Learning Induces Long-Term Potentiation in the Hippocampus

Jonathan R. Whitlock,[1,2]* Arnold J. Heynen,[1]* Marshall G. Shuler,[1] Mark F. Bear[1]†

Years of intensive investigation have yielded a sophisticated understanding of long-term potentiation (LTP) induced in hippocampal area CA1 by high-frequency stimulation (HFS). These efforts have been motivated by the belief that similar synaptic modifications occur during memory formation, but it has never been shown that learning actually induces LTP in CA1. We found that one-trial inhibitory avoidance learning in rats produced the same changes in hippocampal glutamate receptors as induction of LTP with HFS and caused a spatially restricted increase in the amplitude of evoked synaptic transmission in CA1 in vivo. Because the learning-induced synaptic potentiation occluded HFS-induced LTP, we conclude that inhibitory avoidance training induces LTP in CA1.

The phenomenon of LTP, discovered over 30 years ago in the hippocampus (*1*, *2*), has attracted enormous attention. Literally thousands of papers have been published on hippocampal LTP, all predicated on the assumption that LTP reveals an important mechanism for memory in the brain. Remarkably, however, there still has not been a direct demonstration that hippocampal LTP is actually induced by learning.

There may be several reasons why learning-induced LTP has been difficult to demonstrate in hippocampus (*3*). First, many hippocampally dependent learning tasks are iterative and so require many training trials to form a memory. Slight differences in the rate of learning across animals could smear and, therefore, obscure time-sensitive markers of LTP when averaged together. Second, the synaptic changes that underlie hippocampally dependent learning may be sparse and widely distributed, which would make potentiated synapses difficult to detect in a vast sea of unmodified connections. Third, it is now appreciated that information can be stored effectively by long-term depression (LTD), as well as by LTP (*4*). Simultaneous induction of LTP and LTD at different synapses may cancel one another when studied at a population level.

The first two problems were overcome by use of the inhibitory avoidance (IA) paradigm. IA training creates a stable memory trace in a single trial and causes substantial changes in gene expression in area CA1 of dorsal hippocampus, which suggests that this is a site of robust synaptic plasticity (*5*–*7*). The problem of simultaneous bidirectional modifications was overcome by the use of new biomarkers that can detect LTP and LTD occurring at different synapses (*8*–*10*).

Hippocampal glutamate receptors are phosphorylated after IA training. IA memory is rapidly acquired, very stable, and dependent on the hippocampus (*11*–*13*). Training consisted of allowing rats to cross from an illuminated chamber into a dark chamber where a foot shock was delivered (*14*). Memory of this experience was measured as the tendency for the animals to avoid the dark side in subsequent trials (fig. S1A). Acquisition of the avoidance response, like the phenomena of LTP and LTD, required *N*-methyl-D-aspartate (NMDA) receptor activation (fig. S1B). Control cohorts either entered the dark side without receiving a shock (i.e., "walk-through" controls) or were given a foot shock in the dark side and immediately removed from the apparatus before they could form the context-shock association (*15*, *16*) (i.e., "shock-only" controls).

The C terminus of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) GluR1 subunit undergoes distinct phosphorylation and dephosphorylation events following LTP and LTD (*8*). Specifically, serine 831 (Ser^{831}) is phosphorylated after LTP, and Ser^{845} is dephosphorylated after LTD. These changes in phosphorylation alter the function of the AMPAR and contribute to expression of LTP and LTD. More importantly for our purposes, they "mark" synapses as having recently undergone modification and can be used to detect the occurrence of LTP and LTD, even if they occur simultaneously at different synapses.

Therefore, we dissected out the dorsal hippocampus of trained and control animals and assayed the phosphorylation state of GluR1 at Ser^{831} and Ser^{845}. Important features of our experimental design for this and subsequent biochemical experiments included the use of yoked controls (trained versus walk-through, and shocked versus naïve); particular care to be in the linear range of the immunoblot assays to detect small changes; and quantitative analysis of the immunoblots without experimenter knowledge of the experimental condition (*14*).

Although phosphorylation at Ser^{845} did not differ in trained versus walk-through animals [98.3 ± 7.7% of controls; *t* test, $P > 0.05$ (Fig. 1A)], we found an increase in phosphorylation at Ser^{831} 30 min following IA training [126.7 ± 9.4% of controls; *t* test, $P = 0.005$ (Fig. 1B)]. In contrast, no changes in phosphorylation of either site were observed in the shock-only group relative to nonshocked controls. In addition, neither Ser^{831} nor Ser^{845} phosphorylation differed between trained and control animals in samples prepared from the cerebellum.

To see if training-induced phosphorylation of Ser^{831}, like LTP and memory, requires NMDA receptor activation, additional groups of animals were injected intraperitoneally (i.p.) with (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; 10 mg/kg, i.p.) or saline before training. No increase in Ser^{831} phosphorylation was observed in the CPP group (92.5 ± 9.9% of controls; *t* test, $P > 0.05$), but the increase was replicated in animals given pretraining injections of saline [120.7 ± 5.7% of controls; *t* test, $P < 0.025$ (Fig. 1C)]. A time-course analysis determined that the enhancement in Ser^{831} phosphorylation peaked 30 min posttraining, but was indistinguishable from controls by 2 hours posttraining (Fig. 1G).

IA training causes delivery of AMPARs to hippocampal synaptoneurosomes. LTP is associated with the delivery of AMPARs to synapses, and LTD is associated with the removal of AMPARs from synapses (*10*). Because we saw an enhancement in phosphorylation at Ser^{831} and no decrease in phosphorylation at Ser^{845}, we hypothesized that IA training was associated with a net increase in AMPARs at synapses. Therefore, we performed immunoblot analysis of the synaptoneurosome (SNS) biochemical fraction. The SNS preparation provides a modest enrichment for synaptic proteins that is sufficient to detect trafficking of AMPARs after LTP in area CA1 of adult rats in vivo (*10*, *14*).

We found that IA-trained animals showed significantly elevated GluR1 and GluR2 protein levels in the SNS fraction relative to walk-through cohorts 30 min after conditioning [GluR1, 129.9 ± 4.9% of controls; *t* test, $P < 0.002$; GluR2, 128.1 ± 8.2% of controls; *t* test, $P < 0.04$ (Fig. 1, D and E)]. This effect was specific to AMPAR-type glutamate receptors, as we did not detect an increase in the protein levels for the NR1 subunit of NMDA receptors [102.7 ± 9.1% of controls; *t* test, $P > 0.05$ (Fig. 1F)]. Similar to the enhancement in phosphorylation at Ser^{831}, these effects were rapid and transient (Fig. 1, H and I). It is noteworthy that the increases in AMPAR protein levels were specific to associative learning (shock-only samples were 96.7 ± 10.4% of nonshocked controls; for GluR1; 93.5 ± 17.8% of controls for GluR2; *t* test, $P > 0.05$, in both cases). We did not detect

[1]Howard Hughes Medical Institute, The Picower Institute for Learning and Memory, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [2]Department of Neuroscience, Brown University, Providence, RI 02912, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed: mbear@mit.edu

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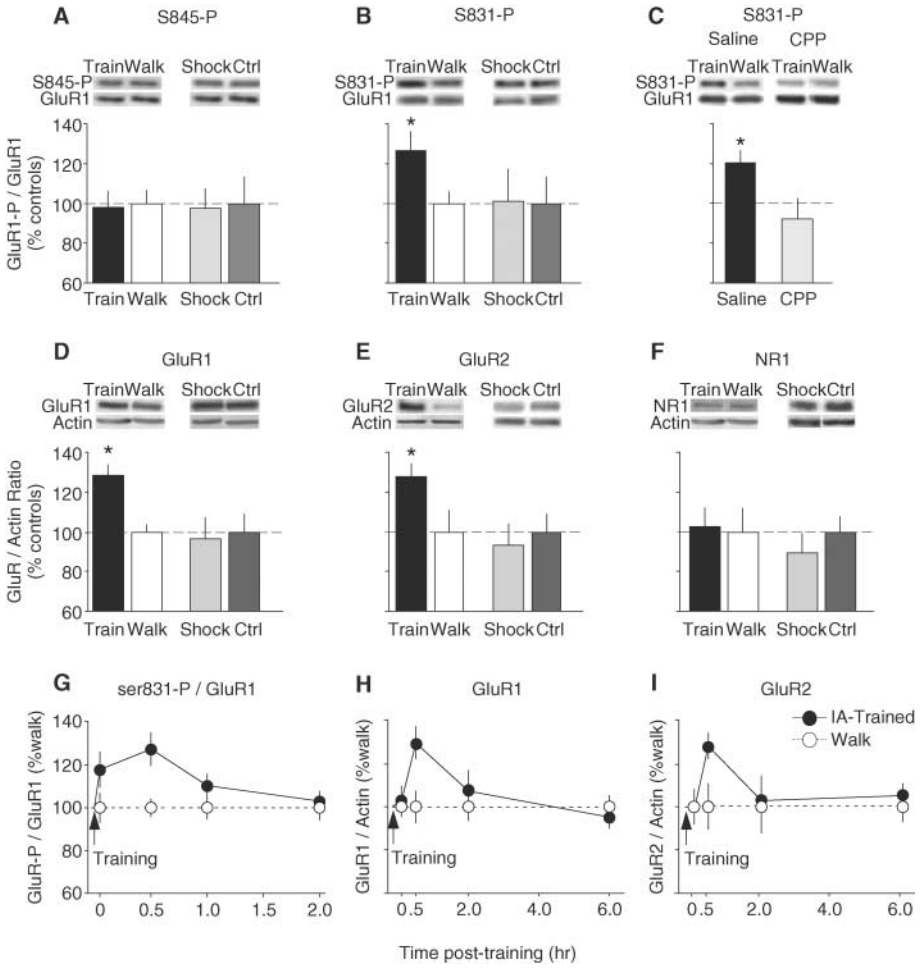

Fig. 1. IA training alters AMPAR phosphorylation and trafficking in the hippocampus. (**A**) Phosphorylation of Ser^{845} in trained animals (black bars, $n = 18$) and shock-only animals (light gray bars, $n = 13$) did not differ from yoked controls (white and dark gray bars, respectively) 30 min after conditioning, whereas (**B**) phosphorylation at Ser^{831} was elevated significantly in trained animals ($n = 14$), but not in shock-only controls ($n = 13$). Error bars indicate SEM in this and all subsequent figures. (**C**) The enhancement in Ser^{831} phosphorylation persisted in animals given pretraining injections of saline (black bar, $n = 18$), but was blocked by pretraining injections of CPP (light gray bar, $n = 19$). (**D** and **E**) Additionally, an increase in GluR1 and GluR2 protein levels in SNS was observed 30 min after IA training ($n = 11$ for GluR1; $n = 12$ for GluR2), but not in shock-only controls ($n = 8$). (**F**) No change was observed for NR1 in IA-trained ($n = 12$) or shock-only control animals ($n = 8$). (**G** to **I**) Time-course analyses demonstrated that training-related changes in Ser^{831} phosphorylation, GluR1 and GluR2 protein levels were rapid and transient, reaching peak values 30 min after training.

training-related changes in AMPAR protein levels in SNS prepared from the cerebellum of trained animals, nor did we detect any changes in the protein levels of GluR1, GluR2, or NR1 in crude hippocampal homogenates.

IA training produces an enhancement of field excitatory postsynaptic potential (fEPSP) slope in area CA1 in vivo. The biochemical changes in AMPARs after IA training are similar to those reported after LTP, not only in direction, but also in magnitude (*10*). This comparison suggested the possibility that we might be able to observe a change in synaptic transmission following IA training using electrophysiology.

We monitored synaptic transmission in CA1 by stimulating the Schaffer collateral axons before and after IA training, as is routinely done in LTP experiments in vivo (*14*). However, unlike an LTP experiment—which has the advantage that the affected synapses are specifically those activated by the stimulating electrode—we did not know a priori where to record changes after IA. We therefore implanted a multielectrode recording array in the apical dendritic layer of CA1 to monitor the strength of synaptic transmission evoked in different locations by stimulation of the Schaffer collateral axons (Fig. 2A).

Baseline fEPSP measurements were first obtained in freely moving animals that were well habituated to the recording box. Data collection was then temporarily suspended while the rats were given IA training, allowed to walk through the apparatus without a shock, or given the shock only. After this experience, the animals were returned to the recording box, and measurements of synaptic transmission resumed. A fourth group of animals ("naïve") remained in the recording box without being handled.

An example of the effect of IA training in one rat is shown in Fig. 2B. Note that although the fEPSP slope in the majority of channels shows a slight decrease after behavioral conditioning, two channels exhibit a substantial increase which is apparent immediately and persists for the duration of the recording session. Consequently, the average response across all channels (seven in this example) is about the same after training as before, but the between-channel variance is greatly increased.

Data from all channels in all animals in all groups are displayed in Fig. 2, C to F. In six trained animals, 12 of 44 electrodes showed increases >10% above baseline. In contrast, of the 140 electrodes comprising all of the recordings from control animals, not one showed an average fEPSP slope enhancement >10% above baseline. Comparison of the cumulative probability distributions of fEPSP slope changes from the 4 behavioral conditions confirmed a significant difference between the IA trained group and all control groups at 30 min, 1 hour, and 2 hours postconditioning [Kolmogorov-Smirnov (K-S) test, $P < 0.05$ at all time points (Fig. 2, G to I)]. Additional animals, receiving different types of experience on successive days (e.g., walk-through on day 1 followed by training on day 2), provided a within-animal comparison of the effects of different types of experience, and yielded comparable results (fig. S2).

We consistently observed a gradual decrease in the fEPSP slope in the postconditioning time period in the majority of electrodes that failed to potentiate after IA training (see Fig. 2, B and C). Thus, when data from all electrodes in all trained animals are averaged, little posttraining change is apparent relative to baseline (Fig. 3A). The postconditioning decreases in fEPSP slope over time do not appear to be a specific consequence of training, however, because they are apparent in the walk-through (Fig. 3B) and shock-only (Fig. 3C) groups. This difference between trained and control groups is particularly clear when the percentage of all channels with responses greater or less than one standard deviation (SD) from the baseline distribution is plotted against time (Fig. 3, E to G). Only in the trained group do we observe an abrupt and persistent increase in the fraction of channels with responses >1 SD above the baseline (Fig. 3E). However, gradual increases in the fraction of channels with responses >1 SD below baseline are seen in trained, walk-through, and shock-only groups (see also fig S3).

Decreases in fEPSP slope tended to occur coherently across electrodes within a given animal and correlated significantly with changes in the electroencephalogram (EEG) [theta/delta

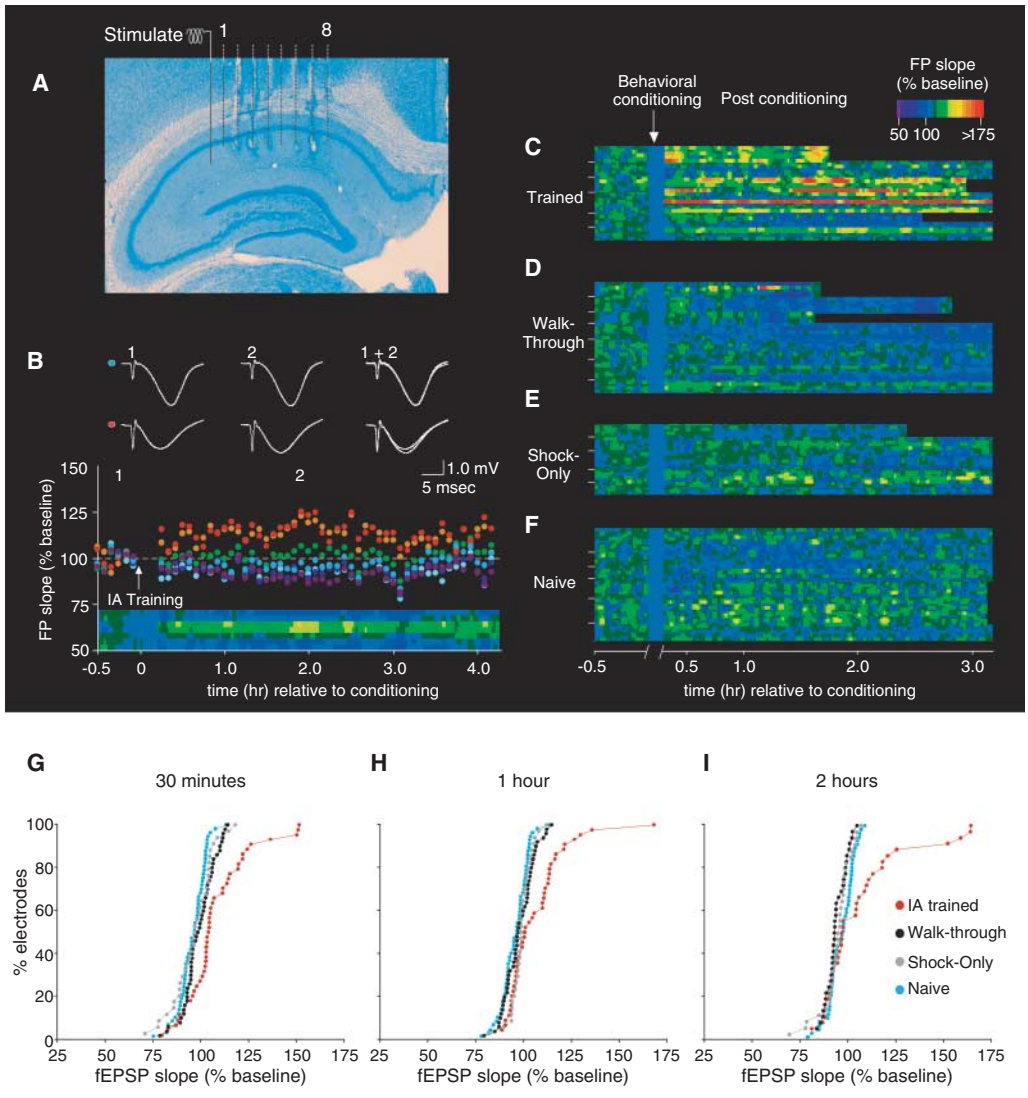

Fig. 2. IA training results in an enhancement of fEPSPs in area CA1 of the hippocampus in vivo. (**A**) Multielectrode recording arrays consisting of eight electrodes were implanted into CA1 of dorsal hippocampus. The Nissl-stained coronal section demonstrates electrode placement in the apical dendritic layer of CA1 (exact recording depths for five of eight electrodes are marked by lesions at electrode tips). (**B**) fEPSP slope measures collected every 30 s (displayed as averages of 5-min bins) from a single animal showing learning-related fEPSP enhancements after IA training. Two electrodes showed fEPSP enhancements ~15% above baseline (red and orange circles). fEPSP slope is represented by color on the inset beneath, where each row on the plot corresponds to individual electrodes. fEPSP waveforms (above) were obtained (top) from an electrode that did not show a training-related enhancement in slope, and (bottom) from an electrode where a 14% enhancement was observed. (**C** to **F**) Color plots representing fEPSP slope measures taken from six trained animals, seven walk-through controls, five shock-only controls, and six naïve controls; white tick-marks indicate individual animals in each group. Each animal was naïve at the time of behavioral conditioning (inclusion of additional animals receiving more than one type of experience appears in fig. S2). Of 44 recording electrodes, 12 showed average fEPSP slope measures >10% above baseline after IA training, although none of the 140 electrodes from control conditions showed such enhancements. Warmer colors indicate fEPSP slope enhancements; cooler colors represent decreases. (**G** to **I**) Cumulative probability distributions of fEPSP slope for IA-trained (red circles, $n = 44$ electrodes), walk-through (black circles, $n = 50$ electrodes), shock-only (light gray, $n = 35$ electrodes), and naïve animals (blue, $n = 55$ electrodes) demonstrate that fEPSP slope measures were enhanced in trained animals relative to controls (K-S test, $P < 0.05$) at 30 min, 60 min, and 120 min.

ratio (*17*); see fig. S4]. We also noted that of the four groups studied, naïve animals—which are not handled during the recording session—showed the least tendency for gradual reductions in fEPSP slope (Fig. 3, D and H). Thus, we interpret the coherent decreases in fEPSP slope as reflecting changes in the behavioral state of the animals over the duration of the recording experiments. However, we cannot rule out the possibility that LTD-like changes also contribute.

Although changes in fEPSP slope that happen coherently across multiple electrodes within an animal may result from changes in brain state, changes recorded at some electrodes but not others must be accounted for by local modifications. Therefore, we calculated the standard deviation of fEPSP slope measures across electrodes within individual animals before and after conditioning. In the naïve group, which remained unhandled in the recording box for 4 hours, the average within-animal variance increased by 43% over the duration of the recording session (Fig. 3L). Comparable values were seen in the walk-through (58% increase) and shock-only (35% increase) conditions (Fig. 3, J and K). However, the average within-animal variance increased to 256% of the baseline interval in IA-trained animals [significantly greater than controls; one-way ANOVA, $F(3,124) = 14.98$, $P < 0.0001$ (Fig. 3I)]. Because increased variability in evoked responses did not correlate with increased across-channel variability in the spontaneous EEG (see fig. S5), we conclude that a specific consequence of IA training is a spatially heterogeneous potentiation of synaptic transmission in some, but not all recording locations in dorsal CA1. Such changes are consistent with theoretical proposals for the structure of distributed associative memories (*18*).

Learning-related enhancements of fEPSP slope occlude subsequent LTP in vivo. The spatially restricted increases in fEPSP slope after IA training were not associated with local changes in the power spectrum of the spontaneous EEG (fig. S6), and were not accompanied by changes in paired-pulse ratio (fig. S7)—changes that might be expected if highly localized changes in temperature were responsible (*19*), rather than LTP. However, the most incisive approach to address the question of whether the modification we observe reflects LTP is to see if the learning-induced change occludes tetanus-induced potentiation.

Therefore, we trained an additional group of animals and compared changes in fEPSP slopes after training with the subsequent enhancements induced by repeated application of high-frequency stimulation (HFS) to saturate LTP. We found a significant inverse correlation

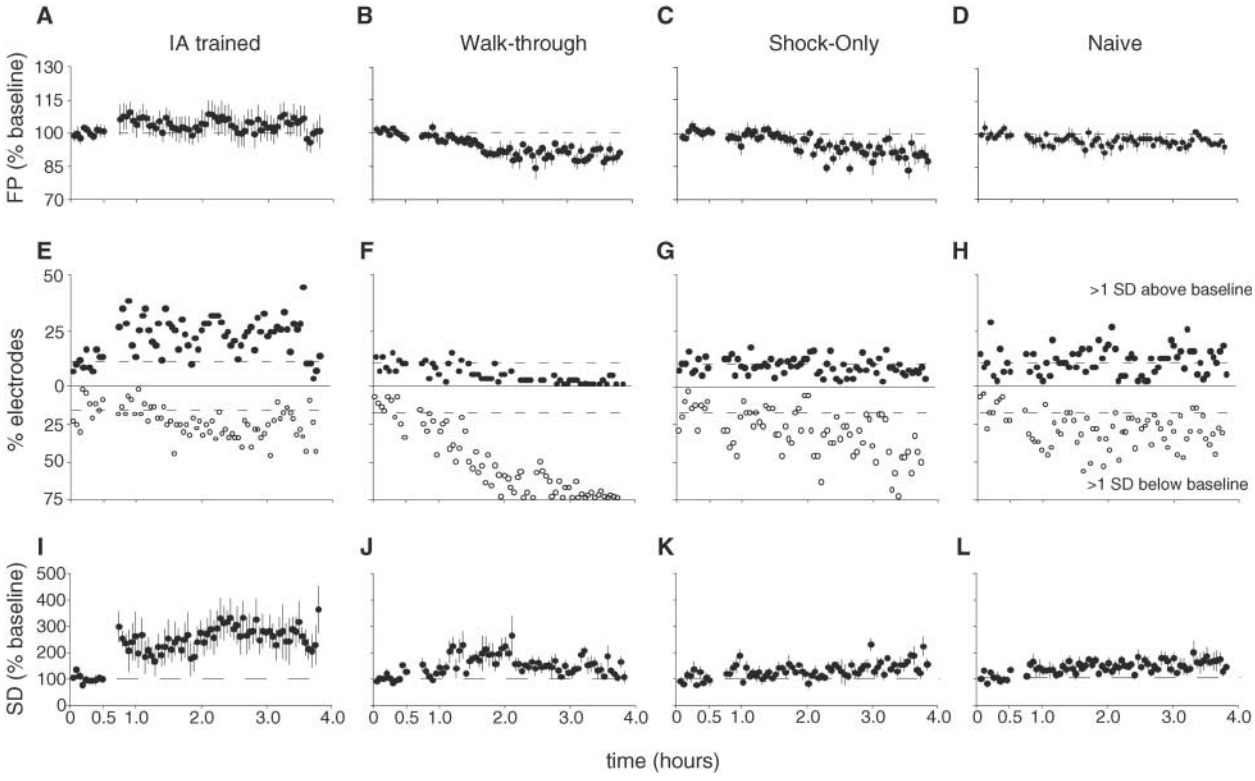

Fig. 3. Learning-related enhancements in IA-trained animals are obscured in group fEPSP averages, but are revealed by analyses of the distribution of responses. (**A** to **D**) Effect of experience on means ± SEM of all electrodes in all animals normalized to baseline. Because IA training affected a subpopulation of electrodes, the group average for fEPSP slope remained within 5% of the preconditioning baseline after IA training (A), whereas average fEPSP slope measures declined over time postconditioning in the walk-through, shock-only, and "naïve" conditions (B to D). (**E** to **H**) Effect of experience on the fraction of electrodes with responses greater or less than 1 SD of the baseline distribution. About 16% of electrodes show responses >1 SD during baseline. However, after training 33.2% of 44 electrodes had fEPSP slope values >1 SD above the baseline distribution [(E), filled circles], whereas fEPSP slope in electrodes from control groups did not increase. The percentage of channels >1 SD below baseline (open symbols) increased over time in all groups (F to H). (**I** to **L**) Within-animal variation in responses across electrodes. Plotted are the mean across-electrode standard deviations (±SEM), normalized to the baseline values. Variability in fEPSP slope measures across electrodes increased to more than 250% of the baseline mean after IA training (I), whereas variability remained within ~60% of the baseline mean for controls (J to L). Each animal included in this analysis was naïve at the time of behavioral conditioning (inclusion of additional animals receiving more than one type of experience appears in fig. S3).

between the amount of behaviorally induced potentiation and the amount of LTP induced by HFS—that is, electrodes where fEPSPs were enhanced after IA showed less subsequent LTP in response to HFS, whereas electrodes that did not exhibit enhancements of fEPSPs after IA training showed a greater magnitude of subsequent LTP [$R = 0.41$, $P < 0.01$ (Fig. 4A)]. This finding contrasts with the expected correlation of initial fEPSP size and LTP magnitude [which reflects cooperativity; see fig. S8 and (*20*)].

The occlusion of LTP by learning can also be illustrated by comparing the time course of average LTP in those electrodes that expressed a >10% training-induced increase in fEPSP slope with those electrodes (in the same animals) that did not (Fig. 4B). Electrodes showing fEPSP enhancements after training had significantly less subsequent LTP (121.1% of renormalized, pre-HFS baseline) than "control" electrodes [136.1%; repeated measures ANOVA, group × time interaction, $F(3,42) = 3.61$, $P < 0.025$; Fisher's protected least significant difference (PLSD) for final HFS epoch, $P < 0.02$]. Furthermore, LTP in IA-enhanced electrodes saturated after the first series of HFS, whereas LTP in "control" electrodes did not saturate until after the second series of HFS (Fig. 4B), which indicated that electrodes showing learning-related fEPSP enhancements were closer to their ceiling for LTP expression before HFS delivery. Importantly, the two groups of electrodes did not differ in their total capacity for LTP expression when the data were expressed as a percentage of the pre-IA training baseline [ANOVA, $F(3,42) = 0.36$, $P > 0.55$].

Discussion. LTP induced in hippocampal area CA1 by HFS of the Schaffer collaterals has properties that have made it the premiere model to study possible memory mechanisms (*21*, *22*). It is induced rapidly by stimulation that appears physiological; it has properties that enable association of temporally contiguous events; and it can be very stable over time (*23*, *24*). Strictly speaking, however, LTP is a "memory" only of having one's brain electrically stimulated. Proving that hippocampal LTP actually reveals the mechanisms of memory therefore remains an important goal (*3*).

Two criteria must be fulfilled to conclude that two events induce a change by a common mechanism: mimicry and occlusion. Here we have shown that IA training mimics the effects of HFS by causing (i) an immediate, NMDA receptor–dependent increase in phosphorylation of GluR1 at Ser^{831} without affecting phosphorylation of GluR1 at Ser^{845}; (ii) delivery of GluR1 and GluR2, but not NR1, to the synaptoneurosome biochemical fraction; and (iii) an increase in the slope of the evoked fEPSP without affecting paired-pulse facilitation. We have also shown that IA-induced increases in the evoked fEPSP partially occlude subsequent LTP by HFS in vivo. We therefore conclude that IA training induces LTP in CA1.

Biochemical changes after IA reported here and in several previous studies (*6*, *25*–*28*) are of the same type and magnitude as those seen after HFS in dorsal hippocampus. However, unlike memory (fig. S1), the changes in AMPARs observed in our study were no longer detectable hours after training (Fig. 1). Perhaps the LTP-like change in synaptic transmission in CA1 is

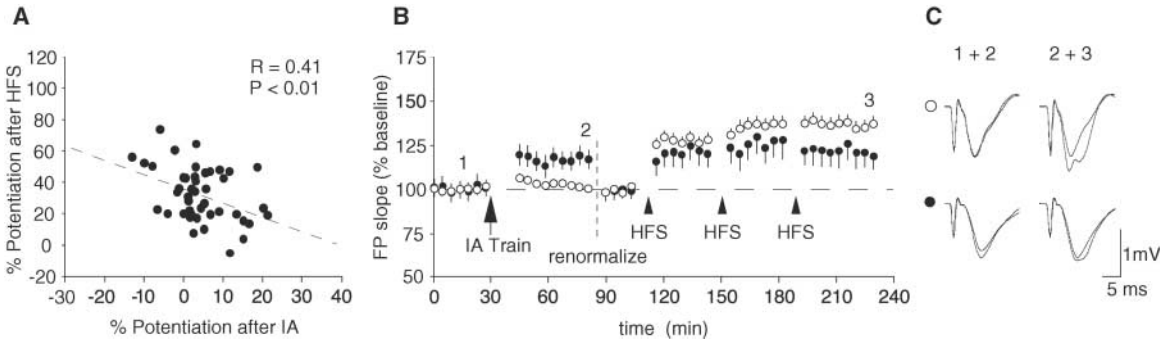

Fig. 4. Learning-associated fEPSP slope enhancements occlude subsequent LTP in the hippocampus of freely behaving animals. (**A**) An inverse correlation was observed between the magnitude of fEPSP slope enhancements after IA training and the magnitude of subsequent LTP in vivo (R = 0.41, $P < 0.01$). (**B**) Of 44 recording electrodes, nine from seven animals were enhanced by >10% above pretraining baseline; the average enhancement was 16.5% above baseline (black circles). Electrodes not showing a training-related enhancement of fEPSP slope ("control" electrodes) averaged 2.4% above pretraining baseline (open circles). LTP was saturated after the first HFS series in electrodes showing learning-related fEPSP enhancements (Fisher's PLSD for HFS 1 versus HFS 2, $P > 0.50$), whereas "control" electrodes showed additional potentiation after the second HFS series (Fisher's PLSD for HFS 1 versus HFS 2, $P = 0.012$). Furthermore, electrodes showing fEPSP enhancements after IA training showed less LTP than control electrodes after the final series of HFS (121.1 versus 136.1%, respectively; Fisher's PLSD, $P < 0.02$). Data from the last 30 min of each epoch was used for statistical comparisons. (**C**) Representative fEPSP traces taken from two electrodes in the same animal before IA training (1), after training (2), and after LTP saturation (3).

only transient. Arguing against this possibility, however, is the direct observation that fEPSPs on some electrodes remained potentiated for ≥3 hours after IA. Another possibility is that the expression mechanism for LTP shifts to the presynaptic side of the synapse over time. Arguing against this hypothesis, however, is the finding that paired-pulse facilitation in potentiated channels remains unaffected >75 min after training (see fig. S7). A third possibility suggested by inspection of the cumulative probability distributions in Fig. 2 and fig. S2 is that the fraction of synapses enhanced relative to the control conditions is winnowed over time. Although some electrodes continue to show a substantial and stable enhancement after 2 hours, this residual, spatially restricted change may be below our biochemical detection threshold.

Experience-dependent changes in evoked responses have been reported previously in the hippocampus (*19*, *29*–*33*). A potential complication in interpreting all such findings, including ours, is that experience can also alter temperature in the hippocampus (via changes in blood flow) which, in turn, alters the properties of synaptic transmission (*34*–*36*). However, there are several reasons that this is an unlikely explanation for our results. First, the potentiation observed after IA is highly local, restricted to a subset of recording electrodes. Second, another physiological measure that is sensitive to temperature, the paired-pulse ratio (*36*), was unchanged at electrodes showing potentiation (fig. S7). Third, and perhaps most definitively, electrodes showing learning-induced increases in fEPSP slope displayed less HFS-induced LTP, which would not be expected if brain temperature were raised.

Less LTP has also been reported in hippocampal slices ex vivo after contextual fear conditioning (*37*). Although this finding is consistent with the hypothesis that learning induces LTP in vivo, an equally plausible explanation is that conditioning causes a generalized inhibition of plasticity in the hippocampus. Our experiments discriminate between these possibilities by showing in the same animal in vivo that the effect of learning on LTP is restricted to those regions that display synaptic potentiation after learning. Such a localized occlusion of LTP by learning is strong evidence that IA and HFS increase synaptic transmission in CA1 by a common mechanism.

A recent report of contemporaneous experiments in mice indicates that another type of aversive associative memory—trace eye-blink conditioning—also causes a detectable (apparently more global) increase in synaptic transmission in CA1 and that induction of LTP by HFS of the Schaffer collaterals during conditioning disrupts memory formation (*33*). Although it has not yet been formally established that trace conditioning induces LTP per se, it is noteworthy that this type of learning is also sufficient to produce increases in hippocampal synaptic transmission large enough to be detected. Perhaps LTP is so robust in CA1 because it plays a special role in formation of memories used to avoid or anticipate danger. More subtle bidirectional modifications might be reserved for memories less basic to survival.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S8
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Learning Induces Long-Term Potentiation in the Hippocampus

Jonathan R. Whitlock *et al.*

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