairing depolarization with postsynaptic action potentials (LTPAP) and LTP induced by pairing depolarization with subthreshold excitatory postsynaptic currents (LTP_EPSC).

The goal of the present study is to examine the induction requirements for low-frequency LTP. During the course of the experiments we established the induction requirements and basic properties of LTPAP and LTP_EPSC. We also describe the effect of NMDAR antagonism on both of them.

**EXPERIMENTAL PROCEDURES**

**Preparation of hippocampal slices**

All experimental procedures were conducted in strict accordance with the international, Canadian (CCAC) and institutional (OHRI) guidelines for the ethical use of laboratory animals. Every effort was made to minimize the number of animals used and their suffering. Coronal brain slices containing the hippocampus were obtained from Sprague–Dawley rats (21–28-days old). Prior to decapitation, the animals were anesthetized with isoflurane inhalation. The brain was removed and placed in an oxygenated (95% O₂/5% CO₂) physiological solution, artificial cerebrospinal fluid (ACSF) at 4 °C, containing (in mM) 126 NaCl, 2.5 KCl, 1 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂ and 10 glucose. The osmolarity of the ACSF was adjusted to 300 mOsm and the pH to 7.2. A block containing the region of interest was prepared, and sections (300 μm) were obtained with a vibrating microtome (Leica VT 1000S, Wetzlar, Germany). The slices were stored for 1 h in an oxygenated chamber at room temperature before they were used for the experiments.

**Data recording**

For recording, the slices were transferred to a submerged-type recording chamber and held securely in place by a nylon mesh. Oxygenated and warmed (32–34 °C) ACSF was continuously superfused at a rate of 2.5 ml min⁻¹. Current- and voltage-clamp recordings were obtained with a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA, USA) under visual control using differential interference contrast and infrared video microscopy (IR-DIC; Leica DMLFSA).

Whole-cell recordings were obtained with borosilicate pipettes filled with a solution containing (in mM) 130 K-gluconate, 10 HEPES, 10 KCl, 2 MgCl₂, 2 ATP-Mg and 0.2 GTP-tris(hydroxy-methyl) aminomethane. The pH was adjusted to 7.2 and osmolarity to 280–290 mOsm. The pipettes had resistances of 5–8 MΩ when filled with this solution. Electroresponsive properties of neurons were studied in a current-clamp by applying 500 ms current pulses from rest. The amplitude of the current pulses was varied in fixed increments of 10 pA. The input resistance was estimated in the linear portion of the current-voltage plots. The evoked postsynaptic response was studied in voltage-clamp from −65 mV holding potential. Voltages have not been corrected for the theoretical liquid junction potential (approximately 10 mV). Aminophosphonovarlic acid (APV) was purchased from RBI (Natick, MA, USA) and 7-chlorokynurenic acid (7-Cl-KYN) was purchased from Tocris (Bristol, UK).

Single electrode whole-cell patch-clamp is prone to error when recording continuously from long multibranching neurons. To minimize space and point clamp errors and maximize uniformity throughout the experiments, the following measures were taken: First, for the analysis, only cells that met the following criteria were included: resting membrane potential (Vₘ) of −65 to −68 mV; spike amplitude of 85–120 mV; input resistance of 90–140 MΩ; spike-afterhyperpolarization of 4–9 mV amplitude. Second, recordings with series resistance higher than 20 MΩ were discarded. Third, recordings were discarded if during the experiment, Vₘ changed spontaneously by more than 2 mV, spike amplitude by more than 8 mV and series resistance by more than 10%. Fourth, excitatory postsynaptic current (EPSC) activation (+2 ms) mediated mainly by amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid receptors (AMPARs) was monitored throughout each experiment.

**Electrical stimulation**

Schaffer collaterals/commissural afferents were stimulated by concentric bipolar electrodes (Frederick Haer Co, Brunswick, ME, USA) placed in the stratum radiatum. Baseline post-synaptic responses were evoked by 100 μs constant current pulses (0.1 Hz) delivered through a current output isolation unit (A-365; World Precision Instruments, Sarasota, FL, USA). Stimulation intensity (0.02–0.15 mA) was adjusted according to EPSC amplitudes and spike threshold.

**LTP induction**

High- and low-frequency LTP induction protocols were used. High-frequency induction (high-frequency LTP) consisted of single (100 or 250 ms) 100 Hz trains paired with or without postsynaptic depolarization (Vₘ = −50 mV). Low-frequency induction (low-frequency LTP) consisted of different types of somatic depolarization (Vₘ = −60, −50, −40, −30, −20 mV) paired with single afferent pulses (0.1 Hz). Stimulation intensity for all induction paradigms was the same as during the baseline period.

The central goal of the study was to examine the threshold requirements for LTP induced by single afferent pulses. To achieve this, it was necessary first to identify the electrophysiological parameters of CA1 pyramidal cells that may influence the impact of synaptic potentials. This was done with the help of current clamp I–V plots and depolarizing steps in the voltage-clamp mode applied from Vₘ = −65 mV. The action potential threshold (approximately −49 mV) was determined by the voltage preceding each spike. This value was relatively stable despite the irregular firing behavior of pyramidal cells. The threshold was further tested and confirmed by prolonged voltage clamp pulses, conditions that mimicked closely our low-frequency induction. In our suboptimal voltage clamp conditions the peak of sodium activation (t = 2 ms) mediated mainly by a-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid receptors (AMPARs) was monitored throughout each experiment.
were paired with somatic depolarization to −50 mV (Fig. 1E). In both cases, potentiation was significant (57.43 ± 8.4%, n=5, P<0.01 for a 25 pulse train and 52.31 ± 7.6%, n=4, P<0.01, for a 10 pulse train) and of maximal magnitude (see Experimental Procedures). Stronger depolarization than that provided by the afferent stimulation must be therefore required for enabling LTP during brief high-frequency synaptic stimulation.

We observed that for LTP induction, the cell must fire prolonged trains of action potentials. We also noticed that in different cells, the bursts were of variable duration. The prolonged action potential discharge during the train led to the buildup of additional depolarization (Fig. 1B, D, lower trace). Thus, depolarization enabled the cell to fire persistently and at levels well above the applied holding potential. The after-depolarization observed here was similar to that reported previously for hippocampal (Jensen et al., 1996) and cortical (Sjöström et al., 2001) pyramidal neurons. The mechanism responsible for the build-up of membrane after-depolarization involves interaction between the membrane time constant and voltage-gated persistent Na+ currents (Azouz et al., 1996; Jensen et al., 1996). The spike after-depolarization buildup during high-frequency stimulation may have contributed to LTP as previously shown for cortical pyramidal neurons (Sjöström et al., 2001).

From the high-frequency LTP experiments summarized in Fig. 1, we conclude that: 1) somatic depolarization enables LTP under conditions where the afferent stimulation alone is insufficient; 2) lowering the threshold for postsynaptic spiking is one possible mechanism depolarization may have contributed to LTP; 3) LTP induction is associated with prolonged trains of action potentials, which lead to the buildup of prolonged after-depolarization; 4) postsynaptic voltage requirements for LTP cannot be determined precisely with the help of high-frequency stimulation due to the variability in postsynaptic spiking and more importantly due to the variability in amplitude and duration of spike after-depolarization.

**Minimal pairing requirements for low-frequency LTP of maximal magnitude**

We used low-frequency pairing protocols to examine the postsynaptic voltage requirements for LTP without the confounding effect of massive, high-frequency induced spike after-depolarization. We tested a number of pairing protocols that differed in duration (1, 5 and 10 min), degree of depolarization (V_m = −60, −50, −40, −30, −20 and 0 mV), and frequency (0.05, 0.1 and 0.2 Hz) of afferent stimulation (data not shown). During these experiments, it was observed that LTP was most easily obtained when the postsynaptic cells fired action potentials during induction (LTP_AP). It was also determined that 60 s of depolarization during which the postsynaptic cell fired consistently in response to the afferent pulses (0.1 Hz) resulted in LTP_AP of maximal magnitude. Pairing depolarization to −40 mV with six, five and four single synthetically initiated action potentials for 60, 50, and 40 s respectively, resulted in LTP_AP of maximal magnitude.

**RESULTS**

**Depolarization enables LTP during brief high-frequency afferent stimuli**

Tetanic stimulation applied to the Schaffer collaterals is a standard procedure for LTP induction in CA1 pyramidal cells (Bliss and Collingridge, 1993). We examined the effects of depolarization on high-frequency LTP with the help of single, 100 Hz, brief (25 and 10 pulses) trains that did not induce LTP when applied alone. The synaptic responses during the tetanic stimulation were continuously monitored (Fig. 1A–D, lower traces). When the cell was clamped at −65 mV during the trains, we did not observe potentiation of the EPSC (Fig. 1A, C). However, when the cell was clamped at −50 mV for the duration of the trains, potentiation invariably was observed (Fig. 1B, D). Pooled data illustrate that LTP was obtained only when the trains were paired with somatic depolarization to −50 mV (Fig. 1E). In both cases, potentiation was significant (57.43 ± 8.4%, n=5, P<0.01 for a 25 pulse train and 52.31 ± 7.6%, n=4, P<0.01, for a 10 pulse train) and of maximal magnitude (see Experimental Procedures). Stronger depolarization than that provided by the afferent stimulation must be therefore required for enabling LTP during brief high-frequency synaptic stimulation.

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magnitude (Fig. 2A–C). Potentiation was: 52.86 ± 7.2%, 
n = 7, P < 0.01, for six action potentials; 52.23 ± 7.8%, 
n = 6, P < 0.01, for five action potentials; 51.1 ± 7.1%, 
n = 5, P < 0.01, for four action potentials (Fig. 2F, G). 
Pairing −40 mV with three and two action potentials 
(Fig. 2D, E) for 30 and 20 s, resulted from rapid reduction 
of LTP size for three action potentials (21.35 ± 9.1%, 
n = 5, P < 0.01), to complete loss of potentiation for two action potentials (1.27 ± 5.6%, 
n = 4; Fig. 2F, G). We conclude that the minimal requirements for obtaining LTP of maximal magnitude is 
$V_m = −40$ mV paired with four to six synaptically initiated single action potentials (0.1 Hz).

**Postsynaptic voltage and the induction of LTP**

The postsynaptic voltage requirements for LTP were 
examined by pairing depolarization of constant duration 
(60 s) but of different amplitude ($V_m = −40, −30$ and 
$−20$ mV) with six suprathreshold synaptic responses (0.1 Hz;
Fig. 2. Minimal pairing requirements for LTP of maximal magnitude. (A) Pairing depolarization to −40 mV with six single (0.1 Hz) suprathreshold synaptic responses leads to LTP of maximal magnitude. Low-frequency induction protocol and a whole cell single response during induction are shown. (B–E) Four different pairing protocols and representative synaptic responses. Depolarization to −40 mV paired with suprathreshold synaptic responses, sequentially reduced from five to two. (F) Summary graph showing potentiation time course under the different induction conditions. (G) Pooled data indicate potentiation for the different pairing protocols. Pairing depolarization to −40 mV with four, five or six synaptically initiated action potentials resulted in LTP of maximal magnitude. Reducing further the number of suprathreshold responses paired with depolarization to three and two led to gradual loss of potentiation.

Fig. 3A–D). In all three conditions, potentiation was of similar and maximal magnitude: 52.86±7.2%, n=7, P<0.01, for −40 mV; 57.17±8.1%, n=5, P<0.01, for −30 mV and 58.22±8.9%, n=5, P<0.01 for −20 mV, respectively (Fig. 3D). These results indicate that once the threshold for action potentials is reached, very little or no additional potentiation is added even when the cell is further depolarized. In the presence of membrane depolarization (V_m=−40), the action potential threshold is also the threshold for obtaining maximal LTP_{AP}.

Pairing postsynaptic depolarization with subthreshold EPSCs results in LTP of submaximal magnitude (LTP_{EPSC})

It has been demonstrated that blockade of the postsynaptic somatic action potential does not prevent the induction of LTP (Kelso et al., 1986; Golding et al., 2002). Although this finding indicates that the generation of somatic sodium spikes is not required for LTP, little is known about LTP obtained with depolarization that is subthreshold for postsynaptic spikes. Here we paired small depolarization with subthreshold EPSCs to examine the properties of LTP obtained in the absence of sodium spikes (LTP_{EPSC}; Fig. 4). Pairing somatic depolarization to −60 mV with six EPSCs (0.1 Hz) did not result in significant potentiation (2.79±4.7%, n=7; Fig. 4A, D). Depolarization to −50 mV was associated with two different types of postsynaptic response during the afferent shocks: either EPSCs or action potentials (Fig. 4B, C). However, the action potentials in most cases did not persist during the entire induction time and a significant number of failures were observed. To control for this difference, we analyzed the cells that fired during induction separately from the cells that did not
ther increase of the depolarization, pure LTP EPSC was present: 16.4 ± 5.9%, n = 5, P < 0.01, for the purely LTP_{EPSC} group and 29.64 ± 7.9%, n = 6, P < 0.01, for the spiking group (Fig. 4B–D).

We found that the size of LTP_{EPSC} obtained with −50 mV is approximately 16%. Since the upper limit of LTP in our conditions is approximately 70%, LTP_{EPSC} (V_m = −50 mV) magnitude translates into 20–25% of total potentiation. LTP_{EPSC} was found to be voltage-dependent since depolarization to −60 mV did not induce any potentiation while at V_m = −50 mV potentiation was maximal. With further increase of the depolarization, pure LTP_{EPSC} was more difficult to obtain due to the appearance of postsynaptic spiking. These results suggest that postsynaptic firing is not a prerequisite for LTP and synapse modification can take place under conditions where local depolarization and synaptic activation coexists with enhanced perisomatic inhibition (Yuste and Tank, 1996; Häusser et al., 2000; Golding et al., 2002).

The effect of NMDAR antagonists APV and 7-Cl-KYN on LTP_{AP} and LTP_{EPSC}

Next we examined the effects of APV (50 μM) on LTP_{AP} and LTP_{EPSC} (Fig. 5). During the experiments, it was observed that although APV did not change baseline EPSC amplitude, it increased the threshold for action potential during depolarization. Pyramidal cells, voltage clamped at −40 mV, usually spike in control ACSF in response to afferent stimulation (Figs. 2 and 3). In the presence of APV, approximately 50% of the cells did not spike (n = 6, data not shown). Depolarizing the cell to −50 mV was often associated with irregular spiking in response to the stimulus. The occasional spiking was absent in the presence of APV (n = 8, data not shown). The effects of APV on the action potential threshold are consistent with a reduction of inward cationic currents mediated by NMDAR (Mayer et al., 1984; Nowak et al., 1984). In the pooled data for the effects of APV on LTP_{EPSC}, cells that did not fire during depolarization to −40 mV were analyzed together with the cells depolarized to −50 mV (Fig. 5A, B).

In the presence of APV, no potentiation was observed when the postsynaptic cell did not spike during depolarization. Pairing depolarization (V_m = −60, −50 and −40 mV) with six subthreshold EPSCs in the presence of APV was found to have no lasting effect on EPSC amplitude (0.62 ± 5.7%, n = 4 for −60 mV and 1.27 ± 5.8%, n = 6 for −50/−40 mV, respectively; Fig. 5A, C). However, APV did not prevent LTP induced by pairing depolarization to −40 mV with six synaptically initiated spikes (LTP_{AP}, 51.17 ± 7.6%, n = 4, P < 0.01; Fig. 5A, B). The magnitude of LTP_{AP} obtained in the presence of APV was not significantly different from that obtained in control ACSF (Fig. 5C). With stronger background depolarization (V_m = −40 mV), APV cannot prevent postsynaptic firing and the induction of maximal LTP_{AP}. Thus, at subthreshold induction conditions, APV through its action on NMDARs increases the threshold for LTP_{AP} and reliably blocks LTP_{EPSC}.

To our knowledge this is the first study to report the resistance of LTP induced by single afferent pulses to NMDAR antagonism. Therefore, we further examined the role of NMDARs in the induction of both types of LTP with the help of 7-Cl-KYN, a selective antagonist for the strychnine-insensitive glycine site of NMDAR (Kemp et al., 1988; Kemp and Priestley, 1991). Glycine is required for NMDAR activation and antagonist blockade of the glycine site prevents NMDAR-mediated responses (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Lerma et al., 1990). 7-Cl-KYN (3 μM) applied during the pairing mimicked the effect of APV on both types of LTP. Blockade of the glycine site during induction eliminated the potentiation obtained by pairing depolarization with subthreshold EPSCs (1.1 ± 5.9%, n = 4 for −60 mV and 0.89 ± 6.2%, n = 5 for −50 mV; Fig. 5B, C). Increasing depolarization to −40 mV in most cases did not prevent postsynaptic firing and the induction of strong LTP (51.67 ± 8.9%, n = 5, P < 0.01;
In the cases where postsynaptic spiking was absent, LTP did not develop (n=3, data not shown). Blockade of LTP_{EPSC} with 3 μM 7-Cl-KYN, which is in the concentration range (0.3–10 μM) previously shown to block the glycine site of NMDARs (Kemp et al., 1988; Izumi et al., 1990; Kemp and Priestley, 1991; Parsons et al., 1993) indicates that the glycine site activation is required for LTPEPSC.

From the experiments with APV and 7-Cl-KYN we conclude that: 1) LTP obtained by pairing depolarization with EPSCs (LTP_{EPSC}) is purely NMDAR-dependent; 2) LTP_{EPSC} requires small depolarization (≤15 mV); 3) bath perfused NMDAR antagonists cannot prevent the development of input specific LTP when the depolarized cell fire action potentials in response to single afferent pulses; 4) NMDAR antagonism has no effect on the magnitude of LTP induced by pairing depolarization (V_m=−40 mV) with synaptically initiated single action potentials.

Thus, by pairing different degrees of somatic depolarization with single sub- or suprathreshold synaptic responses, two different types of LTP can be differentiated: the purely NMDAR-dependent LTP_{EPSC}, which is of sub-maximal magnitude and LTP_{AP}, being of maximal magnitude and resistant to NMDAR antagonism.

**DISCUSSION**

**Pairing requirements for low-frequency LTP**

The goal of the present study was to examine the induction requirements for LTP obtained by pairing depolarization with single afferent pulses. We found that: 1) LTP of maximal magnitude (>40%) was induced when depolarization (V_m=−40 mV, 40–60 s) was paired with four to six single, synaptically initiated action potentials (LTP_{AP}); 2) LTP_{AP} was NMDAR-independent; 3) pairing mild depolarization (V_m=−50 mV, 60 s) with six single subthreshold EPSCs resulted in LTP of submaximal magnitude (<30%; LTP_{EPSC}); 4) LTP_{EPSC} was purely NMDAR-mediated.

In agreement with previous reports (Kelso et al., 1986; Malinow and Miller, 1986; Gustafsson et al., 1987; Debanne et al., 1996; Magee and Johnston, 1997; Sjöström et al., 2001) we found that background depolarization enables high- and low-frequency LTP to be induced under conditions where those same afferent stimuli, when applied alone, did not induce any significant postsynaptic modification. In both types of induction, robust LTP was obtained only when the synaptically initiated depolarization, combined with the background somatic depolarization, was above the threshold for action potential. Our results further demonstrate that in the presence of prolonged depolarization, the number of afferent pulses required for LTP can be reduced to as low as four given at 0.1 Hz. Depolarizing the postsynaptic cell above the threshold for action potential is sufficient for the induction of maximal LTP. NMDAR-mediated component of LTP was obtained by pairing small depolarization with six single subthreshold EPSCs. Collectively, these results demonstrate that postsynaptic depolarization regulates the induc-

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tion and magnitude of LTP during low-frequency afferent stimulation.

NMDARs and low-frequency LTP

NMDAR, often described as a Hebbian molecule, serves as a coincident detector allowing Ca$^{2+}$ influx only when glutamate is present and the postsynaptic membrane is sufficiently depolarized (Bliss and Collingridge, 1993). Functional and calcium imaging studies have demonstrated that NMDARs are active and generate Ca$^{2+}$ signals at potentials close to resting (Mayer et al., 1984; Regehr and Tank, 1994; Swadlow and Hicks, 1997; Mainen et al., 1999; Yuste et al., 1999; Kovalchuk et al., 2000; Sabatini et al., 2002). However, at potentials close to resting, LTP has been difficult to obtain and it was suggested that at that level of postsynaptic activity, LTD is more readily induced (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Ngezahayo et al., 2000; Shouval et al., 2002). The present study demonstrates that pairing mild depolarization with subthreshold EPSCs results in the induction of purely NMDAR-dependent LTP (LTP$_{EPSC}$). This component is of relatively small magnitude (<30%) and can be prevented independently by APV and 7-CI-KYN. The induction parameters of LTP$_{EPSC}$ suggest that while membrane depolarization is required for NMDAR plasticity, clear separation of the NMDAR-dependent component of LTP can be obtained by keeping the synaptic stimulation subthreshold for the generation of somatic action potentials. These findings are in agreement with previous reports and demonstrate that somatic spiking is not necessary for NMDAR-mediated strengthening of the active synapses (Kelso et al., 1986; Golding et al., 2002).

Depolarizing the somatic V_m has been used routinely in plasticity studies for recruitment of synaptic NMDARs blocked at more hyperpolarized potentials (Kelso et al., 1986; Gustafsson et al., 1987; Stricker et al., 1996; Sjöstrom et al., 2001). Our observation that increasing membrane depolarization from −60 to −50 mV led to substantial increase of the NMDAR-mediated component of LTP is consistent with the recruitment of NMDARs relatively inactive at close to resting potentials. The mechanism of electrotonic recruitment of distal NMDARs along the somatodendritic axis was beyond the scope of the study. Accumulating evidence indicates however that enhancement of sodium and calcium channel activity and inactivation of potassium currents contribute to the electrogenesis necessary for activation of synaptic NMDARs (Stuart and Sakmann, 1995; Sjöstrom et al., 2001; Stuart and Haussser, 2001).

A potentially important finding of the present study is that we failed to observe LTD during our subthreshold and suprathreshold induction conditions. It has been demonstrated that LTD is produced in response to "weaker" prolonged low-frequency trains that insufficiently depolarize the post-synaptic membrane and fail to activate NMDARs (Dudek and Bear, 1992; Mulkey and Malenka, 1992). Furthermore, it was proposed that clamping the postsynaptic cell between −60 and −52.5 mV would be sufficient to induce LTD while with more positive potentials LTP would be obtained (Shouval et al., 2002). LTD induction by pairing at even more depolarized potentials has also been reported (Feldman et al., 1998; Ngezahayo et al., 2000). The discrepancies between some of the above studies and our results are most likely due to the different induction parameters used. Specifically, the duration of depolarization used for LTD induction here is smaller to that used by most studies. Another potentially important difference is that we induce LTP by limited number of pairings. Pairing prolonged depolarization with four to six single afferent pulses (0.1 Hz) is sufficient for the development of input specific LTP. The degree of depolarization in the presence of afferent stimulation however, seems to be the most important factor for the induction and magnitude of LTP.

VDCCs (voltage-dependent calcium channels) and low-frequency NMDAR-independent LTP

The synaptic modifications associated with LTP are confined to the excitatory glutamatergic synapses located in dendrites and dendritic spines of CA1 pyramidal cells (Yuste and Denk, 1995; Malenka and Nicoll, 1999; Sabatini et al., 2002). The necessary signal for LTP is Ca$^{2+}$, which may enter the spine through NMDARs, VDCCs, Ca$^{2+}$ permeable AMPA channels (Yuste et al., 1999) or be released from intracellular stores (Empage et al., 1999)

In contrast to the input specific NMDAR-dependent LTP, NMDAR-independent LTP can be input specific (homosynaptic), not input specific (heterosynaptic) or a combination of both (Brown et al., 1990; Kullmann et al., 1992; Bliss and Collingridge, 1993; Hanse and Gustafsson, 1995; Chen et al., 1998). During induction, the input specificity of LTP may be affected by the intensity and type of afferent stimulation and the properties of postsynaptic Ca$^{2+}$ signals. Since for the input specificity of LTP afferent stimulation is required, potentiation obtained without afferent stimulation is not input specific (Kullmann et al., 1992; Chen et al., 1998).

When LTP is obtained by tetanic stimulation, the rate and intensity of stimulation determine the form of LTP through activation of different postsynaptic mechanisms of plasticity. NMDAR-dependent LTP usually requires lower frequency (<30 Hz) afferent stimulation (Grover and Teyler, 1990, 1992; Morgan and Teyler, 2001). Increasing the intensity of afferent stimulation increases the contribution of Ca$^{2+}$ influx via VDCCs. Thus, very high-frequency (>200 Hz) tetanization or high-intensity b-burst stimulation induces potentiation by Ca$^{2+}$ entry through L-type VDCCs (Grover and Teyler, 1990, 1992; Hanse and Gustafsson, 1995; Morgan and Teyler, 2001). Pairing studies have confirmed that VDCC-dependent LTP requires strong postsynaptic depolarization (Kullmann et al., 1992; Chen et al., 1998; Stricker et al., 1999). Our results are in agreement with these studies and further clarify the induction requirements for both types of LTP. To our knowledge, this study presents the first report of NMDAR-independent LTP induced by single afferent pulses. Thus, NMDAR-dependent LTP requires pairing of depolarization (V_m=-50 mV) with subthreshold synaptic stimulation.
whereas NMDAR-independent LTP can be obtained by pairing depolarization ($V_m \geq -40 \text{ mV}$) with suprathreshold stimulation. We have estimated the voltage requirements for LTP through separation of voltage clamp depolarization from that induced by afferent stimulation. This was achieved by reducing the frequency and duration of afferent stimulation. Furthermore, the low-frequency afferent rate allowed us to monitor the postsynaptic response during induction and conclude that NMDAR antagonism is ineffective when the afferent pulses discharge the depolarized pyramidal cell. Thus, the appearance of somatic action potentials during induction is a potential indicator for the occurrence, magnitude and mechanism of LTP. Taken together these findings suggest that NMDAR-independent LTP requires stronger depolarization and the generation of somatic action potentials, conditions most likely occurring during high-frequency tetanization (Grover and Teyler, 1990; Hanse and Gustafsson, 1995; Grover, 1998).

In the present study, low-frequency NMDAR-dependent and NMDAR-independent LTP were induced by single postsynaptic manipulation (depolarization). LTP obtained by postsynaptic current injection is a controlled way of LTP induction. Reducing the rate and number of afferent pulses circumvents a number of confounding mechanisms including altered transmitter release, temporal and spatial integration of EPSCs, buildup of synaptic and extrasynaptic glutamate, and saturation of uptake systems. Most of these mechanisms may affect the input specificity of LTP by recruitment of new release sites. In the present study, we minimized the possibility of heterosynaptic spread of potentiation by two additional measures. First, the number and intensity of afferent pulses were kept to minimal levels and second, potassium- and chloride-mediated inhibitory systems were kept intact. Thus, regardless of the mechanism, our results suggest that the locus of expression of synaptic modification for NMDAR-dependent LTP and NMDAR-independent LTP is postsynaptic and confined to the same set of activated inputs.

Previous studies have suggested that VDCC-dependent LTP and NMDAR-dependent LTP require distinct transduction cascades (Grover and Teyler, 1990; Cavus and Teyler, 1996). Pharmacological treatments with APV during high-frequency tetanization decrease the magnitude of potentiation suggesting that this LTP is a compound potentiation consisting of VDCC-mediated and NMDAR-mediated components (Grover and Teyler, 1990; Cavus and Teyler, 1996; Grover, 1998). In the present study, NMDAR antagonists had no effect on the magnitude of low-frequency NMDAR-independent LTP. This finding indicates that during supramaximal induction conditions postsynaptic Ca$^{2+}$ levels are above the threshold for activation of NMDAR-coupled biochemical pathways. If this is the case, this result would suggest a cross-talk between Ca$^{2+}$ influx through VDCCs and NMDAR-dependent mechanisms of plasticity. This interpretation is consistent with a common expression mechanism of NMDAR-independent and NMDAR-dependent LTP. Indeed, recent evidence suggests that the expression and maintenance of NMDAR-independent LTP appear to require enhancement of postsynaptic AMPAR function (Grover, 1998; Stricker et al., 1999).

### Sodium channels, action potentials and low-frequency LTP

Distal and proximal dendrites of pyramidal neurons contain voltage-gated sodium channels, which main function seems to be the integration of excitatory synaptic input (Magee and Johnston, 1995; Golding and Spruston, 1998; Magee, 2000). Somatic depolarization, similar to that used in the present study, has been the tool for investigating the role of sodium channels in plasticity and in shaping the integrative properties of pyramidal neurons (Deisz et al., 1991; Magee and Johnston, 1995, 1997; Stuart and Sakmann, 1995; Markram et al., 1997; Andreasen and Lambert, 1999; Golding et al., 2002). Thus, activation of axosomatic sodium channels by depolarizing current injection amplifies dendritic excitatory postsynaptic potentials (EPSPs) while TTX prevents the depolarization-induced amplification and reduces the size of large EPSPs (Stuart and Sakmann, 1995; Andreasen and Lambert, 1999).

The contribution of sodium channels to synaptic plasticity has been established by demonstrating that activation of dendritic sodium channels provides the electrogensis required for calcium influx through NMDARs and VDCCs during LTP (Magee and Johnston, 1997; Markram et al., 1997; Magee, 2000; Golding et al., 2002). Although in the present study we have examined the role of sodium channels in LTP indirectly and mostly in respect to the generation of somatic action potential, several observations were consistent with contribution of these channels to plasticity. First, the amount of potentiation was generally proportional to the degree of membrane depolarization. Second, potentiation reached its maximum only when the depolarization was paired with suprathreshold synaptic responses, conditions presumably reflecting full activation of axosomatic and dendritic sodium channels. Third, LTP induced by strong depolarization and somatic action potentials was least affected by NMDAR antagonists. These findings are consistent with other plasticity studies demonstrating the contribution of sodium channels to the generation of dendritic spikes and the spread of backpropagating action potentials (Jaffe et al., 1992; Magee and Johnston, 1995, 1997; Markram et al., 1997; Golding et al., 2002). Our results also confirm however, that depolarization and postsynaptic action potentials without afferent stimulation, does not lead to input specific, Hebbian plasticity (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Conti and Lisman, 2002).

The main source of postsynaptic depolarization under physiological conditions arises from the temporal and spatial integration of dendritic EPSPs (Cash and Yuste, 1998, 1999; Magee and Cook, 2000). Activation of unitary synaptic contacts however, seems to be too weak for plasticity induction and more than one presynaptic cell must fire simultaneously for the postsynaptic membrane to be depolarized sufficiently (Debanne et al., 1996). This is the essence of the ‘cooperativity’ requirement and is best supported by the fact that stronger LTP is obtained when the
number of discharging presynaptic fibers is increased (Mc-
Naughton et al., 1978; Lee, 1983; Debanne et al., 1996).

The back-propagating somatic action potential is an-
other mechanism capable of providing the dendritic depo-
larization necessary for LTP (Stuart and Sakmann, 1994;
Stuart et al., 1997). Action potentials initiated at the soma
could function as an enabling signal for LTP by invading
the distal dendrites and activating NMDARs and VDCCs
(Magee and Johnston, 1997; Markram et al., 1997). Thus,
pairing subthreshold EPSPs with postsynaptic action po-
tentials induced by somatic current injection resulted in
persistent changes in EPSP amplitude (Magee and
Johnston, 1997; Markram et al., 1997). The compound
depolarization from properly timed EPSPs and postsynap-
tic burst firing appears to be equivalent to the background
depolarization used here and in other pairing studies. In-
deed, it has been shown that postsynaptic bursting and
complex spike bursting, which are common in the intact
hippocampus, enable LTP under conditions where single
postsynaptic spikes were ineffective (Thomas et al., 1998;
Pike et al., 1999). Thus, the prolonged somatic depolar-
ization may be a laboratory equivalent of the electrogene-
sis associated with pyramidal cell bursting in intact animals
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