Induction mechanisms for L-LTP at thalamic input synapses to the lateral amygdala: requirement of mGluR5 activation

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Received 4 February 2002; accepted 4 February 2002

L-LTP (late-phase long-term potentiation) at thalamo-amygdala synapses is thought to be critical for auditory fear conditioning, but it has not been clear what kinds of surface receptors and channels are involved in the induction phase of the L-LTP. Here we report that the NMDA receptor antagonist D-AP5 (50 μM), the L-type calcium channel antagonist nifedipine (30 μM) and the metabotropic glutamate receptor 5 antagonist MPEP (10 μM) prevented L-LTP induction when each antagonist was separately applied at saturating concentrations before and during repeated tetanus. By contrast, the mGluRI antagonist CPCCOEt (80 μM) failed to show any effects on L-LTP induction. Neither D-AP5 nor MPEP produced any significant effects on potentiated synaptic responses when applied after L-LTP had been established. Thus, our data suggest that NMDA receptors, L-type calcium channels and mGluR5 are involved in L-LTP induction in the thalamo-amygdala pathway. *NeuroReport* 13:685–691 © 2002 Lippincott Williams & Wilkins.

Key words: Amygdala; Brain slices; Fear conditioning; L-LTP; L-type calcium channel; NMDA receptor; Rat

INTRODUCTION

LTP is believed to be critical for learning and memory, and tremendous efforts have been made to find a link between LTP and learning [1–3]. One of the best examples for the link between LTP and learning in the mammalian brain may be cued conditioning, a form of fear conditioning that requires the lateral amygdala. This form of fear conditioning is produced by the pairing of a neutral tone as a conditioned stimulus (CS) with a shock as an unconditioned stimulus (US). These two stimuli converge onto the lateral amygdala, and the coincidental presentation of the CS and US is thought to induce fear conditioning by potentiating the synaptic strength of the CS pathway by a long-term potentiation-like mechanism [4,5]. The CS alone then could produce a sufficient excitation of the lateral amygdala to elicit conditioned fear. The CS comes into the lateral nucleus of the amygdala via two routes: directly from the medial geniculate nucleus and indirectly from the auditory cortex [6,7]. Although the synapses of both of these projections undergo long-term potentiation, the *in vivo* and *in vitro* studies linking amygdala LTP to fear learning have involved the thalamic pathway to the lateral amygdala [4,5]. However, studies examining amygdala LTP using *in vitro* preparations have focused mainly on the cortical inputs to the LA [8–12].

Mechanisms for LTP (early phase or late phase LTP) in the thalamic input synapses to the lateral amygdala have not been explored up until recently. A study of E-LTP (early phase LTP) induced by pairing pre- and postsynaptic activity reveals that the LTP induction is dependent on L-type voltage-gated calcium channels, but not on NMDA receptors [13]. In addition, an enduring form of LTP (L-LTP) induced by multiple trains of high-frequency stimulation at thalamic input synapses to the lateral amygdala has been shown to be dependent on protein synthesis, and is mediated by protein kinase A and mitogen-activated protein kinase (see Fig. 7 in [14]).

Since L-LTP has an enduring phase, it may be more relevant to study L-LTP as a cellular substrate for conditioned fear memory. Especially, receptors and channels involved in induction mechanisms for L-LTP at thalamic input synapses to the lateral amygdala has not been clearly defined yet. Therefore, we have examined a possible role of NMDA receptors, L-type calcium channels and group I mGluRs in the induction of L-LTP at thalamic input synapses to the lateral amygdala.

MATERIALS AND METHODS

Brain slices were prepared using techniques described previously [15,16]. Sprague–Dawley rat (3–5 weeks old) were decapitated. The isolated whole brains were placed in an ice-cold (0–4°C) modified artificial cerebrospinal fluid (aCSF) solution. The composition of modified aCSF was as

follows (in mM): 175 sucrose, 20 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 11 D-(+)-glucose. Coronal slices (400 μm) containing the amygdala were cut using a vibratome (Campden, UK), and were incubated in aCSF continuously bubbled at room temperature with 95% O_2/5% CO_2 for ≥ 3 h before recordings. Just before transferring the slice to the recording chamber, the cortex overlying the amygdala was cut away with a scalpel so that, in the presence of picrotoxin, cortical epileptic burst discharges would not invade the amygdala [13].

The recording chamber was continuously superfused with aCSF (30–32°C) at a flow rate of 1-2 ml/min. The aCSF contained (in mM): 120 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 2 $CaCl_2$, 11 D-(+)-glucose. Picrotoxin (10 μM) was included in all experiments to minimize fast GABAergic transmission [14]. The slices were incubated in the recording chamber ≥ 30 min before the start of recordings.

To record field potentials at thalamic input synapses to the lateral amygdala, we placed a bipolar stimulating electrode in the thalamic afferent fibers innervating the lateral amygdala, which is located in the ventral part of the striatum, just above the central nucleus of the amygdala, just medial to the lateral amygdala (see Fig. 7 in [14]). A trunk of the thalamic afferent fibers appeared to be well isolated from other structures and it could be easily visualized under our microscope. A stimulating electrode was located specifically on the trunk to elicit the field potential. The recording electrode (>1.0 MΩ) was filled with 0.9% NaCl and placed in the dorsal subregion of the lateral amygdala.

Synaptic responses were elicited at 0.017 Hz. L-LTP was induced by five trains of tetanic stimulation (100 Hz, 1 s at 1 min intervals) with the same intensity and pulse duration as the test stimuli. For the baseline field potential recording 50% of the maximum amplitude was used. The range of stimulus intensity and duration for each pulse is 0.1–0.3 mA and 0.1–0.2 ms, respectively.

Extracellular field potentials were amplified using a DP-301 amplifier (Warner Instrument Co., CT) and the output was digitized with a DIGIDATA 1322A interface (Axon instruments Inc., Foster City, CA). The digitized signals were stored and analyzed with a PC computer using pClamp 8 (Axon Instruments Inc., Foster City, CA).

Drugs used were D-AP5, nifedipine, picrotoxin and kynurenic acid from Sigma-Aldrich (St. Louis, MO). MPEP (2-methyl-6-(phenylethyl)-pyridine) and CPCCOEt (7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester) were from Tocric Cookson (Ballwin, MO). Drugs were made up in stock solutions and diluted more than 1000 times into aCSF. Picrotoxin, CPCCOEt and nifedipine were made up in DMSO.

RESULTS

L-LTP has been defined as a form of LTP that has an enduring phase (> 3 h). In order to achieve a stable recording over 3 h we chose to measure the field potential evoked when the thalamic fibers onto the lateral amygdala were stimulated (see Materials and Methods). In the previous study [14], the field potential at thalamic input synapses to the lateral amygdala has been characterized, and both the E-LTP and L-LTP were studied at this synapse. Consistent with their results, the field potential in our experimental condition had a constant and short latency of about 5 ms, followed high frequency (50 Hz) stimulation reliably and without failure, and it could be blocked by kynurenic acid (5 mM), a non-selective glutamate receptor antagonist (data not shown, see also [14]). These findings suggest that the field potential measured in the present study reflects glutamatergic, monosynaptic responses at thalamic input synapses to the lateral amygdala. As shown in the previous studies, we also included picrotoxin in our recording solution to block feedforward GABAergic inputs to principal neurons in the lateral amygdala [17].

L-LTP at thalamic input synapses to the lateral amygdala has been shown to be induced by ≥ 3 trains of tetanus (100 Hz, 1 s duration) [14]. Therefore, we have examined the effect of 3–5 trains of tetanic stimulation delivered at variable intervals to obtain maximal L-LTP. Five trains at 1 min intervals were found to be most effective for L-LTP induction. One successful example showed L-LTP lasting up to 8 h (data not shown).

In order to determine whether L-LTP induction at thalamic input synapses to the lateral amygdala depends on NMDA receptors, L-type voltage-gated calcium channels or group I mGluRs, we applied antagonists for each candidate molecule before and during L-LTP induction. Each antagonist was applied for a total of 20 min, 15 min prior to repeated tetanus, and an additional 5 min during repeated tetanus. To enable a more reliable comparison, we obtained a pair of recordings for control and antagonist-treated slices from the same animal. We first examined the effect of the NMDA receptor antagonist D-AP5 (50 μM) on induction of L-LTP in field potential recording experiments. D-AP5 prevented a potentiation of field potentials by five trains of tetanus. L-LTP could be induced in paired control slices ($164 \pm 5.5\%$ of control in the amplitude of field potential at 2.5–3 h post-tetanus, $n = 6$), but not in the presence of D-AP5 (Fig. 1a; 50 μM, $95 \pm 3.3\%$ of control in the amplitude of field potentials 2.5–3 h post-tetanus, $p < 0.0001$, paired t-test, $n = 6$). Exposure to D-AP5 completely prevented L-LTP induction.

In order to determine whether L-type voltage-gated calcium channels are involved in the induction of L-LTP, we examined the effect of the L-type voltage-gated calcium channel antagonist nifedipine (30 μM) on the induction of L-LTP. Five trains of tetanus resulted in the induction of L-LTP in paired control slices ($169 \pm 5.8\%$ of control in field potential amplitude 2.5–3 h post-tetanus). In contrast, pretreatment of the L-type voltage-gated calcium channel antagonist nifedipine partially prevented the induction of L-LTP (Fig. 1b; $122 \pm 4.6\%$ of control in field potential amplitude at 2.5–3 h post-tetanus, $p < 0.05$ paired t-test, $n = 6$). The potentiation in the presence of nifedipine was maintained for ≥ 3 h ($p < 0.05$, paired t-test), suggesting that NMDA receptors alone can support tetanus-induced L-LTP. Neither nifedipine (30 μM) nor D-AP5 (50 μM) had any effects on baseline synaptic responses in this pathway (Fig. 1).

An antagonist for NMDA receptors has been shown to be effective in reducing conditioned fear when applied after conditioning [18]. This result raises the possibility that some aspect of potentiation during L-LTP is mediated by NMDA receptors. Therefore, we examined

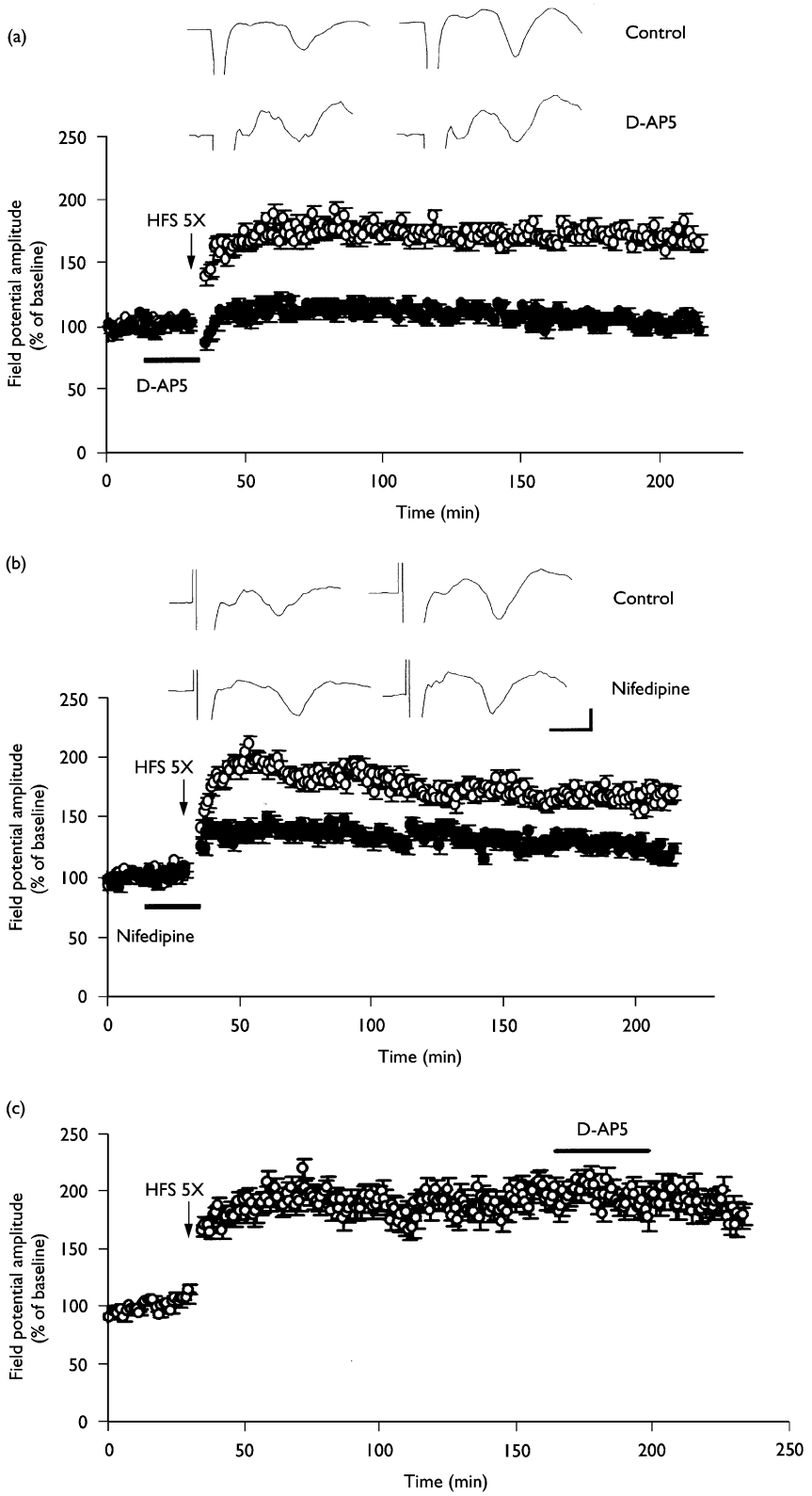

Fig. 1. Involvement of NMDA receptors and L-type voltage-gated calcium channels in the L-LTP induction. (**a**) L-LTP at thalamic input synapses onto the lateral amygdala was completely blocked by the NMDA receptor antagonist D-AP5 (50 μM, $n = 6$; closed circles). (**b**) L-LTP at thalamic input synapses onto the lateral amygdala was partially inhibited by the L-type voltage-gated calcium channel inhibitor nifedipine (30 μM, $n = 6$; closed circles). Please note that L-LTP was maintained even in the presence of nifedipine. (**c**) The potentiated synaptic responses during L-LTP were not altered by D-AP5 (50 μM, $n = 6$). The averaged data traces taken before (left) and 3 h after (right) tetanus were shown at the top of the figure. Calibration = 3 ms, 0.2 mV.

the effect of an antagonist for NMDA receptors on the maintenance phase of L-LTP. We applied D-AP5 (50 μM) to the slices when L-LTP had been stably established (2–2.5 h post-tetanus). However, we failed to observe any significant effects of D-AP5 on the potentiated synaptic responses during L-LTP (Fig. 1c; 104.6 ± 3.4% of control in the amplitude of field potentials, $p > 0.9$, paired t-test, $n = 4$), suggesting that most of the potentiated synaptic responses are mediated by non-NMDA receptors, most likely by AMPA/kainate receptors.

Since D-AP5 (50 μM) completely blocked L-LTP induction, activation of L-type calcium channels alone during repeated tetanus does not appear to support tetanus-induced L-LTP. This would be either because calcium influx through L-type calcium channels during tetanus is too weak to induce L-LTP or because calcium influx especially through NMDA receptors during tetanus is required for the L-LTP induction. In order to determine whether enhanced activity of L-type calcium channels helps to achieve L-LTP upon blockade of NMDA receptors, we examined L-LTP induction in the presence of BAY K 8644 (1 μM), a potentiator for L-type calcium channels, as well as D-AP5 (50 μM), BAY K 8644 did not have significant effects on baseline synaptic responses (Fig. 2a; $n = 3$), whereas L-LTP could be induced. In the presence of 1 μM BAY K 8644 and 50 μM D-AP5 (Fig. 2b; 155.0 ± 4.7% of control in the amplitude of field potentials at 2.5–3 h post-tetanus, $p < 0.05$, paired t-test, $n = 4$). The magnitude of the L-LTP with BAY K 8644 and D-AP5 was similar to that in paired control slices ($p > 0.3$, paired t-test at 2.5–3 h post-tetanus, $n = 4$; L-LTP in paired control = 171.0 ± 6.0% of control in the amplitude of field potential at 2.5–3 h post-tetanus, $p < 0.01$, paired t-test, $n = 4$). One special feature of L-LTP induced in the presence of D-AP5 and BAY K 8644 was that the potentiation after repeated tetanus developed slowly over ~1 h, implying that the early component of L-LTP depends upon activation of NMDA receptors. Thus, our data suggest that L-LTP can be achieved by enhanced calcium influx through L-type calcium channels without intervention of NMDA receptor activity.

Next we examined group I mGluRs (mGluR 1 and 5) on L-LTP induction. We first tested the mGluR5 antagonist MPEP (10 μM) on induction of L-LTP. L-LTP could be induced in paired control slices (185 ± 6.8% of control in the amplitude of field potential at 2.5–3 h post-tetanus, $p < 0.05$, $n = 5$), but not in the presence of MPEP (Fig. 3a; 10 μM, 113 ± 5.4% of control in the amplitude of field potentials at 2.5–3 h post-tetanus, $p > 0.3$, paired t-test, $n = 5$). Similar to the experiment using D-AP5, exposure to MPEP completely prevented L-LTP induction. We next examined the effect of the mGluR1 antagonist CPCCOEt on L-LTP induction. Although applied at a saturating concentration (80 μM), CPCCOEt failed to block L-LTP induction (Fig. 3b; L-LTP in control = 166 ± 5.0%, $n = 5$; L-LTP in CPCCOEt = 173 ± 7.4%, $n = 5$). We also examined the effect of MPEP on L-LTP maintenance. We applied MPEP (10 μM) to the slices when L-LTP had been stably established (2.5–3 h post-tetanus). MPEP failed to show any significant effects on the potentiated synaptic responses during L-LTP (Fig. 3c; $p > 0.2$, paired t-test, $n = 3$), supporting the suggestion that mGluR5 is involved in the induction phase of L-LTP. Neither MPEP (10 μM) nor CPCCOEt (80 μM) had any significant effects on baseline synaptic responses in this pathway (Fig. 3).

DISCUSSION

In the present study, we have found that L-LTP induction at thalamic input synapses onto the lateral amygdala is dependent on activation of NMDA receptors, L-type voltage-gated calcium channels and mGluR5. mGluR1 does not appear to be involved in the induction of L-LTP. Compared to the previous studies of LTP in this pathway [13,14], our findings reveal unique characteristics of L-LTP; (1) tetanus-induced L-LTP at thalamo-amygdala synapses depends upon activation of NMDA receptors, (2) L-type calcium channel-dependent LTP has an enduring phase (> 3 h). (3) L-LTP induction requires mGluR5 activation.

LTP at thalamic input synapses to the lateral amygdala has been proposed as a cellular substrate for conditioned fear [4,5]. One approach to test the hypothesis would be to compare pharmacological and physiological characteristics of LTP and conditioned fear. NMDA receptors, L-type voltage-gated calcium channels and mGluR5, which have been shown to be involved in the induction phase of fear conditioning [18–22], appear to mediate L-LTP induction. Thus, L-LTP and conditioned fear share some of induction mechanisms with each other, supporting the proposal that L-LTP is a cellular substrate for conditioned fear.

Perhaps the most critical finding in the present study is that mGluR5, but not mGluR1, is involved in the induction of L-LTP. In the previous study [23], MPEP showed a selective effect on mGluR5, but not on other glutamate receptors including NMDA receptors at the concentration used herein. No effects of the mGluR1 antagonist CPCCOEt on L-LTP induction further suggest that MPEP selectively antagonized mGluR5, but not mGluR1, in our experiments. It is worthwhile to note that we observed the blocking effect of 80 μM CPCCOEt on the induction of striatal LTD [24], suggesting that 80 μM CPCCOEt is sufficient to block mGluR1 at least in case of striatal slices. Activation of mGluR5 during L-LTP induction can stimulate PI hydrolysis that leads to activation of PKC and increases in intracellular calcium levels, which could contribute to the induction of amygdala L-LTP [25].

Another interesting finding in this study is that L-LTP induction at thalamic input synapses to the lateral amygdala depends upon both the NMDA receptors and L-type voltage-gated calcium channels. The involvement of L-type voltage-gated calcium channels in L-LTP induction would be expected because of its involvement in the pairing-induced E-LTP induction shown in the previous study [13]. However, it is surprising to see the effect of an antagonist for NMDA receptors on the L-LTP induction since it did not show any effects on the induction of E-LTP induced by pairing [13]. One possibility is that the repeated tetanic stimulation used herein produces a more localized depolarization around the synapse, which would be sufficient for maximal activation of NMDA receptors. However, such a local depolarization would allow at most a partial activation of extrasynaptic L-type voltage-gated calcium channels. Thus, one can expect that NMDA receptors play a more important role in initiating calcium entry during the tetanus

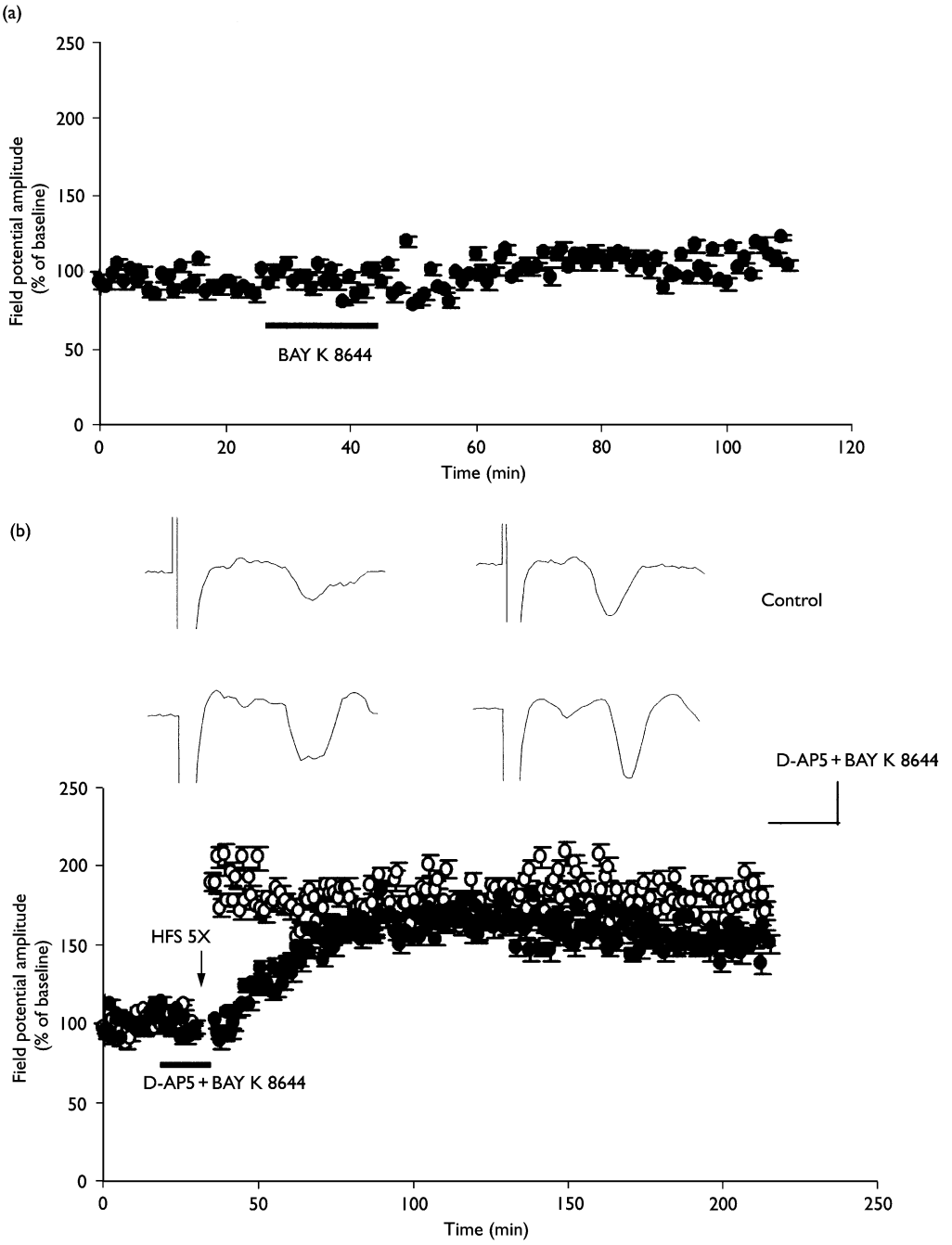

Fig. 2. Restoration of L-LTP by exposure to BAY K 8644 in the presence of D-AP5. (**a**) BAY K 8644 (1 μM) alone did not alter baseline synaptic transmission ($n = 3$). (**b**) L-LTP was induced by repeated tetanus before and during exposure to 1 μM BAY K 8644 and 50 μM D-AP5 ($n = 4$; closed circles). Please note that the potentiation after repeated tetanus develops slowly. The averaged data traces taken before (left) and 3 h after (right) tetanus are shown at the top of the figure. Calibration = 4 ms, 0.2 mV.

than L-type voltage-gated calcium channels do. By contrast, it is possible that the massive postsynaptic depolarization used for the pairing-induced E-LTP in the previous study is so effective at raising calcium levels via L-type calcium channels that it obviates a need for calcium entry through NMDA receptors [13].

Restoration of L-LTP induction by BAY K 8644 upon blockade of NMDA receptors supports the idea that L-type calcium channels play a major role in the induction of NMDA receptor-independent LTP [13]. Furthermore, our data clearly indicate that L-type calcium channel-dependent LTP has an enduring phase in this pathway. Although an

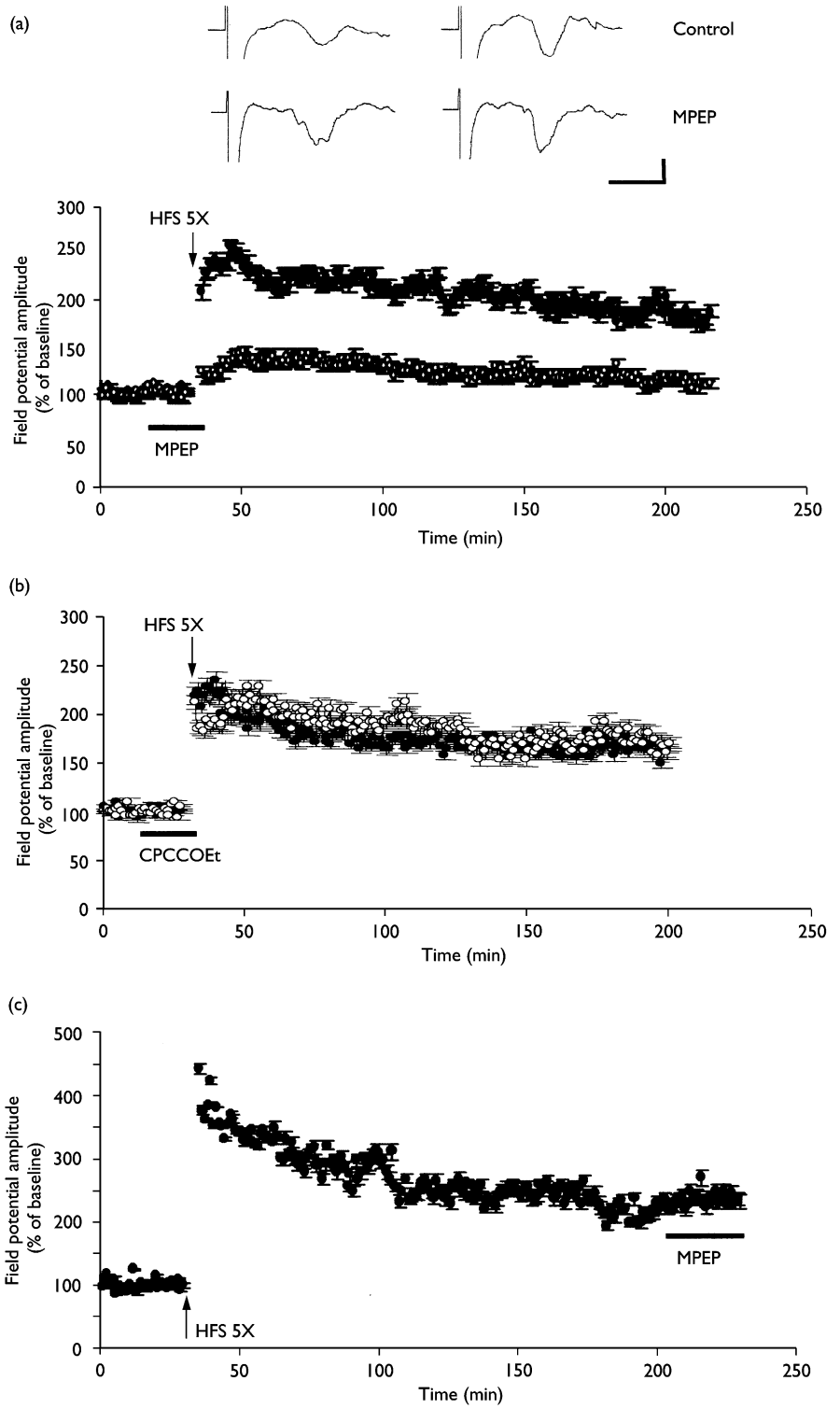

Fig. 3. Involvement of mGluR5, but not mGluRI, in L-LTP induction. (**a**) L-LTP at thalamic input synapses onto the lateral amygdala was completely inhibited by the mGluR5 inhibitor MPEP (10 μM, $n = 5$; open circles). (**b**) The mGluRI antagonist CPCCOEt (80 μM, $n = 5$; open circles) failed to block L-LTP induction at thalamic input synapses onto the lateral amygdala. (**c**) The potentiated synaptic responses during L-LTP were not altered by MPEP (10 μM, $n = 3$). The averaged data traces taken before (left) and 3 h after (right) tetanus are shown at the top of the figure. Calibration = 5 ms, 0.2 mV.

exogenous compound, BAY K 8644 was used in the present experiment, L-type calcium channel activity could be enhanced by a variety of endogenous signal molecules such as G-proteins and protein kinases, so that, at some instances, L-type calcium channels might contribute to L-LTP induction more than NMDA receptors.

It might be odd to see a complete block of L-LTP by D-AP5, compared to a partial block of L-LTP by nifedipine. If both the L-type voltage-dependent channels and NMDA receptors play a role in L-LTP induction by increasing intracellular calcium levels, then specific blockade of either molecule would produce a partial inhibition of L-LTP. One possibility for the complete block by D-AP5 but only partial block by nifedipine is a synergistic rise in calcium involving either sources. Thus, NMDA receptors may be able to raise calcium enough to potentiate some synapses, whereas L-type calcium channels alone may not. However, additional calcium coming through L-type calcium channels might synergize with that coming through NMDA receptors to give the full amount needed for maximal LTP.

At present, we do not understand a precise role of NMDA receptor-dependent, L-type calcium channel-dependent and mGluR5-dependent L-LTP in fear memory, and it remains to be determined in future studies.

CONCLUSION

We have shown that antagonists for NMDA receptors, L-type calcium channels and mGluR5 blocked the induction of amygdala L-LTP. We conclude that activation of these receptors and channels is necessary for the induction of L-LTP at thalamic input synapses to the lateral amygdala.

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Acknowledgements: We thank Dr D.M. Lovinger for his comments on this manuscript. This research was supported by grants from the KMOST grant M1-0108-00-0051 under the neurobiology research program to S.W.C.