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Adenosine A_{2A} Receptors Are Essential for Long-Term Potentiation of NMDA-EPSCs at Hippocampal Mossy Fiber Synapses

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SUMMARY

The physiological conditions under which adenosine A_{2A} receptors modulate synaptic transmission are presently unclear. We show that A_{2A} receptors are localized postsynaptically at synapses between mossy fibers and CA3 pyramidal cells and are essential for a form of long-term potentiation (LTP) of NMDA-EPSCs induced by short bursts of mossy fiber stimulation. This LTP spares AMPA-EPSCs and is likely induced and expressed postsynaptically. It depends on a postsynaptic Ca^{2+} rise, on G protein activation, and on Src kinase. In addition to A_{2A} receptors, LTP of NMDA-EPSCs requires the activation of NMDA and mGluR5 receptors as potential sources of Ca^{2+} increase. LTP of NMDA-EPSCs displays a lower threshold for induction as compared with the conventional presynaptic mossy fiber LTP; however, the two forms of LTP can combine with stronger induction protocols. Thus, postsynaptic A_{2A} receptors may potentially affect information processing in CA3 neuronal networks and memory performance.

INTRODUCTION

Dynamic changes in synaptic efficacy provide a cellular basis for information storage in the nervous system. In its most classical forms, long-term changes in the strength of synaptic transmission are expressed as a modification of the amplitude of AMPA receptor-mediated synaptic currents (AMPA-EPSCs) that require the activation of NMDA receptors (Malenka and Bear, 2004). In turn, little is known about forms of synaptic plasticity that specifically affect NMDA receptors. LTP of NMDA receptor-mediated synaptic transmission has been reported (Bashir et al., 1991; O'Connor et al., 1994; O'Connor et al., 1995; Harney et al., 2006), although this has been a matter of debate (Perkel et al., 1993; Liao et al., 1995; Heynen et al., 2000). It is well established that NMDA receptors and NMDA-EPSCs can be modulated by a variety of cell-signaling molecules converging through G protein coupled receptors to Src family kinases (Salter and Kalia, 2004; Kotecha and MacDonald, 2003). However, the physiological conditions under which these pathways are activated to regulate NMDA EPSCs are presently unclear.

Adenosine is such a modulatory substance since the pharmacological activation of adenosine A_{2A} receptors regulates NMDA receptor function (Tebano et al., 2005; Wirkner et al., 2004). In the central nervous system, the role of adenosine depends mainly on its action on the G protein coupled A_1 and A_{2A} receptors (Fredholm et al., 2005; Dunwiddie and Masino, 2001). A_{2A} receptors are abundantly expressed in the striatum but are also present in the limbic system and neocortex (Tebano et al., 2005; Rebola et al., 2005). Their presence in synapses suggests a role for A_{2A} receptors in controlling synaptic transmission, either by presynaptic mechanisms or by regulating NMDA receptor function (Rebola et al., 2005). The consequences of A_{2A} receptor activation on synaptic transmission in physiological conditions of adenosine release have however remained elusive. The ambient level of adenosine is sufficient to activate A_1 but not A_{2A} receptors, possibly due to differences in affinity (Ciruela et al., 2006). High-frequency bursts of stimulation, which trigger transient release of ATP, may promote conditions where ATP-derived adenosine activates A_{2A} receptors (Cunha, 2001). As these conditions can also induce different forms of short- and long-term synaptic plasticity, activation of A_{2A} receptors by endogenous adenosine may play a role in these processes.

We studied the role of A_{2A} receptors at synapses between hippocampal mossy fibers (Mf) and CA3 pyramidal cells. First, Mf synapses display robust and well-characterized presynaptic forms of synaptic plasticity (Henze et al., 2000; Nicoll and Schmitz, 2005) in response to sustained presynaptic activity, conditions which may promote the increase of adenosine in the extracellular space. Second, while it is known that Mf synapses do not display NMDA-receptor dependent LTP of AMPA-EPSCs, selective changes in NMDA-EPSCs have never been addressed at these synapses.

We found no evidence for functional presynaptic A_{2A} receptors at Mf synapses. In contrast, we report that postsynaptic A_{2A} receptors mediate a form of LTP of NMDA-EPSCs induced by short bursts of Mf stimulation. This LTP is expressed postsynaptically, depends on an elevation of intracellular Ca^{2+} and requires the coactivation of NMDA receptors and mGluR5 in addition to A_{2A} receptors. LTP of NMDA EPSCs displays a low induction

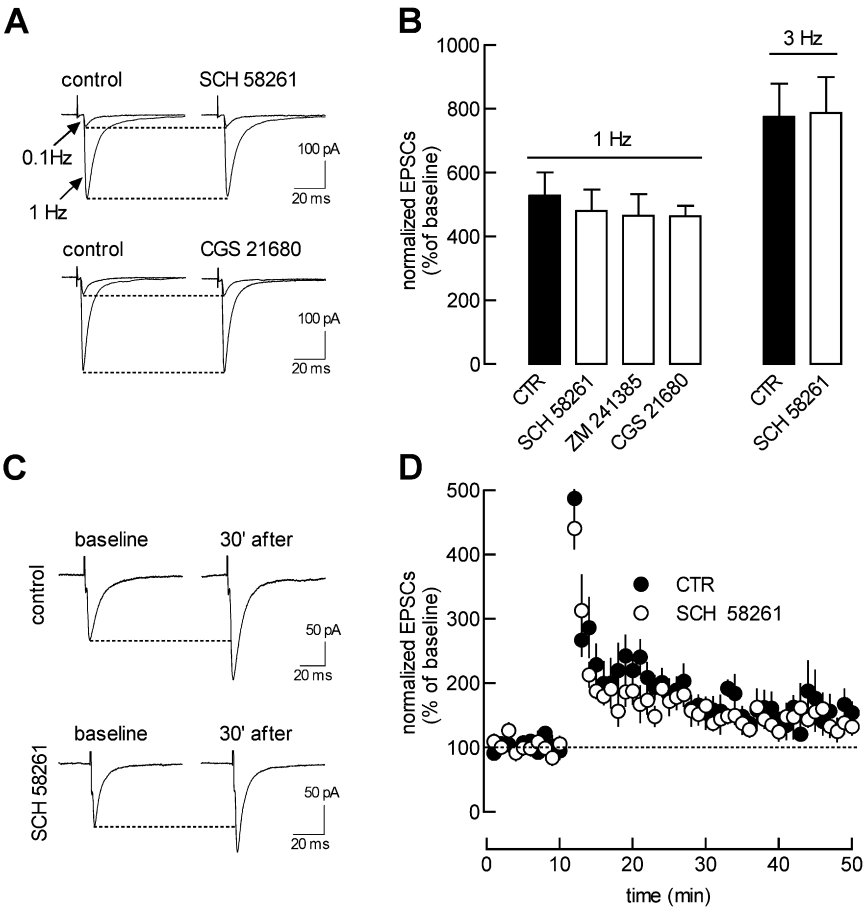

Figure 1. Absence of Functional Presynaptic Adenosine A_{2A} Receptors at Mf-CA3 Pyramidal Cell Synapses

(A) Representative traces of Mf-EPSCs illustrating the lack of effect of the selective A_{2A} receptor antagonist, SCH 58261 (50 nM), and of the selective A_{2A} receptor agonist, CGS 21680 (30 nM) upon stimulation at either 0.1 Hz or 1 Hz.

(B) Mf frequency facilitation, induced by increasing the frequency of stimulation from 0.1 Hz to 1 or 3 Hz, lead to facilitation of Mf-EPSCs. This frequency facilitation was not significantly affected when repeating the same protocol in the presence of 50 nM SCH 58261 ($p > 0.05$) or 50 nM ZM 241385. A_{2A} receptor activation with the selective agonist CGS 21680 (30 nM, n = 6) did not affect Mf frequency facilitation ratio ($p > 0.05$).

(C) Representative traces of Mf-EPSCs before and after the presynaptic LTP induction protocol in control conditions and in the presence of SCH 58261 (50 nM). Mf LTP was induced by 3 bursts of 100 stimuli at a frequency of 100 Hz with a 10 s interburst interval, in the presence of bicuculline (10 μM) and D-AP5 (50 μM).

(D) Summary plot of the effects of the SCH 58261 (50 nM) on Mf LTP. In the presence SCH 58261 (○, n = 8), the amplitude of Mf LTP was not statistical different ($p = 0.8$) from control (●, n = 10). Values are presented as mean ± SEM of n experiments.

threshold as compared to conventional presynaptic Mf LTP. Consequently, protocols used to trigger presynaptic LTP lead to a robust potentiation of NMDA-EPSCs resulting from the combination of pre- and postsynaptic actions.

RESULTS

Adenosine A_{2A} Receptors Are Not Involved in Presynaptic Forms of Synaptic Plasticity

Mf-EPSCs were recorded from CA3 pyramidal cells in the whole-cell voltage-clamp mode. The selective A_{2A} receptor antagonists SCH 58261 (50 nM) or ZM 241385 (50 nM) did not affect AMPA receptor-mediated EPSCs (AMPA-EPSCs) recorded at a rate of stimulation of 0.1 Hz (Figure 1A). Since sustained synaptic activity can increase extracellular adenosine levels, we increased the frequency of stimulation from 0.1 Hz to either 1 Hz or to 3 Hz (528% ± 73%, n = 11, and 775% ± 105%, n = 12, respectively). This lead to a prominent facilitation of Mf-EPSCs that was not significantly affected when repeating the same protocol in the presence of 50 nM SCH 58261 (at 1 Hz, 481% ± 66% of control, n = 11, p = 0.6; at 3 Hz, 788% ± 112% of control, n = 12, p = 0.8) or 50 nM ZM 241385 (at 1 Hz, 464% ± 68%, n = 6; Figures 1A and 1B). SCH 58261 (50 nM) also failed to affect synaptic facilitation triggered by a train of 5 stimuli at 25 Hz (fifth versus first EPSC, control: 813 ± 123, n = 5; SCH 58261: 869 ± 245, n = 5). We further attempted to activate presynaptic A_{2A} receptors at Mf synapses with the A_{2A} receptor agonist, CGS 21680 (30 nM). CGS 21680 failed to alter basal synaptic transmission (0.1 Hz; Figure 1A) and frequency facilitation (at 1 Hz, 465% ± 79%, n = 6; Figures 1A and 1B).

Finally, we evaluated the potential role of A_{2A} receptors in Mf LTP which is presynaptic NMDA-receptor independent form of synaptic plasticity that depends on Ca^{2+} entry in the Mf nerve terminal (Henze et al., 2000; Nicoll and Schmitz, 2005, for review). We induced presynaptic Mf LTP with a high frequency stimulation protocol (100 pulses at a frequency of 100 Hz repeated 3 times with a 10 s intertrain interval), in the presence of bicuculline (10 μM) and D-AP5 (50 μM). In these conditions, the extent of Mf LTP was not significantly ($p = 0.8$) different in control conditions and in the presence of 50 nM SCH 58261 (Figures 1C and 1D). Altogether these data indicate that presynaptic A_{2A} receptors do not modulate glutamatergic synaptic transmission and do not participate in presynaptic forms of synaptic plasticity at Mf synapses.

In contrast, we confirmed that Mf synaptic transmission was highly sensitive to A_1 receptor activation. Application of the mixed A_1/A_2 receptor agonist, CADO (10 μM), reduced EPSCs to 31% ± 5% of control (n = 7; see Figure S1 available online) an effect strongly attenuated by the A_1 receptor antagonist DPCPX (100 nM; 80% ± 9%, n = 10; Figure S1). DPCPX (100 nM) increased Mf-EPSCs to 156% ± 23% (n = 8; Figure S1), indicating that endogenous levels of adenosine tonically inhibit Mf-EPSCs through

A_1 receptor activation. Both A_1 receptor ligands (agonist and antagonist) modified Mf-EPSCs but did not change the frequency facilitation ratio (1 Hz/0.1 Hz; Figure S1), in agreement with previous work (Kukley et al., 2005, but see Moore et al., 2003). Finally, we found that the inhibitory effect of CADO (10 μM) on EPSCs was not affected by the presence of 50 nM SCH 58261 (27% ± 4%, n = 5; p = 0.9 in comparison with the effect of CADO alone; Figure S1), ruling out a possible interaction between the two receptor systems, as described in CA1 synapses (Lopes et al., 2002). Our results indicate that A_{2A} receptors are not functionally present presynaptically at Mf synapses, in contrast with A_1 receptors.

Electron Microscopy Localization of A_1 and A_{2A} Receptors in Hippocampal Mf Synapses

Even though A_{2A} receptors are enriched in biochemically purified presynaptic fractions of the hippocampus, around 30% of the A_{2A} receptor signal is also observed in the postsynaptic fraction (Rebola et al., 2005). We thus directly evaluated by electron microscopy the subcellular distribution of A_1 and A_{2A} receptors in the *stratum lucidum* of the CA3 region of the hippocampus, a region consisting primarily of pyramidal cell dendrites and dendritic spines establishing type I synapses with Mf terminals. Using a pre-embedding immunogold method, gold particles for A_1 receptors were found mainly distributed presynaptically along the extrasynaptic plasma membrane (arrowheads) of Mf terminals (Figures 2A and 2B) as well as along active zones (Figure 2A). Occasionally, a few gold particles labeling A_1 receptors were also detected at postsynaptic sites along the extrasynaptic plasma membrane of dendritic spines of CA3 pyramidal cells (Figure 2A), although this only represented 4.3% (n = 3) of all gold particles examined for A_1 receptors (Figure 2G). In contrast to the predominant presynaptic localization of A_1 receptors, gold particles for A_{2A} receptors were mainly distributed at postsynaptic sites along the extrasynaptic plasma membrane (arrowheads) of dendritic spines of CA3 pyramidal cells (Figures 2C and 2D). Furthermore, some gold particles were also observed at perisynaptic positions or embedded in the postsynaptic densities of dendritic spines of CA3 pyramidal cells (Figure 2D). Occasionally, a few gold particles for A_{2A} receptors were also detected at presynaptic sites along the extrasynaptic plasma membrane of Mf terminals (Figure 2D). Immunoparticles in postsynaptic sites represented 96.4% (n = 3) of all gold particles examined for A_{2A} receptors. The distance between the closest edge of the postsynaptic density and the center of the immunoparticles along the spine membrane was measured, showing that gold particles labeling A_{2A} receptors are localized within a 1 μm diameter from the postsynaptic density (Figure 2H). Using a postembedding immunogold method, the only immunohistochemical technique that allows the unambiguous detection of any protein at postsynaptic densities of type I synapses, we confirmed this different subsynaptic distributions of A_1 and A_{2A} receptors, with A_1 receptors mainly localized presynaptically (Figure 2E) and A_{2A} receptors mainly localized postsynaptically (Figure 2F).

A_{2A} Receptor Activation Potentiates Synaptic NMDA-EPSCs at Mf Synapses

The postsynaptic distribution of A_{2A} receptors at Mf synapses prompted us to examine their potential role in the regulation of synaptic transmission by acting at a postsynaptic level. Our previous experiments (AMPA-EPSCs recorded at 0.1 Hz) showed no effect of the A_{2A} receptor agonist, CGS 21680 on AMPA-EPSCs. However, previous studies have suggested that A_{2A} receptor agonists either inhibit (Wirkner et al., 2004) or positively modulate (Tebano et al., 2005) NMDA receptor function. We thus examined if postsynaptic A_{2A} receptors could selectively control NMDA-EPSCs at Mf synapses. NMDA-EPSCs were recorded in CA3 pyramidal cells voltage clamped at +30 mV in the presence of 20 μM NBQX (to block AMPA and kainate receptors), 10 μM bicuculline and 3 μM CGP55845 (to block $GABA_A$ and $GABA_B$, respectively). Superfusion of the A_{2A} receptor agonist, CGS 21680 (30 nM), increased NMDA-EPSCs by 41% ± 13% (n = 6, p = 0.03; Figures 3A, 3B, and 3D). This effect was blocked by SCH 58261 (50 nM), which by itself did not change the amplitude of NMDA-EPSCs (Figures 3B, 3C, and 3D). CGS 21680 (30 nM) also potentiated NMDA-EPSCs recorded at −50 mV in the presence of 0.3 mM extracellular Mg^{2+} concentration to the same extent (135% ± 9%, n = 4). Thus, activation of A_{2A} receptors selectively potentiates NMDA-EPSCs at Mf synapses. As this effect is not observed for AMPA-EPSCs (see Figure 1), it is unlikely to be mediated by a presynaptic action on glutamate release. Together with the morphological data, this indicates a modulation of NMDA-EPSCs by postsynaptic A_{2A} receptors.

Sustained Mf Stimulation Leads to LTP of NMDA-EPSCs that Depends on A_{2A} Receptor Activation

We then examined the physiological conditions under which extracellular adenosine might regulate NMDA-EPSCs through activation of A_{2A} receptors. The fact that the selective A_{2A} receptor antagonist SCH 58261 failed to affect NMDA-EPSCs under basal stimulation conditions (0.1 Hz) indicates that there is no tonic activation of A_{2A} receptors by ambient adenosine, unlike for presynaptic A_1 receptors. Neurochemical studies in hippocampal slices have indicated that sustained high frequency electrical simulation (60 pulses at 50 Hz repeated 4 times) was required to record an activation of A_{2A} receptors by endogenous adenosine (Pinto-Duarte et al., 2005).

Thus, we tested if high-frequency stimulation of mossy fibers would promote A_{2A} receptor-mediated facilitation of NMDA-EPSCs. A burst of 60 stimuli delivered at a rate of 50 Hz induced a pronounced potentiation of NMDA-EPSCs recorded at + 30 mV (167% ± 7%, 30–40 min after the burst, n = 6) that lasted for the 40 min of recording (Figures 4A and 4D). We examined if this LTP of NMDA-EPSCs depended on A_{2A} receptor activation. In the presence of SCH 58261 (50 nM) a robust posttetanic potentiation was observed but it was not followed by LTP of NMDA-EPSCs (103% ± 7%, 30–40 min after the bursts, n = 5; Figures 4B and 4D). To exclude the possibility that holding the cell at positive potentials contributes to the observed LTP, we recorded NMDA-EPSCs in low Mg^{2+} medium (0.3 mM Mg^{2+}) while holding the cell at −50 mV. In these conditions LTP of NMDA-EPSCs was also observed (172% ± 27%, 30–40 min after the bursts, n = 11; Figures 4C and 4D). In the rest of the study we have recorded NMDA-EPSCs at −50 mV in low Mg^{2+} concentration, unless otherwise stated. The fact that the A_{2A} receptor antagonist blocked LTP of NMDA-EPSCs but did not affect

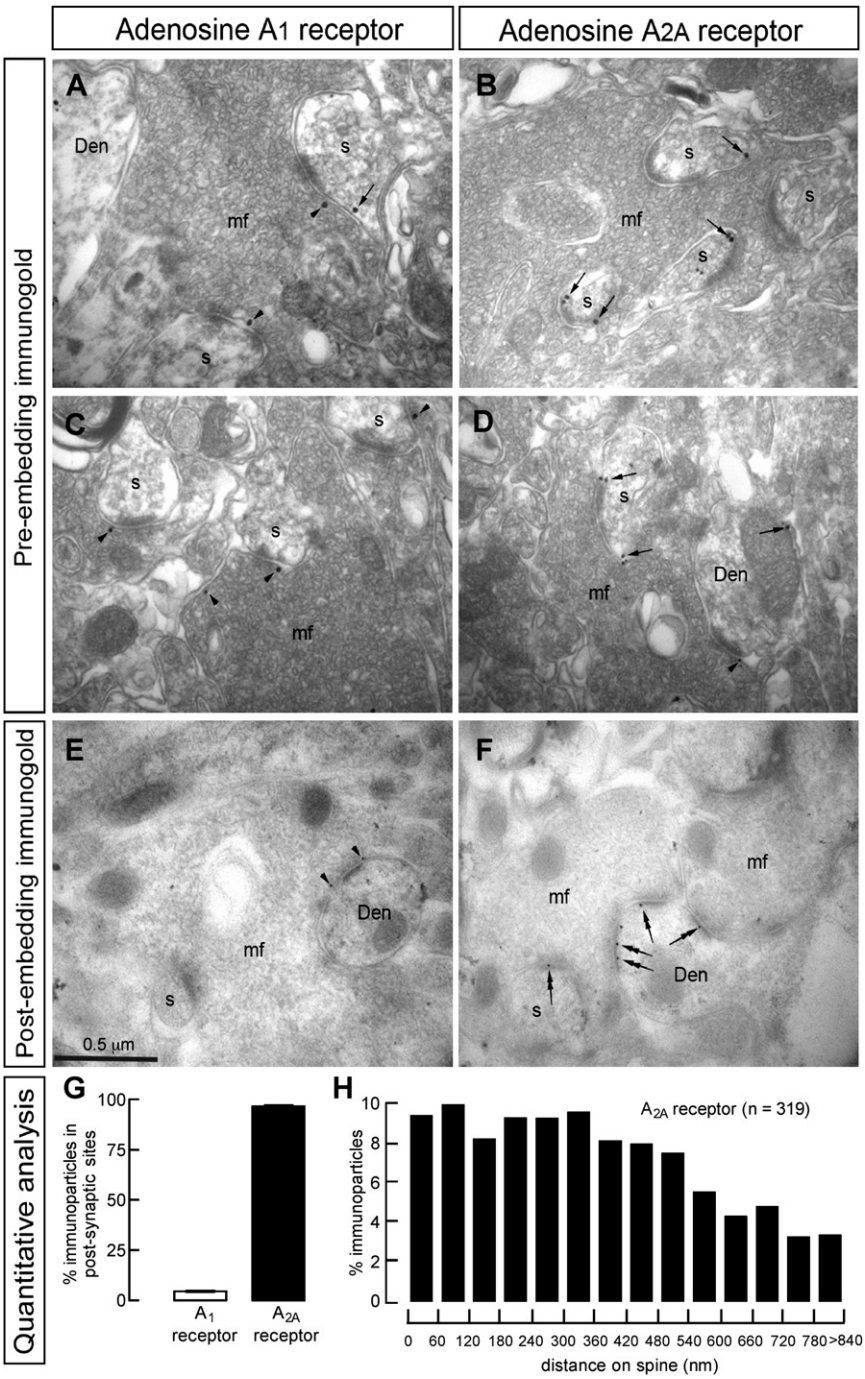

Figure 2. Asymmetric Distribution of Adenosine A_1 and A_{2A} Receptors at Mf Synapses

Electron micrographs of mouse CA3 stratum lucidum region showing immunoreactivity for A_1 and A_{2A} receptors revealed by pre-embedding (A–D) and postembedding (E and F) immunogold methods.

(A and C) Immunoparticles for A_1 receptors were mainly distributed along the extrasynaptic plasma membrane (arrowheads) and active zone of Mf terminals (mf). Very few immunoparticles were also detected at postsynaptic sites along the extrasynaptic plasma membrane (arrow) of CA3 dendritic spines (s).

(B and D) Immunoparticles for A_{2A} receptors were mainly distributed at postsynaptic sites along the extrasynaptic plasma membrane (arrows) of CA3 dendritic spines (s). A few immunoparticles were also detected at presynaptic sites along the extrasynaptic plasma membrane (arrowhead) of Mf terminals (mf).

(E and F) Using the postembedding method, the only technique that allows to detect proteins at synaptic sites, immunoparticles for A_{2A} receptors were mainly located on postsynaptic densities (double arrows) of CA3 dendritic spines (s) establishing type I synapses with Mf terminals, whereas immunoparticles for A_1 receptors were never observed at that location. Instead, immunoparticles for A_1 receptors were observed at presynaptic sites (arrowheads).

(G) Quantitative analysis of the relative number of immunoparticles found in the postsynaptic membrane for A_1 and A_{2A} receptors in Mf synapses. From the total number of immunoparticles detected (n = 1571 for A_1 receptors; n = 1455 for A_{2A} receptors, n = 3 mice), 66 (4.3%) and 1403 (96.4%) were present in postsynaptic sites for A_1 and A_{2A} receptors, respectively. The particles present in glial cells represented less than 0.5% of total labeling.

(H) Quantitative analysis showing the distribution of A_{2A} on dendritic spines of CA3 neurons revealed by pre-embedding immunogold methods. Data are displayed as percentage frequency of particles in 60-nm-wide bins, starting at the edge of the postsynaptic specialization. Scale bars, 0.5 µm. Values are presented as mean ± SEM of n experiments.

conventional presynaptic Mf LTP (see Figure 1) suggests that these two forms of synaptic plasticities operate through different mechanisms. We further analyzed the A_{2A} receptor-dependent LTP of NMDA-EPSCs.

As a first step, we investigated the conditions for triggering LTP of NMDA-EPSCs. The 60 stimuli protocol at 50 Hz was initially chosen based on earlier neurochemical results describing A_{2A} receptor-mediated effects in the hippocampus (Pinto-Duarte et al., 2005). Because dentate granule cells tend to fire in bursts, we used short bursts of 6 stimuli at 50 Hz separated by 150 ms to mimic the frequency of theta oscillation (Figure 5A). Three bursts of 6 stimuli induced LTP of NMDA-EPSCs (133% ± 11%, 20–30 min after the bursts, n = 9; Figures 5A and 5B). The magnitude of LTP was further enhanced with 6 and 10 consecutive bursts at 50 Hz within the same experiment (Figures 5A and 5B). We also quantified the effects of 6 and 10 bursts of stimuli in separate slices (which had not received previous bursts of stimuli) and found that LTP was close to saturation in response to the

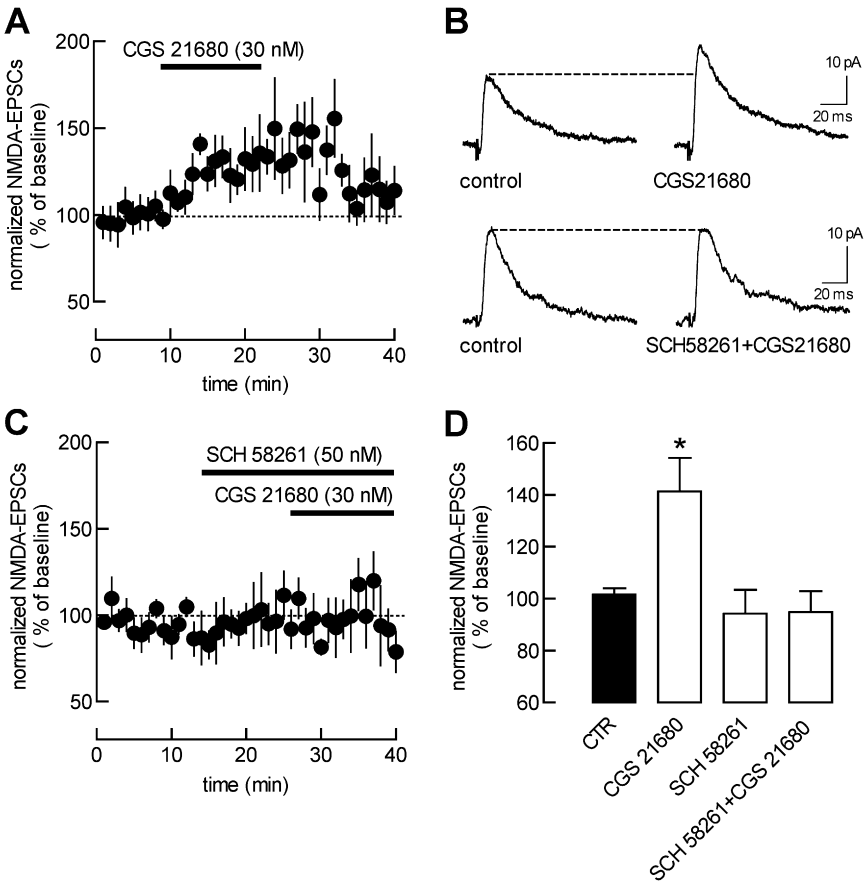

Figure 3. A_{2A} Receptor Activation Potentiates Synaptic NMDA-EPSCs at Mf Synapses

(A) Average time course of the effect of the A_{2A} receptor agonist CGS 21680 (30 nM), on Mf NMDA-EPSCs (n = 6) CGS 21680 was applied through the superfusion solution as indicated by the upper bar.

(B) Representative recordings of Mf NMDA-EPSCs illustrating the potentiation induced by CGS 21680 (30 nM). No potentiation was observed when the slice was preincubated with the A_{2A} receptor antagonist SCH 58261 (50 nM).

(C) Lack of effect of SCH 58261 (50 nM, n = 8) on Mf NMDA-EPSCs by itself and inhibition of CGS 21680-induced potentiation (n = 8).

(D) Summary of effects of the A_{2A} receptor ligands tested on normalized Mf NMDA-EPSCs (*p = 0.03). Values are presented as mean ± SEM of n experiments.

6 bursts stimulation protocol (6 bursts: 174% ± 15%, n = 6; 10 bursts: 191% ± 30%, n = 6, 20–30 min after the bursts; Figure 5C). In the rest of the study, LTP was induced by the 6 bursts stimulation protocol. The A_{2A} receptor antagonist SCH 58261 (50 nM) similarly blocked LTP of NMDA-EPSCs induced by the 6 bursts stimulation protocol (92% ± 8%, 30–40 min after the bursts, n = 6) and by the sustained stimulation at 50 Hz (see Figure 6D).

The six bursts protocol did not induce LTP of AMPA-EPSCs. Indeed the amplitude of AMPA-EPSCs recorded in the presence of D-AP5 (50 μM) was not significantly affected by this protocol (113% ± 20%, 30–40 min after the bursts, n = 6, p = 0.4; Figures S2A and S2B). It could be argued that the lack of LTP for AMPA-EPSCs is linked to the inhibition of NMDA receptors by D-AP5. We thus monitored in parallel the changes in AMPA and NMDA-EPSCs before and after LTP induction with both receptors active during and after the six bursts stimulation protocol. For this, we recorded in the same cells Mf EPSCs at +30 and at −70 mV in 1.3 mM extracellular Mg^{2+} concentration. The cells were held at −70 mV, including during the 6 bursts stimulation protocol, and the potential was changed to +30 mV for less than 1 min to record NMDA-EPSCs. This protocol avoided the potential postsynaptic long-term depression of AMPA-EPSCs induced by sustained depolarization of CA3 pyramidal cells (Lei et al., 2003). In these conditions, the six bursts stimulation protocol did not induce any potentiation of the EPSCs recorded at −70 mV (Figure 5D) (which are mainly mediated by AMPA and kainate receptors in 1.3 mM extracellular Mg^{2+}; Figure S2D). The EPSCs recorded at +30 mV displayed a considerably slower decay time after LTP induction indicating an increase of the NMDA component (Figure 5E). We further confirmed and quantified the selective potentiation of NMDA-EPSCs by recording the composite AMPA/NMDA EPSC at +30 mV after 40 min of recording in cells where LTP was induced and in control cells. In both sets of cells, the NMDA receptor antagonist D-AP5 was then applied and the remaining AMPA-EPSC was recorded. We then measured the NMDA/AMPA ratio at +30 mV by subtracting the AMPA-EPSC from the composite AMPA/NMDA EPSC. In control cells at +30 mV the amplitude of the NMDA-EPSCs represented on average 44% ± 7% (n = 4) of the AMPA-EPSCs (Figure S2). This ratio was significantly increased (82% ± 9%, n = 5) in cells where LTP was induced (Figure S2). These data provide clear evidence for a form of LTP that is selective for NMDA-EPSCs.

Modifications of the paired-pulse ratio are commonly assumed to reflect presynaptic changes in the probability of release. In agreement, conventional presynaptic Mf LTP leads to an increase in the probability of release and concomitantly to a decrease in paired-pulse ratio (Zalutsky and Nicoll, 1990). In contrast, the six bursts stimulation protocol used to trigger LTP of Mf NMDA-EPSCs did not induce any durable changes in the paired-pulse ratio (3.4 ± 0.5 in baseline versus 3.7 ± 0.6, 30–40 min after the bursts, n = 6, p = 0.4; Figures 5F and 5G). The lack of change in the paired-pulse ratio after LTP induction together with the observation that AMPA-EPSCs are not potentiated strongly suggest that this form of synaptic plasticity is expressed postsynaptically and that the mild induction protocol used is not sufficient to trigger presynaptic Mf LTP.

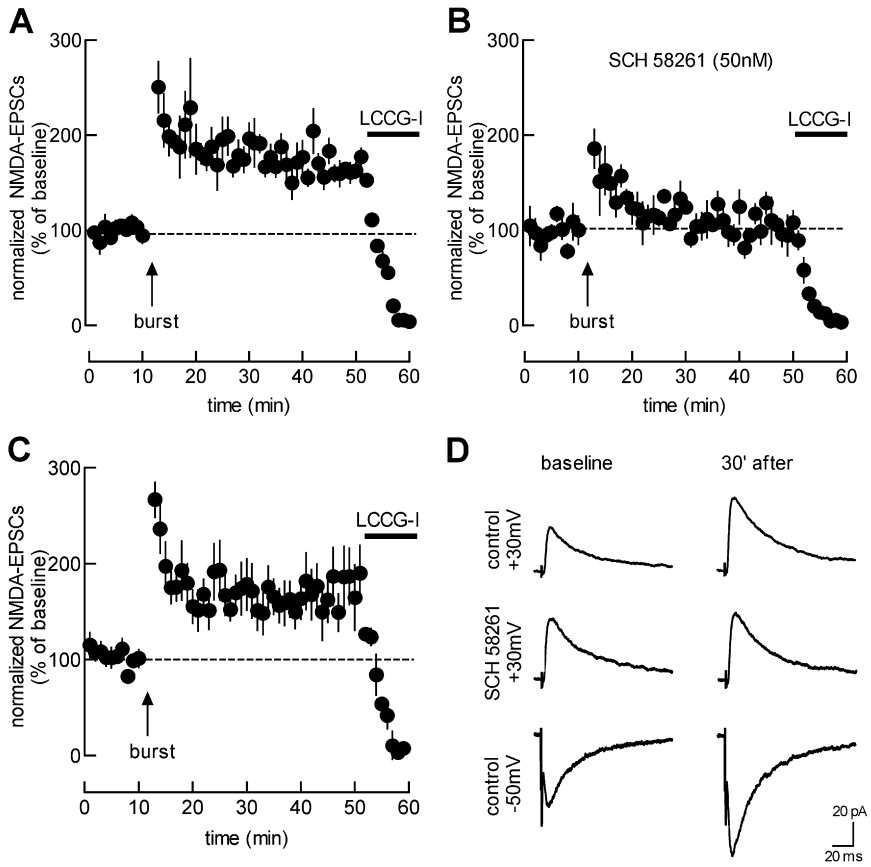

Figure 4. Sustained Mf Stimulation Induces LTP of NMDA-EPSCs Which Is Blocked by A_{2A} Receptor Antagonist

(A) Application of a sustained burst of stimuli to Mfs (60 pulses at 50 Hz) induced LTP of Mf NMDA-EPSCs recorded at +30 mV that lasted for the 40 min of recording (n = 6).

(B) LTP of NMDA-EPSCs was abolished in slices preincubated with the A_{2A} receptor antagonist, SCH 58261 (50 nM, n = 5). The arrow indicates the time of burst stimulation.

(C) Similarly, LTP was observed when recording NMDA-EPSCs at −50 mV with low extracellular Mg^{2+} concentration (0.3 mM Mg^{+2}) (n = 11).

(D) Representative recordings illustrating the effect of burst stimulation on Mf NMDA-EPSCs in the absence or in the presence of SCH 58261. Traces represent average NMDA-EPSCs recorded during 10 min before the burst stimulation ("baseline") or between the 30th and the 40th minute after burst stimulation ("30′ after burst"). As indicated throughout in (A), (B), and (C), the group II mGluR agonist LCCG-I, applied at the end of each experiment inhibited EPSCs (>80%), confirming that they were Mf-EPSCs.

Values are presented as mean ± SEM of n experiments.

A_{2A} Receptor Activation Results from Adenosine Generated by the Catabolism of Adenine Nucleotides

We tested if A_{2A} receptors at Mf synapses are preferentially activated by adenosine originating from the extracellular dephosphorylation of released ATP. For this purpose, we tested two inhibitors of the ectonucleotidase pathway that generates adenosine from ATP: ARL 67156 (100 μM) inhibits extracellular catabolism of ATP, and α,β-methylene ADP (AOPCP) 100 μM, inhibits ecto-5′-nucleotidase, the enzyme converting 5′-AMP into adenosine. Following preincubation of the slices with these ectonucleotidases inhibitors for 60 min, the facilitatory effects of DPCPX on synaptic transmission was decreased indicating that extracellular catabolism of ATP contributes to the ambient level of adenosine, which activates presynaptic A_1 receptors (see also Frenguelli et al., 2007; Figure S1E). In the presence of the inhibitors of the ectonucleotidase pathway, the six bursts stimulation protocol induced a pronounced PTP but failed to induce LTP of NMDA-EPSCs (30–40 min after the bursts: 120% ± 12% in presence of 100 μM ARL67156, n = 8; 101% ± 6% in presence of 100 μM AOPCP, n = 6; p = 0.02 and p = 0.01 as compared to control, respectively; Figure 6). These results support the hypothesis that the adenosine activating postsynaptic A_{2A} receptors originates from the conversion of ATP released by sustained synaptic stimulation.

Mechanisms of LTP of Mf NMDA-EPSCs

We found that LTP of Mf NMDA-EPSCs was abolished when BAPTA (20 mM) was included in the intracellular solution to prevent a postsynaptic rise in Ca^{2+} concentration (100% ± 8.7%, 30–40 min after stimulation, n = 9; Figures 7A and 7F). Given the need for a postsynaptic Ca^{2+} elevation we examined the potential source of Ca^{2+} by evaluating the role of group I mGluRs in the induction of LTP. Since A_{2A} receptors and mGluR5 have been shown to act in synergy to positively modulate NMDA receptor function (Tebano et al., 2005), we tested if LTP was affected by MPEP, an mGluR5 receptor antagonist. In slices preincubated with MPEP (10 μM), the 6 bursts stimulation protocol did not induce LTP of NMDA-EPSCs (93% ± 11%, 30–40 min after the bursts, n = 6, p < 0.01; Figures 7B and 7F). We also examined the role of NMDA receptors in LTP induction by incubating the slices with D-AP5 (50 μM) 5 min prior to delivering the 6 bursts of stimuli. The drug was washed out 2 min after the induction protocol (Figure 7C). In control conditions (no bursts stimulation protocol), washout of D-AP5 was complete within 30 min (Figures 7C and 7D). In cells where the six bursts of stimuli were delivered in the presence of D-AP5, no LTP of NMDA-EPSCs was observed (Figures 7C, 7D, and 7F). These results strongly suggest that the coordinated activation of mGluR5 and NMDA receptors is necessary for the induction of LTP of Mf NMDA-EPSCs. Given the strong dependence of NMDA receptor function on membrane potential, we examined if postsynaptic depolarization was necessary for the induction of LTP. In cells where the membrane potential was voltage-clamped at −90 mV during the 6 bursts stimulation protocol no LTP of NMDA-EPSCs was observed (Figures 7E and 7F). However, the same protocol triggered LTP of NMDA-EPSCs with a membrane potential held at the physiological potential of −70 mV during induction (146% ± 7%, 30–40 min

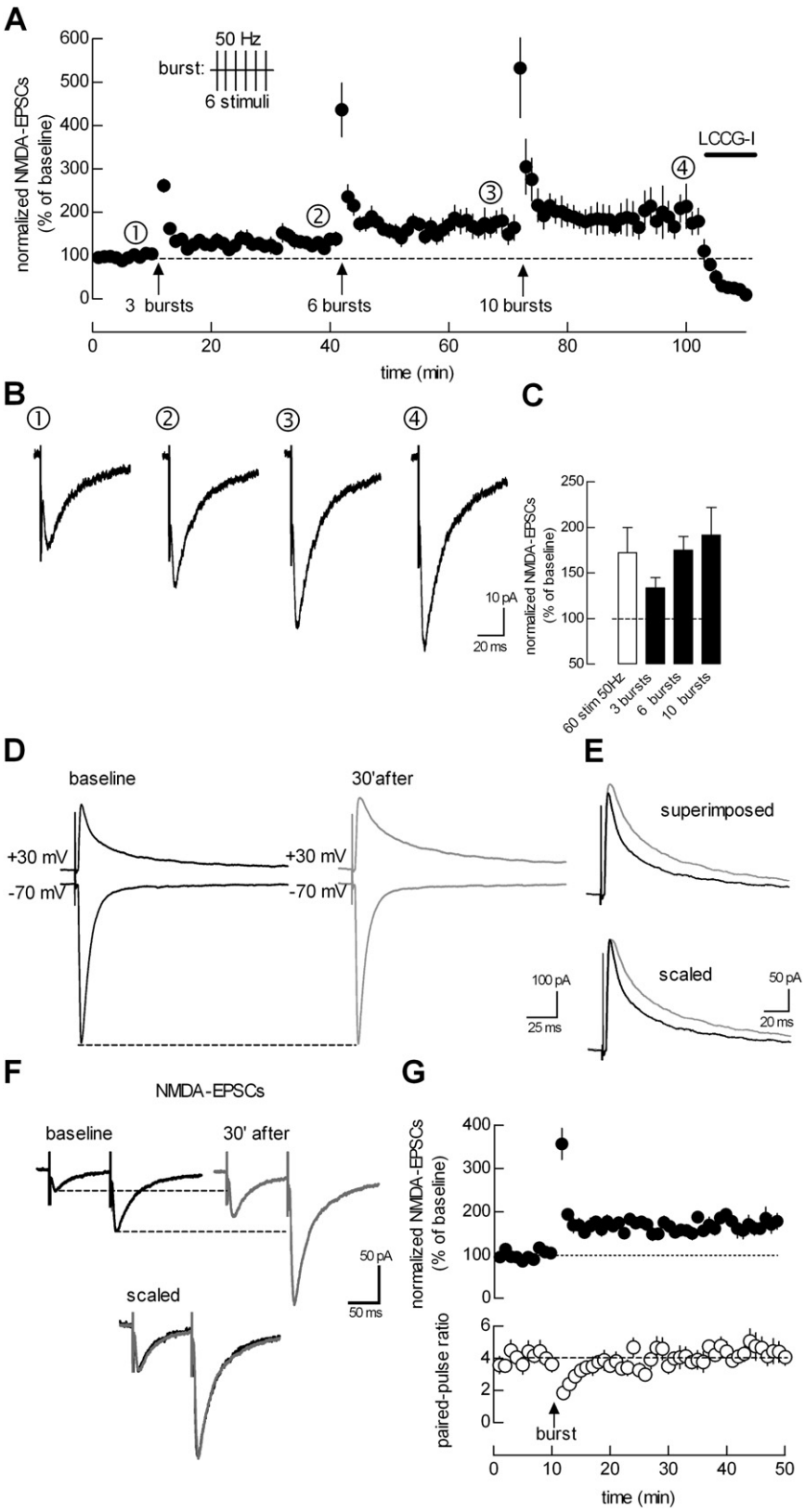

Figure 5. LTP of Mf NMDA-EPSCs Can Be Induced with a Low Number of Short Bursts of Stimuli, Is Selective for NMDA Receptors, and Does Not Induce Changes in Paired-Pulse Ratio

(A) Short bursts of 6 stimuli at 50 Hz with a 150 ms interburst interval (3, 6, and 10 bursts applied consecutively) induce LTP of mossy-fiber NMDA-EPSCs. The magnitude of LTP is enhanced by increasing the number of consecutive bursts within the same experiment.

(B) Representative traces of Mf NMDA-EPSCs obtained during the application of the different protocols of induction. Average traces obtained during a 10 min period of recording at the specific times indicated by the labels that refer to (A).

(C) Mean of normalized NMDA-EPSCs averaged between the 30th and the 40th minute after burst stimulation obtained with the different protocols of stimulation in separate experiments (values from slices that just received one of the burst stimulation protocol).

(D) Representative traces obtained at −70 mV and +30 mV in normal Mg^{2+} concentration (1.3 mM) without any blocker of glutamatergic synaptic transmission, before and after LTP. The LTP protocol did not induce any change in Mf-EPSCs recorded at −70 mV (mediated mainly by AMPA and kainate receptors) but induced changes in the kinetics of Mf-EPSCs recorded at +30 mV (composed of AMPA, kainate, and NMDA receptors).

(E) Superimposing the traces obtained at +30 mV before and after LTP illustrates the slower decay of Mf-EPSCs observed after LTP. This likely corresponds to an increase of the NMDA component.

(F) Representative traces illustrating the lack of changes in the paired-pulse ratio after LTP of Mf NMDA-EPSCs (a six burst stimulation protocol was used).

(G) Average time courses of normalized Mf NMDA-EPSCs and corresponding paired-pulse ratio. The paired-pulse ratio transiently decreases after burst stimulation, reflecting presynaptic short-term plasticity, and returns to baseline a few minutes after, whereas the NMDA-EPSC amplitude remains potentiated until the end of the experiment.

Values are presented as mean ± SEM of n experiments.

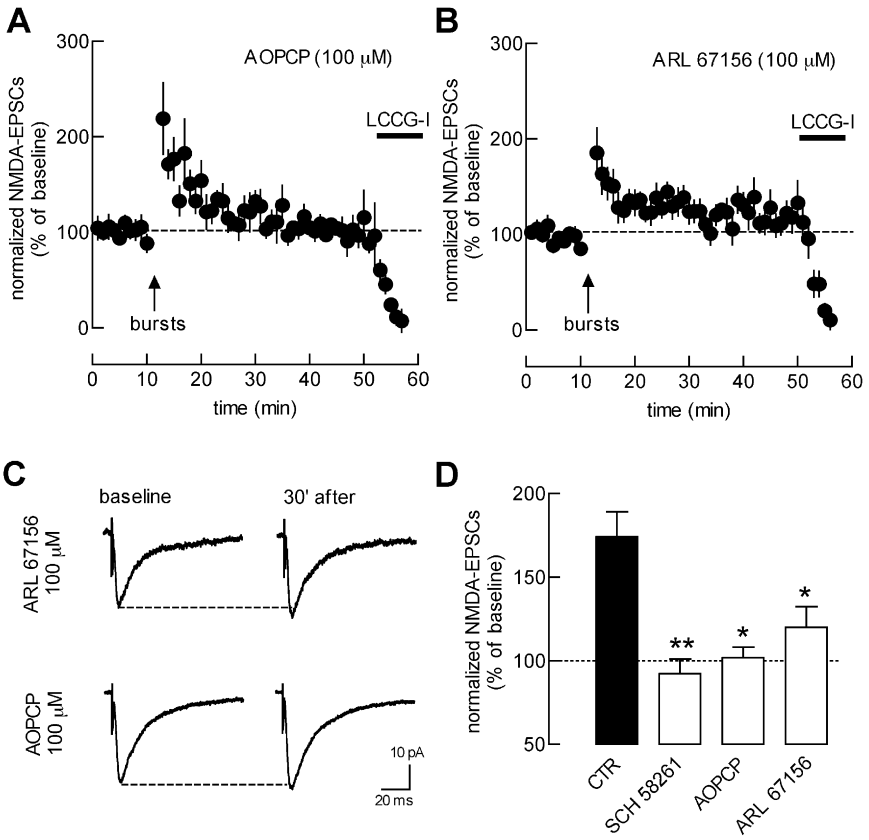

Figure 6. Adenosine Activating Postsynaptic A_{2A} Receptors Likely Originates from the Extracellular Catabolism of Adenine Nucleotides

(A and B) Average time courses of normalized Mf NMDA-EPSCs recorded in the presence of either the ecto-ATPase inhibitor, ARL 67156 (100 μM, n = 8 [A]), or the ecto-5′-nucleotidase inhibitor AOPCP (100 μ, n = 8 [B]). The arrows indicate the time of application of the burst stimulation protocol (6 bursts of 6 stimuli at 50 Hz with a 150 ms interburst interval).

(C) Representative recordings of Mf NMDA-EPSCs before ("baseline") and after burst stimulation. "After burst" represents the average of NMDA-EPSCs recorded between the 30th and the 40th minute after burst stimulation.

(D) Changes in the normalized amplitude of Mf NMDA-EPSCs recorded between the 30th and the 40th minute after burst stimulation in the absence or in the presence of either SCH 58261 (n = 6), AOPCP (n = 6), or ARL67156 (n = 8). LTP of Mf NMDA-EPSCs induced by the 6 bursts stimulation protocol (6 bursts of 6 stimuli at 50 Hz with a 150 ms interburst interval) depends on A_{2A} receptor activation as indicated by inhibition with SCH58261. Both inhibitors of ectonucleotidases significantly (p = 0.02 and p = 0.01 for ARL67156 and AOPCP, respectively) inhibited LTP of Mf NMDA-EPSCs triggered by the 6 bursts stimulation protocol (*p < 0.05; **p < 0.01).

Values are presented as mean ± SEM of n experiments.

after the bursts, n = 5; Figures 7E and 7F). Thus, the induction of LTP depends on the activation of NMDA receptors, and hence on membrane potential. However, a large depolarization of the postsynaptic membrane is not necessary for induction of LTP of NMDA-EPSCs.

We next tested the dependence of LTP of Mf NMDA-EPSCs on G protein activation. The inclusion of the nonhydrolyzable GDP analog, GDP-βS (500 μM) in the patch pipette blocked LTP (102% ± 9%, 30–40 min after the bursts, n = 6; Figures 8A, 8B, and 8F), confirming the involvement of G-proteins in LTP of NMDA-EPSCs. It is well known that Src tyrosine kinases upregulate NMDA receptor function and might act as molecular hubs for the diverse and complex pathways controlling the function of this receptor (Salter and Kalia, 2004). For instance, activation of GPCRs that couple to G_q proteins such as M1 muscarinic (Marino et al., 1998) or PACAP receptors (Macdonald et al., 2005) potentiate NMDA-evoked responses by pathways that converge onto Src kinase. To assess if Src activation was involved in the A_{2A} receptor-mediated LTP of NMDA-EPSCs, we supplemented the perfusion solution with a general tyrosine kinase inhibitor PP1 (30 μM) and delivered the 6 bursts stimulation protocol to mossy fibers. NMDA-EPSCs were transiently potentiated but the stability of the LTP was severely affected and 30 min after the stimulation no significant LTP was observed (111% ± 12%, 30–40 min after stimulation, n = 5, p = 0.03 as compared to control LTP; Figures 8C, 8E, and 8F). Similarly, PP1 inhibited the short-lasting potentiation of NMDA-EPSCs by CGS21680 (30 nM; 114% ± 10%, n = 5). Inhibition of LTP was also obtained when we used a Src inhibitory peptide Src(40–58) (30 μg/ml) infused through the patch pipette (Figures 8D, 8E, and 8F; 120% ± 12%, 30–40 min after stimulation, n = 11, p = 0.04 as compared to control LTP). As a control, we found that the same concentration of a scramble peptide, did not impair LTP of NMDA EPSCs (178% ± 25% 30–40 min after the burst, n = 7; Figures 8D, 8E, and 8F). These data strongly suggest the involvement of Src kinases in LTP of Mf NMDA-EPSCs. These results, the dependence on postsynaptic Ca^{2+} rise, postsynaptic G protein, and Src activation, further strengthen the notion that LTP of NMDA-EPSCs is of postsynaptic origin.

Induction Rules for LTP of Mf NMDA-EPSCs: Comparison with Presynaptic Mf LTP

We have shown that the postsynaptic LTP induction protocol, consisting of 6 bursts of 6 stimuli at 50 Hz (36 stimuli), does not trigger presynaptic Mf LTP. Conversely it is important to assess if the tetanic parameters commonly used to induce conventional presynaptic Mf LTP (3 bursts of 100 stimuli at 100 Hz) also trigger postsynaptic LTP of NMDA-EPSCs. In control conditions, the 3 bursts of tetanic stimulation potentiated NMDA-EPSCs to a level markedly higher than that reached with 6 bursts of 6 stimuli (294% ± 23%, 30–40 min after induction, n = 9, p < 0.01; Figures 9A, 9B, and 9E). The durable decrease of paired-pulse ratio indicates a participation of presynaptic LTP to the enhancement of NMDA-EPSCs induced by 3 bursts of 100 stimuli at 100 Hz (Figures 9B, 9C, and 9D). To test if LTP of NMDA-EPSCs induced by this protocol resulted from the combination of presynaptic

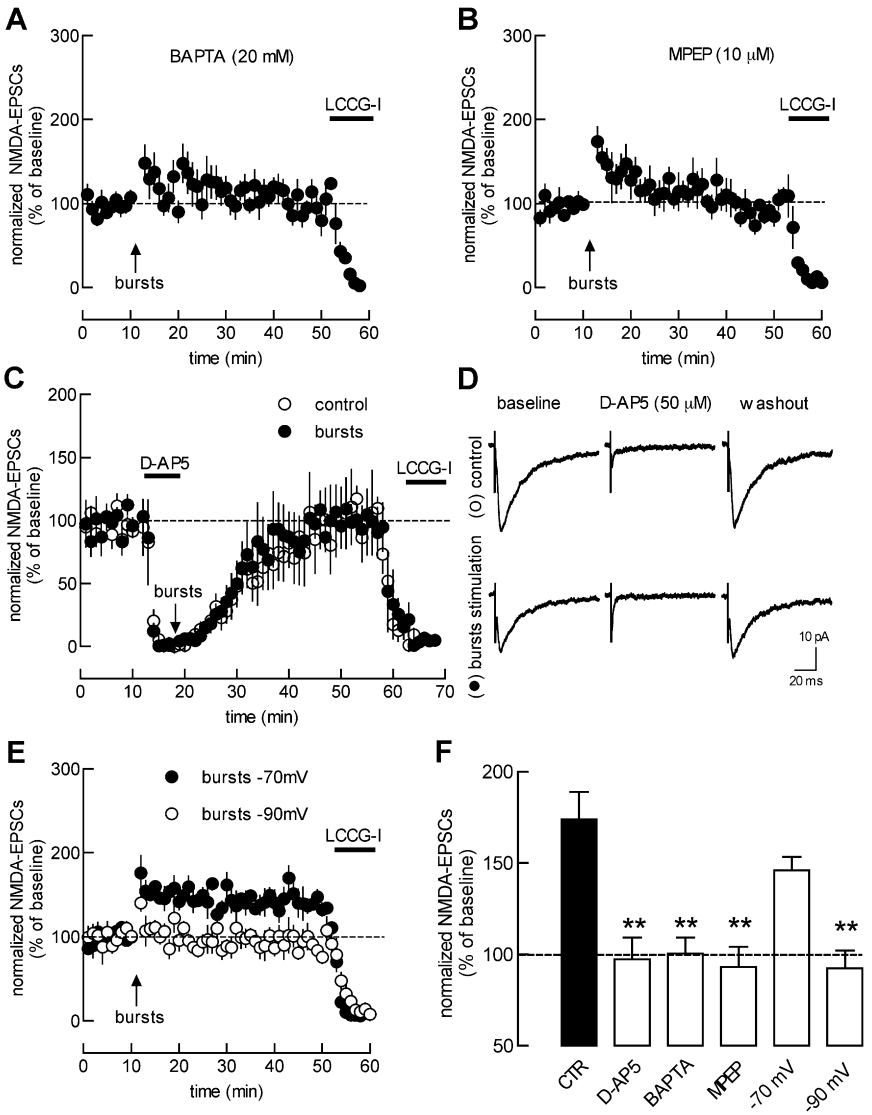

Figure 7. LTP of NMDA-EPSCs Depends on Postsynaptic Calcium Rise, NMDA Receptors, and mGluR5 Activation

(A and B) Average time course of normalized Mf NMDA-EPSCs recorded in the presence of 20 mM BAPTA (n = 9) and 10 μM MPEP (n = 6).

(C) Inhibiting NMDA receptors with D-AP5 (50 μM, during the time indicated by the bar) during the six burst stimulation induction protocol blocks LTP of NMDA-EPSCs. In "control conditions," D-AP5 was applied in the superfusion solution, but no burst stimulation was applied. Washout of D-AP5 was complete within 30 min.

(D) Representative traces of Mf NMDA-EPSCs obtained, during D-AP5 application and washout, in control conditions and in conditions where the burst stimulation was applied while in the presence of D-AP5.

(E) LTP of Mf NMDA-EPSCs can be induced in normal extracellular Mg^{2+} concentration (1.3 mM) and at a physiological resting membrane potential (−70 mV). LTP of Mf NMDA-EPSCs was recorded in the presence of 1.3 mM extracellular Mg^{2+} concentration at +30 mV. This membrane potential was momentarily changed to −70 mV or −90 mV during burst stimulation. LTP of NMDA-EPSCs is observed when applying the six bursts stimulation at −70 mV but not at −90 mV in accordance with the need of NMDA receptor activation.

(F) Relative changes in the amplitude of Mf NMDA-EPSCs recorded between the 30th and the 40th minute after 6 burst stimulation for the different conditions (**$p < 0.01$).

Values are presented as mean ± SEM of n experiments.

and postsynaptic LTP, we repeated the experiment in the presence of BAPTA (20 mM) to block the postsynaptic LTP of NMDA-EPSCs. In these conditions, the three bursts of tetanic stimulation induced LTP of NMDA-EPSCs of markedly lower magnitude (173% ± 20%, 30–40 min after induction, n = 11, $p < 0.01$), consistent with the levels of presynaptic Mf LTP measured in similar experimental conditions (see Figure 1 and Pinheiro et al., 2007). Overall these results strongly suggest that the two forms of Mf LTP can combine resulting in enhanced LTP of NMDA-EPSCs.

In CA3 pyramidal cells, Mf synapses are localized in a confined layer, the stratum lucidum, which approximately corresponds to the proximal 100 μm of the apical dendrites of CA3 pyramidal cells. Because of the proximity of distinct Mf synapses in single CA3 pyramidal cells, it is possible that the induction of postsynaptic LTP not only affects the stimulated Mf synapse but has a general effect on other Mf synapses in a given CA3 pyramidal cell. In all our experiments, Mf synaptic currents were evoked with a minimal stimulation protocol that allows evoking Mf-EPSCs from two independent mossy fibers (Rebola et al., 2007). We checked that there was no crosspotentiation between the two synaptic inputs stimulated as expected for two independent inputs: stimulation of input 1 did not cause any increase in the amplitude of Mf-EPSCs triggered by stimulation of input 2 (and vice-versa; Figure S3). The six bursts stimulation protocol was delivered through one of the stimulating electrodes, and the amplitude of NMDA-EPSCs was monitored for both pathways. No LTP of NMDA-EPSCs was observed in the control pathway that had not received the induction protocol (Figure 9F), strongly suggesting that postsynaptic LTP is a homosynaptic form of synaptic plasticity.

DISCUSSION

Here, we present data relevant to two facets of modulation of synaptic transmission: (1) we describe a functional role for postsynaptic A_{2A} receptors activated in conditions of endogenous release of adenosine, and (2) we uncover a form of LTP at Mf synapses that is selective for NMDA-EPSCs and requires the coactivation of A_{2A} receptors, NMDA receptors, and mGluR5.

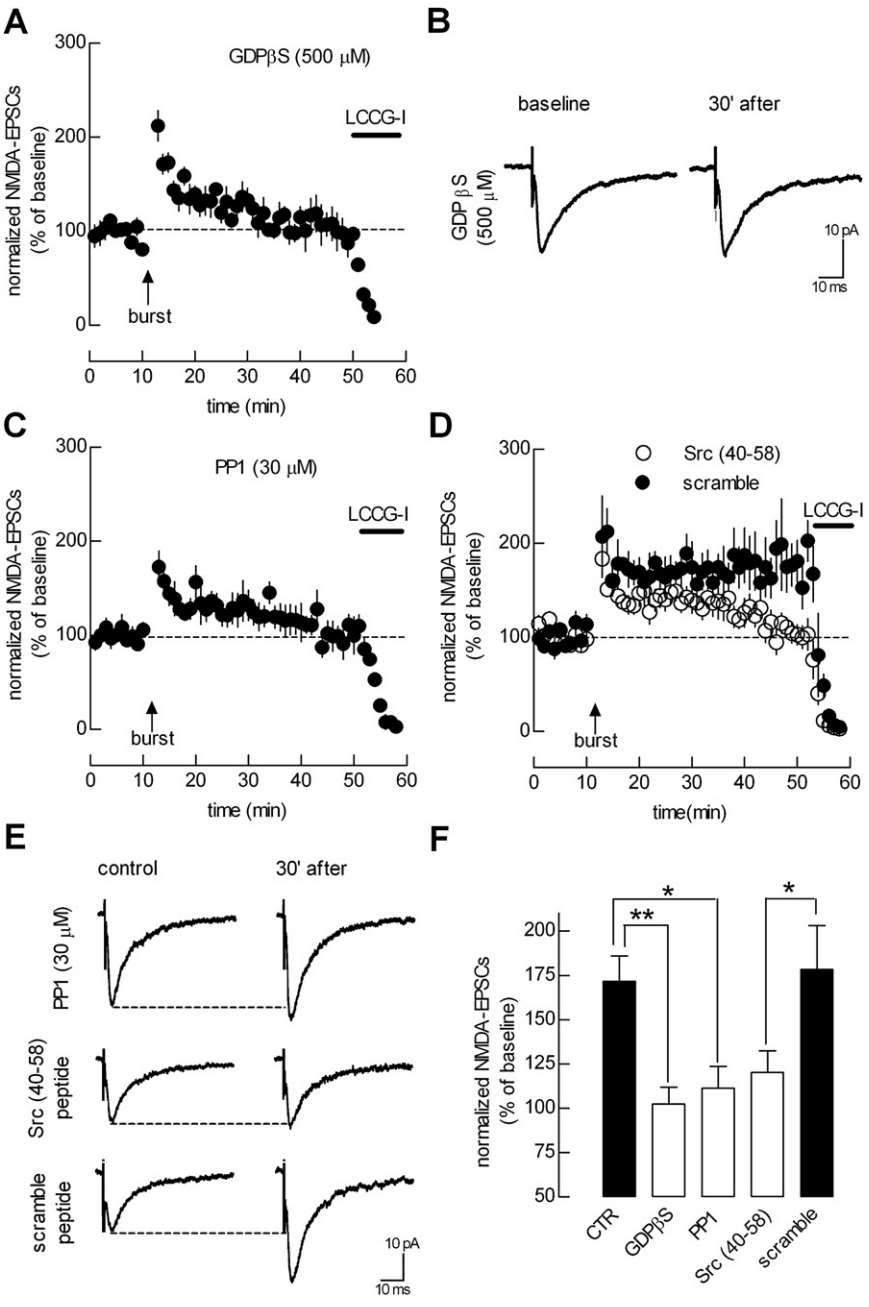

Figure 8. LTP of NMDA-EPSCs Depends on Postsynaptic Activation of G Proteins and Src Family of Tyrosine Kinases

(A) Blocking G protein activation with 500 μM GDPβS (n = 6) in the intracellular patch pipette solution impairs LTP of Mf NMDA-EPSCs.

(B) Representative traces of Mf NMDA-EPSCs recorded in control conditions and after burst stimulation using 500 μM GDPβS.

(C and D) Average time course of normalized Mf NMDA-EPSCs recorded with bath perfusion of the Src inhibitor PP1 ([C] 30 μM, n = 5) and in conditions where Src inhibitor peptide (40–58) (30 μg/ml) ([D] n = 11) was included in the patch pipette. The arrow indicates the time of application of the six burst stimulation protocol.

(E) Representative traces of Mf NMDA-EPSCs obtained in control conditions and after six burst stimulation using the Src inhibitor PP1 (30 μM), the Src inhibitor peptide (40–58) (30 μg/ml), or a scramble peptide (30 μg/ml) in the patch pipette. "After burst" represents the average of NMDA-EPSCs recorded between the 30th and the 40th minute after burst stimulation.

(F) Relative changes in the amplitude of Mf NMDA-EPSCs recorded between the 30th and the 40th minute after 6 burst stimulation for the different conditions (*$p < 0.05$; **$p < 0.01$).

Values are presented as mean ± SEM of n experiments.

The existence of A_{2A} receptors in extrastriatal brain regions like the limbic and neocortex is now well established (reviewed in Fredholm et al., 2005). Although present at a much lower density than in the striatum (Rebola et al., 2005), the pharmacological activation of hippocampal A_{2A} receptors modulates synaptic transmission and glutamate release (Lopes et al., 2002; Tebano et al., 2005; Diogenes et al., 2004). These data contrast with the lack of functional presynaptic A_{2A} receptors in Mf synapses that we now report. Our functional results are corroborated by immunogold electron microscopy studies of A_{2A} receptors which show a predominant postsynaptic localization of these receptors at Mf synapses. In agreement, a substantial amount of hippocampal A_{2A} receptors are found in biochemically purified postsynaptic fractions (Rebola et al., 2005). At Mf synapses, activation of A_{2A} receptors potentiates NMDA-EPSCs likely by a postsynaptic action. In contrast, A_1 receptors inhibit glutamatergic synaptic transmission, most likely through a presynaptic mechanism. We further confirmed this functional segregation of A_1 and A_{2A} receptors at Mf synapses by electron microscopy, which revealed an asymmetric distribution of both receptors with A_1 receptors being mostly presynaptic. Thus, Mf synapses represent an interesting example whereby a neuromodulator can have distinct effects on synaptic transmission by activating two different types of receptors localized either pre or postsynaptically.

We have described the physiological conditions whereby adenosine can activate postsynaptic A_{2A} receptors. Using various protocols of synaptic stimulation, we observed that repeated short bursts of Mf stimulation lead to LTP of NMDA-EPSCs, a process for which A_{2A} receptors activation was essential. In contrast, the presynaptic inhibitory effect of A_1 receptors was effective in conditions of tonic activation by ambient adenosine (Moore et al., 2003; Kukley et al., 2005; this study). Extracellular catabolism of ATP by burst stimulations of mossy fibers likely represents the source of adenosine for the activation of

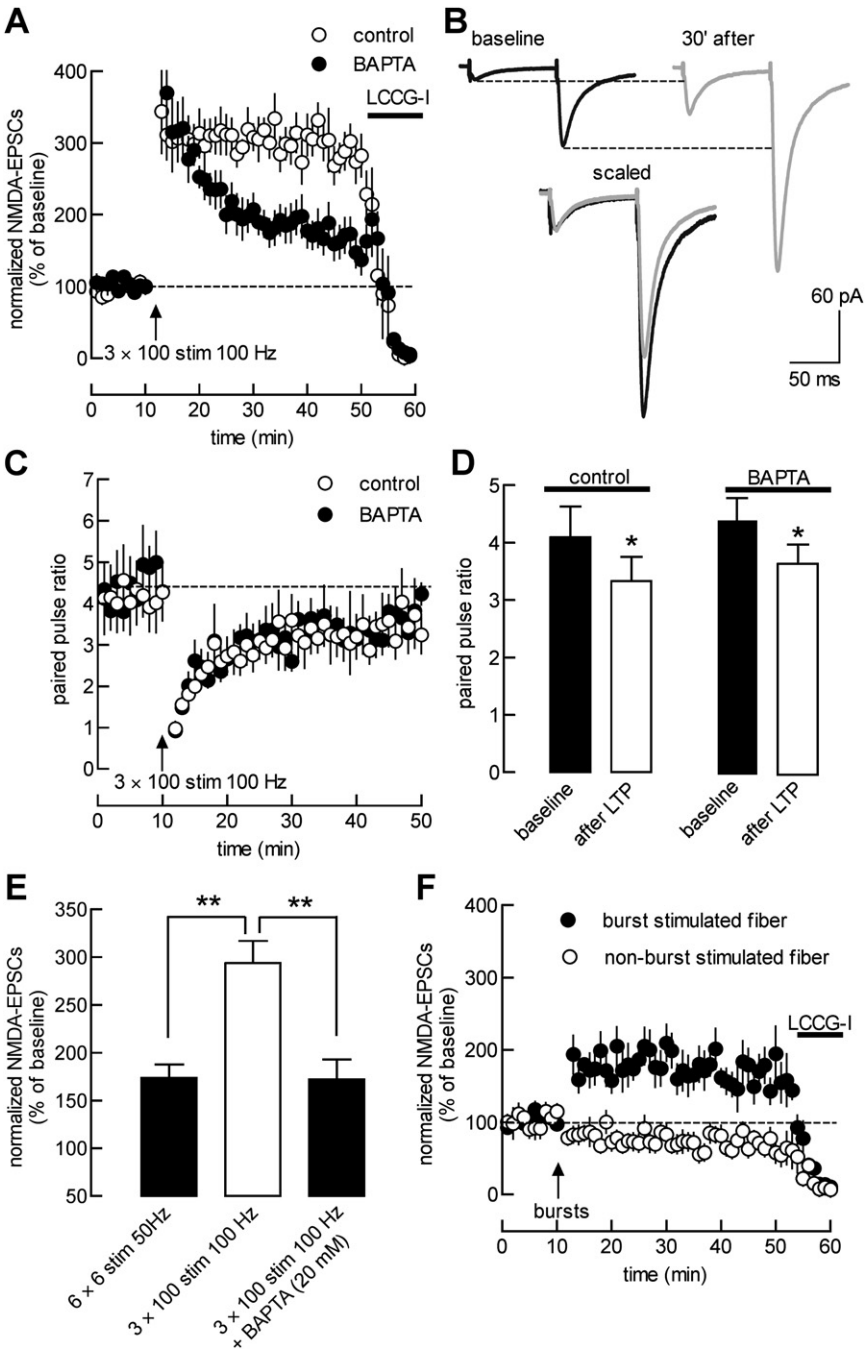

Figure 9. LTP of NMDA-EPSCs Is Induced by Conventional Stimulation Protocols Used to Trigger Presynaptic LTP at Mf Synapses and Is Homosynaptic

(A) A burst of 3 bursts of 100 stimuli at 100 Hz, a conventional simulation protocol used to trigger presynaptic LTP at Mf synapses, potentiates NMDA-EPSCs to a level markedly higher (○, n = 9) than that reached with the 6 bursts stimulation protocol. When the same 3 bursts of 100 stimuli at 100 Hz were applied with 20 mM BAPTA in the patch pipette, LTP of NMDA-EPSCs obtained was significantly reduced (●, n = 11, $p < 0.01$). The arrow indicates the time of application of the induction protocol consisting of 3 bursts of 100 stimuli at 100 Hz separated by 10 s.

(B) Representative traces of Mf NMDA-EPSCs obtained in control conditions and after the application of the 3 bursts of 100 stimuli at 100 Hz.

(C) Average time course of changes in the paired-pulse ratio of Mf NMDA-EPSCs during application of the tetanic stimulation (3 bursts of 100 stimuli at 100 Hz) in control conditions (○) and with 20 mM BAPTA (●) in the patch pipette.

(D) Mean of normalized paired-pulse ratio measured before and after LTP (30–40 min after LTP) in control conditions and with 20 mM BAPTA in the patch pipette.

(E) Mean of normalized NMDA-EPSCs recorded between the 30th to the 40th minute in the different conditions.

(F) LTP of NMDA-EPSCs is homosynaptic. Stimulating two independent Mf pathways (see Figure S3) and applying 6 bursts of 6 stimuli at 50 Hz through to one of the stimulating electrodes while monitoring the amplitude of NMDA-EPSCs for both pathways revealed that LTP of NMDA-EPSCs is only observed in the pathway that has been stimulated with the 6 bursts stimulation protocol. (*$p < 0.05$).

Values are presented as mean ± SEM of n experiments.

postsynaptic A_{2A} receptors. Indeed, inhibition of ecto-5′-nucleotidase or ecto-ATPases significantly reduced the amplitude of LTP of NMDA-EPSCs. This is in accordance with the preferential release of ATP at high frequencies of stimulation in rat hippocampal slices and with its described contribution as a source of adenosine (Cunha, 2001). Thus, it extends to CNS synapses findings obtained at the neuromuscular junction, where acetylcholine release is under control of both A_1 and A_{2A} receptors in a frequency dependent manner (Correia-de-Sá et al., 1996): at low-stimulation frequencies there is a predominant tonic inhibition mediated by A_1 receptors, whereas at higher frequencies there is an increase of ATP-derived adenosine formation that facilitates acetylcholine release through activation of A_{2A} receptors (Correia-de-Sá et al., 1996). These results highlight the differential mode of activation of A_1 and A_{2A} receptors in physiological conditions of synaptic transmission. Further studies should investigate the subsynaptic localization of ectonucleotidase and nucleoside transporters and the mechanisms that regulate local extracellular adenosine levels in the surroundings of synapses, in relation to A_1 and A_{2A} receptors localization. Activation of A_1 receptors by endogenous adenosine derived from the astrocytic syncytium (Pascual et al., 2005) potentially sets a global inhibition of neuronal circuits; in contrast, the activation of A_{2A} receptors upon burst-like high-frequency stimulation promotes synaptic plasticity selectively in potentiated synapses over an increased global inhibitory

tone. Therefore, this cooperation may be a fine-tuning mechanism increasing salience of information processing in brain circuits.

Although NMDA receptors serve as a set point for the frequency-dependent control of synaptic efficacy, relatively little is known about the plasticity of NMDA receptor-mediated synaptic transmission (Malenka and Bear, 2004). Some groups have already reported LTP of NMDA receptor-mediated synaptic transmission (Bashir et al., 1991; O'Connor et al., 1994). In the dentate gyrus, it was suggested that coactivation of mGluRs and NMDA receptors are required for induction of LTP of both the AMPA- and NMDA-mediated synaptic transmission (O'Connor et al., 1995). However, several other studies have found little or no LTP of NMDA-EPSCs under similar experimental conditions (Perkel et al., 1993; Liao et al., 1995; Heynen et al., 2000). More recently, the ability to trigger LTP of NMDA-EPSCs has been proposed to depend on intracellular Ca^{2+} buffering (Harney et al., 2006) in the dentate gyrus. Here, we describe a postsynaptic form of LTP at Mf synapses whereby the short bursts of stimuli induce a marked enhancement of NMDA-EPSCs but not AMPA-EPSCs. This selectivity for NMDA-EPSCs is a strong argument in favor of a postsynaptic mechanism. The LTP of NMDA-EPSCs requires induction protocols that appear much milder that the ones used to trigger conventional presynaptic Mf LTP, especially in terms of the number of stimuli required. Indeed, LTP of NMDA-EPSCs was readily observed with 3 bursts of 6 stimuli at 50 Hz (18 stimuli), whereas induction protocols consisting of up to 60 stimuli at 50 Hz induced no presynaptic LTP (as judged by the lack of potentiation of AMPA-EPSCs). In fact, the tetanic stimulation used for inducing presynaptic Mf LTP (300 stimuli at 100 Hz) additionally triggered postsynaptic LTP of NMDA-EPSCs. With strong induction protocols, both mechanisms of LTP combine to induce a marked increase (300%) in the amplitude of NMDA-EPSCs. The low threshold for induction of the postsynaptic LTP of NMDA-EPSCs suggests that physiological conditions corresponding to the activity of a presynaptic dentate gyrus neuron in an animal exploring its environment (Henze et al., 2002) will possibly induce LTP of NMDA EPSCs.

The postsynaptic distribution of A_{2A} receptors, as well as the blocking effects of infusing the postsynaptic cell with BAPTA, with the Src blocking peptide, or with GDPβS, constitutes additional arguments supporting a postsynaptic mechanism for LTP of NMDA-EPSCs. Pharmacological activation of A_{2A} receptors transiently enhances NMDA-EPSCs, but this is not sufficient to induce long term changes, suggesting that the activation of an additional synaptic mechanism is necessary for the LTP of NMDA-EPSCs. Our results show that a rise of intracellular Ca^{2+} is necessary for LTP of NMDA-EPSCs. This might explain why postsynaptic LTP of NMDA-EPSCs has not yet been observed since higher concentrations of intracellular Ca^{2+} buffers were previously used in experiments where NMDA-EPSCs had been monitored. An increase of postsynaptic Ca^{2+} has been reported to be necessary for at least certain forms of Mf-LTP (Yeckel et al., 1999). However, this study was performed in presence of NMDA receptor antagonists, indicating that it does not relate to the form of LTP reported here.

Postsynaptic Ca^{2+} can increase through different possible mechanisms. Here, we show that coactivation of NMDA receptors and mGluR5, a metabotropic receptor coupled to Ca^{2+} release from internal stores, are necessary for LTP of NMDA-EPSCs. The three receptors thus act in synergy to promote the durable potentiation of NMDA-EPSCs. Along these lines, coactivation of A_{2A} receptors and mGluR5 positively modulate NMDA receptor function (Tebano et al., 2005). Interestingly, pharmacological coactivation of mGluR5 and NMDA receptors potentiates NMDA-EPSCs and induces a long-lasting enhancement of excitatory synaptic transmission in primary cultured hippocampal neurons (Kotecha et al., 2003). It is likely that the coactivation of NMDA receptors and mGluR5 provides the source of Ca^{2+} necessary for the LTP of NMDA-EPSCs. Overall these results suggest a tripartite mechanism (NMDA/mGluR5/A_{2A}) for the induction of LTP of NMDA-EPSCs, with all members being essential.

In contrast to the relative paucity of data on LTP of NMDA-EPSCs, the modulation of NMDA receptors and NMDA-EPSCs by a wide variety of cell-signaling molecules converging through G protein coupled receptors to Src family kinases is well established (Salter and Kalia, 2004; Kotecha and MacDonald, 2003). G protein-coupled receptors regulating NMDA receptor currents include M1 muscarinic receptors (e.g., Marino et al., 1998), LPA receptors, the group I metabotropic glutamate receptor mGluR5 (e.g., Kotecha et al., 2003), and PACAP1 receptors (Macdonald et al., 2005). However, the physiological relevance of such modulation has remained unclear since these studies have relied on an exogenous activation of NMDA modulatory pathways. The present report demonstrates that LTP of NMDA-EPSCs at hippocampal Mf synapses depends on such an endogenous neuromodulator. Indeed, our results using a Src kinase inhibitor and a specific Src inhibitory peptide infused in the patch pipette indicate the involvement of Src family kinases (Salter and Kalia, 2004) in LTP of NMDA-EPSCs.

The current work describes a postsynaptic form of LTP that is selective for NMDA-EPSCs but not AMPA-EPSCs. The reason why the conventional NMDA-dependent LTP of AMPA-EPSCs fails to be triggered at Mf synapse possibly lies in the relatively weaker expression of NMDA receptor channels. However, our preliminary experiments suggest that LTP of AMPA-EPSCs cannot be triggered even following LTP of NMDA-EPSCs. Other molecular or structural reasons (e.g., synaptic architecture) might explain the lack of conventional postsynaptic LTP.

NMDA receptors in CA3 pyramidal cells play a crucial role in memory acquisition (Kishimoto et al., 2006; Nakazawa et al., 2002; Rajji et al., 2006). A long-lasting increase in NMDA receptor function is expected to have important consequences in the activity of CA3 pyramidal neurons. It is not known whether Mf NMDA receptors (and LTP of NMDA-EPSCs now reported) are specifically involved in memory acquisition (Kishimoto et al., 2006; Nakazawa et al., 2003; Rajji et al., 2006). The continuation of this study should result in the identification of new molecular targets whose manipulation can selectively interfere with LTP of Mf NMDA-EPSCs, allowing us to better understand the precise role of this form of synaptic plasticity in information processing in CA3 neuronal network and memory.

EXPERIMENTAL PROCEDURES

Electrophysiology

Experimental procedures followed the recommendations of the CNRS ethics committee and the French Ministry of Agriculture and Forestry concerning

the animal care (authorization number, A33093). Transverse hippocampal slices (350 μm thick) were obtained from 15- to 21-day-old C57Bl/6 mice killed by cervical dislocation. Slices were transferred to a recording chamber in which they were continuously superfused with an oxygenated extracellular medium (95% O_2 and 5% CO_2) containing 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 16 mM glucose (pH 7.4). Slices were used until a maximum of 6 hr after cutting. Whole-cell voltage-clamp recordings were made at ~32°C from CA3 pyramidal cells under infrared differential interference contrast imaging. For studies of presynaptic parameters, the intracellular solution contained 140 mM CsCl, 10 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_2ATP (pH 7.3). When studying the modulation of NMDA receptor-mediated EPSCs (NMDA-EPSCs), the patch electrodes were filled with a solution containing 140 mM cesium methanesulfonate, 2 mM $MgCl_2$, 4 mM NaCl, 5 mM phospho-creatine, 2 mM Na_2ATP, 0.2 mM EGTA, 10 mM HEPES, 0.33 mM GTP (pH 7.3). Bicuculline (10 μM) and CGP 55845 (3 μM) were present in the superfusate of all experiments. To record NMDA-EPSCs, two approaches were used: (1) cells were clamped at +30 mV and NMDA-EPSCs were isolated by blocking AMPA and kainate receptors with 20 μM NBQX; (2) alternatively, cells were clamped at −50 mV, the extracellular Mg^{2+} concentration was reduced to 0.3 mM, and NMDA-EPSCs were recorded in the presence of 20 μM NBQX. The access resistance was <20 MΩ, and cells were discarded if it changed by >20%.

Mf-EPSCs were evoked by minimal intensity stimulation (Marchal and Mulle, 2004) delivering square pulses (200 μs duration, 15–30 μA amplitude) via a glass electrode similar to patch pipettes with a tip diameter no larger than 1 μm, positioned in the dentate granule cell layer. Mf responses were characterized by a marked facilitation of EPSCs after switching stimulus frequency from 0.1 to 1 Hz and by their sensitivity to group II/III mGluR agonist (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine (LCCG-1) (10 μM). Sensitivity to LCCG-I was tested in all cells. On average the inhibition was >90% (91% ± 2%). The rare cells with an inhibition by LCCG-I <80% were not included in the study. Recordings were made using an EPC 9.0 amplifier (HEKA Elektronik, Lambrecht/Pfalz) and were filtered at 0.5–1 kHz, digitized at 1–5 kHz, and stored on a personal computer for additional analysis (IGOR PRO 5.0; Wave-Metrics).

All drugs were obtained from Tocris Cookson or Sigma. SCH 58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) was a generous gift of Scott Weiss (Vernalis, UK). SRC(40–58) and scramble peptides were synthesized by Primm srl. PP1 was obtained from Alexis Biochemicals (San Diego, CA; Coger, Paris, France).

Electron Microscopy

Male C57BL/6 mice (P21–P24) were perfused with 4% paraformaldehyde and 0.05%–0.6% glutaraldehyde in phosphate buffer. Hippocampal sections were treated for electron microscopy immunolabeling as described (Luján et al., 1996). Briefly, for pre-embedding immunogold labeling, brain sections (50–70 mm) were cut on a Vibratome and processed for immunohistochemical detection of adenosine A_1 or A_{2A} receptors using silver-enhanced immunogold techniques. For postembedding immunogold labeling, ultrathin sections (70–90 nm) from three Lowicryl-embedded blocks slices were cut on an Ultramicrotome and processed for immunocytochemical detection of adenosine A_1 or A_{2A} receptors. For ultrastructural analysis, only immunogold particles inside the plasma membrane (closer than 30 nm) of morphologically identifiable terminals (with presynaptic active zone or clear vesicles) and dendrites/spines were assessed. Quantitative analysis of the distribution of adenosine A_{2A} receptor in spines relative to the neurotransmitter release site were performed as described previously (Luján et al., 1996). To test method specificity in the procedures for electron microscopy, the primary antibody was either omitted or replaced with 5% (v/v) normal serum of the species of the primary antibody. Under these conditions, no selective labeling was observed. The antibodies used were a mouse anti-A_{2A}R antibody (2 mg/ml; Upstate Biotechnology) and a rabbit-anti-A_1R antibody (2 mg/ml; Affinity Bioreagents). The specificity of the mouse anti-A_{2A}R antibody used in this study has previously been tested in A_{2A} receptor deficient mice by western blotting (Rebola et al., 2005) and immunohistochemistry (Upstate Biotechnology, antibody data sheet).

Statistical Analysis

Values are presented as mean ± SEM of n experiments. Either a paired or unpaired Student's t test was used to define statistical differences between values.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/57/1/121/DC1/.

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