ception is that of *Trachymyrmex papulatus*. Of two nests examined, one cultivated a lower attine fungus (included in Fig. 1), the other a typical higher attine fungus. This is the only known case of a higher attine ant cultivating a lower attine fungus.

11. Cultivated and free-living *Lepiotaceae* were surveyed in central Panama in November 1995 through December 1996. Most of the free-living fungi appear to be undescribed species ("PA" collection IDs in Fig. 1); a few collections could be associated with published descriptions ("cf." notation).
12. Our Web site (www2.sel.barc.usda.gov/Schultz/agants.html) lists sample sizes by ant species of all 553 cultivar isolates, and additional information on phylogenetic analyses.
13. RFLPs were generated by restricting ITS polymerase chain reaction products [T. White, T. Bruns, S. Lee, J. Taylor, in *PCR Protocols*, M. Innis, D. Gelfand, J. Sninsky, T. White, Eds. (Academic Press, New York, 1990)] with Hae III or Taq I. Fungi were called the same type if RFLPs matched. For each ant species per collection locality, we sequenced at least one representative of each cultivated RFLP type.
14. Forward and reverse sequences were generated on an ABI 377 sequencer for the entire ITS region (680 to 740 bp) (*13*) and the first 610 bp of the 25*S* gene (*7*) (GenBank accession numbers AF079591-AF079754). Sequences of 11 Lepiotaceae were obtained from GenBank (U11921, U85281-U85283, U85287, U85288, U85291, U85292, U85295, U85296, U85306, U85315-U85318, U85321-U85323, U85326, U85327, U85330, U85331). We thank J. Johnson for posting these unpublished sequences in GenBank.
15. Alignments were generated in Megalign 1.1 DNASTAR). Regions of ambiguous alignment (306 of 811 sequence positions in ITS; 5 of 611 positions in 25*S*) were excluded. Phylogenetic analyses were carried out in PAUP* 4.0d61 [D. Swofford, unpublished test version]. Sequence data consisted of 1422 nucleotide sites (minus the 311 unalignable characters), 229 of which were parsimony-informative. Maximum parsimony (MP) analysis identified 180 trees (length = 915, CI = 0.352, RI = 0.663). Successive approximations weighting (SAW) identified five equally parsimonious trees (all members of the original set of 180), the strict consensus of which is presented in Fig. 1. Transition:transversion weighting schemes of 1:2 and 1:5 produced parsimony trees identical in all key features to the tree in Fig. 1. To render maximum-likelihood (ML) analysis computationally tractable, we reduced the data set to 51 taxa by retaining one representative of each set of taxa differing by fewer than five bases. Each of the five SAW trees was "pruned" to retain these 51 taxa, then evaluated under the ML criterion (estimating parameters from the data). The most parameter-rich model (general time-reversible + proportion of sites invariant + rate heterogeneity modeled as a gamma distribution with six rate categories) was significantly better fitting than the next best model. Starting with this model and the most likely of these trees, and using increasingly more efficient branch-swapping algorithms and successively readjusted parameter values, five iterative ML searches identified a single most likely tree, which resembles in all key features the parsimony tree (Fig. 1). See our Web site (*12*) for details of the analyses.
16. A scenario of single domestication 50 million years ago, followed by escapes, is implausible because it stipulates that all free-living fungi (including major Nearctic clades) that arose after the divergence of the most recent common ancestor of all cultivars (Fig. 1) must have descended from "escaped" cultivar ancestors; it is inconsistent with observed levels of allele sequence divergence (ASD); and it contradicts the theoretical prediction that ancient clones will not retain intact the multigene architecture for fruiting [M. Lynch, R. Burger, D. Butcher, *J. Hered.* **84**, 339 (1993)].
17. Tests contrasted unconstrained MP and ML trees with constraint trees, which were inferred using the same methods described above (*15*) for MP and ML, respectively. For MP, tests consisted of all possible pairwise comparisons of multiple equally parsimonious trees. Forcing cultivar monophyly (single domestication) significantly reduced goodness-of-fit [MP: Kishino-Hasegawa (KH), $P < 0.0006$; Templeton's Wilcoxon ranked sums (TWRS), $P < 0.0007$; winning sites (WS), $P < 0.0003$; ML: KH, $P = 0.0005$]. Ad hoc assumptions of two domestications involving (Clade 1 + *Myrm. infuscata* G11) and (Clade 2 + *Myco. smithi* S60) also failed (MP: KH, $P < 0.0122$; TWRS, $P < 0.0122$; WS, $P < 0.0080$; ML: KH, $P = 0.0355$). This failure was due to strong support for the monophyly of Clade 2 excluding *Myco. smithi* S60 (MP: KH, $P < 0.0160$; TWRS, $P < 0.0285$; WS, $P < 0.0428$; ML: KH, $P = 0.0363$). Monophyly of Clade 1 excluding *Myrm. infuscata* G11 was not supported (MP: KH, $P > 0.4398$; TWRS, $P > 0.4534$; WS, $P > 0.3877$; ML: KH, $P = 0.7095$). See our Web site (*12*) for details of the tests.
18. This firm refutation of a single domestication event suggests that further sampling may reveal additional domestications. Promising locations include Amazonian Brazil (putative center of attine origin) and peripheral populations existing under extreme ecological conditions that promote cultivar loss and prompt novel domestications.
19. It is possible that some cases of "intraspecific" cultivar diversity involve unrecognized cryptic species, each specialized on a distinct cultivar.
20. For each of six cases where different ant species farmed cultivars with identical rDNA sequences (*A. auriculatum–C. longiscapus*; *C. minutus–C. rimosus*; *Myco. smithi–Myco. tardus–Myrm. ednaella*; *C. costatus–C. longiscapus*; *Myco. smithi–Myco. sp. nov. 1*; *Myrm. cf. buenzlii–C. faunulus*) (Fig. 1), we fingerprinted the sequenced isolates, and additional isolates of the same RFLP types, with AFLP techniques [U. G. Mueller, S. E. Lipari, M. G. Milgroom, *Mol. Ecol.* **5**, 119 (1996)], permitting highly resolved subdifferentiation into AFLP fingerprint types.
21. Leucocoprinoid fungi use an ephemeral substrate (litter), are relatively short-lived, and are not known to form geographically widespread clones.
22. R. R. Snelling and J. T. Longino, in *Insects of Panama and Mesoamerica*, D. Quintero and A. Aniello, Eds. (Oxford Univ. Press, New York, 1992), pp. 481–494.
23. Asexual cultivar propagation was shown by AFLP fingerprint identities of cultivars from different nests of Floridan *C. minutus* (*20*) and was corroborated by AFLP surveys of lower-attine cultivars from Trinidad (S. A. Rehner and U. G. Mueller, unpublished data).
24. Theory predicts elevated levels of heterozygosity (ASD) for ancient asexual diploid organisms [O. Judson and B. Normark, *Trends Ecol. Evol.* **11**, 41 (1996)]. Without recombination, homologous alleles accumulate mutations independently and diverge with time. Heterozygosity therefore is a relative measure of the time since the origin of asexuality. Recombination (sexuality) purges ASD. Heterozygosities were scored from sequencing contigs as superimposed peaks each of about half intensity (present in forward and reverse sequences), and insertions or deletions (typically involving one base, causing a partial frame-shift at the same position, but in opposite directions, in forward and reverse sequence).
25. We thank the Smithsonian Tropical Research Institute, the National Geographic Society, the Biodiversity of the Guyanas Project, the Smithsonian Scholarly Studies Program, the Laboratory of Molecular Systematics (National Museum of Natural History), and the National Science Foundation (DEB-9707209) for funding; the Bermingham lab for sequencing support; the Instituto Nacional de Recursos Naturales Renovables (INRENARE) of Panama, the Kuna Comarca, the Office of the President of Guyana, the Ministerio de Recursos Naturales Energía y Minas (MIRENEM) and the Instituto Nacional de Biodiversidad (INBIO) of Costa Rica, the Forestry Division and Wildlife Section (FDWS) of Trinidad, and the Conselho Nacional de Pesquisas (CNPq) and the Instituto Nacional de Pesquisas da Amazónia (INPA) of Brazil for collecting and export permits; R. Adams, D. Agosti, V. Aswani, J. Boomsma, M. Braun, M. Chen, C. Currie, G. deAlba, Z. Falin, V. Funk, N. Gomez, J. Heacock, J. Huelsenbeck, J. Hunt, J. Narozniak, N. Knowlton, M. Leone, J. Longino, S. McCafferty, G. Maggiori, B. Norden, D. Piperno, I. Rubinoff, J. Sullivan, D. Swofford, J. Wilgenbusch, R. Wilson, T. Wright, and especially E. Bermingham, C. Delwiche, A. Herre, J. Kays, and B. Wcislo for logistical support, advice, and various other kinds of help. We dedicate this paper to the memory of William L. Brown Jr. (1922–1997).

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Impaired Spatial Learning after Saturation of Long-Term Potentiation

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If information is stored as activity-driven increases in synaptic weights in the hippocampal formation, saturation of hippocampal long-term potentiation (LTP) should impair learning. Here, rats in which one hippocampus had been lesioned were implanted with a multielectrode stimulating array across and into the angular bundle afferent to the other hippocampus. Repeated cross-bundle tetanization caused cumulative potentiation. Residual synaptic plasticity was assessed by tetanizing a naïve test electrode in the center of the bundle. Spatial learning was disrupted in animals with no residual LTP (<10 percent) but not in animals that were capable of further potentiation. Thus, saturation of hippocampal LTP impairs spatial learning.

An important prediction of the hypothesis that activity-dependent synaptic plasticity in the hippocampus (such as LTP) plays a critical role in certain kinds of learning (*1, 2*) is that physiological saturation of synaptic weights should disrupt new memory encoding. Saturation of an intrinsic pathway can be viewed as a neural state in which no further potentiation is feasible, at least for a period of time, at any site in the pathway (*3*). Repeated tetanization at a single site in the perforant path has been reported to block spatial learning when leading to cumulative LTP in the dentate gyrus (*4*), but this result has not been

replicated successfully (*5–7*), and some studies have even found enhanced learning (*8*). The reasons for this failure clearly include the possibility that the hypothesis is wrong but also that tetanization at a single site in the perforant path produces saturation only at selected synaptic loci and only along part of the longitudinal axis of the hippocampal formation (*9*).

Saturation is most likely to be achieved by an electrode array that straddles an afferent pathway and by a stimulation protocol that consists of multiple tetanization episodes with cathodal stimulation at different cross-sectional sites. The variable success of such an arrangement must be assayed by a separate test-stimulation electrode that selectively (but randomly) samples fibers within that pathway. If LTP can still be induced by tetanization of the test electrode, saturation cannot be claimed to have occurred. Thus, to reinvestigate the relation between saturation and spatial learning, we induced LTP through a multielectrode array across the angular bundle of the perforant path fibers in rats.

To increase the sensitivity of animals to the saturation of plasticity at synapses that might be used for learning, we first decided to decrease the volume of hippocampal tissue by making unilateral ibotenic acid lesions of the hippocampus and dentate gyrus (*10*). Two weeks later (day 14), the specially designed array of three bipolar stimulating electrodes and one recording electrode was implanted into the nonlesioned side of the brain. Two electrodes were implanted so that they straddled the angular bundle of the perforant path at the point passed by a high proportion of cortical afferents destined for the dorsal hippocampus (Fig. 1A) (*11*). The vertical placement of each electrode was adjusted so that the use of either the tip or the shaft of one of these concentric electrodes as a cathode and the use of either the tip or the shaft of the other electrode as an anode resulted in high-amplitude dentate field potentials (Fig. 1B). The field potentials were recorded by means of an electrode in the hilar zone of the ipsilateral dentate gyrus. Acute mapping experiments that were conducted under urethane anesthesia in nonlesioned animals revealed that cross-bundle stimulation was able to induce >10-mV amplitude field potentials at sites extending from the septal pole and along the dorsal 60% of the longitudinal axis of the dentate gyrus. We did not record signals in the temporal part of the hippocampus, which is unable to support spatial learning with the present training protocol (*12*).

The third stimulating electrode was positioned between the other two and aimed at the center of the perforant path (Fig. 1A). This served as a low-frequency (LF) test electrode during both baseline recording and induction of cumulative LTP. It also served as the tetanization test electrode to check whether the cumulative LTP that was induced from the other electrodes was saturated. An animal could be said to have saturated LTP if the cumulative LTP had reached an asymptote and if the later attempt to induce LTP from this separate electrode was unsuccessful. Once positioned, the electrodes were cemented in place, and the animals were allowed to recover from the acute effects of surgery for 2 weeks.

High-frequency (HF) tetanization was then conducted on a single day (day 28) with a cathode on one side of the bundle and an anode on the other side. All possible combi-

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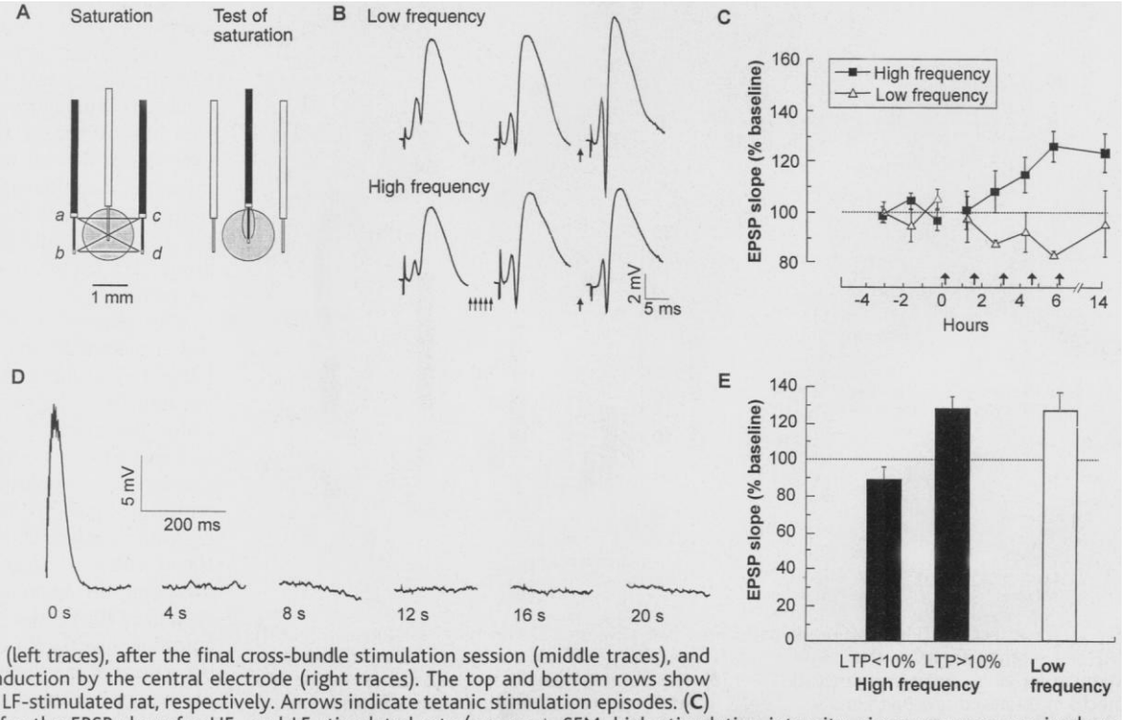

Fig. 1. Saturation of LTP in perforant path synapses of the dentate gyrus. (**A**) The placement of two bipolar electrodes on each side of the medial and lateral parts of the angular bundle (black bars) and one bipolar electrode in the middle (white bar). (Left) Induction of LTP by cross-bundle tetanic stimulation (horizontal and diagonal lines), with anode and cathode sites at different sides of the bundle (a, b, c, and d). (Right) A test of LTP saturation by tetanic stimulation through the naïve central electrode at the end of the experiment. (**B**) Representative evoked potentials shown before cross-bundle stimulation (left traces), after the final cross-bundle stimulation session (middle traces), and after the residual LTP induction by the central electrode (right traces). The top and bottom rows show traces from a HF- and a LF-stimulated rat, respectively. Arrows indicate tetanic stimulation episodes. (**C**) The normalized values for the EPSP slope for HF- and LF-stimulated rats (means ± SEM, high stimulation intensity, six responses per animal per session). The recording was conducted at 1.5-hour intervals, except for the last session, which occurred 7 hours after the end of the last tetanic stimulation. The cross-bundle stimulation (arrows) was delivered immediately after the third through seventh recording sessions at 0, 1.5, 3, 4.5, and 6 hours. The HF stimulation gradually increased the fEPSP slope values. Error bars indicate SEM. The dotted line indicates the mean EPSP slope during baseline recording. (**D**) Representative traces taken during tetanic stimulation at 0 s and at 4-s intervals during the minute after tetanization. There is an absence of afterdischarges. (**E**) The change in the EPSP slope at the highest stimulation intensity for 1 hour after tetanic stimulation at the central stimulation electrode at the end of the experiment. The animals were considered to have residual LTP if the slope of the fEPSP was enhanced by >10% at this pulse intensity. Error bars indicate SEM. The dotted line indicates the mean EPSP slope during baseline (2 min before tetanization).

nations of cathode (tip or shaft) and anode (tip or shaft) were used (Fig. 1A) (*13*). The animals were placed in dark, enclosed chambers in which, to reduce the attenuation of LTP by stress (*14*), they had been familiarized on the preceding 3 days. After baseline responses had been sampled, five series of cross-bundle tetanization episodes were given, starting at 0, 1.5, 3, 4.5, and 6 hours after the last baseline recording. The fifth episode was an anode and cathode arrangement that was identical to the first episode in order to check whether there would be further cumulative potentiation. Low-frequency control animals received the same stimulation sequence of cathode and anode locations, but only single pulses were given at each location. Nonstimulated (NS) controls, with electrodes implanted, were handled and placed in the recording chambers.

The stimulation resulted in cumulative LTP with waveforms showing a gradual increase in the early rising portion of the extracellular field potential (Fig. 1B, middle trace) over the course of the recording period (Fig. 1C). Little change was seen immediately after the first tetanization episode, possibly because the test electrode used for the measurement of the degree of potentiation was in the center of the angular bundle. The field potential slope at 7 hours after the last tetanization session was significantly elevated above the pre-tetanization baseline in the HF group and significantly elevated above the LF group [groups: $F(1,13) = 6.7$, $P < 0.05$; groups × session: $F(4,52) = 3.3$, $P < 0.05$] (F is the variance ratio and P is the probability). The level of LTP after the fifth episode of tetanization was comparable to the level after the fourth episode, with the mean LTP level of fibers in the center of the perforant path being comparable to the level obtained in studies where the tetanization electrodes were placed in the center of the bundle (*4–7*). Thus, cross-bundle stimulation did not induce a greater magnitude of LTP than previous studies did, but the cross-bundle stimulation may have induced LTP on a higher proportion of fibers afferent to the hippocampal formation. The trend toward a slight decline in slope in the LF group may be a temperature effect (*15*), as these animals became less active across the recording sessions; however, no direct recordings of hippocampal temperature with implanted thermistors were made in this study. High-frequency cross-bundle tetanization did not result in seizures in traces recorded at 4-s intervals for 1 min after each tetanization in a subset of eight animals (Fig. 1D) (*16*).

After the last recording session, all animals were trained in an open-field water maze to find a platform that was hidden at a single location in the pool (*17*). All animals showed a decline in escape latency across the 10 trial blocks of training (Fig. 2A). Analysis revealed significant effects of groups [$F(2,24) = 5.4$, $P < 0.01$] and groups × block [$F(18,216) = 2.4$, $P < 0.001$] that reflect the higher mean escape latency of the tetanized group toward the end of training. Probe tests, in which the pneumatic platform was kept submerged for the first 40 s of the trial before raising, showed a gradual increase in time spent in a platform zone of 35-cm radius around the center of the platform in blocks 1, 6, 8, and 11 (the final probe test) (Fig. 2B). Low-frequency and NS test animals showed the most focused searching in the correct zone, with representative swim paths shown in Fig. 2C. High-frequency test animals showed a distribution, with some animals doing quite well but with most animals swimming all over the pool with no spatial bias toward the target area. Statistical analysis revealed significant groups × quadrants [$F(6,72) = 8.5$, $P < 0.001$] and groups × quadrants × probe test [$F(18,216) = 2.2$, $P < 0.005$] interactions.

The reason for the distribution of the search pattern by individual animals in the HF group became apparent when we returned the animals to the recording chamber to examine the extent to which the cumulative LTP that was previously observed reflected a true saturation of synaptic plasticity (*18*). The critical test involved the use of the stimulating electrode located at midbundle as a new site at which to induce LTP (Fig. 1E). Up to this point, the midbundle electrode had only been used for LF test pulses. Midbundle tetanization gave LTP [defined as a >10% enhancement of the slope of the field excitatory postsynaptic potential (fEPSP)] in all LF test animals. The HF group was divided into animals that showed <10% LTP on the test pathway (the saturated subgroup; $n = 7$) and animals that showed >10% LTP (the nonsaturated subgroup; $n = 6$). Analysis of the potentiation induced on the test pathway showed a significant effect [groups $F(2,15)$

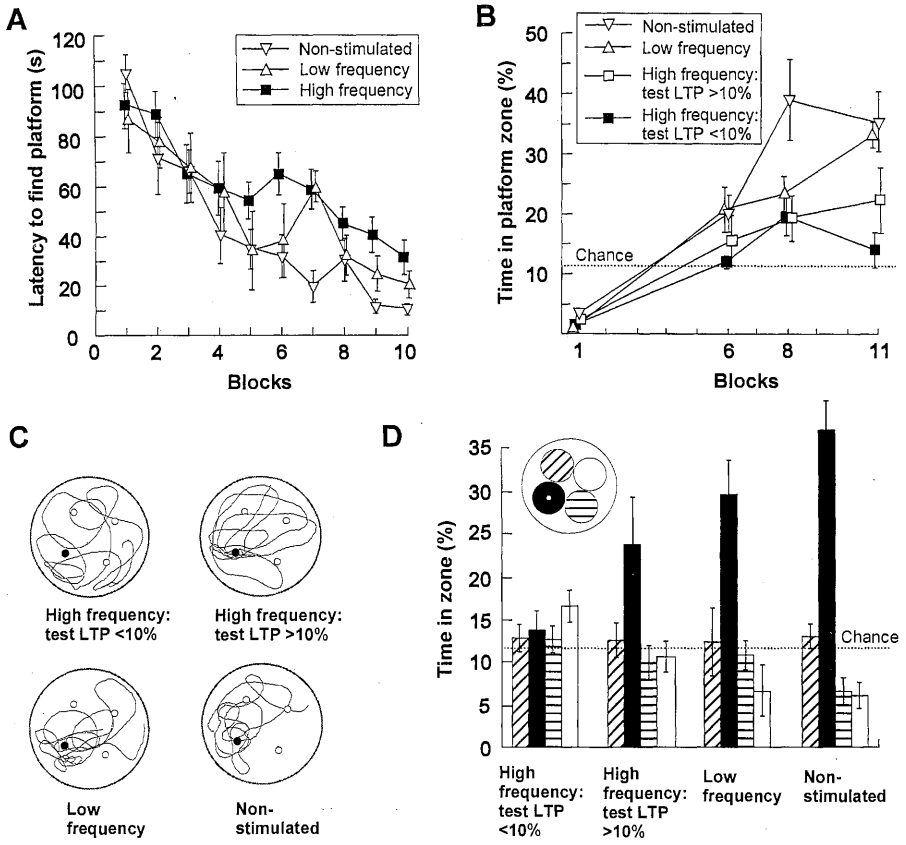

Fig. 2. The effect of LTP saturation on performance in a water maze learning task (means ± SEM). (**A**) The latency to enter the platform of rats receiving HF stimulation, LF stimulation, or no stimulation at all. Error bars indicate SEM. (**B**) The development of spatial behavior across trial blocks in tetanized rats (with and without residual LTP at the central stimulation electrode) and LF and NS control rats. The search time in a circular area (radius of 35 cm) around the platform zone was measured during the first 40 s of four trials with the platform submerged to the bottom of the pool. The dotted line indicates the chance level. Error bars indicate SEM. (**C**) Records of the search pattern of a representative animal from each group during the final spatial probe test (60 s). (**D**) Time spent inside a circle (radius of 35 cm) around the platform position (black bar) and in corresponding, equally large zones in the three other pool quadrants (diagonally striped, horizontally striped, and white bars) during the final spatial probe test (60 s). The dotted line indicates the chance level. Error bars indicate SEM.

$= 10.3$, $P < 0.005$] (*19*). The hypothesis that saturation of LTP will result in a learning deficit predicts that the saturated subgroup should have learned less about the location of the hidden platform than the nonsaturated subgroup. This prediction was upheld (Fig. 2, C and D). An analysis of variance (ANOVA) of the proportion of time spent in the target zone during the final transfer test revealed an overall difference between groups [$F(3,26) = 7.5$, $P < 0.001$]. Subsequent planned orthogonal comparisons revealed that the animals with >10% residual LTP did not differ from the LF group ($F = 1.1$, not significant), but these two groups performed better than the animals with <10% residual LTP ($F = 7.7$, $P < 0.025$). These three groups, all of which had electrodes implanted and were stimulated, also performed more poorly than the NS controls. Thus, successful saturation of LTP did impair spatial learning in the water maze.

These results uphold a key prediction of the "LTP and learning" hypothesis and can explain previous failures to see the effects of cumulative LTP on spatial learning (*5–7*). First, previous studies used only single bipolar tetanization electrodes in the angular bundle of the perforant path and may have activated only a small proportion of the entorhinal afferents. Thus, some studies would succeed in seeing a behavioral effect of tetanization and others would not. Second, our use of animals with a unilateral hippocampus may have increased the sensitivity of the behavioral task to a disturbance of synaptic plasticity in the dorsal hippocampus, which is the region of hippocampal formation whose integrity is essential for this form of spatial learning (*12*). Third, previous studies did not check whether the cumulative LTP was, in practice, saturated. Assuming that our test electrode sampled a representative subset of fibers traveling in the angular bundle, its use constitutes an independent identification of animals that show saturated LTP from those that merely show cumulative LTP. Although it was not possible to induce further LTP by means of the test electrode in the subset of animals that failed to learn where the platform was located, we do not know the proportion of maximally potentiated synapses in these animals (*3*). However, the effects of saturation of LTP on subsequent learning are likely to follow a sigmoidal function where deleterious effects will be observed well before a saturation maximum is achieved (*7*).

The fact that impaired and nonimpaired animals in the tetanized group received identical stimulation suggests that a blockade of learning after saturation of LTP is unlikely to be caused by nonspecific side effects of the HF stimulation of large populations of fibers (*20*). Although such side effects remain a theoretical possibility, their deleterious effects on behavior would have had to covary with the capacity to induce residual LTP on the terminals of the perforant path. The induction of seizures could be such a factor (*6*), but afterdischarges were not seen with our stimulation paradigm.

The procedure of cross-bundle tetanization of the perforant path demonstrably induced LTP in the dentate gyrus, but the procedure may also have induced LTP in the terminal zone of the perforant path in area CA3 or may have affected synaptic transmission at synapses at the outer dendritic portion of area CA1, where fibers emanating from layer III entorhinal cells terminate. Some LTP may also have been induced transsynaptically (*21*). Thus, this tetanization procedure does not speak directly to the issue of whether a blockade of dentate LTP alone is sufficient to impair spatial learning. The possibility that dentate LTP is unimportant has recently been raised by studies of mice harboring mutations of genes that affect dentate but not CA1 LTP (*22*). Further analyses of this mutant have revealed, however, that some residual LTP is present when studied in freely moving mice (*23*).

Several current models of hippocampal function emphasize its role as a distributed associative memory system that is responsible for capturing event-related information online with an LTP-like synaptic mechanism (*2, 24*). In these models, the distributed nature of information representation within the hippocampus and dentate gyrus provides opportunities for pattern completion in response to partial cues. Also, these models predict that artificial saturation of synaptic weights across a substantial proportion of cortical afferents should disrupt the representational capacity of the system and hence disrupt learning. Our results support those models in indicating that saturation of LTP can disrupt one form of hippocampal-dependent learning.

The link between LTP and learning rests on three pillars: blockade, saturation, and erasure. The disruption of spatial learning associated with a blockade of hippocampal LTP is well established (*25*). The present findings reestablish the predicted impairment of learning after saturation of LTP. However, it remains to be shown that an erasure of LTP causes forgetting.

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3. We view saturation of an intrinsic pathway as a neural state in which the pathway cannot be further potentiated in the intact and awake animal. It is unlikely that any method of physiological stimulation will potentiate all synapses of the perforant path to their maximum values. Many synapses are thought to be silent [D. Liao, N. A. Hessler, R. Malinow, *Nature* **375**, 400 (1995); J. T. Isaac, R. A. Nicoll, R. C. Malenka, *Neuron* **15**, 427 (1995)]. Their whole-scale potentiation would render a brain region susceptible to hyperexcitability or even seizures, a state that may ordinarily be prevented by intrinsic inhibitory activity or rapid depotentiation.
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10. Forty-three naïve male Long-Evans rats (300 to 450 g), which were housed in pairs, were on two occasions anesthetized with Equithesin, a mixture containing chloral hydrate and pentobarbital (1.0 ml per 250 g of body weight). During the first surgical session, all rats received complete unilateral hippocampal lesions by the injection of ibotenic acid (Biosearch Technologies, San Rafael, CA) at 14 sites [modified from L. E. Jarrard, *J. Neurosci. Methods* **29**, 251 (1989)]. Ibotenic acid was dissolved in phosphate-buffered saline (pH 7.4) at 10 mg/ml and injected with a 1-μl Hamilton syringe that was mounted to the stereotaxic frame.
11. Two weeks after the induction of the lesions, three bipolar stimulation electrodes (SNEX 100; Rhodes Medical, Woodland Hills, CA) were implanted in the angular bundle of the intact hemisphere 7.0 mm behind and 3.0, 4.0, and 5.0 mm, respectively, lateral to the bregma. A stainless steel recording electrode was placed in the dentate hilus or granule cell layer (3.8 mm behind and 2.4 mm lateral to the bregma). Electrode leads and contacts were encased in dental acrylic, and the animal was allowed 2 weeks for recovery.
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13. Two weeks after implantation, evoked waveforms were recorded in the dentate gyrus at 1.5-hour intersession intervals in response to perforant path stimulation. Recording started 5 min after the rat had been placed in a dark, enclosed recording chamber. Waveforms were sampled in the dentate gyrus in response to constant square-wave pulses (100 μs, 0.1 Hz) delivered to the perforant path at three intensities that were adjusted to give population spikes of 0, 1, and 3 mV, respectively (80 to 1000 μA). The slope of the fEPSP was measured as the amplitude difference at two fixed latencies in the middle of the rising phase of the potential. After the third through seventh recording sessions, the rats received either HF stimulation ($n = 17$), LF stimulation ($n = 12$), or no stimulation at all ($n = 14$). Pilot experiments failed to show more saturation (less residual LTP) in rats receiving LTP across several days than in rats receiving a single day of massed stimulation, possibly because of slowly developing homeostatic changes in synaptic weights in populations undergoing substantial up- or down-regulation of synaptic transmission [G. G. Turrigiano, K. R. Leslie, N. S. Desai, L. C. Rutherford, S. B. Nelson, *Nature* **391**, 892 (1998)]. Thus, a massed stimulation protocol was adopted, with the HF-stimulated rats receiving a total of five episodes of tetanic stimulation at 1.5-hour intervals between the four stimulation sites of the cross-bundle stimulation electrodes. This 1.5-hour interval was used to obtain an optimal saturation of LTP induction in the stimulated pathways, taking into account the fact that LTP does not preclude the further induction of a potentiation during a late

phase of previously induced LTP [U. Frey, K. Schollmeier, K. G. Reymann, T. Seidenbecher, *Neuroscience* **67**, 799 (1995); U. Frey and R. G. M. Morris, *Nature* **385**, 533 (1997)]. The tetanic current was passed between pairs of the four poles of stimulation sites (a, b, c, and d) of the two cross-bundle stimulation electrodes (Fig. 1A, left). The choice of anode and cathode was systematically altered between tetanization episodes, subject to the constraint that anode and cathode were always on opposite sides of the angular bundle. In each episode, eight pulse trains (each consisting of eight stimuli at 400 Hz) were first passed at 2-s intervals between two of the poles (for example, a and c); 1 min later, a similar train was given at the opposite polarity. Then, after another 1-min interval, the whole sequence was repeated with the two other poles (for example, b and d). The choice of the anode and cathode pairs was as follows: ac and bd (episode 1), ad and bc (episode 2), bd and ac (episode 3), and bc and ad (episode 4). The fifth episode was a repetition of the first (ac and bd). Electroencephalogram epochs were recorded at 4-s intervals for 1 min after each tetanization (all combinations of stimulation across the bundle) in a subset of eight tetanized animals, and the samples were screened carefully for afterdischarges. Control rats (also lesioned and implanted) also received eight pulses at 2-s intervals, which were repeated twice within each stimulation episode. In both groups, the intensity was adjusted to evoke fEPSPs at 80 to 90% of the maximum obtained with these electrodes (500 to 2000 μA and 100-μs pulse width). Nonstimulated rats also received a unilateral hippocampal lesion, and 9 rats out of 14 were implanted.

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16. Four of these animals showed <10% on the test for residual LTP (*18*).
17. Behavioral testing was conducted in a water maze, a 198-cm-diameter pool with a featureless white surface, filled to a depth of 40 cm with water at 25° ± 2°C [R. G. M. Morris, *J. Neurosci. Methods* **11**, 47 (1984)]. Latex liquid was added to make the water opaque. A pneumatic escape platform (11 cm in diameter) was located at a fixed position midway between the center and the periphery of the pool. The platform could be moved vertically between an upper available position (1.5 cm below the water surface) and a lower unavailable position (22 cm below the water level) by remote control. Behavioral training started 7 hours after tetanic stimulation was completed. The rats were trained hourly in blocks of two trials, which were separated by 15 s (a total of 10 blocks, corresponding to trials 1 through 20), and were released from one out of eight equally spaced start positions along the perimeter of the pool in a pseudorandom predetermined order. If the rat failed to find the platform within 120 s, the rat was guided onto it. The rat was always left on the platform for 30 s. The position of the black head of the swimming rat was identified and stored at 10 Hz by a video tracking system (VP200, HVS Image, Hampton, UK; Watermaze Software, Edinburgh, UK). Probe tests (with the platform initially unavailable) were conducted on the first trial of blocks 1, 6, and 8 to assess the spatial precision of the search behavior. The platform was kept on the bottom of the pool for the first 40 s and then raised. A final transfer test with the platform submerged for 60 s was conducted at the end of training (called block 11, although consisting of only one trial). On probe trials during training, the latency to cross the platform location was substituted for the actual latency to climb the platform.
18. The extent of saturation at perforant-path/granule-cell synapses was estimated after the completion of the water maze training by tetanizing the fibers activated by the central electrode. The tetanization consisted of two blocks of eight 400-Hz pulses repeated eight times at 2-s intervals and at the same polarity. There was a 1-min interval between the blocks. The tetanization intensity was adjusted to evoke fEPSPs at 80 to 90% of maximum, as above. After the completion of these tests, the rats were killed with an overdose of Equithesin and perfused intracardially with saline and 4% formaldehyde. The brains were stored in formaldehyde for >1 week. Frozen sections were cut coronally (25 μm) and stained with cresyl violet, and the sections were examined for hippocampal and extrahippocampal damage. Sixteen animals (4 HF, 6 LF, and 6 NS) were excluded because of neocortical or thalamic lesions or because of incomplete hippocampal lesions. The exclusion of these animals did not change the pattern of results. Analyses conducted on the entire data set ($n = 43$) gave group [$F(2,40) = 4.3$, $P = 0.02$] and groups × block [$F(18,360) = 2.0$, $P < 0.005$] effects on escape latency and gave a groups × quadrants effect on the probe tests [$F(6,120) = 3.3$, $P < 0.005$].
19. The population spike increased 0.61 ± 0.30 mV (HF group) and 1.34 ± 0.68 mV (LF group). The increase in the HF group was not related to fEPSP enhancement.
20. This conclusion is corroborated by a pilot experiment suggesting that the disruption of spatial learning after LTP saturation is reversible. Six animals receiving HF stimulation were impaired when tested subsequently in a delayed-matching task in the water maze [R. G. M. Morris, J. J. Hagan, J. N. P. Rawlins, *Q. J. Exp. Psychol.* **38B**, 365 (1986)]. These animals showed no improvement in escape latency from the first to the second trial (trial 2 latencies were, on average, 6.5 s longer; there was an intertrial interval of 2 hours). One month later, when LTP had decayed, the animals showed clear evidence of learning from trial 1 to trial 2 on the same test. Latencies were 20.9 s shorter in trial 2 than in trial 1. In NS control rats, the difference was 34.0 s.
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Protein Kinase C Isotypes Controlled by Phosphoinositide 3-Kinase Through the Protein Kinase PDK1

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Phosphorylation sites in members of the protein kinase A (PKA), PKG, and PKC kinase subfamily are conserved. Thus, the PKB kinase PDK1 may be responsible for the phosphorylation of PKC isotypes. PDK1 phosphorylated the activation loop sites of PKCζ and PKCδ in vitro and in a phosphoinositide 3-kinase (PI 3-kinase)–dependent manner in vivo in human embryonic kidney (293) cells. All members of the PKC family tested formed complexes with PDK1. PDK1-dependent phosphorylation of PKCδ in vitro was stimulated by combined PKC and PDK1 activators. The activation loop phosphorylation of PKCδ in response to serum stimulation of cells was PI 3-kinase–dependent and was enhanced by PDK1 coexpression.

Many protein kinases require phosphorylation within their activation loops in order to express full catalytic potential. Such activation loop phosphorylations are also important for protein kinases regulated acutely by allosteric effectors. This is exemplified by PKC, where the Ca^{2+}/diacylglycerol (DAG)–dependent isotypes PKCα and PKCβ display an absolute requirement for phosphorylation in their respective activation loops (*1*, *2*). PKC has been implicated in the control of many cellular processes through the action of the second messenger diacylglycerol and as a receptor for the phorbol ester class of tumor promoters (*3*). There is overlapping specificity for one upstream kinase activity acting on the COOH-terminal hydrophobic sites in PKCα and δ and the equivalent site in PKB (*4*). To assess whether this was also the case for the conserved activation loop sites of PKC and PKB, we tested whether the PKB activation loop kinase PDK1 phosphorylated recombinant PKC.

PKCζ was phosphorylated by recombinant PDK1 (Fig. 1A); incorporation greatly exceeded the basal autophosphorylation of PKCζ itself. The maximum stoichiometry of phosphorylation observed was 2 mol/mol (as deter-

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