Alignments were generated in Megalign 1.1 DNA sequence comparison software. Forward and reverse sequences were generated on an automated sequencer. Our Web site (www2.sel.barc.usda.gov/Schultz/Trachymyrmex papulatus) lists sample sizes by ant species of all 553 cultivar isolates, and additional information on phylogenetic analysis. 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 HaIe 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.

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 25S gene (17) (GenBank accession numbers AF70291-AF70293). Sequences of 11 Lepiotaceae were obtained from GenBank (U11921, U85281-U85283, U85287, U85288, U85291, U85292, U85295, U85296, U85306, U85315, U85318, U85321-U85327, U85326, U85327, U85330, U85331). We thank J. Johnson for posting these unpublished sequences in GenBank.

Alignments were generated in Megalign 1.1 DNASTAR. Regions of ambiguous alignment (306 of 811 sequence positions in ITS; 5 of 611 positions in 25S) were excluded. Phylogenetic analyses were carried out in PAUP* 4.0d61 (D. Swofford, unpublished test version). Sequence data consisted of 1422 nucleotide positions (including ITS sexuality) purges ASD. Heterozygosities were scored time since the origin of asexuality. Recombination late 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).

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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). 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-

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 native 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 EPSP 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 EPSP 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 an 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 $F(2,15)$...
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 \( (F = 1.1, \text{not significant}) \), 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 hippocampal lesion 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 transynaptically (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.

References and Notes


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. Lingo, J. Neurosci. 6, 563 (1986); C. A. Castro, L. H. Silbert, B. L. McNaughton, C. A. Barnes, Nature 342, 545 (1989)].


10. Forty-three naive male Long-Evans rats (300 to 450 g), which were housed in pairs, were on two occasions anesthetized with pentobarbital, 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-ml 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.


13. Two weeks after implantation, evoked waveforms were recorded in the dentate gyrus at 1.5-hour interstimulus 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 EPSP 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 LF than several days after receiving a single day of massed stimulation, possibly because of slowly developing homeostatic changes in synaptic weights in populations undergoing substantial or down-regulation of synaptic transmission [G. C. Turinello, K. R. Leslie, N. S. Desai, L. C. Rutherford, S. B. Nelson, Nature 391, 892 (1998)].

Thus, a massed stimulation protocol was adopted, with 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 stimulus pathways, thereby taking into account the fact that LTP does not preclude the further induction of a potentiation during a late
Phosphorylation sites in members of the protein kinase A (PKA), PKG, and PKC kinase subfamilies are conserved. Thus, the PKB kinase PKD1 may be responsible for the phosphorylation of PKC isotypes. PKD1 phosphorylated the activation loop sites of PKCα 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 PKD1. PKD1–dependent phosphorylation of PKCδ in vitro was stimulated by combined PKC and PKD1 activators. The activation loop phosphorylation of PKCδ in response to serum stimulation of cells was PI 3-kinase–dependent and was enhanced by PKD1 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 associated with cell survival and proliferation. Therefore, the PKB activation loop kinase PDK1 may be responsible for the phosphorylation of PKC isotypes. PKD1 phosphorylated the activation loop sites of PKCα 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 PKD1. PKD1–dependent phosphorylation of PKCδ in vitro was stimulated by combined PKC and PKD1 activators. The activation loop phosphorylation of PKCδ in response to serum stimulation of cells was PI 3-kinase–dependent and was enhanced by PKD1 coexpression.