Bidirectional, Activity-Dependent Regulation of Glutamate Receptors in the Adult Hippocampus In Vivo

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Summary

Experience-dependent regulation of synaptic strength has been suggested as a physiological mechanism by which memory storage occurs in the brain. Although modifications in postsynaptic glutamate receptor levels have long been hypothesized to be a molecular basis for long-lasting regulation of synaptic strength, direct evidence obtained in the intact brain has been lacking. Here we show that in the adult brain in vivo, synaptic glutamate receptor trafficking is bidirectionally, and reversibly, modified by NMDA receptor-dependent synaptic plasticity and that changes in glutamate receptor protein levels accurately predict changes in synaptic strength. These findings support the idea that memories can be encoded by the precise experience-dependent assignment of glutamate receptors to synapses in the brain.

Introduction

Neurons throughout the cerebral cortex, including area CA1 of the hippocampus, have stimulus-selective receptive fields. Chronic recordings from cortical neurons have shown that, as something new is learned, stimulus selectivity changes—some synaptic inputs potentiate and others depress. In CA1, for example, neurons show selectivity for positions in space, and this selectivity shifts rapidly as animals learn a new spatial environment (Breese et al., 1989; Wilson and McNaughton, 1993). Neural network theory suggests that the selectivity shift reflects the creation of new neural representations. The memory is encoded by changing the pattern of synaptic strengths (or "weights") across the network of neurons (Cooper, 1995; Bear, 1996).

When more new information is learned, stimulus selectivity (i.e., the pattern of synaptic weights) shifts further. An implication of this finding is that previously encoded memories can remain stable, even as the pattern of synaptic weights are again modified to create new representations. According to this way of thinking, memory involves repeated, bidirectional modifications of synaptic transmission to fine-tune the patterns of synaptic strengths in the neural network. Of course, in the absence of new learned information, synaptic

weights and neuronal selectivity must remain stable; passive decay of synaptic weight (that is, back to an initial value that might be larger or smaller) leads to a loss of the stored representations.

The bidirectional modification of synaptic transmission requires that individual synapses on neurons be capable of some form of long-term potentiation (LTP) and some form of long-term depression (LTD). However, a theory of memory storage that relies on bidirectional synaptic modification also places an important constraint on the mechanisms underlying LTP and LTD: changes in synaptic weights must be reversible. If the mechanisms underlying LTP and LTD were distinct and irreversible, eventually saturation would occur as synapses were subjected to repeated stimulation. This problem does not occur if the mechanisms underlying LTP and LTD are inversely related.

Over the past several decades, experimental models of LTP and LTD have been developed in hippocampal area CA1 with the aim of revealing the mechanisms of bidirectional synaptic modification that may underlie memory storage. The modifiable synapses between Schaffer collaterals and CA1 pyramidal neurons use glutamate as a neurotransmitter, which activates postsynaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (AMPARs) and NMDA (N-methyl-D-aspartate) receptors (NMDARs). A number of lines of evidence support the idea that LTP and LTD may be due to changes in the function and synaptic expression of postsynaptic AMPARs, which are comprised of GluR1 and GluR2 subunit proteins (for review, see Malenka and Nicoll, 1999; Lüscher et al., 2000). For example, LTP induction in hippocampal slices is associated with an increase in AMPAR function (Kauer et al., 1988; Muller and Lynch, 1988; Isaac et al., 1995; Liao et al., 1995) that correlates (in slice culture) with the delivery of receptors to dendritic spines (Shi et al., 1999; Hayashi et al., 2000) and increased expression of GluR1 protein (Nayak et al., 1998). In addition, research using hippocampal slices (Kandler et al., 1998; Luthi et al., 1999) and cell culture (Carroll et al., 1999; Man et al., 2000) suggests that AMPARs are physically removed from the postsynaptic membrane following LTD.

However, before activity-dependent changes in AMPAR expression can fulfill the criteria required of a synaptic mechanism of memory, three additional, technically challenging questions need to be addressed. First, are the changes in AMPAR expression reversible? Second, do they occur in the adult brain? Third, and most importantly, do they occur in vivo? The answers to these questions are not foregone conclusions. Most of the aforementioned studies have been performed on immature tissue in vitro, often in tissue culture. There is evidence that the expression mechanisms of LTP vary over the course of postnatal development, with regulation of AMPAR expression occurring most prominently in neonates (Durand et al., 1996; Liao et al., 1999). Indeed, evidence for activity-dependent regulation of synaptic glutamate receptor levels in the adult brain in vivo, so far, has been elusive.

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Here we address these questions in the adult hippocampus in vivo using an approach that combines electrophysiology and biochemistry. We find that high-frequency synaptic stimulation (HFS) of the Schaffer collaterals, which induces stable long-term synaptic potentiation, triggers the delivery of glutamate receptor proteins to synaptoneurosomes isolated from CA1, and that low-frequency stimulation (LFS), which induces long-term depression, leads to their removal. Like LTP (Bliss and Collingridge, 1993) and LTD (Bear and Abraham, 1996), these modifications in synaptoneurosomal glutamate receptor levels depend upon activation of NMDARs during conditioning stimulation and are reversible. Thus, synaptic glutamate receptor trafficking is bidirectionally, and reversibly, modified by synaptic activity in the adult brain in vivo. These data demonstrate that changes in glutamate receptor levels accurately reflect both the history of synaptic NMDAR activation and the consequent change in the strength of synaptic transmission and support the idea that long-lasting alterations of glutamate receptor expression at the synapse may serve as a molecular mechanism for memory formation (Lynch and Baudry, 1984).

Results

Redistribution of Glutamate Receptors in Synaptoneurosomes Following Induction of LTP

To test the hypothesis that regulation of glutamate receptor availability at the synapse contributes to long-term modifications of synaptic efficacy in the adult hippocampus in vivo, biochemical analysis of synaptic proteins was performed following manipulation of synaptic strength. Adult, male Long Evans rats (250–500 g) were anesthetized with sodium pentobarbital, and a stimulating electrode was placed unilaterally in the trajectory of the Schaffer collaterals. A recording electrode was placed 200 μm medial to the stimulating electrode in stratum radiatum of area CA1 to record the strength of evoked extracellular excitatory postsynaptic potentials (field EPSPs). Following a stable baseline recording period, multiple trains of high frequency stimulation (100 Hz; HFS) were delivered over the course of 1 hr (see Experimental Procedures). This conditioning protocol induced stable long-term potentiation (LTP) of the field EPSP slope in CA1 ipsilateral to the stimulating electrode (Figure 1a; 132.3% $\pm$ 5.2% of baseline, p $<$ 0.01, n = 8).

The biochemical consequences of conditioning stimulation in vivo occur in a relatively small volume of hippocampus (Barnes et al., 1994; Thiels et al., 1998); therefore, we restricted our biochemical analysis to a small portion of dorsal hippocampus surrounding the recording and stimulating electrodes. Upon termination of the electrophysiological recordings (5.5 hr following the onset of HFS), a small piece of tissue (3 mm[3], 15 mg wet weight) was dissected from each animal and homogenized in preparation for biochemical analysis. In every case, a comparable block of contralateral hippocampus was also dissected, serving as a yoked within-animal control. Quantitative immunoblotting, performed with the experimenter blind to the physiological
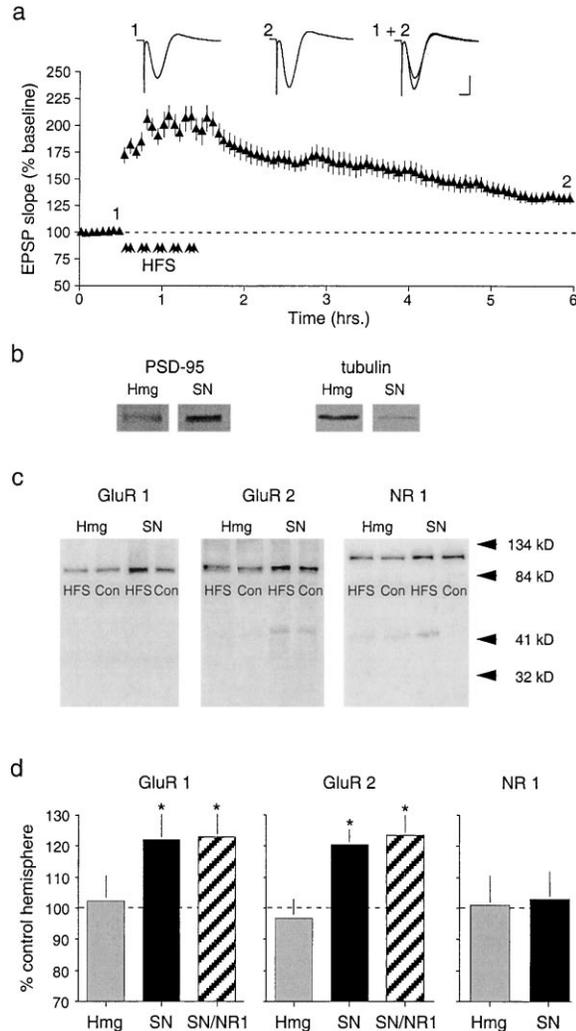

Figure 1. Biochemical Detection of LTP-Associated Increases in Glutamate Receptor Protein Levels in Adult Hippocampus In Vivo Requires Enrichment for Synapses

(a) HFS (five episodes of two 1 s trains of 100 Hz pulses) of the Schaffer collaterals induces long-lasting LTP of the field EPSP slope in area CA1 (n = 8). Field potential traces (average of 20 consecutive sweeps) were obtained from one representative case taken from the times indicated by numerals. Calibration bars in this and subsequent figures: 5 ms, 2 mV.

(b) Biochemical characterization of synaptoneurosomes prepared from adult hippocampus. Representative immunoblots demonstrate an enrichment in the synaptic protein PSD-95 and a decrease in the nonsynaptic protein tubulin in synaptoneurosomes (SN) versus total hippocampal homogenate (Hmg).

(c) Following HFS, immunoblots reveal an increase in the levels of GluR1 and GluR2, but not NR1 proteins, in the stimulated (HFS) versus contralateral control hippocampus (Con) in synaptoneurosomes (SN), but no change in glutamate receptor levels in hippocampal homogenate (Hmg).

(d) Summary of biochemical data for all animals receiving HFS (same animals as in [a]). Following HFS, immunoblot analysis reveals a significant increase in the levels of GluR1 and GluR2, but not NR1 proteins, in synaptoneurosomes (black bars, n = 8), whereas no change in hippocampal homogenate is observed (gray bars, n = 8). A significant increase in GluR1 and GluR2 levels is also observed when expressed as a percentage of NR1 (striped bars, n = 8). *p $<$ 0.05 paired t test versus Con.

stimulation history, was used to measure the levels of GluR1 and GluR2 proteins, the major subunits of AMPARs in hippocampus (Wenthold et al., 1996), and NR1, the obligatory subunit of NMDARs (Mori and Mishina, 1995). Despite the robust expression of LTP, we found no significant differences in the levels of glutamate receptor proteins in homogenates prepared from stimulated versus contralateral control (Con) hippocampi of these animals (Figures 1c and 1d; percent of Con: GluR1 102.5 ± 7.8, $p > 0.05$; GluR2 96.9 ± 6.0, $p > 0.05$; NR1 101.3 ± 10.1, $p > 0.05$).

Although these findings appear to rule out a gross change in glutamate receptor protein levels following induction of LTP, this biochemical preparation is not well suited to resolve changes in the synaptic distribution of glutamate receptors. Therefore, in these same animals, a subset of hippocampal homogenate was further processed into synaptoneurosomes, a subcellular fraction that enriches for synaptic proteins (Hollingsworth et al., 1985; Quinlan et al., 1999a). Biochemical characterization of this preparation confirms that the levels of the synaptic protein PSD-95 are enriched, while the levels of the nonsynaptic protein tubulin are diminished, in synaptoneurosomes compared to hippocampal homogenate (Figure 1b).

Quantitative immunoblotting of synaptoneurosomal proteins now revealed that GluR1 and GluR2 levels were significantly elevated in the hippocampus expressing LTP when compared to the contralateral control hippocampus (Figures 1c and 1d; percent of Con: GluR1 121.8 ± 8.1, $p < 0.05$; GluR2 120.6 ± 4.9, $p < 0.05$). In contrast to the increase in AMPAR subunit proteins, levels of the NMDAR protein NR1 did not differ significantly from control hippocampus (percent of Con: 102.9 ± 9.0, $p > 0.05$). Because NR1 levels were not changed by HFS, we normalized the GluR1 and GluR2 data obtained in each hippocampal sample to the corresponding NR1 value. This procedure yielded the same statistically significant increase in GluR1 and GluR2 in the hippocampus expressing LTP compared to control (percent of Con: GluR1 123.7 ± 11.9, $p < 0.05$; GluR2 122.8 ± 10.8, $p < 0.05$).

These experiments show that activity-induced alterations in synaptic glutamate receptor protein levels are revealed when immunoblot analysis is performed following biochemical enrichment for synapses. In addition, these data suggest that long-lasting increases in synaptic strength in the adult hippocampus in vivo are accompanied by a significant increase in the levels of synaptic AMPAR proteins.

Redistribution of Glutamate Receptors in Synaptoneurosomes Following Induction of LTD

The strength of Schaffer collateral synapses is known to be bidirectionally modifiable (Dudek and Bear, 1993; Mulkey et al., 1993; Heynen et al., 1996), and electrophysiological studies suggest that homosynaptic LTD is the functional inverse of LTP (Dudek and Bear, 1993; Stevens and Wang, 1994; Heynen et al., 1996; Oliet et al., 1996). Therefore, we hypothesized that the biochemical changes associated with the maintenance of LTD would be the opposite of those associated with LTP—namely,

that LTD would be accompanied by a decrease in AMPAR protein levels. In order to test this hypothesis, we induced saturating and stable LTD by applying two episodes of low frequency stimulation (LFS; 900 pulses at 1 Hz separated by 30 min) to the Schaffer collaterals (Figure 2a, $n = 6$). The change in field EPSP slope postconditioning stimulation was $71\% \pm 4\%$ of baseline ($p < 0.05$). Once again, upon termination of the electrophysiological recording sessions (7 hr following the onset of LFS), small pieces of dorsal hippocampus from the stimulated and contralateral control hippocampus were homogenized for immunoblot analysis of glutamate receptor protein levels. The analysis of hippocampal homogenates revealed no significant differences in the levels of GluR1, GluR2, or NR1 proteins in the stimulated versus contralateral control hippocampus (Figures 2b and 2c; percent of Con: GluR1 94.1 ± 4.9, $p > 0.05$; GluR2 111.2 ± 9.6, $p > 0.05$, NR1 94.5 ± 8.5, $p > 0.05$).

As described above, we further refined our biochemical analysis by processing the homogenate into synaptoneurosomes. Now, quantitative immunoblotting of synaptoneurosomal proteins demonstrated that GluR1 and GluR2 levels were significantly decreased in the hippocampus that expressed LTD compared to the contralateral control hippocampus (Figures 2b and 2c; percent of Con: GluR1 83.8 ± 6.5, $p < 0.05$; GluR2 80.7 ± 6.5, $p < 0.05$). The LFS-induced decrease in GluR1 protein was also confirmed using antibodies directed against the extracellular N terminus (data not shown), indicating that the change in AMPAR subunit protein levels was not due to proteolytic cleavage of the intracellular C terminus (Bi et al., 1998).

Interestingly, LFS also resulted in a significant decrease in the level of NR1 protein in synaptoneurosomes (percent of Con: 75.1 ± 8.0, $p < 0.05$). This latter finding was somewhat unexpected and raised the possibility that LFS might induce a nonspecific reduction in total synaptic protein levels. To test this hypothesis, we examined the levels of the cytoskeletal protein actin, which is present in postsynaptic elements. This analysis revealed no significant differences in the levels of synaptoneurosomal actin between stimulated and control hippocampus (percent of Con: 96.1 ± 6.1, $p > 0.05$). Because actin levels were not changed by LFS, we normalized the GluR protein levels obtained from each sample to the corresponding actin value. This procedure yielded the same, statistically significant decrease in GluR1, GluR2, and NR1 in the hippocampus expressing LTD compared to control (Figure 2c; percent of Con: GluR1 82.0 ± 8.1, $p < 0.05$; GluR2 77.1 ± 3.3, $p < 0.05$; NR1 71.2 ± 4.7, $p < 0.05$).

Activity-Dependent Redistribution of Glutamate Receptor Proteins Requires NMDAR Activation

Induction of LTP and LTD in the adult hippocampus in vivo requires activation of NMDARs during conditioning stimulation (e.g., Abraham and Mason, 1988; Heynen et al., 1996; Thiels et al., 1998). To investigate whether the HFS-induced increase in glutamate receptor protein levels is also dependent upon NMDAR activation, HFS was applied following administration of the competitive NMDA receptor antagonist CPP [(±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; 10 mg/kg i.p.]. As
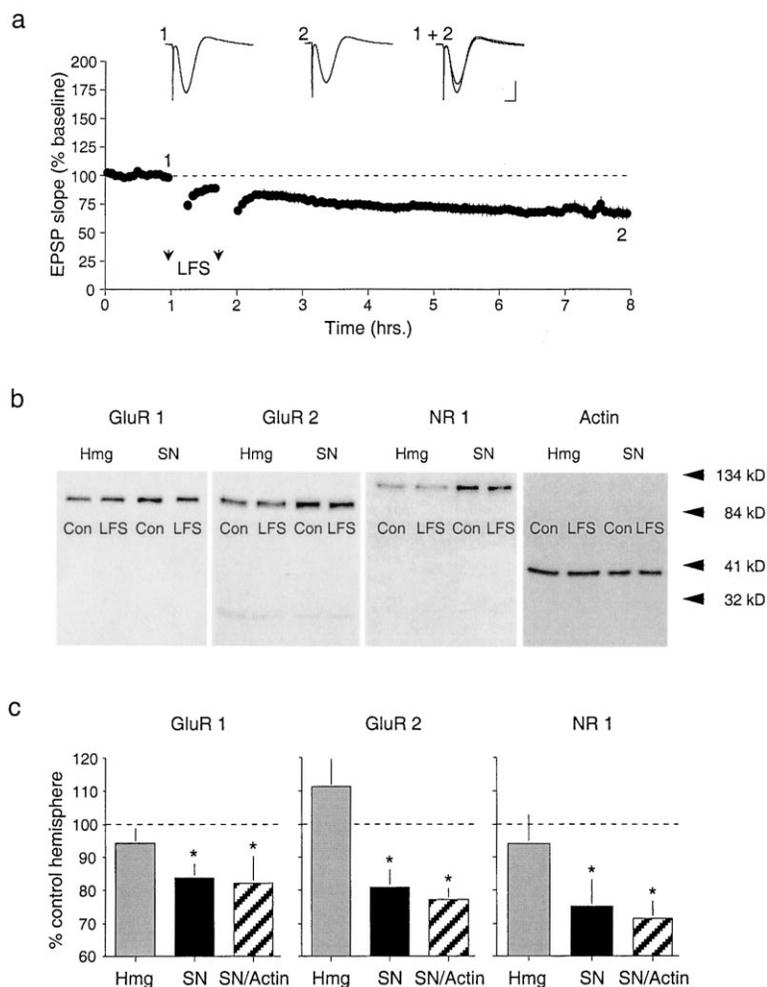
Figure 2. Biochemical Detection of LTD-Associated Decreases in Glutamate Receptor Protein Levels in Adult Hippocampus In Vivo Requires Enrichment for Synapses

(a) LFS (two episodes of 1 Hz, 900 pulses) of the Schaffer collaterals induces stable LTD of the field EPSP slope in area CA1 (n = 6). Field potential traces (average of 20 consecutive sweeps) were obtained from one representative case taken from the times indicated by numerals.
(b) Following LFS, immunoblots reveal a decrease in the levels of GluR1, GluR2, and NR1 proteins in the stimulated (LFS) versus contralateral control hippocampus (Con) in synaptoneurosomes (SN) but no change in glutamate receptor levels in hippocampal homogenate (Hmg).
(c) Summary of biochemical data for all animals receiving LFS (same animals as in [a]). Following LFS, immunoblot analysis reveals a significant decrease in the levels of GluR1, GluR2, and NR1 proteins in synaptoneurosomes (black bars, n = 6), but no change in total hippocampal homogenate (gray bars, n = 6). A significant decrease in glutamate receptor protein levels is also observed when expressed as a percentage of actin (striped bars, n = 6). *p < 0.05 paired t test versus Con.

expected, CPP-treated animals failed to show LTP following HFS (Figure 3b; 94.7% ± 5.3% of baseline, p > 0.05, n = 7), whereas HFS induced a significant increase in synaptic strength in interleaved, untreated control animals (Figures 3a and 3b; 130.6% ± 4.3% of baseline, p < 0.05; n = 8). Pretreatment with CPP also blocked the HFS-induced increase in synaptoneurosomal GluR1 and GluR2 levels (Figure 3c; percent of Con: GluR1 104.1 ± 5.5, p > 0.05; GluR2 93.6 ± 12.0, p > 0.05; NR1 92.6 ± 9.5, p > 0.05). In interleaved control animals, again we observed an HFS-induced increase in the levels of synaptoneurosomal GluR1 and GluR2 (Figure 3c; percent of Con: GluR1 118.0 ± 5.7, p < 0.05; GluR2 115.6 ± 4.2, p < 0.05; NR1 108.0 ± 6.7, p > 0.05), which persisted when the data were normalized to actin protein levels (GluR1 116.7 ± 9.5, p < 0.05; GluR2 124.5 ± 9.1, p < 0.05; NR1 109.2 ± 8.9, p > 0.05). Thus, like the induction of LTP, the activity-dependent delivery of AMPAR proteins to the synaptoneurosomal fraction requires NMDAR activation.

We next investigated the NMDAR dependence of the LFS-induced decrease in glutamate receptor protein levels. As expected from previous work (Thiels et al., 1994; Heynen et al., 1996; Manahan-Vaughan, 1997), CPP-treated animals failed to show significant LTD following LFS (Figure 4b; field EPSP slope 93.8% ± 4.7%

of baseline p > 0.5; n = 5), whereas LFS induced a significant decrease in synaptic strength in interleaved, untreated control animals (Figures 4a and 4b; field EPSP slope 68.7% ± 6.2% of baseline; p < 0.05; n = 7). No significant decrease in synaptoneurosomal GluR1, GluR2, and NR1 protein levels was observed following LFS in animals pretreated with CPP (Figure 4c; percent of Con: GluR1 108.6 ± 5.8, p > 0.05; GluR2 98.4 ± 3, p > 0.05; NR1 109.3 ± 3.6, p > 0.05), whereas the decrease in glutamate receptor proteins was again observed in interleaved control animals (Figure 4c; percent of Con: GluR1 76.5 ± 7.4, p < 0.05; GluR2 78.0 ± 6.6, p < 0.05; NR1 71.8 ± 9.2, p < 0.05). The significant decrease in glutamate receptor protein levels persisted when these data were normalized to actin (GluR1 78.1 ± 7.9, p < 0.05; GluR2 81.2 ± 7.1, p < 0.05; NR1 76.6 ± 5.7, p < 0.05). Thus, like the induction of LTD, activity-dependent removal of glutamate receptor proteins from the synaptoneurosomal fraction requires NMDAR activation.

Activity-Dependent Changes in Glutamate Receptors and Synaptic Strength Are Reversible
LTP and LTD have been shown to be reversible modifications of synaptic transmission. Therefore, we next examined whether reversing LTP and LTD was accom-
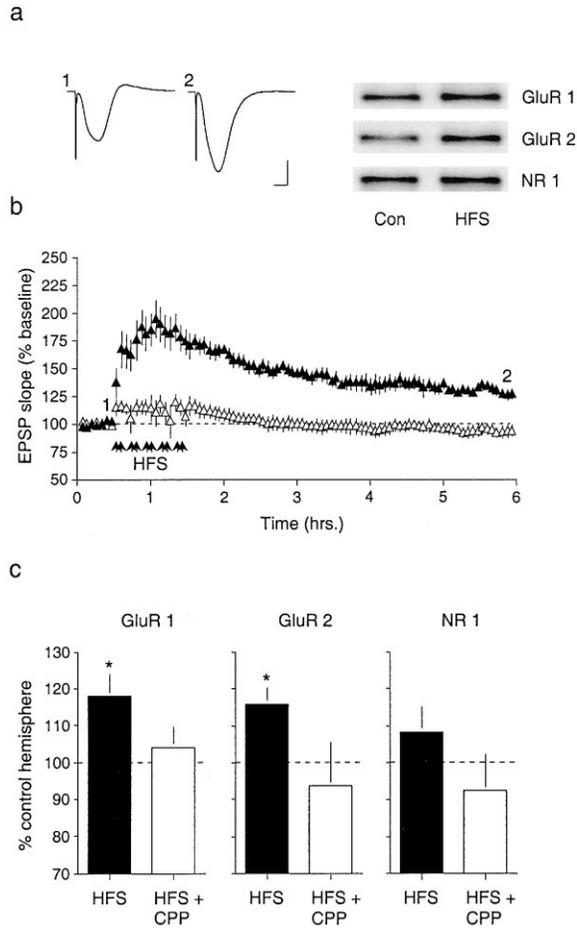
Figure 3. Activation of NMDARs Is Required for HFS-Induced Increases in Synaptic Strength and Synaptoneurosomal AMPAR Protein Levels

(a) Field potential traces (average of 20 consecutive sweeps; taken from times indicated by numerals in [b]) and immunoblots were obtained from one representative case in which HFS is delivered in the absence of the NMDA receptor antagonist CPP.
(b) Summary of electrophysiological data for animals receiving HFS (five episodes of two 1 s trains of 100 Hz pulses) in the absence (closed triangles, n = 8) and presence (open triangles, n = 7) of CPP (10 mg/kg i.p., 30 min prior to onset of recording). Note that LTP is blocked when HFS is delivered in the presence of the NMDA receptor antagonist CPP, whereas stable LTP is observed in interleaved, untreated control animals.
(c) Summary of biochemical data for animals receiving HFS in the absence and presence of CPP (same animals as in [b]). Following HFS, a significant increase in the levels of GluR1 and GluR2, but not NR1 proteins, was observed in stimulated relative to contralateral control hippocampus (closed bars). The HFS-induced increase in AMPAR protein levels is blocked when HFS is delivered in the presence of CPP (open bars). *p < 0.05 paired t test versus Con.
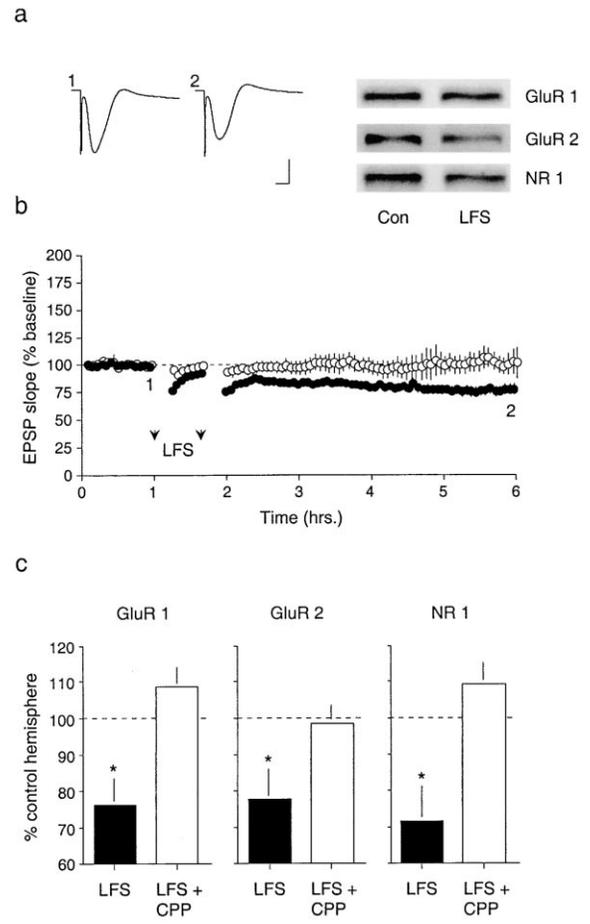
Figure 4. Activation of NMDARs Is Required for LFS-Induced Decreases in Synaptic Strength and Synaptoneurosomal Glutamate Receptor Protein Levels

(a) Field potential traces (average of 20 consecutive sweeps; taken from times indicated by numerals in [b]) and immunoblots obtained from one representative case in which LFS is delivered in the absence of the NMDA receptor antagonist CPP.
(b) Summary of electrophysiological data for animals receiving LFS in the absence (closed circles, n = 7) and presence (open circles, n = 5) of CPP. LTD is blocked when LFS is delivered in the presence of the NMDA receptor antagonist CPP, whereas stable LTD is observed in interleaved, untreated control animals.
(c) Summary of biochemical data for animals receiving LFS in the absence and presence of CPP (same animals as in [b]). Following LFS, a significant decrease in the levels of GluR1, GluR2, and NR1 proteins was observed in stimulated relative to contralateral control hippocampus (closed bars). The LFS-induced decrease in glutamate receptor protein levels is blocked when LFS is delivered in the presence of CPP (open bars). *p < 0.05 paired t test versus Con.

panied by a concomitant reversal of glutamate receptor protein changes.

To determine if the LFS-induced decrease in synaptoneurosomal glutamate receptor levels are prevented or reversed by subsequent HFS, stable LTD was induced using the conditioning protocol described above. One hour following LFS, HFS was administered to the synaptically depressed pathway. This intervening conditioning stimulation completely reversed LTD (Figures 5a and

5b; field EPSP slope 102.0% ± 3.7% of baseline, p > 0.05, n = 5). In these same animals, we did not observe a significant difference in the levels of glutamate receptor proteins in stimulated versus control hippocampus 5 hr after the onset of conditioning stimulation (Figure 5c; percent of Con: GluR1 116.8 ± 12.7, p > 0.05; GluR2 102.8 ± 9.7, p > 0.05; NR1 105.1 ± 12.9, p > 0.05). Thus, the intervening HFS prevented expression of both LTD and the decrease in synaptoneurosomal glutamate receptor levels expected following LFS (Figures 2 and 4). Moreover, compared to LFS-only animals, glutamate
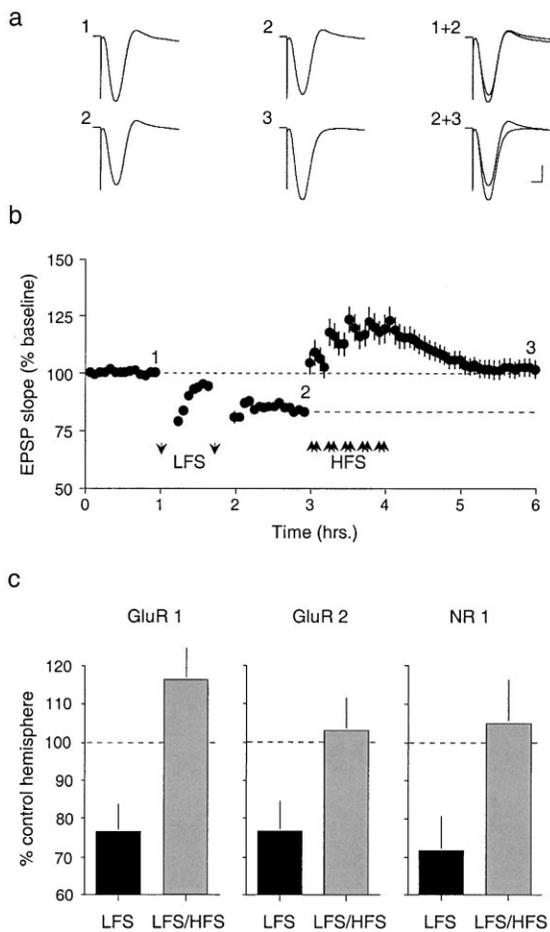
Figure 5. LFS-Induced Decrease in Synaptic Strength and Synapto-neurosomal Glutamate Receptor Proteins Are Reversed by Intervening HFS

(a) Representative field potentials from an animal in which, following the induction of stable LTD, subsequent application of HFS fully reversed the decrease in field EPSP slope. Traces of field potentials (average of 20 consecutive sweeps) were taken at times indicated by numerals in (b).

(b) Summary of electrophysiological data for all animals receiving HFS 1 hr following LTD induction (n = 5).

(c) LFS-induced decrease in glutamate receptor protein levels (black bars; same data as in Figure 4c) are prevented by subsequent application of HFS (gray bars, same animals as in [b]).

Figure 6. HFS-Induced Increase in Synaptic Strength and Synapto-neurosomal Glutamate Receptor Proteins Are Reversed by Intervening LFS

(a) Representative field potentials from an animal in which, following the induction of stable LTP, subsequent application of LFS fully reversed the increase in field EPSP slope. Traces of field potentials (average of 20 consecutive sweeps) were taken at times indicated by numerals in (b).

(b) Summary of electrophysiological data for all animals receiving LFS 1 hr following LTP induction (n = 7). Asterisk denotes a significant decrease in field EPSP slope as compared to original pre-HFS baseline.

(c) HFS-induced increase in glutamate receptor protein levels (black bars; same data as in Figure 3c) are prevented by subsequent application of LFS (gray bars, same animals as in [b]). *$p < 0.05$ paired t test versus Con.

receptor protein levels were elevated when LFS was followed by HFS [ANOVA; $F_{(5,29)} = 3.73$, $p < 0.01$; GluR1 $p < 0.05$, Bonferroni planned post hoc comparison].

To investigate whether the HFS-induced increase in synaptoneurosomal AMPAR levels are prevented or reversed by subsequent LFS, LTP was induced as described above. One hour following HFS, LFS was administered to the synaptically potentiated pathway, which resulted in a complete reversal of LTP and significant additional LTD relative to baseline (Figures 6a and 6b; field EPSP slope 83.0% ± 7.3% of baseline, $p < 0.05$, n = 5). Correlated with this decrease in synaptic strength, we found that the levels of GluR1, GluR2, and NR1 proteins were all significantly reduced in stimulated versus control hippocampus 5.5 hr following the onset of conditioning stimulation (Figure 6c; percent of Con:
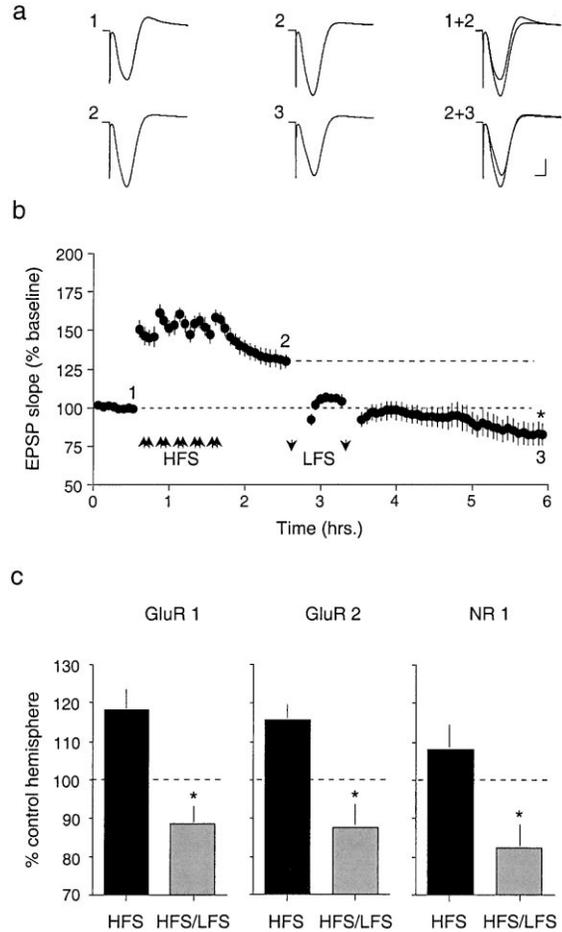
GluR1 88.5 ± 5.3, $p < 0.05$; GluR2 87.8 ± 8.0, $p < 0.05$; NR1 82.2 ± 7.1, $p < 0.05$). Moreover, compared to HFS-only animals, levels of GluR1, GluR2, and NR1 were all significantly decreased when HFS was followed by LFS [ANOVA; $F_{(5,39)} = 6.30$, $p < 0.001$, Bonferroni planned post hoc comparison, $p < 0.05$].

Changes in Synaptic Strength Correlate with Changes in Synaptoneurosomal Glutamate Receptor Levels

Taken together, the data suggest that changes in synaptic AMPAR levels correlate with changes in synaptic strength. To further examine the degree to which regulation of synaptic transmission can be predicted by
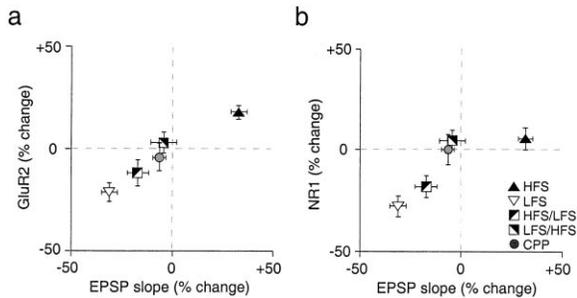
Figure 7. Changes in AMPAR, but Not NMDAR, Protein Levels Predict the Magnitude and Direction of Changes in Synaptic Strength

Scatter plots depicting the relationship between change in synaptic strength (x axis: EPSP slope, percent of preconditioning baseline) and change in synaptoneurosomal GluR 2 (a) and NR1 (b) protein levels (y axis: percent of control hemisphere). Data are averages for each experimental group ± SEM.

changes in the level of glutamate receptor proteins, we performed a hierarchical multiple regression analysis on data derived from all animals from all experimental conditions (n = 51). The stepwise model included GluR2 first (present in the majority of AMPARs), followed by GluR1 and NR1. At each step, the overall fit of the model was assessed, as well as the significance of the improvement as each new protein was included. The relationship between changes in GluR2 protein levels and changes in synaptic transmission was highly significant [R^2 = 0.30, $F(1,49)$ = 21.30, $p < 0.001$]. Inclusion of GluR1 into the model significantly improved the relationship between GluR protein levels and synaptic strength [R^2 (GluR2 + GluR1) = 0.41, $F(2,48)$ = 17.28, $p < 0.001$; R^2 change with addition of GluR1 = 0.12, $F(1,48)$ = 9.55, $p < 0.003$]. However, as expected, addition of NR1 did not significantly improve the relationship [R^2 (GluR2 + GluR1 + NR1) = 0.42, $F(3,47)$ = 11.34, $p < 0.001$; R^2 change with addition of NR1 = 0.010, $F(1,47)$ = 0.75, $p > 0.7$]. The relationship between the change in synaptic strength relative to baseline, and the change in GluR2 and NR1, is plotted in Figure 7 for each of the experimental groups. This graph clearly illustrates that knowledge of the change in the levels of GluR2, but not NR1, is highly predictive of changes in synaptic strength.

Discussion

By combining electrophysiology and synaptic biochemistry analysis, we have obtained evidence that LTP in vivo is associated with the delivery of glutamate receptor proteins to CA1 synapses, while LTD is associated with their removal. Like LTP and LTD, the changes in glutamate receptors depend on NMDAR activation during conditioning stimulation and are reversible. Although LTP results in an increase in synaptoneurosomal glutamate receptor protein levels, while LTD results in a decrease, these changes are not perfectly symmetric. LTP de novo correlates with an increase in AMPAR protein in CA1 synaptoneurosomes without a detectable change in NMDAR protein levels, while LTD de novo correlates with a decrease in both AMPAR and NMDAR protein in this biochemical fraction. These data demonstrate, for

the first time, that a bidirectional redistribution of glutamate receptors accompanies bidirectional synaptic plasticity in the adult hippocampus in vivo.

Technical Considerations
Prior to this study, evidence for glutamate receptor changes associated with synaptic plasticity in vivo was scarce and confined to studies of dentate gyrus (Maren et al., 1993; Thomas et al., 1996; Williams et al., 1998). Detection of biochemical changes using LTP/D paradigms is challenging because only a subset of synapses is affected by the stimulation. An important feature of our experimental design was the use of synaptoneurosomes. Activity-dependent changes in glutamate receptor protein levels were not detected in crude homogenates of our hippocampal samples. Subcellular fractionation was necessary, and synaptoneurosomes were sufficient, to detect reliable changes. Synaptoneurosomes are a preparation designed to preserve metabolically active, resealed pre- and postsynaptic compartments of synapses (Hollingsworth et al., 1985). Further purification of the synaptic membrane in our hippocampal synaptoneurosomes was unnecessary to reveal activity-dependent changes in glutamate receptor levels.

A second important feature of our experimental design was the use of yoked, within-animal controls that were immunoblotted in parallel with experimental tissue. This procedure enabled the use of a pairwise statistical comparison of the optical densities of immunoreactive bands reflecting the levels of glutamate receptor protein present in stimulated and control hippocampal tissue samples. To aid in the detection of small amounts of protein, enhanced chemiluminescence was used; however, we were careful to work in the linear range of the assay, such that differences in immunoblot intensity were proportional to differences in protein concentration. To eliminate the possibility that experimenter bias (e.g., a systematic difference in protein loading) contributed to our results, all samples were run and analyzed "blind" without experimenter knowledge of the stimulation history.

A third important feature of the design was the use of six different experimental manipulations to bidirectionally, and reversibly, modify synaptic transmission. The power of this approach is demonstrated by the significant linear relationship between changes in AMPAR proteins and changes in synaptic transmission (Figure 7).

**Functional Significance of Changes
in Synaptoneurosomal Glutamate
Receptor Expression**
Electron microscopy reveals that synaptoneurosomes resemble isolated glutamatergic synapses located on dendritic spines, with resealed pre- and postsynaptic compartments (Hollingsworth et al., 1985; Quinlan et al., 1999a). GluR1, GluR2 (Hampson et al., 1992), and NR1 (Petralia et al., 1994) are not observed in presynaptic elements. A change in synaptoneurosomal glutamate receptors, therefore, likely reflects an alteration in receptor availability in dendritic spines.

We find that NMDAR activation during HFS and LFS

produce symmetric changes in both synaptic strength in vivo and in the levels of GluR1 and GluR2 proteins measured ex vivo in synaptoneurosomes. Because the majority of AMPARs in the adult hippocampus contain both GluR1 and GluR2 subunits (Wenthold et al., 1996), these results suggest that the level of AMPARs within the synaptoneurosome fraction, like the efficacy of synaptic transmission, is bidirectionally modifiable by HFS and LFS. Our study therefore supports the idea that NMDAR activation during HFS results in the delivery of AMPARs to spines, while NMDAR activation during LFS results in AMPAR removal.

These activity-dependent alterations in AMPAR localization are likely to directly impact synaptic transmission. In visual cortex, for example, small changes in synaptoneurosomal glutamate receptor protein have been shown to be highly predictive of changes in synaptic transmission (Quinlan et al., 1999b). Moreover, recent work on hippocampal neurons in vitro has provided compelling support for the idea that AMPARs are inserted into the postsynaptic membrane following induction of LTP (Shi et al., 1999; Hayashi et al., 2000) and that AMPARs are removed following induction of LTD (Carroll et al., 1999; Luthi et al., 1999; Man et al., 2000). We suggest that very similar changes occur in the adult hippocampus in vivo and that they are detectable as alterations in the level of AMPAR protein in synaptoneurosomes.

Although NR1 protein levels were unaffected when LTP was induced de novo, our experiments consistently revealed a decrease in synaptoneurosomal NR1 when synaptic transmission was depressed relative to the initial baseline level. Although this finding would not be anticipated based on work performed recently in cell culture (Carroll et al., 1999) that shows a selective effect on AMPARs after LTD-inducing stimulation, it is consistent with data obtained in acutely prepared hippocampal slices, which show a parallel decrease in AMPAR- and NMDAR-mediated synaptic transmission after LFS (Xiao et al., 1994; Selig et al., 1995). Our data suggest that synaptic expression of both AMPARs and NMDARs are modified by LFS in vivo.

A novel aspect of the present study concerns the reversibility of the observed changes in glutamate receptor expression in synaptoneurosomes. If LFS is followed by HFS, producing dedepression, then no decrease in GluR1, GluR2, or NR1 levels are observed relative to the control hemisphere. Similarly, if HFS is followed by LFS, producing depotentiation, no increase in GluR1 or GluR2 levels are observed; indeed, GluR1, GluR2, and NR1 are often decreased below control levels, in parallel with the depression of synaptic transmission below the initial baseline value. Our experiments do not allow us to determine if receptor changes were first induced by the initial conditioning stimulation and then reversed by the subsequent stimulation, or whether the subsequent conditioning interfered with the delayed expression of changes set in motion by the initial stimulation. However, assuming that the data reflect a true reversal of glutamate receptor changes, they indicate that the consequences of HFS, in particular, depend on the initial state of the synapse. When HFS is delivered de novo, only AMPAR protein levels are increased; however, when HFS is delivered after prior induction of LTD,

both NMDAR and AMPAR protein levels are increased (relative to LFS-only controls). This asymmetry is consistent with a recent study showing differential phosphorylation of GluR1 by HFS depending on the previous activation history of the synapse (Lee et al., 2000).

Another distinctive feature of our experiments was that the biochemical measurements were made many hours after conditioning stimulation, when the expression of synaptic plasticity was stable. The original rationale for this approach was to increase the likelihood that we could detect changes in glutamate receptors that might require protein synthesis (Nayak et al., 1998). We did not address the question of whether protein synthesis is involved in the changes in vivo, because in pilot studies we found that hippocampal infusion of mRNA translation inhibitors produces instability in baseline synaptic transmission. Nonetheless, the fact that we observe a redistribution of glutamate receptors in the late, stable phase of synaptic plasticity suggests that this reflects a mechanism for long-term maintenance of activity-dependent synaptic modifications.

Conclusion

In neural network models, memories can be encoded in the pattern of synaptic strengths distributed among many neurons. The same synapses can participate in many different memories if their weights can be continually, and bidirectionally, adjusted as new experiences occur (Cooper, 1995). Our results show that glutamate receptor localization, like synaptic strength, can be adjusted by synaptic activity in a highly predictable fashion. This is the first demonstration that synaptic glutamate receptor trafficking is bidirectionally, and reversibly, modified by synaptic activity in the adult brain in vivo. We propose that memories are encoded by the precise experience-dependent assignment of glutamate receptors to synapses in the hippocampal neural network.

Experimental Procedures

Electrophysiology

Adult, male Long Evans rats (250–500 g) were anesthetized with sodium pentobarbital (65 mg/kg, i.p.), tracheotomized, and then placed in a stereotaxic frame. Animals were artificially ventilated (100% O_2, 40 bpm) and maintained at 37°C $\pm$ 0.5°C. Anesthesia was maintained by continuous administration of sodium pentobarbital (6–10 mg/hr) through an intraperitoneal catheter. A monopolar recording electrode was positioned in the CA1 stratum radiatum of the hippocampus of one hemisphere (coordinates from bregma and the midline: 3.6 mm posterior; 2.3–2.5 mm lateral). A monopolar stimulating electrode was positioned approximately 0.2 mm lateral to the recording electrode to activate the ipsilateral Schaffer collaterals. All electrodes were constructed from Teflon-insulated stainless steel wire (75 μm) cut flat at the tip. Final depths of recording and stimulating electrodes were adjusted to optimize the magnitude of the evoked response. Screws inserted into the skull overlying the cerebellum and frontal cortex served as recording ground and stimulus anode, respectively. Field EPSPs were elicited using stimuli of 0.2 ms duration. Evoked responses were amplified and filtered at 0.1 and 3.0 kHz (1/2 amplitude), digitized at 160 kHz, and stored on a computer. The initial slope of the field EPSP was used as a measure of the magnitude of the response. At the beginning of each experiment a full input/output curve was generated, and a stimulus strength eliciting an EPSP slope which equaled 50%–60% of maximum was used for the remainder of the experiment (20–55 μA). Baseline measurements were collected using single stimuli applied

every 30 s. Saturating, long-lasting LTP was induced by two 1 s trains of 100 Hz separated by 30 s, applied every 15 min for 1 hr, for a total of ten trains. Saturating, long-lasting LTD was induced using LFS, consisting of two episodes of 900 pulses delivered at 1 Hz, separated by 30 min. Simultaneous recordings from CA1 in the contralateral hemisphere confirmed that the synaptic modifications elicited by conditioning stimulation were confined primarily to the hippocampus ipsilateral to the stimulating electrode. Electrophysiological data are expressed as a percentage of the mean response magnitude recorded during the baseline period.

Synaptoneurosome Preparation

Tissue was harvested for biochemical analysis 5–7 hr following the onset of conditioning stimulation, and synaptoneurosomes were prepared as previously described (Quinlan et al., 1999a, 1999b). Tissue blocks were collected in ice-cold dissection buffer containing (in mM: sucrose, 212.7; KCl, 2.6; NaH_2PO_4, 1.23; $NaHCO_3$, 26; dextrose, 10; $MgCl_2$, 1.0; $CaCl_2$, 0.5; CNQX, 0.02; AP5, 0.1; saturated with 95% O_2 and 5% CO_2). One block of tissue encompassed the recording and stimulation sites in the experimental hemisphere; the other block was from the homotopic region of the contralateral hemisphere and served as the internal control. The hippocampi were immediately homogenized in ice-cold homogenization buffer (10 mM HEPES, 1.0 mM EDTA, 2.0 mM EGTA, 0.5 mM DTT, 0.1 mM PMSF, 10 mg/l leupeptin, 50 mg/l soybean trypsin inhibitor, 100 nM microcystin) in a glass/glass tissue homogenizer (Kontes, Vineland, NJ). Synaptoneurosomes were prepared from homogenate by sequential filtration through two 100 μm pore nylon mesh filters, followed by a 5 μm pore filter and centrifugation at 1000 × g for 10 min at 4°C. The resulting pellets (synaptoneurosomes) were resuspended in boiling 1% SDS and stored at −80°C.

Quantitative Immunoblotting

Equal concentrations of hippocampal homogenate or synaptoneurosomal proteins (12.5 μg/sample) were resolved on polyacrylamide gels, transferred to nitrocellulose, and probed with either an anti-GluR1 (anti-C-terminal, 1:1000 Upstate Biotechnology; anti-N-terminal 1:100 Chemicon), an anti-GluR2 (1:200 Calbiochem), anti-NR1 (1:1000, clone 54.1 Pharmingen), or anti-actin (1:1000, clone 1501 Chemicon) antibody, followed by the appropriate secondary antibody coupled to horseradish peroxidase (1:3500, Sigma Immunochemicals) in Tris-buffered saline (pH 7.3) containing 1% bovine serum albumin and 0.1% Triton X-100. Visualization of immunoreactive bands was induced by enhanced chemiluminescence (Amersham ECL) captured on autoradiography film (Amersham Hyperfilm ECL). Construction of a standard curve, in which the amount of synaptoneurosomal protein is varied systematically, revealed that we operate within the linear range of the ECL detection method (see supplemental addendum at http://www.neuron.org/cgi/content/full/28/2/527/DC1). In addition, each immunoblot was exposed to autoradiography film multiple times (average 5; minimum 3), for varying durations, to ensure that we operate in the linear range of the ECL film for each experiment. Digital images, produced by densitometric scans of autoradiographs on a ScanJet IIcx (Hewlett Packard) with DeskScan II software (Hewlett Packard), were quantified using NIH Image 1.60 software. The optical density (OD) of each immunoreactive band from samples of hippocampus receiving conditioning stimulation, relative to a baseline immediately above and below the band within the same lane, was compared to the OD of the corresponding band from samples of the yoked, contralateral control hippocampus. Our preliminary control experiments revealed that, in the absence of conditioning stimulation, the total levels of glutamate receptor proteins is comparable in right versus left dorsal hippocampus. Group data are represented as the mean ± SEM of the optical density of samples from stimulated hippocampus, normalized to the within-animal control hippocampus. With the exception of the initial pilot experiments, all immunoblots and densitometry were performed with the experimenter blind to the physiological stimulation history.

Statistical Analyses

For electrophysiological experiments, the magnitude of the field EPSP slope obtained during the last 5 min of the baseline recording period was compared to the final 5 min postconditioning stimulation using paired two-tailed t tests. For immunoblot analysis, GluR protein levels in the hippocampus receiving conditioning stimulation were compared to GluR levels of the contralateral control hippocampus (Con) from the same animal run on the same gel. Within-group comparisons were performed using paired two-tailed t tests, differences between groups were compared using ANOVA with Bonferroni planned post hoc comparisons. Differences were considered significant when $p < 0.05$. For display purposes, GluR protein levels for the hippocampus receiving conditioning stimulation are expressed as a percentage of contralateral control hippocampus.

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