region next to the plastid body, especially if the tubules encircle other organelles such as mitochondria.

REFERENCES AND NOTES

1. Reviewed in J. K. Hoober, *Chloroplasts* (Plenum, New York, 1984), and J. T. O. Kirk and R. A. E. Tilney-Bassett, *The Plastids: Their Chemistry, Structure, Growth and Inheritance* (Elsevier/North-Holland, Amsterdam, ed. 2, 1978).
2. Reviewed in R. Cline and R. Henry, *Annu. Rev. Cell Dev. Biol.* **12**, 1 (1996).
3. R. H. Köhler, W. R. Zipfel, W. W. Webb, M. R. Hanson, *Plant J.* **11**, 613 (1997).
4. The modified GFP4 coding region [J. Haseloff and B. Amos, *Trends Genet.* **111**, 328 (1995)], which was further altered (*3*) by a Ser-to-Thr mutation at codon 65 (S65T mGFP4), was fused in frame with the *Arabidopsis recA* transit peptide (CT) [H. Cerutti, M. Osman, P. Grandoni, A. T. Jagendorf, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8068 (1992)].
5. The CT-GFP fusion was cloned into the Bam HI and Sst I sites of pBIN19-based binary vector pCT37 [C. M. Tobias and J. B. Nasrallah, *Plant J.* **10**, 523 (1996)], introduced into *A. tumefaciens* pCIB542/A136 [E. E. Hood, G. L. Helmer, R. T. Fraley, M. D. Chilton, *J. Bacteriol.* **168**, 1291 (1986)], and used to transform plants [R. B. Horsch *et al.*, *Science* **227**, 1229 (1985)].
6. We prepared the chloroplasts by breaking tobacco leaf cells with a mortar and pestle, followed by differential centrifugation and separation on a Percoll gradient according to J. Huang, E. Hack, R. W. Thornburg, and A. M. Meyer [*Plant Cell* **2**, 1249 (1990)], except that a 15%/40%/80% Percoll gradient was used.
7. A BioRad MRC-600 CLSM with a standard K1/K2 filter set was used. Pseudocolor similar to the color observed by eye with the Olympus microscope was added to the images by the import of data collected in the green and red channels from the BioRad microscope into Adobe Photoshop. Optical sections were taken along the optical axis and projected into one image with the COMOS software (BioRad).
8. Purified chloroplasts were lysed for 10 min at 4°C in 50 mM Hepes at pH 7.5 and 1 mM phenymethylsulfonyl fluoride and separated by 15 min of centrifugation at 4°C into a soluble and a membrane fraction. The membrane fraction was washed twice in lysis buffer. Protein was quantified by the Bradford protein assay (Bio-Rad) and separated by SDS–polyacrylamide gel electrophoresis on a 12% gel, transferred to nitrocellulose, and probed with 1:1000 dilutions of the different antibodies as described in (*3*).
9. J. Zhao and R. L. Last, *J. Biol. Chem.* **270**, 6081 (1995).
10. L. A. Payan and K. Cline, *J. Cell Biol.* **112**, 603 (1991).
11. R. H. Köhler, J. Cao, W. R. Zipfel, W. W. Webb, M. R. Hanson, data not shown.
12. W. Denk, J. H. Strickler, W. W. Webb, *Science* **248**, 73 (1990); R. M. Williams, D. W. Piston, W. W. Webb, *FASEB J.* **8**, 804 (1994). The images were obtained with a two-photon microscope consisting of a Zeiss IM-35 microscope, a modified BioRad MRC-600, and a Spectra-Physics Tsunami Ti:S laser. GFP fluorescence was collected in non-descanned mode through a 500 dichroic long pass mirror and a 520DF35 emission filter; excitation was at 800 nm (with a 100-fs pulse width and an 80-MHz repetition rate). A Zeiss 40/1.2 water immersion objective lens was used. Localized photobleaching was carried out by repetitive rapid line scanning of the targeted subcellular structure.
13. S. G. Wildman, T. Hongladarom, S. I. Honda, *Science* **138**, 434 (1962); S. G. Wildman, in *Biochemistry of Chloroplasts*, T. W. Goodwin, Ed. (Academic, Press, New York, 1967), vol. 2, pp. 295–319; T. Hongladarom, I. Shigeru, S. I. Honda, S. G. Wildman, "Organelles in Living Plant Cells," color sound film produced by P. Kahana and Y. Kahana (Extension Media Center, University of California, Berkeley, 1965); D. Spencer and S. G. Wildman, *Aust. J. Biol. Sci.* **15**, 599 (1962).
14. M. Vesk, F. V. Mercer, J. V. Possingham, *Aust. J. Bot.* **13**, 161 (1965); T. E. Weier and W. W. Thomson, *Am. J. Bot.* **49**, 807 (1962).
15. D. Menzel, *Protoplasma* **179**, 166 (1994).
16. T. Osafune, T. Ehara, E. Hase, J. A. Schiff, *J. Electron Microsc.* **42**, 201 (1993); T. Ehara, T. Osafune, E. Hase, *Exp. Cell Res.* **190**, 104 (1990).
17. M. W. Gray, *Int. Rev. Cytol.* **141**, 233 (1992).
18. Differential interference contrast microscopic images were collected with an Olympus BX50 microscope equipped with a cooled charge-coupled device camera [Optronics (Chelmsford, MA) DEI-750T].

19. We thank J. Haseloff for the mGFP4 gene and T. Horiagon, R. Last, S. Lawrence, and K. Cline for antibodies to GFP, AS, and LHCP. Supported by DOE Energy Biosciences Program grant DE-FG02-89ER14030 to M.R.H. and grants to W.W.W. and Developmental Resources for Biophysical Imaging and Opto-electronics by NSF (DIR8800278) and NIH (RR04224 and R07719).

15 January 1997; accepted 9 May 1997

Regulatory Phosphorylation of AMPA-Type Glutamate Receptors by CaM-KII During Long-Term Potentiation

Andres Barria, Dominique Muller, Victor Derkach, Leslie C. Griffith, Thomas R. Soderling*

Long-term potentiation (LTP), a cellular model of learning and memory, requires calcium-dependent protein kinases. Induction of LTP increased the phosphorus-32 labeling of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–type glutamate receptors (AMPA-Rs), which mediate rapid excitatory synaptic transmission. This AMPA-R phosphorylation appeared to be catalyzed by Ca^{2+}- and calmodulin-dependent protein kinase II (CaM-KII): (i) it correlated with the activation and autophosphorylation of CaM-KII, (ii) it was blocked by the CaM-KII inhibitor KN-62, and (iii) its phosphorus-32 peptide map was the same as that of GluR1 coexpressed with activated CaM-KII in HEK-293 cells. This covalent modulation of AMPA-Rs in LTP provides a postsynaptic molecular mechanism for synaptic plasticity.

Long-term potentiation is a prolonged enhancement in synaptic efficacy that may underlie certain types of learning and memory, but its molecular mechanisms are unclear (*1*). Considerable evidence implicates changes in presynaptic transmitter release, postsynaptic responses, and synaptic structural changes. Postsynaptic elevations in Ca^{2+} and Ca^{2+}-dependent protein kinases are required for establishment of LTP, and a likely target of these kinases is AMPA-Rs (*2*) because (i) their responsiveness is enhanced after elevations of postsynaptic Ca^{2+} (*3*) or by LTP induction (*4*), and (ii) these changes are blocked by KN-62 (*3*, *5*), a CaM-K inhibitor (*6*). Activated CaM-KII enhances AMPA-R responsiveness in CA1 neurons in hippocampal slices and many other systems (*7*–*9*). In the slices, expression or infusion of activated CaM-KII also increases synaptic current and occludes subsequent induction of LTP. However, a key observation, direct phosphorylation of AMPA-Rs in response to LTP, has not been previously demonstrated.

Induction of LTP produces a small increase in the Ca^{2+}-independent or constitutive activity of CaM-KII (*10*). CaM-KII can autophosphorylate on multiple sites (*2*, *11*), so to confirm that the constitutive CaM-KII activity was due to autophosphorylation of Thr^{286}, we used a phosphospecific antibody, $Ab_{P\text{-}Thr286}$. This antibody was specific for P-Thr^{286} in CaM-KII, reacting with autophosphorylated wild-type CaM-KII but not with the autophosphorylated Thr^{286}-Ala mutant (Fig. 1A). Protein immunoblot analyses with $Ab_{P\text{-}Thr286}$ of a hippocampal slice extract detected multiple immunoreactive bands, but only the one corresponding to the 50-kD α-CaM-KII was selectively blocked by preadsorption with the phosphopeptide antigen (Fig. 1B). Induction of LTP in the CA1 region of hippocampal slices with theta-burst stimulation (*10*) resulted in a small, stable increase in immunoreactivity to $Ab_{P\text{-}Th286}$ (Fig. 1C). The magnitude of the increase is in general agreement with the estimate that about 10% of synapses are potentiated by theta-burst stimulation (*12*). The enhanced phosphorylation of Thr^{286} was not due to an LTP-mediated increase in CaM-KII protein, because immunoreactivity with a general CaM-KII antibody ($n = 4$) did not increase at 5 min

A. Barria, V. Derkach, T. R. Soderling, Vollum Institute, Oregon Health Sciences University, Portland, OR 97201, USA.
D. Muller, Department of Neuropharmacology, Centre Medical Universitaire, 1211 Geneva 4, Switzerland.
L. C. Griffith, Department of Biology, Brandeis University, Waltham, MA 02254, USA.
*To whom correspondence should be addressed.

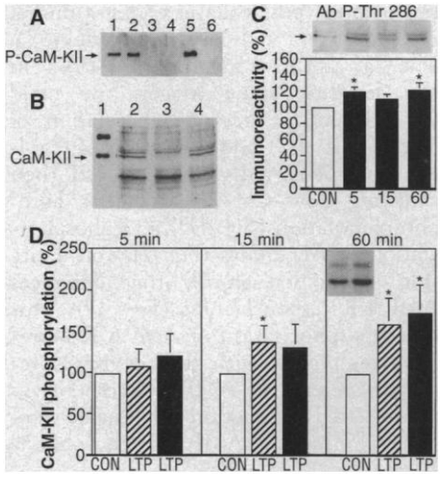

Fig. 1. LTP increased phosphorylation of CaM-KII. (**A**) Protein immunoblot analysis of autophosphorylated CaM-KII using $Ab_{P\text{-}Thr286}$ (*27*). Wild-type CaM-KII (lanes 1, 2, and 5) or mutant Thr^{286}-Ala (lanes 3, 4, and 6) were subjected to Ca^{2+}- and CaM-dependent autophosphorylation (*11*) at 5°C for 5 min (lanes 1 and 3) or 10 min (lanes 2 and 4) and then at 30°C for 30 min (lanes 5 and 6). (**B**) Protein immunoblot of hippocampal slice homogenate with either a general CaM-KII antibody (lane 1) or $Ab_{P\text{-}Thr286}$ without (lane 2) or with preadsorption with the phosphopeptide antigen (lane 3) or non-phosphopeptide (lane 4). Arrow shows the position of α-CaM-KII. (**C**) $Ab_{P\text{-}Thr286}$ protein immunoblot of hippocampal slices at 5, 15, or 60 min after theta-burst stimulation to induce LTP (*28*) or 60 min after low-frequency stimulation (CON). Top panel, illustrative example; bar graph, composite values normalized per microgram of homogenate protein (mean ± SE, $n = 4$, $^*P < 0.05$). (**D**) Homogenates from ^{32}P-labeled hippocampal slices (LTP or CON) were immunoprecipitated with general CaM-KII antibody and subjected to SDS-PAGE and autoradiography (inset), and ^{32}P–CaM-KII was quantitated per microgram of protein (mean ± SE, $n = 5$ to 8 homogenates, $^*P < 0.05$). Slashed bars represent the α subunit; solid bars, the β subunit.

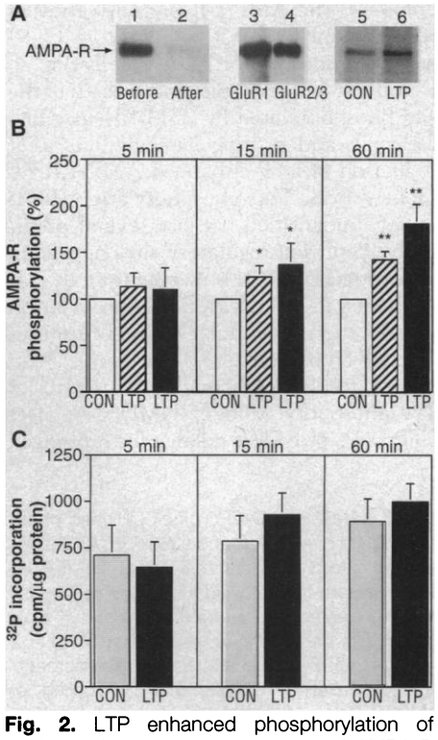

Fig. 2. LTP enhanced phosphorylation of AMPA-R. (**A**) Immunoprecipitation of AMPA-R complex (*28*). Protein immunoblots with GluR1 antibody (lanes 1 through 3) or GluR2/3 antibody (lane 4) of either slice supernatants before (lane 1) and after (lane 2) immunoprecipitation with GluR1 antibody or of GluR1 immunoprecipitates (lanes 3 and 4). Representative autoradiographs from SDS-PAGE of immunoprecipitated AMPA-R (lanes 5 and 6) from 60-min control and LTP ^{32}P-labeled slices. (**B**) AMPA-R immunoprecipitates from CON or LTP slices at the indicated times were quantitated for ^{32}P incorporation (mean ± SE, $n = 5$ to 8 homogenates, $^*P < 0.05$, $^{**}P < 0.01$). Data were normalized to GluR1 protein immunoblots from the same gel (slashed bars) or 1 μg of total homogenate protein per sample (solid bars). No significant difference in AMPA-R immunoreactivity was detected between CON and LTP samples at any time points. (**C**) ^{32}P incorporation into total cellular proteins was determined in ^{32}P-labeled hippocampal slices at the indicated times ($n = 5$ to 8). Aliquots of slice homogenates were spotted on P81 paper and analyzed for total ^{32}P-protein (*28*).

(93.6 ± 11.5% of control) or 15 min (98.7 ± 1.5%), and the small increase at 60 min was not significant (122 ± 16%, $P > 0.1$).

When total autophosphorylation of CaM-KII was measured in ^{32}P-labeled hippocampal slices by immunoprecipitation with a general CaM-KII antibody, LTP induction produced a significant increase in ^{32}P–CaM-KII at 15 min that was further elevated at 60 min (Fig. 1D). The fact that phosphorylation of Thr^{286} was maximal at 5 min (Fig. 1C) whereas total autophosphorylation was slower (Fig. 1D) is consistent with the fact that autophosphorylation of Thr^{286} is much faster than autophosphorylation of other sites or of exogenous substrate proteins (*11*).

We next examined whether induction of LTP increased ^{32}P labeling of AMPA-Rs. Conditions were optimized to immunoprecipitate more than 90% of GluR1 from the homogenate (Fig. 2A, lanes 1 and 2). The immunoprecipitate was also reactive to a GluR2/3 antibody (*13*) (Fig. 2A, lanes 3 and 4), indicating immunoprecipitation of the hetero-oligomeric AMPA-R complex. The amount of ^{32}P–AMPA-R was significantly elevated 15 min (24.0 ± 11.6% over control, $P < 0.05$, $n = 5$, normalized by immunoreactivity) and 60 min (42.5 ± 8.6%, $P < 0.01$, $n = 8$) after induction of LTP compared with the low-frequency stimulated control (Fig. 2B). This enhanced phosphorylation of AMPA-Rs, as well as the phosphorylation of CaM-KII, was not due to an LTP-induced increase in total protein phosphorylation (Fig. 2C, $n = 5$ to 8), thereby ruling out an increase in adenosine triphosphate (ATP)–specific activity.

To examine whether AMPA-R phosphorylation was catalyzed by the activated CaM-KII, we used the CaM-kinase inhibitor KN-62, which blocks induction of LTP (*5*) as well as Ca^{2+}-dependent potentiation (*3*) in hippocampal slices. The KN-62 did not affect basal synaptic transmission or suppress short-term potentiation (STP) (that is, the first 10 min of potentiation), but it blocked the expression of LTP (Fig. 3A) and the enhanced phosphorylations of CaM-KII and AMPA-Rs (Fig. 3B). Because KN-62 inhibits CaM-KII competitively with Ca^{2+} and CaM and does not inhibit protein kinase A or C (PKA or PKC) in vitro or in vivo (*6*, *14*), the data of Fig. 3, A and B, strongly support the conclusion that CaM-KII was the catalyst of the AMPA-R phosphorylation. This conclusion is important, because PKA and PKC can also be activated during LTP (*15*, *16*), and both can phosphorylate AMPA-Rs in cultured cells (*17*, *18*). However, activation of PKA is transient (5 to 15 min) and requires stimuli much stronger than theta-burst stimulation (*16*), and effects of PKC on AMPA-R responsiveness are controversial (*19*). As expected, the *N*-methyl-D-aspartate receptor (NMDA-R) antagonist D-AP5 also blocked AMPA-R phosphorylation (Fig. 3C).

When peptide mapping was performed on ^{32}P–AMPA-Rs from control and LTP slices, both peptide maps appeared identical (Fig. 4, A and B), suggesting that LTP increased phosphorylation of the same site. This result is similar to our previous observation on AMPA-R phosphorylation upon stimulation of NMDA-R in cultured hippocampal neurons (*20*). To further confirm that phosphorylation of AMPA-R in hippocampal slices was catalyzed by CaM-KII, GluR1 was expressed in HEK-293 cells with or without CaM-KII. Little basal ^{32}P labeling of the GluR1 was observed, but coexpression of GluR1 with CaM-KII gave a 10-fold increase in ^{32}P-GluR1 (Fig. 4C, top panel) that showed a major ^{32}P-peptide (Fig. 4C, bottom panel), which must have been the same as that observed in the hippocampal slices because a mixture of the two did not reveal any differences (Fig. 4D). CaM-KII

enhanced the AMPA-R responsiveness of recombinant and native receptors (*7*–*9*) and the response in HEK-293 cells (Fig. 4, E and F). These results indicate that the site phosphorylated by CaM-KII in HEK-293 cells and in hippocampal slices after induction of LTP enhanced AMPA-R responsiveness. This phosphorylation site is as yet unidentified because several previously identified regulatory sites appear to be extracellular (*21*). A recent study has identified PKA and PKC phosphorylation sites in the intracellular COOH-terminus of GluR1 (*18*).

Our results, combined with reports of Tyr phosphorylation of NMDA-Rs after LTP (*22*), provide evidence for prolonged biochemical postsynaptic changes during LTP consistent with a current model (*23*). The Ca^{2+} influx through the NMDA-R in the dendritic spine triggers the rapid Ca^{2+}-dependent autophosphorylation of Thr^{286} (Fig. 1C) and activation of CaM-KII (*10*). This activated CaM-KII then catalyzes slow Ca^{2+}-independent autophosphorylation (Fig. 1D) and phosphorylation of AMPA-Rs (Fig. 2B) on a site (Fig. 4, B through D) that enhances AMPA-R responsiveness (Fig. 4F). This slow phosphorylation of AMPA-R correlated temporally with the previously reported increase in AMPA-R responsiveness after LTP induction (*4*). The delay of 10 to 15 min in AMPA-R phosphorylation by CaM-KII suggests that this mechanism is not involved in STP, consistent with the observation that KN-62 also does not block STP but is only inhibitory after about 10 to 15 min (Fig. 3A). Although ours and other data (*7*–*9*) implicate a critical role for AMPA-R phosphorylation by CaM-KII in LTP, it is likely that CaM-KII modulates additional aspects of synaptic plasticity. Mutant mice lacking α-CaM-KII show deficits in LTP induction and spatial learning, but mice overexpressing a partially active CaM-KII mutant exhibit more complex alterations in long-term potentiation and depression (*24*). Thus, it is likely that additional mechanisms, regulated by CaM-KII and other enzymes, are also essential for the multiple facets (for example, neurotransmitter release and increased gene expression) of this complex phenomenon.

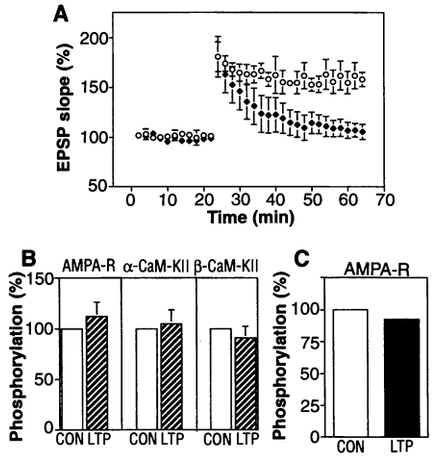

Fig. 3. KN-62 and D-AP5 block LTP and phosphorylation of AMPA-R and CaM-KII. (**A**) Changes in excitatory postsynaptic potential (EPSP) slope before or after induction of LTP (*10*) in slices treated without (open circles; $n = 4$) or with (solid diamonds; $n = 5$) 10 μM KN-62 (Seigakagu). Data are expressed as a percentage of baseline values. (**B**) ^{32}P labeling of AMPA-R ($n = 12$ homogenates, normalized to AMPA-R immunoreactivity) and CaM-KII ($n = 11$ homogenates, normalized to protein) subunits were analyzed at 60 min in slices treated with 10 μM KN-62. Slices were incubated in phosphate-free medium plus KN-62 for 30 min and ^{32}P-labeled in the presence of KN-62 for another 30 min before theta-burst stimulation. Treatment with KN-62 had no effect on total ^{32}P-protein. (**C**) Effect of AP5 on AMPA-R phosphorylation. Slices were treated as in (B) but with 50 μM D-AP5. Data are the average of two experiments.

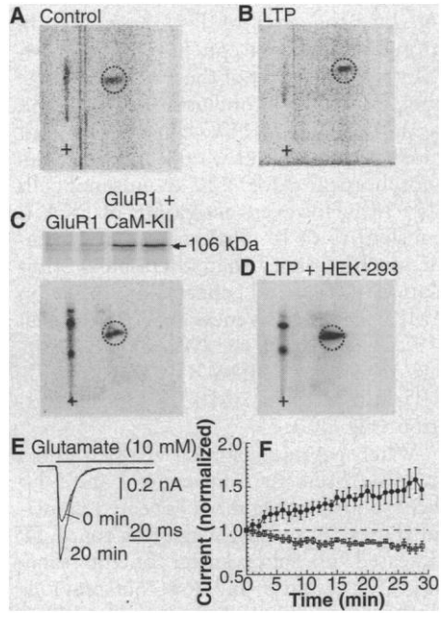

Fig. 4. ^{32}P-peptide mapping of AMPA-Rs. (**A** and **B**) Peptide maps of AMPA-Rs from hippocampal slices. Immunoprecipitated ^{32}P–AMPA-Rs from 60-min CON or LTP slices were excised from the SDS-PAGE, subjected to complete tryptic digestion, and resolved by two-dimensional (2D) peptide mapping (*29*). The main phosphopeptide is circled. (**C** and **D**) Phosphorylation of GluR1 (flip) expressed in HEK-293 cells (*30*) with or without coexpression of the CaM-KII mutant H282R (*26*), which has 20% constitutive activity. Two days after transfection, cells were ^{32}P-labeled for 1.5 hours, GluR1 was immunoprecipitated and analyzed by SDS-PAGE and autoradiography [(C), duplicates shown], and the ^{32}P-GluR1 from (C) was subjected to 2D mapping as in (A) and (B). The main phosphopeptide is circled. Trypsin-digested hippocampal slice (LTP-induced) AMPA-Rs were mixed with equal amounts (in counts per minute) of HEK-293 cell GluR1 (plus CaM-KII H282R) and subjected to 2D peptide mapping (D). (**E** and **F**) Effect of CaM-KII on whole cell currents recorded from HEK-293 cells expressing GluR1 (flip) (*30*). (E) Responses elicited by application of 10 mM glutamate (100-ms pulses, −80 mV) immediately after formation of the patch (0 min) and 20 min after infusion of activated CaM-KII (*30*). (F) Peak currents, normalized to 0 time, for cells infused with activated (solid circles, $n = 9$) or heat-inactivated (open squares, $n = 10$) CaM-KII.

REFERENCES AND NOTES

1. T. V. P. Bliss and G. L. Collingridge, *Nature* **361**, 31 (1993); R. Malinow, *Science* **266**, 1195 (1994); A. U. Larkman and J. J. B. Jack, *Curr. Opin. Neurobiol.* **5**, 324 (1995).
2. T. R. Soderling, *Adv. Second Messenger Phosphoprotein Res.* **30**, 175 (1995).
3. D. J. A. Wyllie and R. A. Nicoll, *Neuron* **13**, 635 (1994).
4. S. A. Davies, R. A. J. Lester, K. G. Reymann, G. L. Collingridge, *Nature* **338**, 500 (1989).
5. I. Ito, H. Hidaka, H. Sugiyama, *Neurosci. Lett.* **121**, 119 (1991).
6. H. Tokumitsu *et al.*, *J. Biol. Chem.* **265**, 4315 (1990).
7. E. McGlade-McCulloh, H. Yamamoto, S. E. Tan, D. A. Brickey, T. R. Soderling, *Nature* **362**, 640 (1993); M. Kolaj, R. Cerne, D. A. Brickey, M. Randic, *J. Neurophysiol.* **72**, 2525 (1994); D. L. Pettit, S. Perlman, R. Malinow, *Science* **266**, 1881 (1994).
8. P. M. Lledo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11175 (1995).
9. J. Yeakel, P. Vissavajjhala, V. Derkach, D. Brickey, T. R. Soderling, *ibid.*, p. 1376.
10. K. Fukunaga, L. Stoppini, E. Miyamoto, D. Muller, *J. Biol. Chem.* **268**, 7863 (1993); K. Fukunaga, D. Muller, E. Miyamoto, *ibid.* **270**, 6119 (1995).
11. S. G. Miller and M. B. Kennedy, *Cell* **44**, 861 (1986); Y. L. Fong, W. L. Taylor, A. R. Means, T. R. Soderling, *J. Biol. Chem.* **264**, 16759 (1989).
12. P. A. Buchs and D. Muller, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8040 (1996).
13. R. J. Wenthold, N. Yokotani, K. Doi, K. Wada, *J. Biol. Chem.* **267**, 501 (1992).
14. H. Enslen and T. R. Soderling, *ibid.* **269**, 20872 (1994).
15. U. Frey, Y. Y. Huang, E. R. Kandel, *Science* **260**, 1661 (1993); G. Y. Hu *et al.*, *Nature* **328**, 426 (1987); E. Klann, S. J. Chen, J. D. Sweatt, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8337 (1993); T. C. Sactor *et al.*, *ibid.*, p. 8342.
16. E. D. Roberson and J. D. Sweatt, *J. Biol. Chem.* **271**, 30436 (1996).
17. P. Greengard, J. Jen, A. C. Nairn, C. F. Stevens, *Science* **253**, 1135 (1991); C. Rosenmund *et al.*, *Nature* **368**, 853 (1994).
18. K. W. Roche, R. J. O'Brien, A. L. Mammen, J. Bernhardt, R. L. Huganir, *Neuron* **16**, 1179 (1996).
19. H. Urushihara, M. Tohda, Y. Nomura, *J. Biol. Chem.* **267**, 11697 (1992); L. Chen and L. Y. M. Huang, *Nature* **356**, 521 (1992); L. Y. Wang, E. M. Dudek, M. D. Browning, J. F. McDonald, *J. Physiol. (London)* **475**, 431 (1994); O. Hvalby *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4761 (1994).
20. S. E. Tan, R. J. Wenthold, T. R. Soderling, *J. Neurosci.* **14**, 1123 (1994).

21. M. Hollmann, in *The Ionotropic Glutamate Receptors*, D. T. Monaghan and R. J. Wenthold, Eds. (Humana, Totowa, NJ, 1997), pp. 39–80; T. R. Soderling, *ibid.*, pp. 121–134.
22. J. A. P. Rostas *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 10452 (1996); K. Rosenblum, Y. Dudai, G. Richter-Levin, *ibid.*, p. 10457.
23. J. Lisman, *Trends Neurosci.* **17**, 406 (1994).
24. A. J. Silva, R. Paylor, J. M. Wehner, S. Tonegawa, *Science* **257**, 206 (1992); A. J. Silva, C. F. Stevens, S. Tonegawa, Y. Wang, *ibid.*, p. 201; M. Mayford, J. Wang, E. R. Kandel, T. J. O'Dell, *Cell* **81**, 891 (1995); M. Mayford *et al.*, *Science* **274**, 1678 (1996).
25. T. Suzuki, K. Okumura-Noji, A. Ogura, Y. Kudo, R. Tanka, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 109 (1992); B. L. Patton, S. S. Malloy, M. B. Kennedy, *Mol. Biol. Cell* **4**, 159 (1993).
26. D. A. Brickey *et al.*, *J. Biol. Chem.* **269**, 29047 (1994).
27. Polyclonal $Ab_{P\text{-}Thr286}$ was generated in rabbits using as antigen phosphopeptide His-Arg-Gln-Glu-Thr(PO_4)-Val-Asp-Cys-Leu, corresponding to residues 282 to 290 of α-CaM-KII, conjugated to keyhole limpet hemocyanin. Serum was passed over a column of nonphosphopeptide coupled to agarose, and the flow-through was affinity purified on a phosphopeptide column. A similar antibody has been previously described (*25*). For protein immunoblots using $Ab_{P\text{-}Thr286}$, 1 μM microcystine was included in the blotting buffer to inhibit protein phosphatases in the Carnation milk.
28. Hippocampal slices from male Sprague-Dawley rats (4 to 6 weeks old) were prepared and maintained at 33°C and pH 7.4 in an interface chamber under continuous perfusion as previously described (*10*). The ^{32}P-labeled slices were incubated in phosphate-free medium for 15 min and then in $^{32}PO_4$ (0.5 mCi/ml) for 45 min before induction of LTP. The LTP was induced by theta-burst stimulation, whereas control slices received low-frequency stimulation; both protocols were applied by coactivation of two independent groups of Schaffer-commissural fibers (*10*). At 5, 15, or 60 min after stimulation, the CA1 region was dissected and frozen in liquid nitrogen. Three CA1 slices (either from LTP or CON) were pooled together and homogenized at 5°C in 500 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 50 mM tris-HCl, 200 mM EDTA (pH 7.5), 200 mM EGTA, 100 mM Na_3VO_4, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, soybean trypsin inhibitor (20 μg/μl), 1 μM microcystine-LR, 0.5% Triton X-100, and 0.1% deoxycholate. Samples were sonicated and centrifuged at 10,000 rpm for 15 min, and 10 μl of supernatant was spotted onto P81 papers, which were then washed in 10% trichloroacetic acid to determine total ^{32}P-protein. Supernatants were diluted to give equal amounts (in counts per minute) of ^{32}P-protein, incubated with GluR1 antibody (*13*) and protein A–Sepharose at 4°C for 5 hours, and centrifuged. CaM-KII was then immunoprecipitated from the supernatant (*20*). Immunoprecipitates were run on SDS–polyacrylamide gel electrophoresis (PAGE) and exposed on a Phosphor Screen (Molecular Dynamics) for analyses. The SDS-PAGE of the GluR1 immunoprecipitates were also subjected to protein immunoblot analyses to quantitate the amount of GluR1. Protein content of homogenates was determined by protein assay (Bio-Rad).
29. ^{32}P–AMPA-R was immunoprecipitated (GluR1 antibody) from hippocampal slices or HEK-293 cells expressing GluR1 ± CaM-KII H282R (*26*). Immunoprecipitated AMPA-R was isolated by SDS-PAGE; ^{32}P–AMPA-R was cut from the gel, subjected to two digestions with trypsin (16 and 4 hours with 75 μg of trypsin per milliliter), oxidized, and subjected to two-dimensional peptide mapping (cathode on the right) (*20*).
30. AMPA GluR1(flip) was expressed in HEK-293 cells by the lipofectine method (Glbco protocol) with the use of 2 μg of plasmid per 35-mm dish for transfection of 5×10^4 to 7×10^4 cells. Whole cell recordings were made 16 to 20 hours after transfection. Patch pipette solution was 160 mM CsCl, 2 mM $MgCl_2$, 4 mM Na-ATP, 1 mM EGTA, and 10 mM Hepes. Series resistance of 10 to 12 megohms was 80% compensated and monitored through the recordings. Extracellular solution contained 165 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 5 mM Hepes. When added to intracellular solution, activated CaM-KII [truncated at residue 316 and autothiophosphorylated (*8*)] was at 0.4 μM. Glutamate (10 mM) was delivered to single cells by rapid application (*9*). Currents were filtered at 2 kHz and digitized at 10 kHz.

31. This work was supported by NIH (NS27037, T.R.S.), the Swiss National Research Foundation (3100-040815.94, D.M.), the International Human Frontier Science Program (RG-30/96B, D.M. and T.R.S.), and an Alfred P. Sloan and Klingenstein fellowship (L.G.). We thank D. Brickey for providing the activated CaM-KII, and Z. Wang for making the P-Thr^{286} antibody.

16 August 1996; accepted 30 April 1997

Mutation in the α-Synuclein Gene Identified in Families with Parkinson's Disease

Mihael H. Polymeropoulos,* Christian Lavedan†, Elisabeth Leroy†, Susan E. Ide, Anindya Dehejia, Amalia Dutra, Brian Pike, Holly Root, Jeffrey Rubenstein, Rebecca Boyer, Edward S. Stenroos, Settara Chandrasekharappa, Aglaia Athanassiadou, Theodore Papapetropoulos, William G. Johnson, Alice M. Lazzarini, Roger C. Duvoisin, Giuseppe Di Iorio, Lawrence I. Golbe, Robert L. Nussbaum

Parkinson's disease (PD) is a common neurodegenerative disorder with a lifetime incidence of approximately 2 percent. A pattern of familial aggregation has been documented for the disorder, and it was recently reported that a PD susceptibility gene in a large Italian kindred is located on the long arm of human chromosome 4. A mutation was identified in the α-synuclein gene, which codes for a presynaptic protein thought to be involved in neuronal plasticity, in the Italian kindred and in three unrelated families of Greek origin with autosomal dominant inheritance for the PD phenotype. This finding of a specific molecular alteration associated with PD will facilitate the detailed understanding of the pathophysiology of the disorder.

Parkinson's disease (PD) was first described by James Parkinson in 1817 (*1*). The clinical manifestations of this neurodegenerative disorder include resting tremor, muscular rigidity, bradykinesia, and postural instability. A relatively specific pathological feature accompanying the neuronal degeneration is an intracytoplasmic inclusion body, known as the Lewy body, which is found in many regions, including the substantia nigra, locus ceruleus, nucleus basalis, hypothalamus, cerebral cortex, cranial nerve motor nuclei, and the central and peripheral divisions of the autonomic nervous system (*1*).

In many cases a heritable factor predisposes to the development of the clinical syndrome (*2*). We have recently shown that genetic markers on human chromosome 4q21–q23 segregate with the PD phenotype in a large family of Italian descent (*3*). The clinical picture of the PD phenotype in the Italian kindred has been well documented to be typical for PD, including Lewy bodies, with the exception of a relatively earlier age of onset of illness at 46 ± 13 years. In this family the penetrance of the gene (the proportion of people with the genotype who actually manifest the disease) has been estimated to be 85%, suggesting that a single gene defect will be sufficient to determine the PD phenotype.

Alpha-synuclein, a presynaptic nerve terminal protein, was originally identified as the precursor protein for the non–β amyloid component of Alzheimer's disease amyloid plaques NAC (*4*). The human α synuclein gene was previously mapped in the 4q21–q22 region (*5*). Genotype analysis in the Italian PD kindred with additional genetic markers showed recombination events. One recombination was observed for genetic marker D4S2371 at the centromeric end of the PD interval and one recombination was inferred for marker

M. H. Polymeropoulos, C. Lavedan, E. Leroy, S. E. Ide, A. Dehejia, J. Rubenstein, R. Boyer, R. L. Nussbaum, Laboratory of Genetic Disease Research, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892–1430, USA.
S. Chandrasekharappa, B. Pike, H. Root, A. Dutra, Laboratory of Gene Transfer, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892–1430, USA.
L. I. Golbe, W. G. Johnson, E. S. Stenroos, R. C. Duvoisin, A. M. Lazzarini, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA.
G. Di Iorio, Instituto di Scienze Neurologiche, Faculta di Medicina, Seconda Universita degli Studi di Napoli, Naples, Italy.
T. Papapetropoulos and A. Athanassiadou, University of Patras Medical School, Patras, Greece.

*To whom correspondence should be addressed.
†These authors contributed equally in this work.