pant's waist. The lamp housing was placed beneath the table and under the skirt, so that any light escaping through the housing vents was obscured from the participant's eyes. Illumination at the participant's eye level never exceeded 20 lux. Each BiliBlanket provided ~13,000 lux to the popliteal region. An exhaust fan (in addition to those in each BiliBlanket housing) was placed beneath the skirt to evacuate any heat produced by the halogen light source.
14. Body core temperature was recorded in 2-min epochs, with disposable rectal thermistors (Yellow Springs) attached to Mini-logger ambulatory recording devices (Mini-Mitter, Sun River, OR).
15. Saliva samples were collected under dim light from 1800 until 2400 on night 2 (before light exposure) and on night 4. Melatonin concentrations were measured by radioimmunoassay (ALPCO, Windham, NH) with the Kennaway G280 antibody [G. M. Vaughan *et al.*, *J. Pineal Res.* **15**, 88 (1993)]. All samples from a given participant during a given laboratory session were analyzed in the same assay. We have calculated an intra-assay coefficient of variation of 2.1%; the interassay precision has been reported as 10.4% (*26*).
16. The raw temperature data set for each participant was divided into 24-hour subsets and demasked {to account for any evoked effect of sleep on body temperature [D. S. Minors and J. M. Waterhouse, *Chronobiol. Int.* **6**, 29 (1989)]}, and then each subset was fit with a complex cosine curve with a 24-hour and a 12-hour harmonic. All temperature curves reached a goodness-of-fit criterion of $r^2 \geq 0.8$ (variance accounted for); one participant whose baseline temperature curve did not meet this requirement was excluded from the analyses. The nadirs (fitted minima) of the fitted curves were used to determine the phase of the body core temperature rhythm. The DLMO was defined as the time at which salivary melatonin concentrations exceeded 3.33 pg/ml. This threshold was based on a conventional definition of a 10 pg/ml threshold for plasma melatonin concentrations [A. J. Lewy and R. L. Sack, *ibid.*, p. 93] and published evidence that the radioimmunoassay used here yields salivary melatonin concentrations ~30% of those obtained in plasma (*26*).
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Postsynaptic Membrane Fusion and Long-Term Potentiation

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The possibility that membrane fusion events in the postsynaptic cell may be required for the change in synaptic strength resulting from long-term potentiation (LTP) was examined. Introducing substances into the postsynaptic cell that block membrane fusion at a number of different steps reduced LTP. Introducing SNAP, a protein that promotes membrane fusion, into cells enhanced synaptic transmission, and this enhancement was significantly less when generated in synapses that expressed LTP. Thus, postsynaptic fusion events, which could be involved either in retrograde signaling or in regulating postsynaptic receptor function or both, contribute to LTP.

Brief repetitive stimulation of excitatory synapses in many regions of the central nervous system results in a long-lasting increase in synaptic strength referred to as long-term potentiation (LTP). Although LTP at most synapses is known to require the activation of the *N*-methyl-D-aspartate (NMDA) subclass of glutamate receptor and a subsequent rise in postsynaptic calcium concentration, the steps involved in generating the persistent increase in synaptic strength are poorly understood (*1*). Thus, it is still unresolved whether the increase in synaptic strength results primarily from a persistent increase in the release of glutamate (the transmitter at excitatory synapses) or from a persistent increase in the sensitivity of the postsynaptic cell to glutamate.

Regardless of which mechanism proves to be correct, an attractive hypothesis is that membrane fusion events in the postsynaptic cell play an important role in LTP. A presynaptic LTP expression mechanism requires the release of retrograde messengers from the postsynaptic cell, a process that could involve either membrane-permeant messengers or the exocytosis of messenger from the postsynaptic cell (*2*). A proposed postsynaptic expression mechanism involves the all-or-none up-regulation of glutamate receptors, possibly by the insertion of membrane containing glutamate receptors (*3*). Thus, membrane fusion events in the postsynaptic cell could be required for generation of both the pre- and postsynaptic modifications that have been proposed to occur during LTP. To test this possibility we examined the effects on LTP of several agents that disrupt membrane fusion by interrupting different steps in the protein-protein interaction cascade involved in membrane fusion. In addition, we examined the effects of introducing a recombinant protein into the postsynaptic cell that promotes fusion.

Standard hippocampal slice and electrophysiological recording techniques were used for all experiments (*4*). All compounds were introduced directly into the postsynaptic cell through sharp, intracellular recording microelectrodes, which were used to prevent the washout of LTP that occurs with whole-cell recording (*5*). In all experiments we compared the responses recorded intracellularly with those recorded simultaneously from an extracellular recording electrode placed nearby in the stratum radiatum. This permitted us to monitor the stability of the preparation and, importantly, the generation of LTP in the cells surrounding the manipulated cell simultaneously.

First, we tested the effects of *N*-ethylmaleimide (NEM), which blocks NEM-sensitive factor (NSF), a cytosolic adenosine triphosphate–binding protein that, by interacting with SNAPs (soluble NSF-attachment proteins), is required for a large number of membrane fusion reactions (*6*). NEM (5 mM) was dissolved in the electrode solution (2 M potassium acetate) and loaded into the postsynaptic cell by diffusion from the intracellular electrode. To allow sufficient time for NEM to diffuse into the cell, we waited 30 to 50 min before attempting to elicit LTP with tetanic stimulation (Fig. 1). Although tetanic stimulation produced a large LTP in the field potential recording,

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averaging 145 ± 37% (n = 9) (Fig. 1, B and D), it produced a potentiation of only 24 ± 19% (n = 9) in cells loaded with NEM (Fig. 1, A and C) ($P < 0.0001$, unpaired t test). The lack of LTP was not simply due to the prolonged recording with a sharp electrode, because other control experiments with the same duration of recording (Fig. 2C) showed normal LTP. Importantly, NEM had minimal effect on baseline synaptic transmission.

Although NEM, which reacts with free sulfhydryl groups in cysteine residues, is a powerful tool in the study of membrane fusion machinery, it might disrupt LTP by an action unrelated to the blockade of NSF, for instance, by inactivating NMDA receptors (NMDARs). Thus, we tested the effect of NEM on NMDAR-mediated synaptic currents by adding 5 mM NEM to the whole-cell pipette solution and measuring the amplitude of excitatory postsynaptic currents (EPSCs) over time in the presence of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor antagonist NBQX. Whole-cell recording was used because voltage control of the NMDAR-mediated response is superior with this approach. The use of the same concentration of NEM as was used with the intracellular recording experiments would be expected to result in a much greater intracellular concentration. Under these recording conditions, no reduction in NMDAR EPSCs was observed when measured 50 to 60 min after starting whole-cell recording (117 ± 4%, n = 4).

The binding of NSF to SNAP is required for many membrane fusion events. Because NEM may have effects other than interfering with NSF, it was important to determine whether a different inhibitor of NSF-SNAP interaction blocked LTP. For the squid giant synapse it has been shown that presynaptic injection of a short peptide that has the same sequence as the site on SNAP to which NSF binds blocks the release of transmitter, presumably by competing with NSF for binding to SNAP (7). We prepared a similar peptide (referred to as N19) and examined its effect on the ability of the

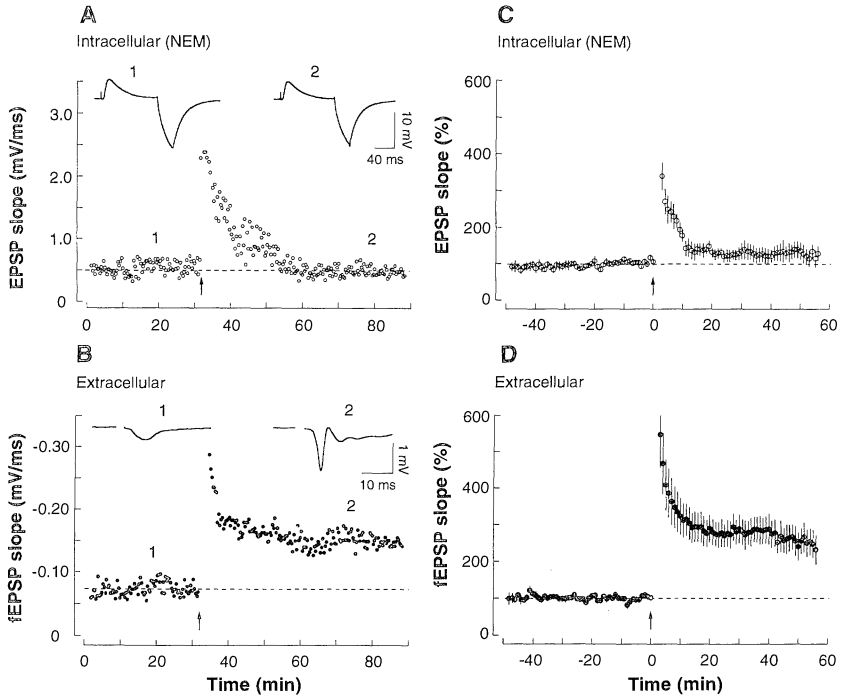

Fig. 1. Postsynaptic loading of NEM reduces LTP in the CA1 region of the hippocampus. (**A** and **B**) Results from a single experiment that compare intracellular recordings of excitatory postsynaptic potentials (EPSPs) from microelectrodes containing 5 mM NEM (A) with simultaneous recordings of field potentials (fEPSPs) (B). Insets show averages of five consecutive traces recorded before (trace 1) and 50 min after (trace 2) tetanization (arrows). (**C** and **D**) Comparison of LTP in cells loaded with NEM (C) (n = 9) with simultaneous recordings of fEPSPs (D).

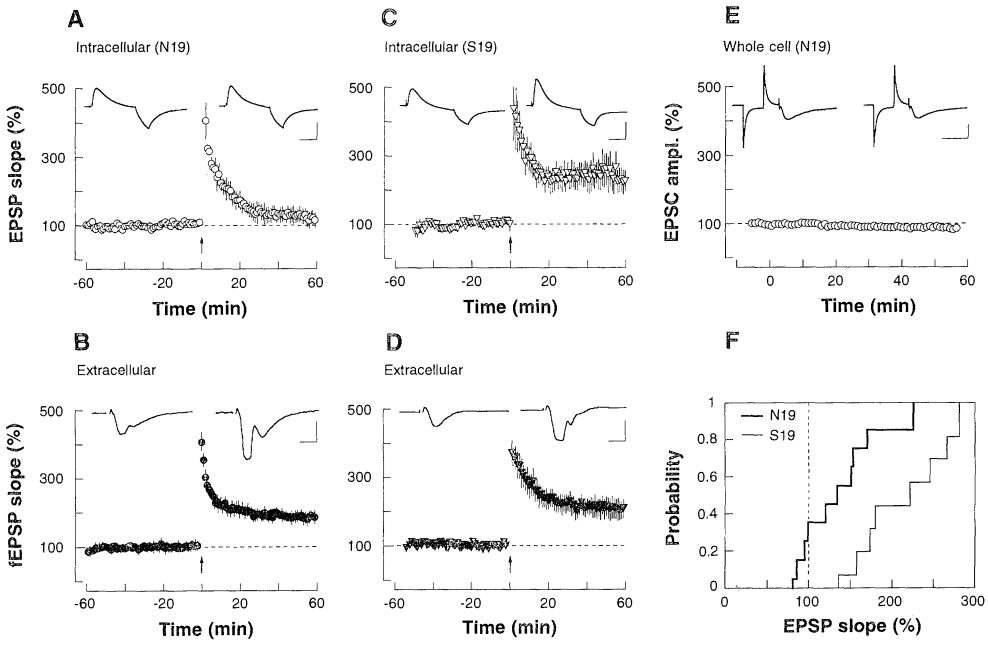

Fig. 2. Postsynaptic loading of a peptide that mimics the NH_2-terminal domain of SNAP reduces LTP. Graphs of LTP in cells recorded with microelectrodes containing (**A**) N19 (1 mM) (n = 12) or (**C**) S19 (n = 8). (**B** and **D**) Graphs of LTP in extracellular field potential recordings from experiments in (A) and (C), respectively. Arrows indicate the time of tetanic stimulation. Insets show representative traces recorded 30 min before (left) and after (right) the tetanic stimulation. Scale bars: 10 mV and 50 ms for (A) and (C); 0.4 mV and 10 ms for (B) and (D). (**E**) Graph (n = 4) of normalized NMDA current amplitudes recorded with N19 (1 mM) in the whole-cell patch pipette solution. Inset shows representative NMDAR EPSCs recorded at 5 and 50 min after recording was begun. Scale bars: 40 pA and 50 ms. (**F**) Cumulative histograms that compare the magnitude of LTP obtained during intracellular recordings with either N19 (thick line) or S19 (thin line) peptides.

postsynaptic cell to generate LTP (8). Again, to ensure adequate diffusion of the peptide into the postsynaptic cell, the tetanus was given only after 1 hour of intracellular recording. As was the case for NEM, N19 had no effect on baseline transmission, but the magnitude of LTP recorded intracellularly (30 ± 18%) was much less than that recorded extracellularly (100 ± 16%) (Fig. 2, A and B) (n = 12).

Two additional experiments were conducted to rule out nonspecific effects of N19. First, we made another peptide using a random sequence of the same 19 amino acids (9). This scrambled peptide (referred to as S19) applied at an identical concentration with the same protocol had no effect on LTP (138 ± 40% and 106 ± 19% for intracellular and extracellular recordings, respectively, n = 8) (Fig. 2, C and D). It is important to note that during the course of these experiments the investigator did not know whether the electrode contained the N19 or S19 peptide. For a more detailed comparison of the results with N19 and S19, we plotted the results from each cell as a cumulative probability distribution (Fig. 2F). The graph of the N19 experiments is shifted to the left ($P < 0.005$, unpaired t test), and a number of cells loaded with N19 showed no LTP, whereas all cells loaded with S19 generated LTP. The second control experiment examined whether N19 had any effect on NMDAR-mediated synaptic responses because blockade of NMDARs would also block LTP. Use of the same concentration of N19 in the whole-cell pipette had no significant effect on the amplitude of the NMDAR EPSC (94 ± 5% measured between 50 to 60 min, n = 5) (Fig. 2E). Furthermore, the envelope of depolarization during the tetanus was unaltered by N19. Thus, the effects of N19 on LTP were not due to some disruption in the initial triggering events that are required for LTP.

Perhaps the most selective way to disrupt membrane fusion machinery is with the clostridial neurotoxins, which, depending on the specific serotype, proteolytically cleave three proteins, synaptobrevin (or VAMP), SNAP-25, and syntaxin (6, 10). These three proteins form a complex that serves as a receptor for α-SNAP and NSF. We chose to use botulinum toxin (serotype B), which acts on the vesicular membrane proteins of the synaptobrevin family (10). The tetanic stimulation was given only after 2 hours of intracellular recording with electrodes containing 0.5 μM botulinum toxin. The light chain of botulinum toxin had no effect on baseline transmission (Fig. 3A) but greatly reduced the magnitude of LTP (11 ± 15% and 110 ± 11% for intracellular and extracellular recordings, respectively, n = 5) ($P < 0.0001$, unpaired t test). This effect presumably resulted from its enzymatic activity, because the heat-inactivated enzyme (11) had no effect on LTP (72 ± 25% and 82 ± 43% for intracellular and extracellular recordings, respectively, n = 5) (Fig. 3, C and D). A cumulative frequency plot of each of the experiments in the two groups indicates that the number of cells showing LTP was considerably reduced by the active toxin (Fig. 3F) ($P < 0.015$, unpaired t test). The effect of botulinum toxin on LTP was not due to a blockade of NMDAR function, because application of 0.5 μM botulinum toxin through whole-cell recording pipettes had no effect on the NMDAR EPSC (Fig. 3E) (102 ± 2% measured at 50 to 60 min, n = 4), nor was it due to a modification of the 100-Hz stimulus–induced depolarization, because the depolarizing envelope was the same.

It has been proposed that SNAP may be a limiting component in the transmitter release process, because addition of SNAP to the squid giant synapse results in the enhanced release of transmitter (7). If LTP involves a similar mechanism, postsynaptic loading of CA1 cells with SNAP might result in an enhancement in synaptic strength. We loaded CA1 pyramidal cells with recombinant SNAP through whole-cell recording pipettes. After the whole-cell recording was established, the EPSC gradually increased in size and reached a maximal potentiation of 66 ± 21% (n = 12) when measured at 50 to 60 min (Fig. 4A, upper graph). In contrast, the simultaneously recorded extracellular synaptic responses remained stable during the experiment (Fig. 4A, lower graph). In interleaved control experiments that used the same pipette solution but without SNAP, there was no change in the synaptic responses (Fig. 4A). Throughout these experiments the investigator did not know the nature of the solution contained in the pipette.

A critical question is whether the enhancement of synaptic strength by postsynaptic loading of SNAP is due to mechanisms that also occur during LTP. We com-

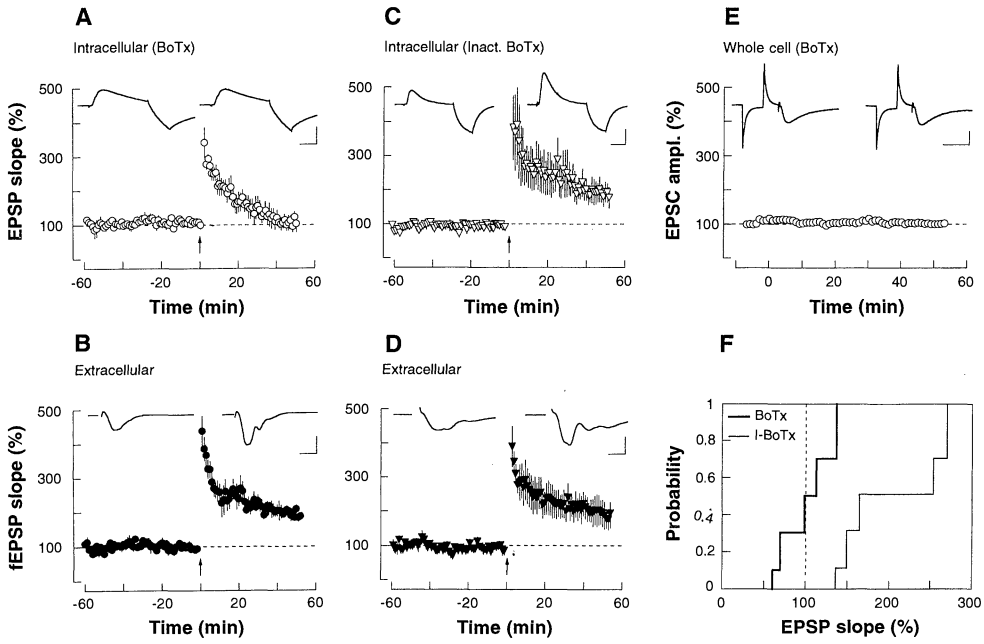

Fig. 3. Postsynaptic loading of the light chain from botulinum toxin (BoTx) reduces LTP. Graphs of LTP in cells recorded with microelectrodes containing (**A**) 0.5 μM BoTx (n = 5) (serotype B) or (**C**) heat-inactivated BoTx (n = 5). (**B** and **D**) Graphs of LTP in extracellular field potential recordings from experiments in (A) and (C), respectively. Arrows indicate the time of tetanic stimulation. Insets show representative traces recorded 30 min before (left) and after (right) the tetanic stimulation. Scale bars: 10 mV and 30 ms for (A) and (C) ; 0.3 mV and 10 ms for (B) and (D). (**E**) Normalized NMDA current amplitudes (ampl.) recorded with 0.5 μM BoTx in the whole-cell patch pipette solution (n = 4). Inset shows representative NMDAR EPSCs recorded 5 and 50 min after recordings were begun. Scale bars: 40 pA and 50 ms. (**F**) Cumulative probability plot that compares the magnitude of LTP obtained during intracellular recordings with either BoTx (thick line) or heat-inactivated BoTx (thin line) (96 ± 14% and 194 ± 28% for BoTx and heat-inactivated BoTx, respectively; P = 0.0133, unpaired t test). Experiments were done at 30°C to increase the enzymatic activity of BoTx.

pared the effects of SNAP on a pathway in which LTP had been saturated with the effects on a control pathway to the same cell. If the SNAP-induced enhancement shares features with LTP, then the magnitude of this enhancement at the potentiated synapses should be smaller than that at the control synapses on the same cell. We first saturated LTP in one pathway by applying repetitive tetani that had no effect on the control pathway (Fig. 4B). A whole-cell recording was then made with a pipette solution that contained SNAP. The results showed a significant increase in synaptic strength in the control pathway, but a minimal effect in the potentiated pathway (Fig. 4C). A summary of these experiments (n = 8) is shown in Fig. 4D. On average, SNAP elicited an enhancement of 58 ± 11% in the naïve pathway but only 22 ± 12% in the potentiated pathway ($P < 0.05$, unpaired t test). Thus, prior saturation of LTP significantly reduced the enhancement of synaptic strength that is elicited by loading CA1 pyramidal cells with SNAP.

Our results show that three mechanistically independent inhibitors of membrane fusion blocked or strongly reduced LTP when loaded into CA1 pyramidal cells. Furthermore, an increase in synaptic strength was elicited when membrane fusion was facilitated by loading CA1 cells with SNAP, and this enhancement appeared to share mechanisms with LTP. Importantly, the inhibitors had no effect on basal synaptic transmission, resting membrane potential, input resistance, or the NMDAR-mediated component of synaptic responses. Thus, it is very unlikely that the effects of the inhibitors on LTP were due to some nonspecific deterioration of the cell, and the lack of change in membrane potential makes it unlikely that some constitutive exocytotic pathway is required for LTP. Instead, we favor the conclusion that the molecular machinery involved in regulated exocytosis, such as that responsible for the release of transmitter from presynaptic terminals, may also be present in dendrites of neurons and there play an important role in LTP and perhaps other forms of synaptic plasticity.

A number of lines of evidence support the suggestion that vesicle exocytosis may occur in pyramidal cell dendrites. Electron microscopic studies have identified smooth vesicles (*12*) and coated vesicles (*13*) in dendritic spines. The synaptic vesicle protein synaptobrevin also appears to be present in the dendrites of hippocampal neurons (*14*), as does NSF (*15*), which may interact with the COOH-terminal tail of GluR2 (*15*, *16*). In addition, imaging studies with the fluorescent dye FM1-43 suggest that calcium-dependent, tetanus toxin–sensitive exocytosis can occur in the dendrites of hippocampal neurons in culture (*17*). The presence of calcium-dependent exocytosis in both muscle cells (*18*) and nonneuronal cells (*19*) indicates that this process occurs in many different cell types.

How might postsynaptic-regulated exocytosis contribute to LTP? One possibility is that it is required for delivery of retrograde messengers (*20*). Alternatively, membrane fusion may be required for delivery or insertion of glutamate receptors into the postsynaptic membrane, a mechanism that could explain the conversion of silent to functional synapses during LTP (*3*, *21*). The finding of coated pits at the postsynaptic density that are immunoreactive for AMPA receptors (*22*) is consistent with such a proposal. Regulated membrane insertion of proteins appears to be a common process in cell biology. Two well-established examples include the insulin-stimulated insertion of glucose transporters into adipocyte and myocyte membranes (*23*) and the vasopressin-stimulated delivery of water channels to kidney or bladder membrane (*24*), both of which involve vesicle fusion machinery. In fact, such a mechanism might account for the recently reported recruitment of γ-aminobutyric acid type A receptors to synapses by insulin (*25*). If structural remodeling of the synapse occurs during LTP, membrane fusion could also play an important role in the delivery of the appropriate synaptic constituents or components. Whatever its exact role, membrane fusion machinery, in addition to being important for the presynaptic release of neurotransmitter, appears to play an important role in the postsynaptic cell to generate LTP.

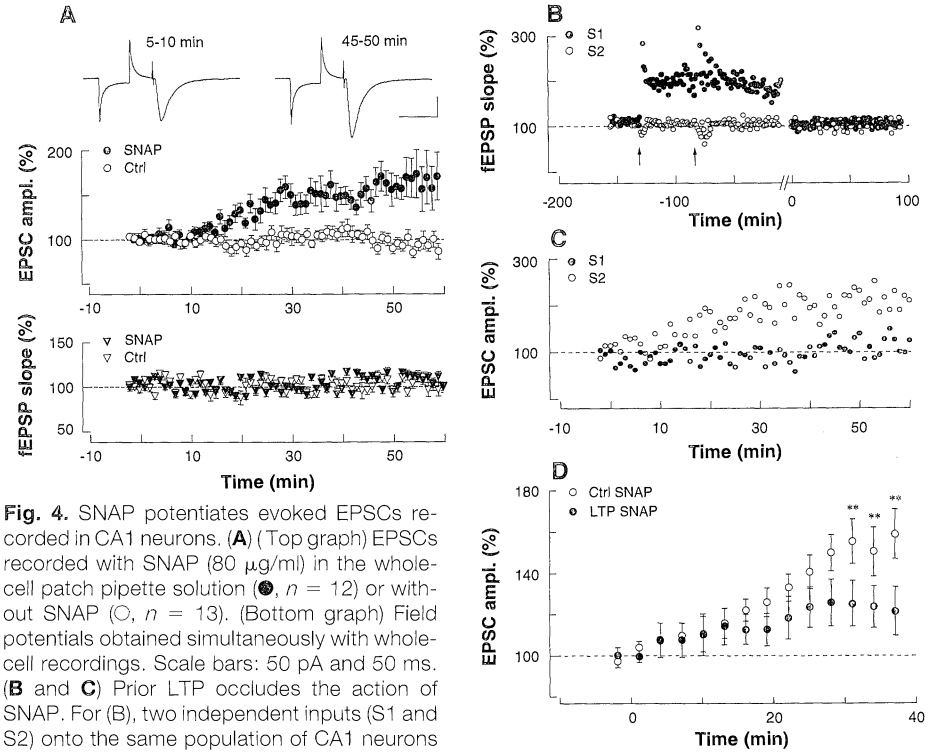

Fig. 4. SNAP potentiates evoked EPSCs recorded in CA1 neurons. (**A**) (Top graph) EPSCs recorded with SNAP (80 μg/ml) in the whole-cell patch pipette solution (●, n = 12) or without SNAP (○, n = 13). (Bottom graph) Field potentials obtained simultaneously with whole-cell recordings. Scale bars: 50 pA and 50 ms. (**B** and **C**) Prior LTP occludes the action of SNAP. For (B), two independent inputs (S1 and S2) onto the same population of CA1 neurons were alternately stimulated and the fEPSPs recorded. Arrows indicate when tetanic stimulation to one pathway was given. Stimulus strength to the tetanized pathway was reduced at time 0 so that the size of the fEPSP was the same as for the control pathway. For (C), a whole-cell recording with a pipette containing SNAP was made from a neuron in the same region recorded with the field electrode. There was a gradual growth in the EPSC of the control pathway, but little change in the pathway expressing LTP. (**D**) Graph of eight experiments, similar to that in (B) and (C), showing the effect of prior LTP on the enhancing action of SNAP (**$P < 0.005$; unpaired t test).

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4. We followed standard procedures for preparing and maintaining hippocampal slices [P.-M. Lledo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11175 (1995); P. E. Castillo, M. G. Weisskopf, R. A. Nicoll, *Neuron* **12**, 261 (1994)]. Slices (400 μm thick) were obtained from 1- to 3-week-old Hartley guinea pigs and maintained at room temperature for at least 1 hour in a submersion chamber containing artificial cerebrospinal fluid (ACSF) that was saturated with

95% O_2 and 5% CO_2. Slices were then transferred one at a time to a superfusing chamber for recording. The ACSF contained 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM D-glucose, and 0.1 mM picrotoxin and was equilibrated with 95% O_2 and 5% CO_2. All recordings were made in the CA1 region after removal of the CA3 region. Monosynaptic excitatory postsynaptic potentials (EPSPs) were evoked by stimulating close to the CA1 pyramidal cell layer, and neurons were recorded in bridge mode with sharp microelectrodes containing 2 M potassium acetate (pH 7.3; impedance of 50 to 80 megohms).

5. Depending on the experiment, the microelectrodes contained 5 mM NEM, 1 mM N19 peptide, 1 mM S19 peptide, 0.5 μM botulinum toxin (BoTx), or 0.5 μM heat-inactivated BoTx. We recorded extracellular fields with glass electrodes containing 1 M NaCl (impedance of 5 to 20 megohms) using an Axoclamp-2B amplifier (Axon Instruments) and stimulated Schaffer collateral afferents (100-μs duration) with a bipolar tungsten stimulating electrode. Baseline responses were obtained every 20 s with a stimulation intensity that yielded a half-maximal response. Tetanus-induced LTP was obtained by using a 100-Hz stimulus for 1 s performed four times at 20-s intervals. The magnitude of LTP was measured 50 to 60 min after applying the tetanus. Responses were filtered at 1 kHz, digitized at 4 kHz on a TL-1 interface (Axon Instruments), and collected on a 486 IBM compatible computer. A modification of pClamp software was used for all analyses. Whole-cell, patch-clamp recordings were made with the blind recording technique [M. G. Blanton, J. J. Lo Turco, A. R. Kriegstein, *J. Neurosci. Methods* **30**, 203 (1989)]. Patch pipettes (5 megohms) pulled from borosilicate glass contained 123 mM cesium gluconate, 10 mM CsCl, 10 mM Hepes, 10 mM cesium-EGTA, 8 mM NaCl, 1 mM $CaCl_2$, 2 mM adenosine triphosphate (Mg^{2+} salt), 0.3 mM guanosine triphosphate, 0.2 mM adenosine 3′,5′-monophosphate, and 10 mM D-glucose (pH 7.3, 290 mosM). Stimulus-evoked NMDA currents were recorded at a holding membrane potential of −60 mV in the presence of the non-NMDA receptor antagonist NBQX (10 μM). Baseline values of NMDA EPSCs were obtained from averages of responses during the first 3 min (time 0 on graphs) and defined as 100% for subsequent analyses. Series and input resistances were monitored throughout each experiment with a −3-mV calibration pulse given at a frequency of 0.1 Hz and were typically in the range of 10 to 20 and 150 to 200 megohms, respectively. Experiments were stopped if the series resistance changed more than 15%. The dissolving buffer for SNAP contained 137 mM NaCl, 2.68 mM KCl, 10 mM NaH_2PO_4, 1.76 mM KH_2PO_4, and 250 mM imidazole at pH 7.5. This medium was dissolved 1/26 with the patch pipette solution to give a final concentration for SNAP of 80 μg/ml, then briefly sonicated and placed in the tips of the whole-cell pipettes. The pipettes were then backfilled with the standard whole-cell pipette solution. Baseline values of EPSCs were obtained from averages of responses during the first 2 min (time 0 on the graph) and defined as 100% for subsequent analyses.
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26. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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A Potassium Channel Mutation in Neonatal Human Epilepsy

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Benign familial neonatal convulsions (BFNC) is an autosomal dominant epilepsy of infancy, with loci mapped to human chromosomes 20q13.3 and 8q24. By positional cloning, a potassium channel gene (*KCNQ2*) located on 20q13.3 was isolated and found to be expressed in brain. Expression of *KCNQ2* in frog (*Xenopus laevis*) oocytes led to potassium-selective currents that activated slowly with depolarization. In a large pedigree with BFNC, a five–base pair insertion would delete more than 300 amino acids from the KCNQ2 carboxyl terminus. Expression of the mutant channel did not yield measurable currents. Thus, impairment of potassium-dependent repolarization is likely to cause this age-specific epileptic syndrome.

Although most forms of idiopathic epilepsy have a genetic component, only a few specific syndromes are single-gene disorders (*1*). BFNC is an autosomal dominant idiopathic epilepsy characterized by unprovoked partial or generalized clonic convulsions, sometimes with apneic spells, which occur during wakefulness and sleep. Seizures typically start around day 3 of life and most often disappear after several weeks or months (*2*). However, about 10 to 15% of patients have febrile or afebrile seizures later in childhood. Gene loci for BFNC have been mapped to chromosome 20q13.3 (*3*) and to chromosome 8q24 (*4*). Most families in which the disorder occurs are linked to chromosome 20.

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