$\beta APP_{649-664}$. On the basis of its size, this protein may be a derivative that contains only the cytoplasmic region of the βAPP.

The multiple COOH-terminal βAPP derivatives that we have identified are depicted schematically in Fig. 3B. In transfected 293 cells, the smaller two fragments are most abundant (Fig. 2B), and one of these most likely corresponds to the derivative beginning at βAP_{17} (*6*). Proteins essentially identical to the two large βAP-bearing derivatives have been shown to (i) aggregate in vitro (*16*), (ii) form deposits when overexpressed in cultured cells (*10, 18*), and (iii) cause degeneration of neurite-producing PC12 cells (*19*). Thus these derivatives are likely to be important intermediates in the pathway leading to amyloid deposition in AD.

REFERENCES AND NOTES

1. M. R. Palmert *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6338 (1989).
2. M. R. Palmert *et al.*, *Biochem. Biophys. Res. Commun.* **165**, 182 (1989).
3. A. Weidemann *et al.*, *Cell* **57**, 115 (1989).
4. D. Schubert *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2066 (1989).
5. S. S. Sisodia *et al.*, *Science* **248**, 492 (1990).
6. F. S. Esch *et al.*, *ibid.*, p. 1122; J. P. Anderson *et al.*, *Neurosci. Lett.* **128**, 126 (1991).
7. Pellets containing membrane-associated proteins, prepared from cortical gray matter of control subjects or subjects with histologically proven AD (postmortem interval <5 hours) as described (*1*), were homogenized in TBS (150 mM NaCl, 10 mM tris, pH 8.0)-LPT (1 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 1 μg/ml 7-amino-1-chloro-3-tosylamido-2-heptanone) containing 2% Nonidet P-40, 2% Triton X-100, 2 mM EDTA, and 1 mM phenylmethylsufonyl fluoride. After 20 min on ice, homogenates were centrifuged for 1 hour at 100,000*g*, and the supernatant was applied to a protein A–agarose column. The void volume was then passed over an anti-C_{24} column made by dimethylpimelimidate cross-link of 5 ml of antiserum to protein A–agarose (*20*). The bound proteins were washed extensively with TBST-LPT (TBS-LPT containing 0.05% Tween-20), RIPA (150 mM NaCl, 10 mM tris, pH 8.0, 1% Nonidet P-40, 0.5% cholic acid, 0.1% SDS)-LPT, TBST-LPT, and then eluted with 0.1 M glycine-LPT (pH 2.6) in 0.05% Tween-20. The pH of the eluant was neutralized, and it was applied to a second protein A–agarose column. Immunoblotting and immunoabsorptions (50 μg peptide) were carried out as described (*1*). Anti-C_{24} was induced by injecting a rabbit with 100 μg of $\beta APP_{672-695}$ conjugated to keyhole limpet hemocyanin in complete Freund's adjuvant and then injecting biweekly 250 μg $\beta APP_{672-695}$ plus 1 mg of heat killed *Mycobacterium tuberculosis*. Figure 1, A through D, represents βAPP derivatives purified from approximately 0.5 g of human brain. The immunoprecipitations shown in Fig. 1, F and G, were carried out by incubating 10 to 20 μl of the indicated antibody with affinity-purified protein in 600 μl (final volume) RIPA-LPT. $M\beta AP_1$-COOH was generated with a subclone of the transcription vector pSP72 that contained an Eco R1–Mae 1 βAPP cDNA fragment ($\beta APP_{1795-2085}$). Translation was initiated from an ATG site located within oligonucleotide adaptors.
8. H. Schagger and G. von Jagow, *Anal. Biochem.* **166**, 368 (1987).
9. D. J. Selkoe *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7341 (1988).
10. D. Wolf *et al.*, *EMBO J.* **9**, 2079 (1990).
11. J. D. Buxbaum *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6003 (1990).
12. T. Oltersdorf *et al.*, *J. Biol. Chem.* **265**, 4492 (1990).
13. J. Hambor *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4010 (1988).
14. βAPP_{695} was overexpressed in 293 cells by using an episomal replicon (CEP4β) similar to that described by Hambor and co-workers (*13*) except that expression was driven by a cytomegalovirus promoter. Stable lines containing CEP4β or CEP4β-βAPP_{695} were established by transfecting 10^6 cells with lipofectin (BRL) (20 μg/ml) and cesium chloride (10 μg/ml)–purified plasmid DNA in Opti-MEM 1 (BRL) medium. After 4 hours in the lipofectin DNA mix, cells recovered for 24 hours in fresh medium [Opti-MEM 1 plus 5% calf serum, supplemented and iron enriched (BRL)], were trypsinized, and stable lines were selected by 2-week exposure to hygromycin B (Boehringer Mannheim) (100 μg/ml). Stable lines were maintained in hygromycin B (200 μg/ml). Approximately 5×10^6 cells (80% confluent grown at 37°C in 5% CO_2) were rinsed twice with Hanks balanced salt solution and labeled with TRAN^{35}S-LABEL (ICN, 400 μCi/ml [^{35}S]methionine and 60 μCi/ml [^{35}S]cysteine) in 2 ml of methionine and cysteine-deficient DMEM for 10 min. Chases were performed by rinsing twice with serum-free media and incubating in 5 ml of fresh serum-free media for the indicated times. Cells were then lysed in RIPA-LPT (7), the supernatants cleared with protein A–agarose, and the proteins immunoprecipitated with 4 μl of anti-C_{20} and protein A–agarose (*20*).
15. Samples (1.5 g) of adult rat cerebrum or human tissue (control sample, 4 hours after death, flash frozen at autopsy and stored at −80°C until use) were thawed, Dounce-homogenized in RIPA-LPT, cleared with protein A–agarose, immunoprecipitated with 10 μl of anti-C_{20} and 20 μl of protein A–agarose (*20*), separated by 10 to 16% tris-tricine SDS-PAGE, and immunoblotted with anti-C_{20}.
16. T. Dyrks *et al.*, *EMBO J.* **7**, 949 (1988).
17. S. Gandy, A. J. Czernik, P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6218 (1988).
18. K. Maruyama *et al.*, *Nature* **347**, 566 (1990).
19. B. A. Yankner *et al.*, *Science* **245**, 417 (1989).
20. E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Press, Cold Spring Harbor, NY, 1988).

21. We thank D. Selkoe for anti-βAP_{1-40} (DS1280), anti-C_{20} (rabbit C7), and anti-$\beta APP_{649-664}$, G. Perry for anti-βAP_{1-42} (rabbit RGP9), and R. Groger and M. Tykocinski for the CEP4β expression construct. Supported by NIH grants AG06656, AG08012, and AG08992.

28 August 1991; accepted 4 December 1991

Processing of the Amyloid Protein Precursor to Potentially Amyloidogenic Derivatives

TODD E. GOLDE, STEVEN ESTUS, LINDA H. YOUNKIN, DENNIS J. SELKOE, STEVEN G. YOUNKIN

The ~120-kilodalton amyloid β protein precursor (βAPP) is processed into a complex set of 8- to 12-kilodalton carboxyl-terminal derivatives that includes potentially amyloidogenic forms with the ~4-kilodalton amyloid β protein (βAP) at or near their amino terminus. In order to determine if these derivatives are processed in a secretory pathway or by the endosomal-lysosomal system, (i) deletion mutants that produce the normal set of carboxyl-terminal derivatives and shortened secreted derivatives were analyzed and (ii) the effect of inhibitors of endosomal-lysosomal processing was examined. In the secretory pathway, cleavage of the βAPP occurs at a single site within the βAP to generate one secreted derivative and one nonamyloidogenic carboxyl-terminal fragment, whereas, in the endosomal-lysosomal system, a complex set of carboxyl-terminal derivatives is produced that includes the potentially amyloidogenic forms.

THE βAPP IS NORMALLY CLEAVED within the 43–amino acid βAP to produce a large secreted derivative ending at amino acid 15 of βAP and a small membrane-associated COOH-terminal derivative beginning at amino acid 17 (*1*). If βAPP secretase cleaves full-length βAPP at additional minor sites, then it could generate the entire set of COOH-terminal derivatives that have been identified (*2*). In this process, a corresponding set of secreted derivatives would be produced. These secreted derivatives of over 100 kD, differing by only several kilodaltons, would be difficult to separate by SDS–polyacrylamide gel electrophoresis (PAGE). To avoid this difficulty and to determine whether βAPP secretase produces multiple secreted derivatives, we made βAPP_{695} deletion constructs (Fig. 1A) similar to those described by Sisodia and co-workers (*3*). These constructs secrete shortened derivatives in which small length differences are readily detectable by SDS-PAGE.

Comparison of control (CEP4β) 293 cells and cells transfected with these deletion constructs showed the production of shortened βAPPs (Fig. 1B). These shortened βAPPs were processed into (i) a set of 8- to 12-kD COOH-terminal derivatives essentially identical to that observed in cells transfected with a βAPP_{695} expression construct (Fig. 1C) and (ii) secreted derivatives that, like secreted βAPP_{695}, were ~10 kD smaller than the full-length forms (compare

T. E. Golde, S. Estus, L. H. Younkin, S. G. Younkin, Division of Neuropathology, Institute of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH 44106.
D. J. Selkoe, Department of Neurology (Neuroscience), Harvard Medical School, and Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA 02115.

βAPP_{438} in Fig. 1, B and D). Significantly, the derivatives secreted by cells transfected with these deletion constructs were well separated by 10% tris-tricine SDS-PAGE (Fig. 1D) even though they differ by as few as four amino acids. To analyze the fluorogram showing these secreted derivatives (Fig. 1D), we exposed it so that the intensity of the major secreted derivatives matched or exceeded the intensity of the major COOH-terminal forms (Fig. 1C). Under this condition, any minor secreted derivatives produced by βAPP secretase should be readily detected at approximately the same intensity as the minor COOH-terminal forms (Fig. 1C). However, only a single secreted derivative was detected for each construct (Fig. 1D), and this was the case even with much longer exposure of the fluorogram. Thus our data show that βAPP secretase cleaves at only one site to produce one secreted NH_2-terminal derivative and one COOH-terminal form. This indicates that an alternative pathway is required to produce the complex set of COOH-terminal derivatives that includes potentially amyloidogenic forms.

To assess the possibility that endosomal-lysosomal processing produces this complex set of derivatives, we treated 293 cells transfected with βAPP_{695} with ammonium chloride (50 mM) or leupeptin (100 μg/ml), inhibitors of endosomal-lysosomal proteolysis that act by different mechanisms (*4*). In transfected cells incubated with radioactive amino acids for 1 hour and then with unlabeled amino acids for 1 or 4 hours (chase), ammonium chloride substantially reduced the entire set of 8- to 12-kD COOH-terminal derivatives and almost completely abolished the two largest forms (Fig. 2A). In addition, ammonium chloride markedly augmented the amount of full-length, cell-associated βAPP remaining after a 4-hour chase (Fig. 2C). To assess the possibility

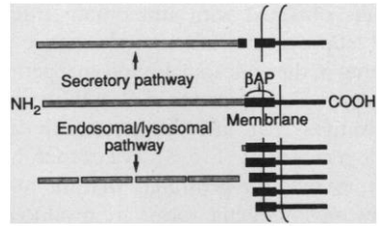

Fig. 3. Schematic summary of βAPP processing.

that some of the effect of ammonium chloride, particularly on the small COOH-terminal derivatives, might be due to inhibition of βAPP secretase, we examined secreted βAPP derivatives after treatment with ammonium chloride. Ammonium chloride caused no reduction in the secreted derivatives that accumulated during a 4-hour chase and, consistent with this, permitted continued production of a COOH-terminal derivative with a size appropriate for the secretase cleavage at βAP_{16} (Fig. 2B). Thus our data indicate that endosomal-lysosomal processing generates a complex set of COOH-terminal derivatives that includes potentially amyloidogenic forms as well as smaller derivatives similar to the nonamyloidogenic derivative produced by βAPP secretase.

Leupeptin had no effect on the secreted derivatives that accumulated during a 4-hour chase (Fig. 2B), and it also diminished the COOH-terminal derivatives produced during 1 hour of chase to an extent similar to that observed with ammonium chloride (Fig. 2A). Thus leupeptin, like ammonium chloride, inhibits production of COOH-terminal derivatives without affecting βAPP secretase. However, leupeptin caused COOH-terminal derivatives to accumulate between 1 and 4 hours of chase (Fig. 2A), and it did not cause accumulation of full-length βAPP (Fig. 2C). Thus the effect of leupeptin on βAPP processing in the endosomal-lysosomal system is more complex than that of ammonium chloride. Like ammonium chloride, leupeptin decreases the initial production of COOH-terminal derivatives. However, leupeptin permits partial degradation of full-length forms and causes COOH-terminal derivatives to eventually accumulate, possibly because it inhibits not only the production of COOH-terminal derivatives but also their degradation. In support of this interpretation, we found that leupeptin increases and ammonium chloride decreases cell-associated (~15 to 75 kD) NH_2-terminal βAPP derivatives recognized by antibody to βAPP_{17-611} and augmented in transfected cells.

Our analysis of deletion mutants indicates that in 293 cells βAPP secretase cleaves at a single site previously shown to be within the βAP to preclude amyloid deposition. The

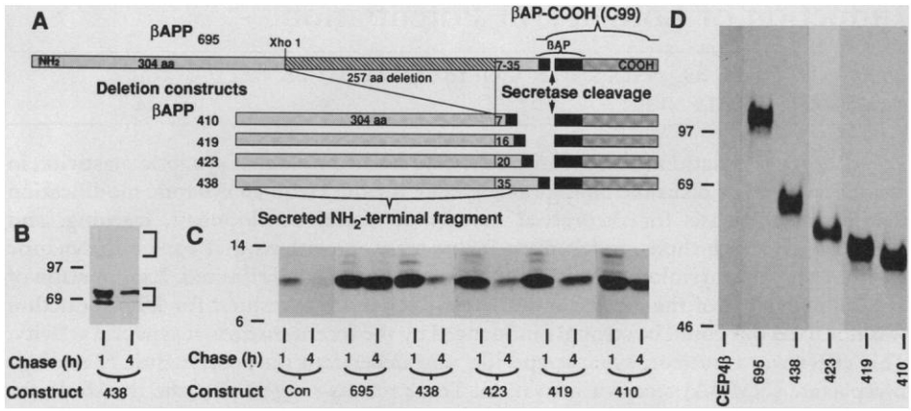

Fig. 1. (**A**) βAPP deletion mutants. These mutants encode βAPPs that contain (i) the first 304 amino acids (aa) in βAPP_{695}, (ii) the 7 (βAPP_{410}), 16 (βAPP_{419}), 20 (βAPP_{423}), or 35 (βAPP_{438}) amino acids on the NH_2-terminal side of βAP_1, and the last 99 amino acids (C99 or βAP_1-COOH) of wild-type βAPP (*10*). (**B**) Full-length βAPP_{438}. Upper bracket shows endogenous $\beta APP_{751/770}$. All other deletion mutants showed similar shortened full-length forms. Exposure time, 4 hours; 10% to 16.5% SDS-PAGE. (**C**) COOH-terminal derivatives in stable cell lines expressing βAPP deletion constructs. Exposure times: 72 hours; 10% to 16.5% tris-tricine SDS-PAGE. (**D**) Secreted derivatives produced by stable cell lines expressing βAPP deletion constructs. Media were collected for 4 hours after 1-hour pulse. Exposure times: 423 construct, 12 hours, all others, 4 hours; 10% tris-tricine SDS-PAGE. Approximately 5×10^6 cells were labeled for 1 hour (250 μCi [^{35}S]methionine) and chased for 1 or 4 hours. COOH-terminal derivatives were immunoprecipitated with anti-C_{20} as described (*1*). To immunoprecipitate secreted derivatives, the medium was made 1× in RIPA buffer (*1*), boiled for 10 min, cleared with protein A–agarose for 1 hour, and immunoprecipitated with 10 μl of antibody to βAPP_{45-62} cross-linked to protein A–agarose beads. Samples were then washed and loaded on gels as described (*1*).

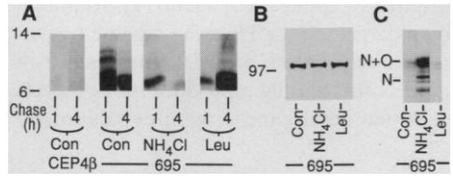

Fig. 2. Effects of ammonium chloride and leupeptin on βAPP processing. Each panel shows untreated (Con), ammonium chloride–treated (NH_4Cl), and leupeptin-treated (Leu) cells transfected with a βAPP_{695} expression construct as well as untreated cells transfected with the CEP4β alone (Con, CEP4β). (**A**) COOH-terminal derivatives. Exposure time, 72 hours; 16.5% tris-tricine SDS-PAGE. (**B**) Secreted derivatives accumulated during a 4-hour chase. Exposure time, 2 hours; 10% tris-tricine SDS-PAGE. (**C**) Full-length βAPP. Exposure time, 4 hours; 10% tris-tricine SDS-PAGE. Approximately 5×10^6 cells were pretreated for 30 min with either 50 mM ammonium chloride or 100 μg of leupeptin per milliliter (diluted from 250× stocks in water). Labeling with [^{35}S]methionine (250 μCi, 1 hour) and chases (1 or 4 hours in serum-free media) were performed in the presence of these agents as described (*1*). Immunoprecipitations were performed as in Fig. 1.

results obtained with ammonium chloride and leupeptin indicate that alternative processing in the endosomal-lysosomal pathway generates a complex set of COOH-terminal derivatives that includes potentially amyloidogenic forms (Fig. 3). We cannot, however, exclude the possibility that the potentially amyloidogenic forms are produced to some extent by an atypical nonlysosomal protease inhibited by both ammonium chloride and leupeptin.

Our finding that the βAPP is processed by the endosomal-lysosomal system is supported by studies showing punctate intracellular labeling by antibodies to βAPP consistent with localization of βAPP to lysosomes (*5*), and reports by Cole and co-workers (*6*) demonstrating that inhibitors of endosomal-lysosomal processing increase the steady-state level of βAPP and βAPP derivatives. Moreover, the βAPP contains a cytoplasmic sequence known to target membrane-associated glycoproteins for endocytosis (7).

The specific proteases that produce amyloid are potential therapeutic targets in Alzheimer's disease (AD). Our data showing that endosomal-lysosomal processing produces potentially amyloidogenic βAP-bearing COOH-terminal derivatives focus the search for these proteases on the endosomal-lysosomal system. βAP-bearing COOH-terminal derivatives are probably further processed in this system to produce small fragments similar or identical to the βAP. These fragments could be quite stable because they would tend to aggregate into a β-pleated sheet structure, but fragments like this have not yet been detected in normal cells.

The apparent localization of the βAPP to lysosomes (*5*) and the immunocytochemical detection of lysosomal proteases in senile plaques (*8*) have independently led several groups to propose that aberrant lysosomal processing may play a role in AD. Our data indicate that normal endosomal-lysosomal processing produces potentially amyloidogenic βAPP fragments, and they support the hypothesis that dysfunctional endosomal-lysosomal processing may play a critical role in the pathogenesis of AD. There is evidence that amyloid deposition arises through mutations in the βAPP gene in familial AD (*9*), but it is not yet known whether these mutations alter endosomal-lysosomal processing in a way that is amyloidogenic.

REFERENCES AND NOTES

1. F. S. Esch *et al.*, *Science* **248**, 1122 (1990).
2. S. Estus *et al.*, *ibid.* **255**, 726 (1992).
3. S. S. Sisodia *et al.*, *ibid.* **248**, 492 (1990).
4. P. O. Seglen, *Methods Enzymol.* **96**, 737 (1983). Ammonium chloride is a weak base amine that is concentrated in acidic compartments where it inhibits endosomal-lysosomal proteolysis by raising the pH of these compartments. It is generally considered the most specific inhibitor of endosomal-lysosomal proteolysis. Leupeptin is a dipeptidyl protease inhibitor. It effectively inhibits endosomal-lysosomal proteolysis by inhibiting serine and cysteine proteases. Because protease inhibitors like leupeptin only affect a specific class of proteases, they are usually not as effective as the weak base amines. However, because lysosomal enzymes work sequentially, as much as 80% of the amine-sensitive degradation can be inhibited with agents like leupeptin.
5. G. M. Cole *et al.*, *Neurochem. Res.* **14**, 933 (1989); L. I. Benowitz *et al.*, *Exp. Neurol.* **106**, 237 (1989); G. M. Cole *et al.*, *Neurobiol. Aging* 12, **85** (1991); M. Kawai *et al.*, *Am. J. Pathol.*, in press.
6. G. M. Cole *et al.*, in *Molecular Biology and Genetics of Alzheimer's Disease*, T. Miyatake *et al.*, Eds. (Elsevier, Amsterdam, 1990), pp. 113–122.
7. W.-J. Chen *et al.*, *J. Biol. Chem.* **265**, 3116 (1990).
8. A. M. Cataldo *et al.*, *Brain Res.* **513**, 181 (1990); A. M. Cataldo and R. A. Nixon, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3861 (1990).
9. A. Goate *et al.*, *Nature* **349**, 704 (1991); G. Lucotte *et al.*, *ibid.* **351**, 530 (1991); J. Murrell *et al.*, *Science* **254**, 97 (1991); M.-C. Chartier-Harlin, *Nature* **353**, 844 (1991).
10. The forward oligonucleotides used to generate the deletion mutants are βAPP410 (5′-GTATCTCGAGATCTCTGAAGTGAAGATGG-3′); βAPP419 (5′-GTATCTCGAGGGGTTGACAAATATCAAGA-3′); βAPP423 (5′-GTATCTCGAGCGACCAGGTTCTGGGTTGA-3′); and βAPP438 (5′-GTATCTCGAGGAGCCTGTTGATGCCCGCC-3′). These primers correspond to nucleotides 1771–1789, 1744–1762, 1732–1750, and 1687–1705, respectively, of the βAPP_{695} sequence [J. Kang *et al.*, *Nature* **325**, 733 (1987)] and contain a common 5′ sequence, GTATCTCGAG, that allows cleavage with Xho 1 and in-frame fusion of the amplified DNAs encoding the various COOH-terminal regions with the DNA encoding the 304 residues at the NH_2-terminal end of the deletion mutants. The reverse primer used for each mutant was βAPP2121 (5′-GACTCGAGTCGACGGATCCGTGTCCAACTTCAGAGGCTGC-3′), which contains a 5′ adaptor sequence of 20 base pairs followed by nucleotides 2121–2101 of βAPP_{695} (*11*).
11. We thank G. Perry for antibody to βAPP_{45-62}.

28 August 1991; accepted 4 December 1991

The Influence of Prior Synaptic Activity on the Induction of Long-Term Potentiation

YAN-YOU HUANG, ASUNCION COLINO, DAVID K. SELIG, ROBERT C. MALENKA*

Long-term potentiation (LTP) is an extensively studied model of synaptic plasticity, in part because it is a plausible biological correlate for the Hebbian synaptic modification that forms the basis for theoretical models of neural development, learning, and memory. Although these models must incorporate algorithms that constrain synaptic weight changes, physiological evidence for such mechanisms is limited. Examination of LTP in area CA1 of the hippocampus revealed that the threshold for LTP induction was not fixed but could be strongly influenced by the recent history of synaptic activity. This effect was transient, synapse-specific, and dependent on postsynaptic *N*-methyl-D-aspartate (NMDA) receptor activation. These results suggest that the threshold for LTP induction may be continually adjusted according to the recent history of NMDA receptor activation and provide a physiological mechanism by which LTP can be transiently inhibited.

ACTIVITY-DEPENDENT MODIFICATIONS in synaptic strength are critical for the development of neural networks and for certain forms of learning and memory. Most attention has focused on one form of LTP that is dependent on activation of postsynaptic NMDA receptors, because its induction requires coincident pre- and postsynaptic activity, thus fulfilling the criteria for a synaptic plasticity mechanism originally postulated by Hebb (*1*). Although by incorporating a Hebbian scheme of synaptic modification, artificial neural network models successfully reproduce many aspects of neural development, learning, and memory, they also require a means for limiting changes in synaptic weights (*2*). One theoretical mechanism, which has been incorporated into some neural network models, permits an adjustment of the threshold for synaptic modification according to the recent history of postsynaptic cell activity (*3*). We have tested whether the history of synaptic activation influences the induction of LTP in the CA1 region of the hippocampus.

In all experiments we simultaneously recorded excitatory postsynaptic potentials (EPSPs) in response to two independent inputs synapsing on the same cell or population of cells (*4*). When a weak tetanus (30 Hz; 0.1 to 0.2 s) that elicits decremental, NMDA receptor–dependent short-term potentiation (STP) (*5, 6*) was given repetitively (four to six times) at 2-min intervals, a subsequent stronger tetanus (100 Hz; 0.5 s) capable of eliciting LTP in a control pathway did not elicit LTP in the test pathway

Y.-Y. Huang, A. Colino, D. K. Selig, Department of Psychiatry, University of California, San Francisco, CA 94143.
R. C. Malenka, Departments of Psychiatry and Physiology, University of California, San Francisco, CA 94143.

*To whom correspondence should be addressed.