15. J. F. Parkinson *et al.*, *J. Biol. Chem.* **265**, 12602 (1990).
16. Human protein C (500 nM) or factor XI (60 nM) was incubated with recombinant thrombomodulin (15 nM) in 200 μl of tris-NaCl, 25 μM ZnCl for 60 min at 37°C in the presence or absence of thrombin (2 nM). Calcium, at previously determined optimal concentrations for each type of rTM, was included in samples containing protein C (*15*). Reactions were terminated by addition of hirudin to a final concentration of 25 units/ml and factor XIa production was determined by chromogenic assay (*18*). A higher concentration of thrombomodulin did not increase the production of factor XIa.
17. R. E. Thompson, R. Mandle, A. P. Kaplan, *J. Clin. Invest.* **60**, 1376 (1977).
18. Activation of factor XI by thrombin or factor XIIa was measured by chromogenic assay. Incubations without dextran sulfate were done in 200 μl of tris-NaCl and 1% bovine serum albumin (TBSA) at 37°C. Reactions containing thrombin were terminated by addition of hirudin to a final concentration of 25 units/ml and those with factor XIIa by addition of partially purified trypsin inhibitor from corn (CTI). Neither hirudin nor CTI inhibited factor XIa. After dilution to a final volume of 1 ml with TBSA containing 600 μM S-2366 (Kabi), the generation of free para-nitroanalid was determined by measuring the change in absorbance at 405 nm (A_{405}). The amount of factor XIa produced was determined by comparing the change in A_{405} to a standard curve constructed with the use of purified factor XIa. Reactions with dextran sulfate were 50 μl in size and were terminated by dilution in 950 μl of TBSA, hirudin (25 units/ml), polybrene (5 μg/ml), and 600 μM S-2366. The generation of para-nitroanalid was stopped by addition of 200 μl of 10% acetic acid. A_{405} was measured and compared to a standard curve.
19. S. D. Revak, C. G. Cochrane, J. H. Griffin, *J. Clin. Invest.* **59**, 1167 (1977).
20. R. Radcliffe and Y. Nemerson, *J. Biol. Chem.* **250**, 388 (1974).
21. B. Osterud and S. I. Rapaport, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5260 (1977).
22. M. Hultin and Y. Nemerson, *Blood* **52**, 928 (1978).
23. Since the original submission of this manuscript, Naito and Fujikawa have independently reported similar results concerning factor XIIa–independent activation of factor XI [K. Naito and K. Fujikawa, *J. Biol. Chem.* **266**, 7353 (1991)].
24. J. M. Gershoni and G. E. Palade, *Anal. Biochem.* **131**, 1 (1983).
25. The authors thank D. Coleman for preparation of the manuscript and J. F. Parkinson for recombinant Thrombomodulins.

4 March 1991; accepted 11 June 1991

Stimulation of Protein Tyrosine Phosphorylation by NMDA Receptor Activation

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The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptors, plays a key role in synaptic plasticity in the nervous system. After NMDA receptor activation, calcium entry into the postsynaptic neuron is a critical initial event. However, the subsequent mechanisms by which the NMDA receptor signal is processed are incompletely understood. Stimulation of cultured rat hippocampal cells with glutamate resulted in the rapid and transient tyrosine phosphorylation of a 39-kilodalton protein (p39). Tyrosine phosphorylation of p39 was triggered by the NMDA receptor and required an influx of Ca^{2+} from the extracellular medium. Because p39 was found to be highly related or identical to the microtubule-associated protein 2 kinase, the NMDA receptor signal may be processed by a sequential activation of protein kinases.

GLUTAMATE IS THE PRINCIPAL EXCITATORY neurotransmitter in the mammalian central nervous system and acts on postsynaptic neurons through glutamate receptors that fall into two main categories, the NMDA and non-NMDA subtypes (*1*). NMDA receptors are critical for long-lasting physiological modifications of neurons (*2*) and neuronal degeneration (*3*). However, the intracellular mechanisms of NMDA receptor signaling and the biochemical processes through which these receptors modify neuronal properties and connections during development and in the mature nervous system are unclear. Alterations in neuronal phenotype have been suggested to involve protein phosphorylation (*4*). Because tyrosine kinases are particularly abundant in neurons (*5*), we tested the hypothesis that protein tyrosine phosphorylation plays a role in propagating the NMDA receptor signal.

Phosphotyrosine-containing proteins in primary rat hippocampal cells were examined before and after glutamate stimulation (*6*). Immunoblot analyses with two distinct antibodies to phosphotyrosine demonstrated that stimulation of hippocampal cells with 10 μM glutamate rapidly induced an increase in tyrosine phosphorylation of a 39-kD protein (p39) (Fig. 1A). Several other phosphotyrosine-containing polypeptides were also detected, but these did not undergo any apparent change in the amount of tyrosine phosphorylation upon glutamate stimulation. The specificity of the polyclonal antibodies for phosphotyrosine was demonstrated by incubation of the antibody with 5 mM phosphotyrosine, which prevented subsequent binding to p39. In contrast, treatment with 5 mM phosphoserine or 5 mM phosphothreonine had little effect. In addition, incubation of hippocampal cells with the specific tyrosine kinase inhibitor genistein (*7*) completely blocked glutamate-induced tyrosine phosphorylation of p39 (*8*). The kinetics of p39 tyrosine phosphorylation were rapid and transient. An increase in tyrosine phosphorylation was detectable within 1 min after glutamate stimulation (Fig. 1D). Maximal phosphorylation occurred after 3 min and returned to basal levels by 60 min (Fig. 1D). Because hippocampal cultures contain about 10 to 20% glial cells, we investigated the possibility that the increased phosphorylation of p39 on tyrosine occurs in glial cells. However, glutamate stimulation failed to induce p39 tyrosine phosphorylation in pure glial cell populations.

The hippocampal cells we used express functional NMDA and non-NMDA receptors (*9*). Therefore, the receptor specificity of glutamate-stimulated tyrosine phosphor-

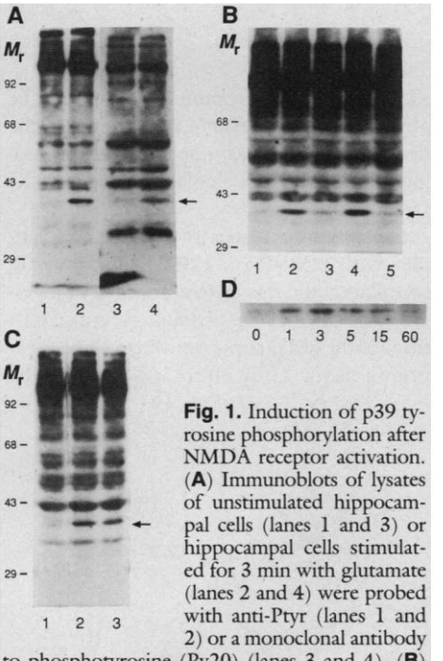

Fig. 1. Induction of p39 tyrosine phosphorylation after NMDA receptor activation. (**A**) Immunoblots of lysates of unstimulated hippocampal cells (lanes 1 and 3) or hippocampal cells stimulated for 3 min with glutamate (lanes 2 and 4) were probed with anti-Ptyr (lanes 1 and 2) or a monoclonal antibody to phosphotyrosine (Py20) (lanes 3 and 4). (**B**) Anti-Ptyr immunoblot analyses of unstimulated hippocampal cells (lane 1), cells stimulated for 3 min with glutamate (lane 2), or cells stimulated for 3 min with glutamate in the presence of 100 μM APV (lane 3), 40 μM CNQX (lane 4), or 1 mM sodium kynurenate and 11.3 mM $MgCl_2$ (lane 5). Cells were pretreated with APV, CNQX, or sodium kynurenate and 11.3 mM $MgCl_2$ for 10 min before glutamate stimulation. (**C**) Anti-Ptyr immunoblot analyses of unstimulated hippocampal cells (lane 1) and cells stimulated for 3 min (lane 2) and 5 min (lane 3) with 50 μM NMDA. The arrows in (A), (B), and (C) indicate p39. M_r, ^{14}C-labeled protein molecular weight standards ($\times 10^{-3}$). (**D**) Time course (in minutes) of the glutamate-induced increase in p39 tyrosine phosphorylation. Only part of the immunoblot is shown.

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ylation was examined. The selective NMDA receptor antagonist D(−)-2-amino-5-phosphonovalerate (APV) inhibited the glutamate-induced tyrosine phosphorylation of p39, whereas 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), which blocks the non-NMDA type of glutamate receptors, had no detectable effect (Fig. 1B). Under conditions where both subtypes of glutamate receptors were blocked with sodium kynurenate and a high concentration of Mg^{2+}, glutamate treatment failed to stimulate tyrosine phosphorylation (Fig. 1B). These results demonstrate that NMDA receptor activation is required for the glutamate-induced increase in p39 tyrosine phosphorylation. In addition, selective activation of the NMDA receptor with the synthetic agonist NMDA was sufficient to stimulate p39 tyrosine phosphorylation (Fig. 1C).

A key function of the NMDA receptor is its ability to conduct Ca^{2+} ions (*10*). An influx of Ca^{2+} from the extracellular medium was required for glutamate-induced tyrosine phosphorylation of p39 because addition of 2 mM EGTA to the medium completely blocked the increase in p39 phosphorylation (Fig. 2A). A possible route for Ca^{2+} entry other than through the NMDA receptor is through voltage-gated Ca^{2+}-channels (*11*). However, treatment of hippocampal neurons with the N-type Ca^{2+}-channel antagonist ω-conotoxin or the dihydropyridine-sensitive Ca^{2+}-channel antagonist nifedipine independently, or in combination, had no detectable effect on p39 tyrosine phosphorylation (Fig. 2B). Therefore, it is likely that glutamate-stimulated Ca^{2+} influx via the NMDA receptor triggers p39 tyrosine phosphorylation.

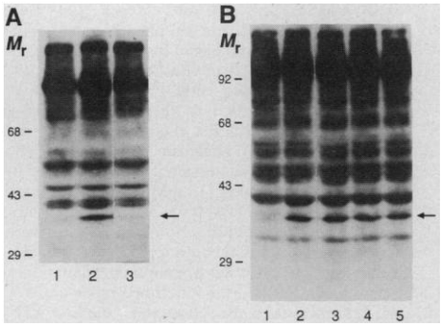

Fig. 2. (**A** and **B**) Glutamate-induced p39 tyrosine phosphorylation requires a transmembrane Ca^{2+} influx and is independent of the activation of voltage-sensitive Ca^{2+} channels. Anti-Ptyr immunoblot analyses of unstimulated hippocampal cells (A, lane 1; B, lane 1), cells stimulated for 3 min with glutamate (A, lane 2; B, lane 2), cells stimulated for 3 min with glutamate in the presence of 2 mM EGTA (A, lane 3), 4 μM ω-conotoxin (B, lane 3), 5 μM of nifedipine (B, lane 4), or 4 μM ω-conotoxin plus 5 μM nifedipine (B, lane 5). Cells were treated with ω-conotoxin or nifedipine for 10 min before glutamate stimulation. Cells were treated with EGTA for 5 min before glutamate stimulation. The arrows indicate p39. M_r, ^{14}C-labeled protein molecular weight standards (× 10^{-3}).

We next determined the identity of p39. A serine-threonine kinase of approximately 42 kD, microtubule-associated protein 2 (MAP-2) kinase (also called extracellular signal regulated kinase) (*12*), becomes rapidly phosphorylated on tyrosine in fibroblasts and adipocytes upon growth factor stimulation and in chromaffin cells in response to various secretagogues (*13*). The possibility that p39 from hippocampal cells is related or identical to MAP-2 kinase was examined by immunoblot analyses with two distinct rabbit polyclonal antibodies, antibody to a p44^{MPK} peptide sequence (anti-GEGA) (*14*), both of which recognize the mammalian MAP-2 kinase (*15*). When immunoblots of extracts obtained from unstimulated or glutamate-stimulated hippocampal cells were probed with the antibodies that recognize the MAP-2 kinase, a protein that comigrated with p39 was detected (Fig. 3A). On immunoblots, anti-p44^{MPK} preferentially recognizes the tyrosine-phosphorylated form of the fibroblast MAP-2 kinase relative to the form that is not tyrosine-phosphorylated (*15*). We found that anti-p44^{MPK} also recognized p39 from glutamate-stimulated hippocampal cells better than it recognized p39 from untreated cells (Fig. 3A, lanes 3 and 4). This is consistent with phosphorylation on tyrosine of the p39 protein from stimulated hippocampal neurons and suggests that this protein is related to the MAP-2 kinase. To further establish the identity of p39, we compared the partial proteolysis pattern of p39 recognized by the antibody to phosphotyrosine to the pattern recognized by antibodies that bind MAP-2

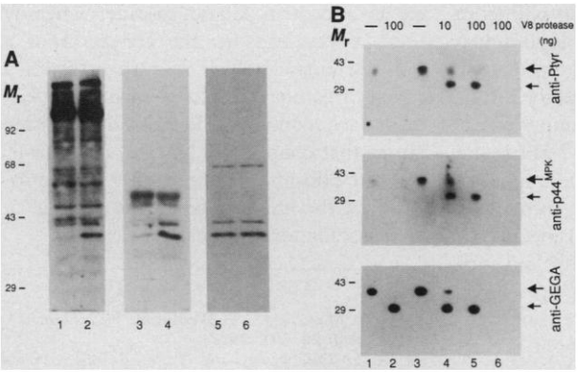

Fig. 3. Relation or identity of p39 and MAP-2 kinase. (**A**) Immunoblot analysis of lysates from unstimulated hippocampal cells (lanes 1, 3, and 5) or from cells stimulated for 3 min with glutamate (lanes 2, 4, and 6) using anti-Ptyr (lanes 1 and 2), anti-p44^{MPK} (lanes 3 and 4), or anti-GEGA (lanes 5 and 6). The arrow indicates p39. (**B**) Immunoblot analysis of peptides generated by V8 protease cleavage of p39 from unstimulated hippocampal cells (lanes 1 and 2) or from cells stimulated for 3 min with glutamate (lanes 3 to 5) using anti-Ptyr (top panel), anti-p44^{MPK} (middle panel), or anti-GEGA (bottom panel). Lane 6 contains 100 ng V8 protease but no p39. The large arrow indicates p39; the small arrow indicates its 30-kD V8 cleavage product. M_r, ^{14}C-labeled protein molecular weight standards (× 10^{-3}).

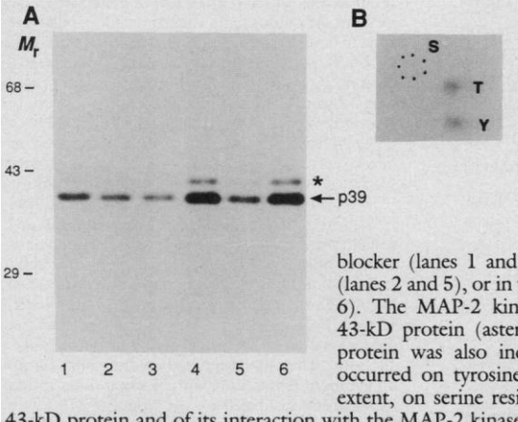

Fig. 4. Immunoprecipitation of ^{32}P-labeled p39 and phosphoamino acid analysis. (**A**) ^{32}P-labeled p39 was immunoprecipitated from lysates prepared from unstimulated (lanes 1 to 3) or glutamate-stimulated hippocampal neurons (lanes 4 to 6) using an antibody to a peptide sequence from the MAP-2 kinase (*20*). Labeling with [^{32}P]orthophosphate was carried out in the absence of glutamate receptor blocker (lanes 1 and 4), or in the presence of 100 μM APV (lanes 2 and 5), or in the presence of 40 μM CNQX (lanes 3 and 6). The MAP-2 kinase antibody also immunoprecipitated a 43-kD protein (asterisk). The phosphorylation of the 43-kD protein was also inducible upon glutamate stimulation and occurred on tyrosine and threonine residues and, to a lesser extent, on serine residues (*8*). On the basis of the size of the 43-kD protein and of its interaction with the MAP-2 kinase antibody, it seems likely that this protein is another member of the MAP-2 kinase family (*12*). M_r, ^{14}C-labeled protein molecular weight standards (× 10^{-3}). (**B**) Phosphoamino acid analyses of ^{32}P-labeled p39 immunoprecipitated from glutamate-stimulated hippocampal neurons [corresponding to lane 4 in (A)]. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. The p39 from unstimulated hippocampal neurons [corresponding to lanes 1 to 3 in (A)] also contained phosphotyrosine and phosphothreonine, but to a lesser degree than did stimulated hippocampal neurons (*8*).

kinase. SDS gel slices containing p39 from unstimulated and stimulated hippocampal cells were subjected to partial proteolytic digest with V8 protease (*16*), and the resulting peptides were analyzed by immunoblotting (*17*). If p39 and the MAP-2 kinase are related or identical proteins, one might expect that V8 cleavage products, recognized by the antibody to phosphotyrosine, would also be recognized by anti-p44^{MPK} and anti-GEGA. We found that partial proteolytic digests of p39 from glutamate-stimulated cells gave rise to a peptide of about 30 kD, which was detected by the antibody to phosphotyrosine, anti-p44^{MPK}, and anti-GEGA (Fig. 3B, lanes 3 to 5). A second proteolytic fragment of about 28 kD was recognized by each of the three antibodies and became detectable after prolonged exposure of the immunoblots. Anti-GEGA, which recognizes the tyrosine-phosphorylated and unphosphorylated forms of p39 equally well (Fig. 3A, lanes 5 and 6), also bound to p39 and the 30-kD cleavage product from unstimulated cells, whereas these antigens reacted poorly with the antibody to phosphotyrosine and anti-p44^{MPK} (Fig. 3B, lanes 1 and 2). Taken together, these peptide-mapping experiments indicate that p39 is related or identical to MAP-2 kinase.

We next investigated if glutamate-induced phosphorylation of p39 plays a role in the activation of this kinase. Phosphorylation of both tyrosine and threonine residues is required for the induction of MAP-2 kinase activity (*18*). To determine if glutamate stimulation induces phosphorylation of p39 on both tyrosine and threonine residues, hippocampal neurons were labeled with ^{32}P-labeled orthophosphate (*19*) and p39 was immunoprecipitated with an antibody to the MAP-2 kinase (*20*). This experiment confirmed that the phosphorylation of p39 is induced by glutamate in an NMDA receptor–specific manner (Fig. 4A). Phosphoamino acid analyses (*21*) revealed that p39 was phosphorylated on both tyrosine and threonine residues (Fig. 4B), suggesting that its kinase activity is induced upon NMDA receptor activation. Direct measurements of MAP-2 kinase activity in crude cell extracts, with MAP-2 protein and myelin basic protein used as substrates, showed that the kinase activity in hippocampal neurons increases after glutamate stimulation (*8*). The glutamate induction of MAP-2 kinase activity was dependent on activation of the NMDA receptor (*8*).

Taken together, these findings suggest that the NMDA receptor signal is propagated as a protein kinase cascade, whereby Ca^{2+} influx leads to the activation of a tyrosine kinase, which in turn activates a serine-threonine kinase. Further experiments are required to identify the tyrosine kinase that couples the NMDA receptor to tyrosine phosphorylation of p39 and to investigate the importance of this protein kinase cascade for neuronal plasticity.

REFERENCES AND NOTES

1. M. L. Mayer and C. L. Westbrook, *Prog. Neurobiol. (Oxford)* **28**, 197 (1987).
2. G. L. Collingridge and T. V. P. Bliss, *Trends Neurosci.* **10**, 288 (1987); A. Artola and W. Singer, *Nature* **330**, 649 (1987); H. T. Cline, E. A. Debski, M. Constantine-Paton, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4342 (1987); A. Kleinschmidt, M. F. Bear, W. Singer, *Science* **238**, 355 (1987); R. G. M. Morris *et al.*, *Nature* **319**, 774 (1986); M. J. D. Miserendino, C. B. Sananes, K. R. Melia, M. Davis, *ibid.* **345**, 716 (1990).
3. S. M. Rothman and J. W. Olney, *Trends Neurosci.* **10**, 299 (1987); D. W. Choi, *Neuron* **1**, 623 (1988).
4. P. Greengard, *Mol. Neurobiol.* **1**, 81 (1987); R. C. Malenka *et al.*, *Nature* **340**, 554 (1989); R. Malinow, H. Schulman, R. W. Tsien, *Science* **245**, 862 (1989); R. F. Akers *et al.*, *ibid.* **231**, 587 (1986).
5. P. C. Cotton and J. S. Brugge, *Mol. Cell. Biol.* **3**, 1157 (1983); M. Sudol and H. Hanafusa, *ibid.* **6**, 2839 (1986); A. A. Hirano, P. Greengard, R. L. Huganir, *J. Neurochem.* **50**, 1447 (1988); D. T. Pang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 762 (1988); K. R. Stratton, P. F. Worley, R. L. Huganir, J. M. Baraban, *ibid.* **86**, 2498 (1989).
6. Isolation of hippocampal formations from newborn Long-Evans rats and the dissociation of neurons was performed as described [E. J. Furshpan and D. D. Potter, *Neuron* **3**, 199 (1989)] with the following modifications: dissociation medium contained 1.4 mM Hepes (pH 7.4), 73.6 mM Na_2SO_4, 27 mM K_2SO_4, 15.2 mM $MgCl_2$, 0.23 mM $CaCl_2$, 1 mM sodium kynurenate, 18 mM glucose, and 0.0011% phenol red; the glucose concentration in L-15 CO_2 growth medium was 30 mM. The Ca^{2+} and Mg^{2+} concentrations in L-15 CO_2 growth medium were 0.9 mM and 1.3 mM, respectively. Cells were plated on poly(D-lysine)–and laminin-coated 60-mm tissue culture dishes at a density of 0.05 to 0.06 hippocampi per square centimeter and were grown at 37°C in a humidified atmosphere consisting of 5% CO_2 plus 95% air. To inhibit proliferation of nonneuronal cells, 2.4 μM cytosine-β-D-arabinofuranoside was added to the cultures 1 to 2 days after plating. Nine days after plating the neurons were stimulated by addition of 10 μM of glutamate to the medium. At various times after stimulation, cells were lysed in boiling SDS sample buffer [160 mM tris-HCl (pH 6.8), 4% SDS, 30% glycerol, 5% β-mercaptoethanol, 10 mM dithiothreitol, 0.02% bromophenol blue]. Lysates corresponding to equal numbers of cells were separated by electrophoresis on 7.5% or 8.5% SDS–polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose membranes were incubated at 37°C for 2 hours in blocking solution [3% bovine serum albumin, 50 mM tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween-20, 20 μM sodium orthovanadate], then incubated for 2 hours at 37°C in blocking solution containing either a rabbit polyclonal antibody to phosphotyrosine (anti-Ptyr) (L. M. Kozma, A. J. Rossomando, M. J. Weber, *Methods Enzymol.*, in press), diluted 1:600, or the monoclonal antibody Py20 (ICN Biomedicals), diluted 1:400. The blots were washed four times for 10 min at room temperature with blocking solution and subsequently incubated for 1.5 hours at 37°C with ^{125}I-labeled protein A at a concentration of 1 μCi/ml in blocking solution. After four washes for 10 min with blocking solution and three washes for 10 min with phosphate-buffered saline containing 20 μM sodium orthovanadate, the membranes were air-dried and analyzed by autoradiography.
7. T. Akiyama *et al.*, *J. Biol. Chem.* **262**, 5592 (1987).
8. H. Bading and M. E. Greenberg, unpublished observations.
9. H. Bading, M. M. Segal, M. E. Greenberg, unpublished observation.
10. P. Ascher and L. Nowack, *Trends Neurosci.* **10**, 284 (1987).
11. R. W. Tsien, D. Lipscombe, D. V. Madison, K. R. Bley, A. P. Fox, *ibid.* **11**, 431 (1988).
12. T. G. Boulton *et al.*, *Science* **249**, 64 (1990); T. G. Boulton *et al.*, *Cell* **65**, 663 (1991).
13. L. B. Ray and T. W. Sturgill, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1502 (1987); *ibid.* **85**, 3753 (1988); A. J. Rossomando *et al.*, *ibid.* **86**, 6940 (1989); L. B. Ray and T. W. Sturgill, *J. Biol. Chem.* **263**, 12721 (1988); T. W. Sturgill, L. B. Ray, E. Erikson, J. L. Maller, *Nature* **334**, 715 (1988); C. M. Ely *et al.*, *J. Cell Biol.* **110**, 731 (1990).
14. Anti-p44^{MPK} was generated against purified myelin basic protein kinase from sea star oocytes; anti-GEGA was raised against a peptide corresponding to an amino acid sequence of the same enzyme determined by partial microprotein sequencing [J. S. Sanghera, H. B. Paddon, S. L. Pelech, *J. Biol. Chem.* **266**, 6700 (1991)]. Immunoblots were carried out as described (*6*), except that both antibodies were used at a 1:1000 dilution, and incubation was performed overnight at room temperature.
15. A. J. Rossamondo *et al.*, *J. Biol. Chem.*, in press.
16. D. W. Cleveland, S. G. Fischer, M. W. Kirschner, U. K. Laemmli, *ibid.* **252**, 1102 (1977).
17. The generated peptides were separated by electrophoresis on 15% SDS polyacrylamide gels and transferred to nitrocellulose. Parallel blots were probed with anti-Ptyr and anti-GEGA (*14*). Subsequently, the anti-Ptyr blot was stripped by four successive 15-min incubations with a solution containing 5 M sodium iodide and 1 mM sodium thiosulfate and then rinsed twice, briefly, with distilled water. After incubation with blocking solution, the filter was reincubated with ^{125}I-labeled protein A (*6*) and subjected to autoradiography for confirmation that the anti-Ptyr had been removed. The filter was then probed with anti-p44^{MPK}.
18. N. G. Anderson *et al.*, *Nature* **343**, 651 (1990).
19. The neurons were incubated in labeling medium [114 mM NaCl, 26.1 mM $NaHCO_3$, 3.3 mM KCl, 1.3 mM $MgCl_2$, 1 mM $CaCl_2$, 24.5 mM glucose, 0.82 mM glycine, 10 mM Hepes (pH 7.4), 0.0008% phenol red, and 1% phosphate-free minimum essential medium Eagle] for 2 hours at 37°C in a humidified atmosphere consisting of 7.5% CO_2 plus 92.5% air. Subsequently, [^{32}P]orthophosphate (1 mCi/ml) was added to the medium. After a labeling period of 3 hours, the neurons were stimulated with 10 μM glutamate for 3 min, briefly washed with phosphate-buffered saline, and lysed in boiling SDS lysis buffer [10 mM sodium phosphate (pH 7.2), 2 mM EDTA, 0.5% SDS, 2 mM dithiothreitol, and Trasylol (20 μg/ml)]. The lysates were adjusted to radioimmunoprecipitation assay buffer conditions and subjected to immunoprecipitation.
20. J. Chung and J. Blenis, unpublished observation.
21. M. P. Kamps and B. M. Sefton, *Anal. Biochem.* **176**, 22 (1989).

22. We thank M. J. Weber, S. L. Pelech, and J. Blenis for providing antibodies and for communicating results before publication; M. M. Segal for help in culturing hippocampal neurons; D. M. Payne for advice on the immunoblots; and T. Hunter and M. H. Cobb for helpful discussion. Supported by the Deutsche Forschungsgemeinschaft (H.B.), by NIH grants CA 43855 and NS 28829 (M.E.G.). M.E.G. is the recipient of an American Cancer Society Faculty Research Award (FRA-379) and a Scholar's Award from the McKnight Endowment Fund for Neuroscience.

25 February 1991; accepted 19 June 1991