INTRODUCTION

A salient feature of \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) signaling in the developing hippocampus is the relative ease with which prolonged synaptic depression is induced, this depression being a possible preliminary to the synapse elimination important for the developing brain (Baistrikova et al. 2008; Shinoda et al. 2005). Both \( N \)-methyl-\( \alpha \)-aspartate/metabotropic glutamate receptors (NMDAR/mGluRs) although the depression was smaller (to \(~55\% \) of naïve level). Using separate blockade of either NMDARs or mGluRs, we found that this impairment of the depression resulted from the NMDAR, and not from the mGluR, blockade. In fact, during NMDAR blockade alone, depression was smaller even than that observed during combined blockade. We also found that mGluR blockade alone facilitated the LFS-induced depression. In conclusion, test-pulse stimulation produced as much depression as LFS when applied to naïve synapses even when allowing for NMDAR and mGluR activation. Our results seem in line with the notion that NMDARs and mGluRs may exert a bidirectional control on AMPA receptor recruitment to synapses.

The CA3–CA1 glutamate synapse is commonly assumed to start out as AMPA silent, AMPA signaling then being acquired during development as a consequence of Hebbian induction (Durand et al. 1996; Isaac et al. 1995; Liao et al. 1995). More recent studies have suggested, however, that the developing CA3–CA1 synapses exhibit AMPA signaling prior to being activated, i.e., when in its naïve state (reviewed by Groc et al. 2006). When activated at test-pulse frequencies (such as 0.05–0.2 Hz), the CA3–CA1 synapses can rapidly become AMPA silent, this AMPA lability then explains the common observation of AMPA-silent synapses in the developing hippocampus. When exposed to Hebbian induction conditions, these synapses return to their AMPA signaling state, i.e., long-term potentiation (LTP) at these developing synapses (< postnatal day 12) is no more than a restoration and stabilization of the naïve AMPA signaling state (Abrahamsson et al. 2008). The test-pulse-induced depression thus seems, in common with LTD, to act as complementary to LTP. In contrast to LTD, however, the test-pulse-induced depression does not require NMDAR or mGluR activation for its induction, and it spontaneously reverses within 30 min when stimulation is interrupted (Abrahamsson et al. 2007).

The developing CA3–CA1 synapse thus does not only exhibit LTD but, from its naïve AMPA signaling state, also a depression that does not require NMDARs or mGluRs for its induction and that interacts with LTD. Whether this test-pulse-induced depression is a more easily induced and more unstable version of some form of LTD, or a distinct process, is an open question. Nonetheless, the test-pulse-induced depression is induced under conditions commonly used to monitor the synaptic response prior to and after a low-frequency stimulation (LFS, usually 1 Hz) used to evoke LTD. NMDAR-\( ^{-} \) or mGluR-\( ^{-} \) dependent LTD is thus evoked against a background of already induced synaptic depression that may, or may not, have properties in common with LTD. What is observed as LTD in the developing CA3–CA1 synapse may thus be conditioned by the extent to which this test pulse-induced depression has been already induced.

In LTD experiments quite prolonged LFS activation, often 15 min at 1 Hz, i.e., 900 stimuli, is used. This contrasts to the test pulse-induced depression that appears to reach a plateau within 100 stimuli (Abrahamsson et al. 2007; Xiao et al. 2004). However, whether this apparent plateau represents a saturation of test pulse-induced depression has not been systematically examined. To explore relations between the depressions evoked by test-pulse stimulation and LFS, respectively, we have evoked these depressions using the same number of stimuli (900) and under similar conditions of NMDAR and/or mGluR blockade. It will be shown that the depressions evoked...
during test-pulse stimulation and LFS differ little in magnitude. Moreover, while NMDAR or mGluR activations are not required for these depressions, these receptor activations were found to play a modulatory role both for test-pulse- and LFS-induced depression. These results, indicating a similarity between test-pulse- and LFS-induced depression, suggest that the use of conventional stimulation protocols to examine LTD does not give an accurate account of the capability for and properties of depression at developing CA3–CA1 synapses, this depression being rather determined by the number of stimuli than depending on mGluR or NMDAR activation for its induction.

METHODS

Slice preparation and solutions

Experiments were performed on hippocampal slices from 8- to 12-day-old Wistar rats. The animals were kept and killed in accordance with the guidelines of the Gothenburg ethical committee for animal research. The rats were anesthetized with isoflurane (Abbott Scandinavia AB) prior to decapitation. The brain was removed and placed in an ice-cold solution containing (in mM) 140 cholineCl, 2.5 KCl, 0.5 CaCl2, 7 MgCl2, 25 NaHCO3, 1.25 NaH2PO4, 0.5 ascorbic acid, and 7 dextrose. Transverse hippocampal slices (400 μm thick) were cut with a Vibratome (HM 650 V, Microm) in the same ice-cold solution, and they were subsequently stored in artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 3 KCl, 2 CaCl2, 4 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, 0.5 ascorbic acid, 3 myo-inositol, 4 d,l-lactic acid, and 10 d-glucose. After 1–8 h, typically 2–5 h, of storage at 25°C, a single slice was transferred to a recording chamber where it was kept submerged in a constant flow (~2 ml min⁻¹) at ~30°C. It has previously been shown that there is no relationship between the storage time and the amount of synaptic depression observed (Abrahamsson et al. 2007). The slice was allowed to equilibrate in the recording chamber, and the stimulating and recording electrodes were put in place, 20 and 10 min, respectively, before the recording started. For each slice, there was only one recording session, and from each rat only one to four slices were used. The perfusion ACSF contained (in mM) 124 NaCl, 3 KCl, 4 CaCl2, 4 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, 0.5 ascorbic acid, and 10 d-glucose. Picrottin (100 μM) was always present in the perfusion ACSF to block GABA_A receptor-mediated activity. All solutions were continuously bubbled with 95% O_2-5% CO_2 (pH ~7.4). A cut between CA3 and CA1 and the higher than normal Ca²⁺ and Mg²⁺ concentrations were used to prevent spontaneous network activity.

Recording and analysis

Electrical stimulation of Schaffer collateral afferents was carried out in the stratum radiatum. Stimuli consisted of biphasic constant current pulses (200 + 200 μs, 20–50 μA; STG 1002, Multi Channel Systems MCS Gmbh, Reutlingen, Germany) delivered through an insulated tungsten microelectrode (resistance: ~0.1 MΩ). In most experiments, two stimulating electrodes were positioned on either side of the recording electrode to provide for two independent synaptic inputs to the same dendritic region. Field excitatory postsynaptic potential (EPSP) recordings were made by means of a glass micropipette (~2 MΩ, filled with 1 M NaCl) in the stratum radiatum. Field EPSPs were recorded at a sampling frequency of 10 kHz and filtered at 3 kHz, using an AC/DC differential amplifier (Model 3000, A-M Systems, Carlsborg, WA). Field EPSPs were analyzed off-line using custom-made IGOR Pro (WaveMetrics, Lake Oswego, OR) software. Field EPSP magnitude was measured, using linear regression over the first 0.8 ms, as the initial slope of the field EPSP rising phase. With the stimulation intensities used (20–50 μA depending on animal age) the naïve field EPSPs were generally subthreshold for spike initiation, i.e., the field recordings did not exhibit signs of population spike activity. If field EPSPs showed signs of population spike activity, the experiment was discontinued. Synaptic depression in the individual experiments was calculated as the percentage decrease between the first evoked field EPSP and the average of 20 field EPSPs at the time point indicated. The magnitude of the presynaptic volley was estimated by linear regression of the negative slope of the initial positive-negative deflection. During a recording session, the presynaptic volley was not always stable, and changes ±20% were accepted. To diminish the error introduced in the field EPSP measurements by accepting ±20% changes in the presynaptic volley, the field EPSP slope measurements in each experiment were linearly adjusted for changes in the magnitude of the presynaptic volley (using a bin width of 20 volleys). Such a linear adjustment is based on the about linear relation between field EPSP slope and presynaptic volley observed experimentally (Wigstrom and Gustafsson 1985). To verify this linearity under the present experimental conditions, in nine experiments stimulus strength was lowered to produce volley variation. This showed that a lowering of the volley to 57 ± 3% of control led to a field EPSP decrease to 51 ± 3% of control, i.e., there was a close to linear change. To illustrate the effect of this “volley correction” on the present data, the depressions obtained by test-pulse stimulation (0.2-0.05 Hz) shown in Fig. 1D was used. Thus while the uncorrected depressions on average reached to 36 ± 2.1% of the naïve level (n = 28), the “volley corrected” depressions reached on average to 40 ± 1.9% of the naïve level (n = 28). When selecting the subpopulation of these experiments displaying the most stable volleys, the volleys having decreased, on average, 3 ± 1.5% (n = 13) during the recording session, the uncorrected depressions reached on average to 40 ± 3.3% (n = 13) of the naïve level. That is, after volley correction, the average depression for the whole material agreed well with that obtained using the “best” experiments. With the exception of the experiments in which a control input was kept nonstimulated for 120 min to examine for field EPSP stability, only values from one of the two stimulated inputs were included in the final material. Data are expressed as means ± SE. Statistical significance for independent samples was evaluated using Student’s t-test.

The data presented on LTD induced using a conventional LTD protocol (e.g., Fig. 1A) were obtained in conjunction with a previous study on paired-pulse-induced depression (Washling et al. 2002) and represent previously unpublished data from that study.

Drugs

Chemicals were from Sigma–Aldrich (Stockholm) except for AIDA, d-2-amino-5-phosphonopentanoic acid (d-AP5), LY 341495, and MSPG (Tocris Cookson, Bristol, UK).

RESULTS

To study LTD, synapses are first activated at low frequency (e.g., 0.1 or 0.05 Hz) until a seemingly stable baseline is obtained, after which an LFS (1 Hz, 900 stimuli) is given. Figure 1A illustrates results from P8–P12 rats using this conventional stimulation protocol (test-pulse frequency: 0.1 Hz), demonstrating an LFS-induced LTD of field EPSPs to 66 ± 3.0% of baseline (measured 20 min after the LFS, n = 13 experiments). At this animal age, however, test-pulse stimulation itself has been found to produce depression when applied to previously nonstimulated (naïve) CA3–CA1 synapses (Abrahamsson et al. 2007; Xiao et al. 2004). Thus to establish the baseline before the LFS, the LFS-induced depression must have been preceded by a substantial depression. Figure 1B illustrates experiments in which the 0.1-Hz test-pulse stimulation was given to previously naïve synaptic inputs during 140 min (840 stimuli). This stimulation resulted in a depression with an initial faster decay within the first 100 stimuli followed by a slower continuous decline. After the 140 min of
stimulation, the field EPSP was reduced to less than half of its naïve level (39 ± 3.3% of the naïve field EPSP, n = 10). This field EPSP decrease was stimulation-dependent as shown by the unaltered field EPSP of control inputs after 120 min of stimulus interruption (97 ± 1.8% of the naïve field EPSP, n = 9, P = 0.18; Fig. 1B). These inputs had been stimulated only thrice initially to establish the naïve level. As further shown in Fig. 1B, the field EPSP decrease was not either associated with a decrease in the fiber volley.

As noted in the preceding text, the field EPSP decline during the 0.1-Hz stimulation seems continuous, the depression yet to reach a plateau even after 2 h of stimulation. In fact, a linear regression over the last 20 min of stimulation produced a significant (P < 0.02) negative slope corresponding to ~2% decline per 100 stimuli. Such continuous decline of the field EPSP by test-pulse stimulation when establishing a baseline implies that a switch during such a decline from a lower to a higher frequency in itself should result in an apparent LTD. This should be true even if the higher frequency does not recruit more depression per stimulus than the lower frequency. This is because the successive field EPSP values are plotted against time, not against stimulus number, which results in an apparent acceleration of the decline when stimulation frequency is raised. This effect is illustrated in Fig. 1C where the field EPSP depression shown in B is re-plotted. The plot in Fig. 1C starts at 40 min after onset of stimulation, and the 420 stimuli from 50 to 120 min are compressed to a 7-min period, that is, a 10× compression to simulate a frequency increase from 0.1 to 1 Hz.

LTD at these developing synapses is thus normally induced against a background of depression that may, or may not, be related to LTD. In addition, as indicated in Fig. 1C, LTD at these synapses may partly be explained by such test-pulse depression itself. We thus wished to compare the capabilities of test-pulse stimulation and LFS, respectively, to induce depression during stimulation. To do this, previously naïve synaptic inputs were subjected to either test pulse or LTD-inducing LFS (1 Hz) activations, and the depressions induced during the same number of stimulations (900) were compared. To evaluate the possible importance of which test-pulse frequency to use, we compared depressions induced by 0.05 Hz (n = 3) and 0.2 Hz (n = 15) with 0.05 Hz (n = 3). Included in this graph is also the 0.1 Hz-induced depression (grey circles) shown in B, and - - - , baseline and naïve levels, respectively, as indicated in A and B, in this and all the following figures.

Test pulse- and LFS-induced field EPSP depressions of previously naïve CA3–CA1 synapses in P8–P12 rats

The field EPSP depression produced by the test-pulse stimulation in Fig. 1D was to 39 ± 2.7% (n = 15) of the naïve level. To compare this depression with a depression
elicited under conditions that should produce LTD, previously naïve synaptic inputs were also subjected to an LFS. This LFS resulted in a depression that reached to 38 ± 1.1% (n = 14) of the naïve level, i.e., that resulted in no more depression than that obtained using the test-pulse stimulation. The time course of the depressions evoked by test-pulse stimulation and LFS, respectively, differed however. When plotted superimposed (Fig. 2A), the LFS led to an initial potentiation, the two depressions thereafter slowly converging toward each other during the 900 stimuli. As will be shown in the following text, this initial difference in depression is related to two processes. First, it was related to an initial NMDAR-dependent potentiation produced to a larger extent by LFS than by test-pulse stimulation (Fig. 4, C–E). Second, it was related to a transient frequency facilitation produced by LFS as compared with test-pulse stimulation as indicated by the initial transient field EPSP increase observed when switching from test-pulse stimulation to LFS (Fig. 1A, see also Fig. 3A).

The field EPSP depression obtained both during test-pulse stimulation and LFS varied among the experiments, from 41 to 76% and from 57 to 69% for test-pulse stimulation and LFS, respectively. As also found previously (Abrahamsson et al. 2007), this variability in depression was not explained by the variation in the naïve field EPSP peak amplitude among the experiments (Fig. 2B). This result suggests that the amount of depression obtained is not critically dependent on the depolarizing effect of the field EPSP. However, field EPSP magnitudes obtained in different experiments may not be exactly comparable. We therefore considered an additional manner to judge the possible variation in the depolarizing effect of the field EPSPs among the experiments. As described in the preceding text, the LFS produced, on average, an initial NMDAR-dependent potentiation, that, however, varied in magnitude among the experiments. In fact, such an initial potentiation was also sometimes observed using 0.2-Hz stimulation as well as using 0.05-Hz stimulation (Fig. 1D). Assuming that the appearance of such an initial potentiation, or of a flattening of the initial

![Field EPSP depression graph](http://jn.physiology.org/)

**FIG. 2.** LFS- and test pulse-induced field EPSP depression in control solution. A: depression from a previously nonstimulated (naïve) level using either 0.2 Hz (○, n = 15) or 1 Hz (○, n = 15) stimulation. The 0.2-Hz-induced depression is the same as that shown previously in Fig. 1D. Example field EPSPs (average of 20 records) taken at time points indicated in the figure. B: the final amount of depression reached after 900 stimuli of 0.2 Hz (0.2 Hz) and 1 Hz (○) activation is plotted against the peak amplitude of the naïve field EPSP used in that experiment. The linear regression lines indicated in the graph did not indicate any linear relation between the amount of depression and naïve field EPSP peak amplitude (P = 0.54 and P = 0.11 for 0.2- and 1-Hz data, respectively). C: the test-pulse-induced depression shown in A was subdivided in 2 groups according to the initial amount of potentiation/flattening of the depression. This value was estimated as the ratio between the average of the slopes of the first 20 field EPSPs and the slope of the very first field EPSP following stimulation onset. D: same as C, but for the LFS-induced depression. E and F: expanded versions of C and D.
blockade was true not only with respect to the LTD but also with respect to the depression taking place during the LFS. That is, while LFS in control solution (Fig. 1A) gave rise to an initial facilitation followed by a depression that about equaled that of the subsequent LTD, during the combined NMDAR/mGluR blockade, this depression largely vanished together with LTD (96 ± 3.0% of baseline, \(P = 0.22\); Fig. 3A). Thus also the depression induced during the LFS in a conventional LTD experiment is NMDAR and/or mGluR-dependent.

Figure 3A also shows that the switch from test-pulse stimulation to an LFS during combined NMDAR/mGluR blockade gave rise to a transient frequency facilitation that vanished before the end of the LFS. We have not explored what may explain this transient facilitation, assuming that an increased stimulus rate first leading to an enhanced vesicle release probability because of intraterminal calcium accumulation and, later to more slowly developing vesicle depletion, may plausibly account for this behavior (Zucker and Regehr 2002).

Importantly, however, this return to baseline level at the end of the LFS suggests that any difference in final depression of previously naïve inputs produced by test-pulse stimulation and LFS, respectively, should not reflect differences in release probability produced in an NMDAR- and mGluR-independent manner by these stimulations.

In contrast to LTD, test-pulse-induced depression has not been found to be significantly affected by blockade of these glutamate receptors (Abrahamsson et al. 2007; Xiao et al. 2004). However, when during combined NMDAR/mGluR blockade previously naïve synaptic inputs were subjected to either test-pulse stimulation or LFS, the depression was in both cases significantly impaired by this blockade. The depressions were now to 54 ± 2.3% (\(n = 9\)) and to 53 ± 2.1% (\(n = 15\)) of the naïve level for test-pulse stimulation and LFS, respectively (Fig. 3B). These depressions were significantly smaller (\(P < 0.001\) in both cases) than the corresponding depressions to ~40% of the naïve level obtained in control solution (Fig. 2A). Thus while the larger part of the depression induced by 900 stimulations of previously naïve synaptic inputs was obtained in an NMDAR- and mGluR-independent manner, the depression is affected by NMDAR and/or mGluR activation. Interestingly, our results show that this effect of NMDAR and/or mGluR activation was the same, irrespective of stimulation frequency, indicating that test-pulse stimulation and LFS make equally good use of NMDARs and/or mGluRs to produce additional depression during the stimulation. As expected from the transient frequency facilitation observed in Fig. 3A when switching from test-pulse stimulation to LFS, superposition of the 0.2- and 1-Hz depression curves observed during the combined NMDAR/mGluR blockade (Fig. 3B) indicates some initial discrepancy between the two depression curves. In fact, when measured at an initial part of these curves (see horizontal bars, Fig. 4, C and D), the field EPSP was significantly more depressed using test-pulse stimulation (to 80 ± 2.1%, \(n = 9\)) than using LFS (to 89 ± 2.1%, \(n = 15\); \(P < 0.01\)).

When superimposing the depressions observed in control solution with those obtained during combined NMDAR/mGluR blockade using either test-pulse stimulation (Fig. 4, A and C) or LFS (B and D), the NMDAR/mGluR blockade appears in both cases to result in depressions that initially are larger and thereafter smaller than the depressions observed in control solution. Thus when measuring the field EPSP magni-

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**Effect of combined NMDAR/mGluR blockade on LTD, and on test-pulse- and LFS- induced field EPSP depressions of previously naïve CA3–CA1 synapses**

When using the conventional LTD protocol in the combined presence of the NMDAR antagonist d-AP5 (50 \(\mu M\)) and the broad spectrum mGluR antagonist LY 341495 (100 \(\mu M\)), there was no significant depression when measured 20 min after the LFS (97 ± 4.7% of baseline, \(n = 6, P = 0.61\); Fig. 3A). This depression, reflected the use of field EPSPs reaching closer toward firing threshold, we subdivided both the test-pulse and LFS-induced depressions into two groups according to the amount of such an initial potentiation/flattening (see text-Fig. 2 for details). This subdivision created curves that, except for their initial parts (Fig. 2, E and F), were super imposable, reaching the same final amounts of depression (Fig. 2, C and D).

**FIG. 3.** Stimulation-induced field EPSP depression during combined N-methyl-D-aspartate receptor (NMDAR)/mGluR blockade. A: an LFS (900 stimuli at 1 Hz) is applied after prolonged test-pulse stimulation (0.1 Hz) to establish a steady baseline \((n = 6)\). Note the blockade of depression not only after the LFS but also of the depression at the end of the LFS. B: depression from a previously nonstimulated (naïve) level using either 0.2 Hz \((0.2, n = 9)\) or 1 Hz \((1, n = 15)\) stimulation. Example field EPSPs (average of 20 records) taken at time points indicated in the figure. All experiments were performed in the presence of 50 \(\mu M\) 2-amino-5-phosphonopentanoic acid (d-AP5) and 100 \(\mu M\) LY 341495.
tudes at an initial phase of these curves (horizontal bars in Fig. 4, C and D), the field EPSPs were more depressed during combined NMDAR/mGluR blockade than in control, both using LFS (to 89 ± 2.1%, n = 15 vs. 104 ± 2.2%, n = 14, of the naïve level, \( P < 0.001 \)), and using test-pulse stimulation (to 80 ± 2.1%, n = 9 vs. 86 ± 2.8%, n = 15, of the naïve level), this latter effect, however, not being statistically significant (\( P = 0.11 \)). It would thus appear that NMDAR and/or mGluR activation produce an initial potentiation that masks the initial depression. This initial potentiation was found to be largely NMDAR-dependent, as it was not observed in the presence of d-AP5 alone (Fig. 4E). While the preceding statistics would suggest that such an initial NMDAR-dependent potentiation is restricted to LFS, the results previously presented (Figs. 1D and 2E) show that such an initial potentiation effect, as indicated in Fig. 4C, can also be observed using test-pulse stimulation although to a more limited and variable extent.

**Effects of NMDAR versus mGluR activation on LFS-induced LTD**

The effect of NMDAR activation on LTD was evaluated by blocking mGluRs alone (100 μM LY 341495), the LFS under these conditions reducing the field EPSP to 88 ± 2.4% of baseline (\( n = 5 \); Fig. 5A). Thus there was a statistically significant (\( P < 0.01 \)), but small, LTD, suggesting only a minor role for NMDAR activation itself for LTD at these developing synapses. When instead blocking NMDARs alone (50 μM d-AP5) to evaluate mGluR contribution to LTD induction, the LFS-induced LTD was larger, and reached to
80 ± 1.7% of baseline ($n = 10$; Fig. 5B). This value significantly differs both from that obtained when allowing for NMDAR activation alone ($P < 0.02$) and that obtained in control conditions ($P < 0.001$). Thus mGluR activation alone contributes more to this LTD than does NMDAR activation alone but cannot fully account for the LTD.

Experiments using d-AP5, or LY 341495, alone can be described as evaluating the effect of NMDAR, or mGluR, activation alone, when compared with data obtained in control solution. However, such experiments can equally well be described as instead evaluating the effect of mGluR, or NMDAR, activation alone when compared with data obtained using combined...
NMDAR/mGluR blockade. As can be noted in the preceding text, this latter way of describing our results was used in the preceding text, and will be used in the following. This is because we wished to evaluate the modulatory actions of NMDARs, or mGluRs, alone on the depression that is evoked when none of these receptors were activated.

Effects of NMDAR versus mGluR activation on test-pulse- and LFS-induced field EPSP depressions of previously naïve CA3–CA1 synapses

When instead subjecting previously naïve synaptic inputs to test-pulse stimulation, or LFS, in the presence of either LY 341495, or d-AP5, the resulting depressions were quite differently affected by these drugs as compared with the effect of these drugs on LFS-induced LTD. Thus when allowing for NMDAR activation alone (in LY 341495) test-pulse stimulation (Fig. 5C) and LFS (D) reduced the field EPSP to 42 ± 1.9% (n = 15) and to 31 ± 1.5% (n = 13) of the naïve level, respectively. These depressions were significantly larger (P < 0.001 and P < 0.001, respectively) than those obtained during combined NMDAR/mGluR blockade and were similar to, or with respect to LFS even significantly larger (P < 0.001) than, the corresponding depressions obtained in control solution. It would thus seem that NMDAR activation explains essentially all the positive modulation of the depression provided in control solution. Moreover, at least when using LFS, mGluR activation rather opposes the depression because larger depression is observed in the presence of LY 341495 alone than in control solution.

When instead performing these experiments in the presence of d-AP5, the resulting depressions were to 68 ± 3.0% (n = 7) (Fig. 5 C) and to 59 ± 1.7% (n = 17; Fig. 5D) of the naïve level. These depressions were thus no greater than those observed during combined NMDAR/mGluR blockade (to 54 and to 53%, respectively). In fact, these depressions obtained in the presence of d-AP5 alone were even significantly smaller (P < 0.005 and P < 0.05) than the depressions obtained during the combined NMDAR/mGluR blockade by test-pulse stimulation and LFS, respectively. Thus mGluR activation appears not only to oppose the depression obtained in control solution (see preceding text) but also the depression obtained in the absence of NMDA and mGluR activation. This opposing effect was, however, greater using test-pulse stimulation than using LFS (P < 0.01).

Effects of mGluR group I versus mGluR group II/III activation on test-pulse- and LFS-induced field EPSP depressions of previously naïve CA3–CA1 synapses

Because LY 341495 is a broad spectrum mGluR antagonist, it does not tell which types of mGluRs that are involved in opposing the depression. To examine this issue, the preceding experiments were performed using the specific group I antagonist AIDA (500 µM) to allow for mGluR group II/III activation alone, and the specific group II/III antagonist MSPG (200 µM) to allow for mGluR group I activation alone. These experiments were first performed in the combined presence of d-AP5 to examine the opposing influence of mGluR activation on the test pulse-induced depression observed during NMDAR blockade (Fig. 5C). When previously naïve synaptic inputs were subjected to test-pulse stimulation, they were depressed to 50 ± 1.3% (n = 6) and to 62 ± 3.3% (n = 6) of the naïve level when allowing for group II/III and group I activation alone, respectively (Fig. 6A). This result rules out mGluR group II/III activation as responsible for opposing test pulse-induced depression. On the other hand, the impairment when allowing for mGluR group I activation alone (54% to 62%) did not fully reach that observed when allowing for mGluR activation alone (in d-AP5 alone; 54% to 68%) and did not reach statistical significance (P = 0.056). These experiments were also performed in the absence of d-AP5 to examine the opposing influence of mGluR activation on LFS-induced depression when allowing for NMDAR activation (Fig. 6B). As shown in the preceding text, in the presence of LY 341495, the LFS-induced depression was to 31 ± 1.5% (n = 13) of the naïve level compared with 38 ± 1.1% (n = 14) in control solution. When performed in the presence of AIDA, the depression

**FIG. 6.** Stimulation-induced field EPSP depressions during blockade of either group I or group II/III mGluRs. A: test-pulse-induced depression from a previously naïve level in the presence of either 50 µM d-AP5 and the specific group I antagonist AIDA (500 µM; ○, n = 6), or of 50 µM d-AP5 and the specific group II/III antagonist MSPG (200 µM; ●, n = 6); B: LFS-induced depression from a previously naïve level in the presence of the specific group I antagonist AIDA (500 µM; ○, n = 9), or the specific group II/III antagonist MSPG (200 µM; ●, n = 6). Example field EPSPs (average of 20 records) in A and B are taken at time points indicated in the figure.
The test-pulse-induced depression described previously did seemingly saturate after some 100 stimuli and was explained by AMPA silencing (Xiao et al. 2004). The present study shows that more prolonged test-pulse stimulation leads to a further slower progressive decline of the synaptic response. After 900 stimuli, i.e., after the same number of stimuli commonly used to evoke LTD, the synaptic response had decreased to less than half of the naïve level, this number of stimuli still not saturating the depression. Because field responses were used, we have no direct evidence that this further depression is an AMPA silencing of synapses more resistant to silencing than those silenced during the first rapid phase of depression. Nonetheless, considering the basic similarities in properties between these two phases, i.e., that both are evoked to the same extent by various sub-LFS frequencies (0.2–0.05 Hz, Fig. 1D) and do not require NMDAR and/or mGluR activation (Fig. 3B), it seems plausible that this later slower phase is a continuation of the AMPA silencing previously demonstrated (Xiao et al. 2004).

Modulation of test-pulse-induced depression by NMDAR and/or mGluR activity

The test pulse-induced depression to ~40% of the naïve level (after 900 stimuli) was reduced to ~55% of the naïve level by combined NMDAR/mGluR blockade. Thus while the major part of the depression (~75%) did not require NMDARs or mGluRs for its induction, the depression evoked during sub-LFS frequency activation was thus clearly influenced by NMDAR and/or mGluR activations. To what extent this effect of NMDARs or mGluRs results from receptor activations produced by the test-pulse stimulation itself or reflects tonic activation of these receptors by ambient glutamate cannot be determined from the present results. Considering that both these receptor types, in particular mGluRs, have been linked to LTD at these developing synapses (Bolshakov and Siegelbaum 1994; Li et al. 2002; Pavlov et al. 2004), one might have expected both these receptor types to contribute to the induction of the depression. In contrast to this expectation, NMDAR activation alone was found to explain all of the remaining 25% of the depression. Furthermore, mGluR activation alone was found to actually oppose the test pulse-induced depression, resulting in a depression that was only ~70% of that observed during combined NMDAR/mGluR blockade. Use of subgroup specific mGluR antagonists suggested that group I mGluRs rather than group II/III mGluRs should be involved in this opposing action. While such a differential effect of NMDARs and mGluRs may seem surprising, such differential modulatory effects are consistent with recent studies on the developing glutamate synapse (reviewed by Hall and Ghosh 2008). As described by these authors, while NMDARs have been found to act negatively on AMPARs on several levels, including suppression of transcription and of translation, reduction in surface localization, and promotion of degradation, group I mGluRs have been found to act to positively regulate local levels of AMPARs. Such results have led to the suggestion of a bidirectional regulation of AMPAR availability by NMDARs and mGluRs (Hall and Ghosh 2008). The present findings seem clearly in line with such a bidirectional control of these receptors on AMPAR recruitment to synapses.

It should be noted, however, that test pulse-induced depression, when allowing for both NMDAR and mGluR activation, did not differ from that obtained when allowing for NMDAR activation alone, implying a nonopposing role for mGluRs when activated, under these conditions, together with NMDARs. Such a dual rule for mGluRs on the synaptic plasticity depending on stimulation conditions would not be unique. For example, NMDARs are capable of producing both depression as well as potentiation of these synapses dependent on stimulation conditions (Malenka and Bear 2004).

Although supported by these recent studies reviewed by Hall and Ghosh, we have no direct evidence that these modulatory actions of NMDARs and mGluRs are a modulation of the AMPA silencing that is produced in an NMDAR- and mGluR-independent manner by the test-pulse stimulation. When examining the time course of the depressions evoked under the various induction conditions, the depressions evoked when allowing for NMDAR activation did not develop as fast, but rather slower, than those evoked in the absence of NMDAR activation, despite reaching a larger final value (Fig. 4, A and C). This result indicating a differential effect of NMDARs on the initial faster versus the later slower phase of the depression may be taken to suggest different expression mechanisms for these two phases. We do not think such a suggestion is warranted. When activating a naïve input using an LFS, there was an initial plateau phase (or even slight potentiation) preceding the depression that was blocked in the presence of n-APS (Fig. 4E), indicating that, initially, an NMDAR-dependent potentiation
masks the depression. This potentiation was also evoked, to a variable extent, using a test-pulse stimulation (Fig. 2, C and E), and thus explains the slower onset when allowing for NMDAR activation. Moreover, when comparing the test-pulse-induced depressions, when allowing for NMDAR activation alone versus allowing for mGluR activation alone, these depressions were different already within 50 stimuli (Fig. 5C), i.e., within the initial faster decay phase previously found to be associated with AMPA silencing. We thus find it reasonable to tentatively conclude that the modulatory actions of NMDARs and mGluRs on the test-pulse-induced depression presently observed is a modulation of a basically NMDAR- and mGluR-independent AMPA silencing process.

**LFS- versus test-pulse-induced depression**

Our finding that LFS activation of a naive input during combined NMDAR/mGluR blockade produced no more depression than did test-pulse stimulation agrees with previous results (Abrahamsson et al. 2007; Xiao et al. 2004) that this depression should only depend on the number of stimuli not their frequency. However, it extends this finding to a much larger number of stimuli (900 vs. ~100). Surprisingly, however, this same result was also observed in control solution, i.e., when an LFS, compared with a test-pulse stimulation, should be a potent inducer of LTD. The simplest explanation for this result would be that this LTD-inducing stimulation then operates on the same expression mechanism, i.e., AMPA silencing, as does test-pulse stimulation, with about the same efficacy (per stimulus). On the other hand, when allowing for mGluR, or NMDAR, activations alone, LFS produced more depression than did test-pulse stimulation. This difference seems, however, not to be a consequence of LFS adding a new depression component under these circumstances but rather to reflect the variable opposing action of mGluR activation on the depression under these various induction conditions. Thus also when using LFS, less depression was produced when allowing for mGluR activation alone than when NMDAR/mGluRs were both blocked. This opposing action was, however, smaller using LFS than using test-pulse stimulation, resulting in a larger LFS- than test-pulse-induced depression. Similarly, when allowing for NMDAR activation alone, the LFS-induced depression was, in contrast to the test-pulse-induced depression, larger than in control solution. That is, the opposing action of mGluRs on the depression was now greater during an LFS than during the test-pulse stimulation. It thus seems reasonable to propose that NMDAR and mGluR activations during LFS of a naive input produce no more than a modulation of the AMPA silencing produced by the test-pulse stimulation.

**Depression of a naive input versus conventional LTD**

The present results suggest that NMDAR rather than mGluR activation is most involved in synaptic depression of naive synapses. This result may seem unexpected because mGluRs are thought to have a more prominent role than NMDARs for LTD in these neonatal animals (Bolshakov and Siegelbaum 1994; Li et al. 2002). In fact, when presently examined after prior baseline stimulation, our results agreed with this notion. Thus while a combined NMDAR/mGluR blockade was needed to fully block this LTD (Fig. 3A), as also previously described (Pavlov et al. 2004), a significantly larger LTD was observed when mGluR activation was allowed than when NMDAR activation was allowed (Fig. 5, A and B). However, the depression in focus in the present study was obtained using previously nonstimulated, naive, synapses. Moreover, what we report here is not LTD but depression occurring during the stimulation. While the depression occurring during the LFS in our conventional LTD experiments was blocked by combined NMDAR/mGluR blockade in parallel with LTD (compare Figs. 1A and 3A), this does not necessarily mean that this depression induced during stimulation is the same as LTD. Nevertheless the fact that this depression is evoked to much the same extent by test-pulse stimulation, as by an LFS, does imply that conventional LTD experiments on developing synapses produce an LTD that is biased by the amount of baseline stimulation that has preceded the LFS as well as by the receptor activations allowed during this baseline activation. In fact, an “LTD” may even be constructed from test-pulse stimulation alone if simulating an LTD by plotting some of the 0.1-Hz test-pulse stimuli on the x axis, as if they were given at 1 Hz (Fig. 1C).

It would then appear that the use of naive synapses should give a more accurate view of the capacity and induction properties for depression at these developing synapses. At their naïve level (using hippocampal slices, see last section of DISCUSSION) all the synapses are AMPA signaling, i.e., there are no AMPA silent synapses (Groc et al. 2006). This naïve level is also a ceiling for LTP at these developing synapses (± P12), Hebbian induction producing no more than a return to and stabilization of this naïve level (Abrahamsson et al. 2008). In other words, using this naïve level as a reference level, depressions evoked under various activations and pharmacological conditions can be dealt with not only as percentage changes from a prior baseline but also as absolute changes with respect to this reference level. This point can be schematically illustrated by data from the present study. Thus the mGluR-dependent LTD induced after prior baseline stimulation was to 80% of a baseline that could be ~70% of the naïve level if taking the level reached by 900 pulses of test-pulse stimulation in the presence of D-AP5 as the baseline level. The total depression when allowing for mGluR activation would then be to ~56% (80% of 70%) of the naïve level, which is no more than the 53% obtained by 900 stimuli in the absence of NMDAR and mGluR activation. The NMDAR-dependent LTD, on the other hand, induced after prior baseline stimulation was to 90% of a baseline level that, by the same reckoning as in the preceding text, could be ~40% of the naïve level. That is, the total depression allowing for NMDAR activation would be to ~36% (90% of 40%) of the naïve level. That is, the LTD produced by LFS in conventional LTD experiments may underestimate the participation of NMDARs in producing activity-dependent depression while overestimating that of mGluRs. By eliminating NMDARs during synaptogenesis, it has been found that NMDAR activation plays an important role in keeping the synapses AMPA silent (Adesnik et al. 2008). Such an important role for NMDAR activation during development seems well compatible with the results presented here.

**Functional aspects on test-pulse-induced depression**

Considering the relatively rapid recovery from test-pulse-induced AMPA silencing by stimulus interruption (7 min time...
constant following ~100 stimuli) (Abrahamsson et al. 2007), the relative proportion of AMPA silence in a synaptic population will be decisively dependent on the activity of the preparation used. In this regard, the hippocampal slice preparation may constitute an extreme, producing virtually no steady-state level of AMPA silent synapses in the absence of evoked synaptic activity. This would be especially true when using higher than normal Ca\(^{2+}\) and Mg\(^{2+}\) concentrations and a section between the CA3 and CA1 regions. On the other hand, in an active (developing) brain, synapses may be expected to be exposed to AMPA silencing activity far more often than they can recover. They may then either become silenced for a while, or, if active under Hebbian induction conditions produced by other synapses, become stabilized. What is shown presently is that this depression obtained by evoked stimulation at frequencies far below those normally used to induce LTD, is not only a consequence of the AMPAR signaling itself. That is, the induction of this depression is modulated by NMDAR and mGluR activity, NMDAR activity facilitating the depression and mGluR activity opposing the depression. Whether these synaptic activations in the sub-LFS range, that are not associated with Hebbian induction conditions, ultimately lead to stabilization of this silencing and (later) to subsequent elimination of the synapse or whether such stabilization of depression requires higher (LFS) frequencies remains to be investigated.

ACKNOWLEDGMENTS

We thank members of the lab for helpful comments on the manuscript.

GRANTS

This project was supported by the Swedish Research Council (Project Number 01580) and Stiftelsen Wilhelm och Martina Lundgrens Vetenskapsfond.

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