0026-895X/05/6704-1158–1165$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 67:1158–1165, 2005

Vol. 67, No. 4
5447/1197148
Printed in U.S.A.

N-Methyl-D-aspartate and Brain-Derived Neurotrophic Factor Induce Distinct Profiles of Extracellular Signal-Regulated Kinase, Mitogen- and Stress-Activated Kinase, and Ribosomal S6 Kinase Phosphorylation in Cortical Neurons

S. Rakhit, C. J. Clark, C. T. O'Shaughnessy, and B. J. Morris

Division of Neuroscience and Biomedical Systems, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, Scotland (S.R., C.J.C., B.J.M.); and Neurology CEDD, GlaxoSmithKline, Harlow, Herts, United Kingdom (C.T.O.)

Received July 27, 2004; accepted December 21, 2004

ABSTRACT

Stimulation of *N*-methyl-D-aspartate (NMDA) receptors is believed to underlie long-term memory formation, and excessive NMDA receptor activation has been linked to several neuropathological conditions. Phosphorylation and activation of p42/44 mitogen-activated protein kinase (ERK) is believed to mediate many of these effects, but the downstream targets of ERK in response to NMDA activation have not been determined. In primary cultures of rat cortical neurons, we found that NMDA was able to elevate phosphorylation of mitogen- and stress-activated kinase 1 (MSK1) as well as ERK. Likewise, brain-derived neurotrophic factor (BDNF) treatment increased phosphorylation of MSK1 and ERKs. The NMDA-induced MSK1 phosphorylation was sensitive to the MEK inhibitor 2′-amino-3′-methoxyflavone (PD98059) and the p38 inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580). MSK1 activation by NMDA was transient, although ERK remained phosphorylated within the neuronal cytoplasm for several hours. Although BDNF increased ribosomal S6 kinase (RSK) phosphorylation, NMDA had no discernable effect on the phosphorylation of RSKs. Thus, phosphorylation and activation of MSK1 but not RSK could be an important step in the pathway linking NMDA-induced ERK phosphorylation to the activation of transcription factors required for the formation of long-term memory.

Activation of the *N*-methyl-D-aspartate (NMDA) class of ionotropic glutamate receptor is a key stimulus for the induction of long-term plasticity in neuronal cells. Stimulation of NMDA receptors in neurons not only underlies activity-dependent changes in synaptic strength such as long-term potentiation (LTP) but is linked to pathological changes leading to neurotoxic damage, such as during stroke.

The NMDA receptor is a ligand-gated sodium/calcium ion channel, and maximum ion flow requires the coincidence of both presynaptic activity, which releases glutamate to bind the receptor, and postsynaptic depolarization, which is needed to relieve blockade of the receptor channel by extracellular magnesium. Calcium entry leads to the activation of signaling intermediates such as protein kinase C, ERKs, and Ca^{2+}/calmodulin-dependent kinases, which are necessary for LTP induction (English and Sweatt, 1996, 1997; Morris, 2004). Activation of these pathways subsequently leads to phosphorylation of the transcription factor cAMP-response element binding protein (CREB), which, together with CREB-binding protein, may initiate transcription of immediate early genes required for LTP.

The later phases of LTP are dependent on gene transcription and protein synthesis, and CREB has been implicated in mediating enhanced gene transcription in response to NMDA receptor stimulation (Impey et al., 1996, 1998). However, the signaling pathways that link activation of NMDA receptors to phosphorylation of CREB have only begun to be understood. The best-characterized CREB kinases belong to the ribosomal S6 kinase (RSK) family, consisting of RSKs 1 to 4. The fact that phospho-ERKs are activators of RSK phosphor-

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.005447.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; RSK, ribosomal S6 kinase; BDNF, brain-derived neurotrophic factor; ERK, p42/44 mitogen-activated protein kinase; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; LTP, long-term potentiation; CREB, cAMP-response element binding protein; EGF, epidermal growth factor; MAP, mitogen-activated protein; TBST, Tris-buffered saline/Tween 20; O.D., optical density; MK801, dizocilpine maleate; TNFα, tumor necrosis factor-α; 8BrcGMP, 8-bromo-cGMP; NGF, nerve growth factor; MEK, mitogen-activated protein kinase kinase; MAK, mitogen- and stress-activated kinase; Bad, Bcl-2–associated death protein; p-, phospho-.

ylation in many cell types (Frodin and Gammeltoft, 1999), coupled with the observation that NMDA induces ERK phosphorylation in neurons, has resulted in the general assumption that RSKs are activated in response to NMDA receptor stimulation and mediate NMDA-induced CREB phosphorylation (West et al., 2002). This has been reinforced by the observation that massive depolarization of hippocampal neurons causes RSK to phosphorylate CREB (Impey et al., 1998).

Brain-derived neurotrophic factor (BDNF) can mimic the long-term effects of NMDA receptor stimulation, in that application of BDNF to cortical or hippocampal tissue induces LTP (Messaoudi et al., 2002; Ying et al., 2003). As with the results of NMDA receptor stimulation, ERKs and CREB are also activated by BDNF. However, there is a similar lack of direct evidence that BDNF can induce phosphorylation and activation of RSKs.

The idea of RSKs being the major CREB kinases within neurons has been challenged with the recent discovery of the mitogen- and stress-activated kinase (MSK) proteins, MSK1 and MSK2 (Deak et al., 1998; Pierrat et al., 1998; New et al., 1999). MSKs are activated by EGF and fibroblast growth factor in various cell types, and the activation can occur either via ERK or p38 MAP kinase (p38) (Deak et al., 1998; Pierrat et al., 1998; New et al., 1999; Wiggin et al., 2002). There is good evidence that MSKs are highly efficient and physiologically important CREB kinases (Deak et al., 1998; Arthur and Cohen, 2000; Wiggin et al., 2002). The evidence for compromised CREB phosphorylation in MSK-deficient cells (Wiggin et al., 2002) is complemented by evidence that CREB phosphorylation is normal in RSK2-deficient cells (Bruning et al., 2000). This raises the possibility that some of the downstream effects of NMDA stimulation or BDNF exposure might be mediated via MSK rather than RSK. Indeed, recent evidence suggests that neuronal CREB phosphorylation in response to BDNF may involve MSKs rather than RSKs (Arthur et al., 2004). We therefore investigated the characteristics of both MSK and RSK phosphorylation to see to what extent they were activated in response to NMDA and BDNF in cultured rat cortical neurons.

Materials and Methods

Antibodies toward phospho-ERK (pERK), total ERK, phospho-p38 (p-p38), phospho-T581-MSK1 (pMSK1), phospho-S298-MEK (pMEK), phospho-S380-Rsk, and phospho-S112-Bad (pBad) were purchased from Cell Signaling Technology, Inc. (Beverly, MA); the antibody toward total MSK1 was provided by GlaxoSmithKline (Uxbridge, Middlesex, UK), and the antibody for MEK was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation, Dissociation, and Culture of Embryonic Rat Cortical Cells. Primary neuronal cultures were prepared as described previously (Morris, 1995; Simpson and Morris, 2000). The cortex was dissected out from between 10 and 15 embryos at the 17th day of pregnancy and pooled. The tissue was collected in minimal essential medium (Invitrogen, Carlsbad, CA) and minced finely, then dispersed in a solution of trypsin/EDTA for 1 h at 37°C. After trituration through a sterile Pastette, the cells were suspended in Dulbecco's modified Eagle's medium (Invitrogen) containing penicillin/streptomycin and 20% fetal calf serum. Cortical cells were plated on laminin/poly-D-lysine–coated sterile culture plates, and after 24 h, the medium was changed to Neurobasal medium (Invitrogen) with B27 supplement to promote selective growth of neuronal cells. Cells were routinely stimulated after 7 days in culture (Morris, 1995; Simpson and Morris, 2000).

Preparation of Nuclear Fractions. After stimulation, cells were washed in phosphate-buffered saline and scraped into 1 ml of phosphate-buffered saline. Cells were centrifuged at 500*g*, and the supernatant was discarded; the cell pellet was dissolved in nuclear extraction buffer (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 26% glycerol, 300 mM NaCl, 0.1% dithiothreitol, and 0.025% Nonidet P-40). Pestles were used to aid the grinding of cellular material. This was then frozen and thawed three times in quick succession and then centrifuged (2100*g*, 20 min). The supernatant, which contains nuclear material, was added to X2 gel-loading sample buffer (0.25 M Tris, 2 mM $Na_4P_2O_7$, 5 mM EDTA, 5% glycerol, and 2% SDS, pH 6.7), boiled, and then loaded onto a gel.

SDS-Polyacrylamide Gel Electrophoresis and Western Analysis of Samples. Neurons in Neurobasal medium grown on culture plates were stimulated for various times and with different drugs as specified, after which the medium was aspirated off and replaced with X2 gel-loading sample buffer, boiled, and loaded onto a gel.

Proteins were separated by the Laemmli method using a 10% polyacrylamide gel and Western blotted onto nitrocellulose paper. The blots were incubated in TBST (10 mM Tris, 0.1 M NaCl, and 0.1% Tween 20, pH 7.5) plus 5% nonfat milk for 1 h to block nonspecific binding. This was followed by incubation in primary antibody made in TBST in 1% bovine serum albumin for 18 h at 4°C. The blots were then washed three times for 5 min each in TBST and incubated with secondary antibody conjugated to horseradish peroxidase for 1 h, washed again, and bands were detected by a chemiluminescence kit (Amersham Biosciences, Piscataway, NJ). Integrative optical density readings for bands were obtained using NIH Image Software (W. Rasband, National Institutes of Health, Bethesda, MD). To normalize data between experiments, optical density (O.D.) values after drug treatment were expressed as the change in O.D. (ΔO.D.) after subtraction of the corresponding value after vehicle treatment. Data for kinase inhibitor studies were log-transformed before analysis because of the large range of absolute values obtained. Results were analyzed using paired *t* test (data within a single culture were paired) or ANOVA (Minitab Software; Minitab Inc., State College, PA).

Results

Phosphorylated ERK1 and -2 appeared as two bands at 42 and 44 kDa, respectively, with the 42-kDa band predominating. Both NMDA (in the presence of 15 mM K^+, to provide a slight depolarization) and BDNF produced dramatic increases in the levels of pERK immunoreactivity (Fig. 1). Either agent seemed to saturate the response, because the combination of NMDA and BDNF together did not produce any further increase in pERK levels (Fig. 1). Pretreatment with the selective NMDA channel blocker MK801 abolished this ERK1/2 phosphorylation response to NMDA, although the cells still responded normally to the neurotrophin BDNF (Fig. 1).

Whole-cell ERK1/2 phosphorylation increased rapidly within 5 min of adding NMDA, and the elevation was maintained for at least 2 h (Fig. 2b). Phosphorylation of the upstream activator of ERK1/2, MEK, was observed as a characteristic 45-kDa band (Fig. 2a). Basal phosphorylation of MEK as with ERK in these cells was very low. MEK was already maximally phosphorylated by 5 min of NMDA stimulation, and this decreased gradually over the course of the hour.

Possible phosphorylation of MSK1 in response to NMDA was then investigated. The pMSK1 band was detected at approximately 90 kDa. Treatment with NMDA induced a

clear, transient increase in MSK phosphorylation (Fig. 2c). MSK seemed to be phosphorylated rapidly upon the addition of NMDA and reached a peak within 10 min, after which it declined. In the cortical neurons, NMDA could consistently induce MSK1 phosphorylation at concentrations greater than 10 μM (Fig. 3a). The MSK response was also sensitive to MK108, thus verifying that the kinase is selectively activated by NMDA receptors. Both BDNF and TNFα could also activate MSK1 phosphorylation (Fig. 3a). NMDA and BDNF were then tested for their ability to activate p38 MAP kinase in cortical neurons. Although H_2O_2, a known activator of p38, produced robust p38 phosphorylation, no bands were detected in response to BDNF or NMDA stimulation (Fig. 3b).

Because NMDA and BDNF clearly stimulate phosphorylation of ERK1/2 and its downstream target MSK1 in cortical neurons, we investigated whether phosphorylation of another ERK target, RSK, could be also detected. We compared the effects of NMDA and BDNF with those of a known RSK activator, 8-bromo-cGMP (8BrcGMP) (Ho et al., 2003).

Figure 4 shows the phosphorylation levels of ERK, RSK, and MSK1 in whole-cell lysates of cortical neurons in response to BDNF and NMDA. ERK was phosphorylated robustly and consistently by both BDNF and NMDA. The levels of pERK, however, seemed unaffected by exogenous addition of 8-bromo-cGMP (Fig. 4a). BDNF and NMDA also induced MSK1 phosphorylation, but no response to 8-bromo-cGMP was observed (Fig. 4b). In contrast, pRSK levels were increased by 8-bromo-cGMP and by BDNF but were not affected by NMDA treatment (Fig. 4c). For comparison, PC12 cells were treated with vehicle or NGF, and the cell lysates were processed similarly. NGF induced marked phosphorylation of RSK and ERK, with a slight increase in pMSK levels (Fig. 4, d–f).

Semiquantitative analysis of the Western blots confirmed that BDNF significantly increased phosphorylation of both MSK1 and RSK. In contrast, NMDA receptor stimulation resulted in phosphorylation of ERK and MSK1, but not RSK, in cortical neurons (Fig. 5, a and b). The potency of NMDA in inducing MSK1 phosphorylation seemed slightly lower than its potency in inducing ERK phosphorylation (Fig. 5c).

As expected, the MEK inhibitor PD98059 reduced the phosphorylation of ERK in response to NMDA and BDNF, whereas the p38 inhibitor SB203580 had little effect on ERK phosphorylation (Fig. 6, a, d, and e). However, PD98059 and SB203580 were both able to attenuate MSK1 activation by NMDA (Fig. 6, b, d, and e). In comparison, BDNF-induced phosphorylation of pMSK1 seemed largely via the ERK pathway, because PD98059 but not SB203580 was able to attenuate the increased phosphorylation. The same blot reprobed for total MSK1 shows that there was equal loading throughout the lanes (Fig. 6c).

To confirm that the cortical cultures exhibited some response that was representative of RSK/MSK activation, we tested the ability of BDNF to induce phosphorylation of Bcl-

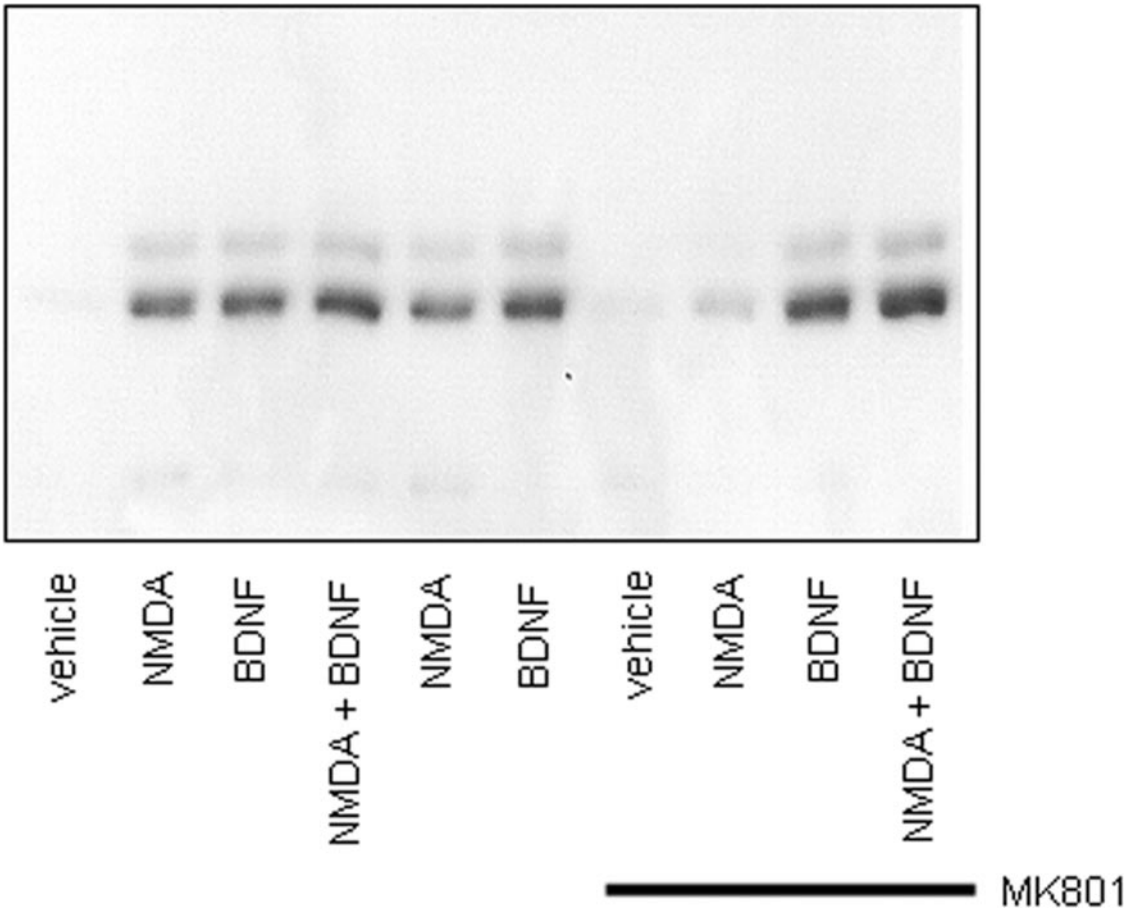

Fig. 1. Effect of NMDA or BDNF treatment on ERK phosphorylation. Cortical neurons were exposed to 100 μM NMDA and/or 20 ng/ml BDNF, for 10 min in the absence or presence of 10 μM MK801, and the whole-cell lysates were processed for Western blotting with anti-pERK antibody. Results shown are from a typical experiment that was replicated three times.

2–associated death protein (Bad). Both Msk1 and Rsk2 have been suggested to be involved in Bad phosphorylation (Tan et al., 1999; She et al., 2002). We detected a clear increase in phospho-Bad (pBad) levels after exposure of the cortical neurons to BDNF (Fig. 7).

Discussion

In cortical or hippocampal neurons, ERK activation is believed to mediate many of the long-term effects of stimulating NMDA receptors, such as the changes in synaptic efficiency associated with LTP. There are also reports which suggest that NMDA-induced excitotoxicity in primary neurons is mediated by activated ERKs. However, although ERK seems to be an intermediate in these diverse signals in neurons, it is not understood how, for instance, the NMDA-mediated ERK signal that causes LTP differs from the ERK activity that signals excitotoxic apoptosis. Thus, it is important to investigate both the mechanism by which NMDA generates ERK phosphorylation and the characteristics and profile of this signal, as well as the subsequent activations of downstream elements that result.

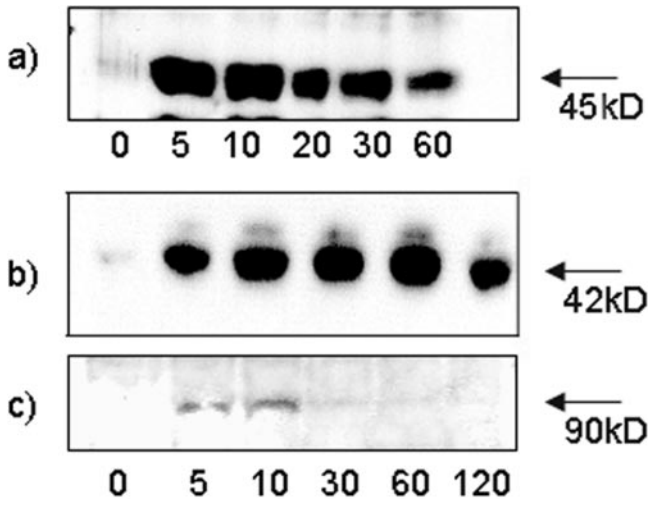

Fig. 2. Time course of NMDA effects on MEK, ERK, and MSK1 phosphorylation. Cortical neurons were exposed to 100 μM NMDA for the times shown (in minutes), and the whole-cell lysates processed for Western blotting with anti-pMEK antibody (a), anti-pERK antibody (b), or anti-pMSK1 antibody (c). Results shown are from a typical experiment that was replicated three times.

MSK1 phosphorylation has been characterized previously in terms of the response to growth factors such as EGF and fibroblast growth factor in peripheral cell lines. In addition, MSK can be activated by oxidative or osmotic stress or by heat shock. We report here that MSK1 can be phosphorylated in neurons in response to stimulation of NMDA receptors—a finding that implicates MSK1 in the signaling responses to excitatory synaptic stimulation.

MSKs have a structure similar to that of RSKs, containing two kinase domains in a single polypeptide chain and multiple phosphorylation sites. Because of the high degree of similarity between RSKs and MSKs and conservation of the four key phosphorylation sites, it has been largely assumed that the activation mechanism of MSKs corresponds broadly to that for RSKs (Deak et al., 1998). This would imply that activation of the C-terminal kinase domain by ERK or p38 would then be essential for autophosphorylation and activation of the N-terminal domain of MSK1. The antibody used here to detect phosphorylated MSK recognizes MSK1 phosphorylated at Thr581, which corresponds to Thr573 on RSK1 within the activation loop of the C-terminal kinase domain, a residue believed to be critical for kinase activation and predicted to be phosphorylated by ERK, or possibly by p38 (Dalby et al., 1998; Deak et al., 1998; Pierrat et al., 1998).

It has been observed previously that Ca^{2+} influx (via a Ca^{2+} ionophore) is a powerful stimulus for MSK1 phosphorylation (Tomas-Zuber et al., 2000), so it is likely that the influx of Ca^{2+} ions through the activated NMDA receptor channel triggers the phosphorylation of MSK1. It has also become clear that either ERK or p38 can phosphorylate and activate MSKs (Deak et al., 1998; Tomas-Zuber et al., 2000, 2001; Markou and Lazou, 2002; Wiggin et al., 2002). We confirmed many previous reports that neuronal NMDA receptor stimulation results in pronounced phosphorylation of ERKs (English and Sweatt, 1996, 1997;

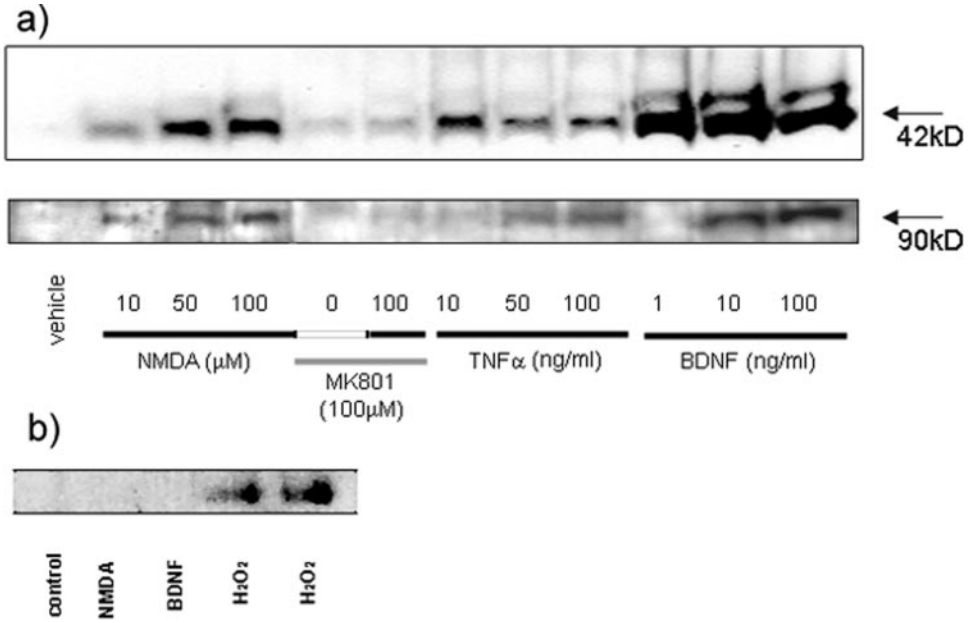

Fig. 3. a, effect of NMDA, TNFα, or BDNF treatment on phosphorylation of ERK and MSK1. Cortical neurons were exposed to NMDA, BDNF, or TNFα for 10 min, and the whole-cell lysates were processed for Western blotting with anti-pERK antibody (top) or anti-pMSK1 antibody (bottom). Results shown are from a typical experiment that was replicated three times. b, effect of NMDA, BDNF, or hydrogen peroxide treatment on p38 MAP kinase phosphorylation. Cortical neurons were exposed to 100 μM NMDA, 20 ng/ml BDNF, or 200 μM H_2O_2, and the whole-cell lysates were processed for Western blotting with anti-phospho-p38 antibody.

Fuller et al., 2001; Iida et al., 2001). However, we did not detect any increased phosphorylation of p38 after NMDA treatment, a finding that is also consistent with previous reports (Fuller et al., 2001). Hence, stimulation of NMDA receptors in cortical neurons activates ERKs but not p38. It is interesting that the phosphorylation of MSK1 resulting from NMDA receptor stimulation was attenuated by both the MEK inhibitor PD98059 and the p38 inhibitor SB203580. Because a dramatic increase in pERK was induced by treatment with NMDA, this implies that although ERK activation is probably the major influence driving MSK1 phosphorylation in cortical neurons, some basal level of p38 activity is necessary for this effect of NMDA to be observed.

SB203580 inhibits both α and β forms of p38 MAP kinase with submicromolar IC_{50} values (Clerk and Sugden, 1998; Lee et al., 1999; Lali et al., 2000; Davies et al., 2001). There is evidence that SB203580 can also inhibit a small number of other kinases, including c-Jun NH_2-terminal kinase 2 (Clerk and Sugden, 1998; Lee et al., 1999), Lck (Davies et al., 2001), and protein kinase B/Akt (Lali et al., 2000), although with less potency. Certainly, at concentrations greater than 5 μM, there is a substantial risk of loss of selectivity. Therefore, we cannot unequivocally ascribe the effect of SB203580 on MSK1 phosphorylation observed here to p38 inhibition. However, this apparent permissive effect of p38 on activation of MSK1 by ERK has also been reported previously in human embryonic kidney 293 cells (Tomas-Zuber et al., 2001), in which it was suggested that the targeting of Thr687 of MSK by p38 is necessary for activation by ERK (via phosphorylation at Thr581), suggesting that p38 inhibition is the most likely mechanism. However, because SB203580 did not seem to cause any appreciable inhibition of the ability of BDNF to phosphorylate MSK1 in our experiments, some aspect of BDNF signaling may overcome the permissive effect of p38 activity. Further investigation is required to characterize the mechanisms for these effects.

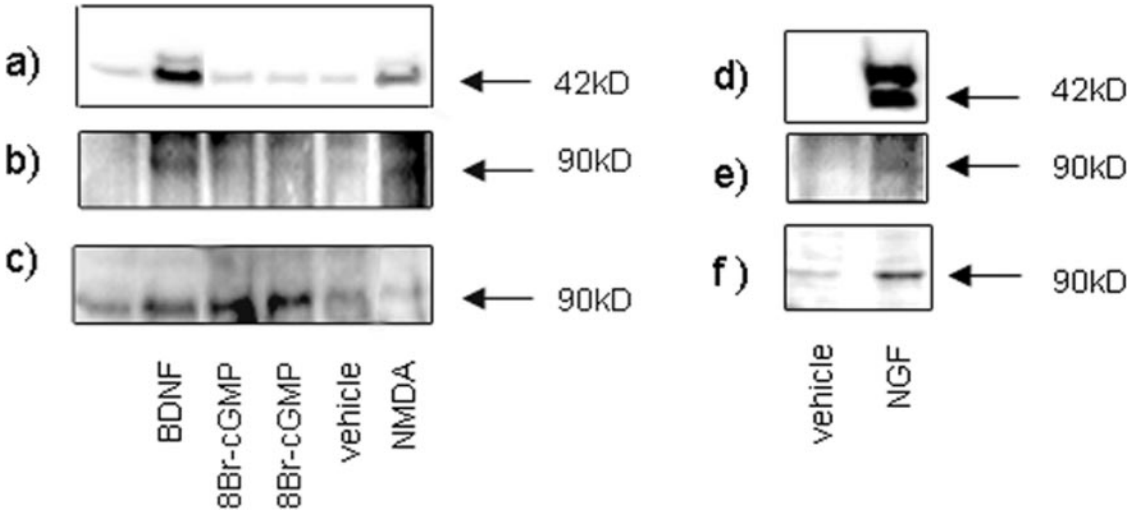

Fig. 4. Effect of NMDA, BDNF, and 8BrcGMP on phosphorylation of ERK, MSK1, and RSK. Cortical neurons were exposed to vehicle or 100 μM NMDA, 20 ng/ml BDNF, or 10 μM 8BrcGMP for 10 min, and the whole-cell lysates were processed for Western blotting with anti-pERK antibody (a), anti-pMSK1 antibody (b), or anti-p90RSK antibody (c). Results shown are from a typical experiment that was replicated three times. Likewise, PC12 cells were exposed to vehicle or 10 ng/ml NGF for 10 min and then processed for Western blotting with anti-pERK antibody (d), anti-pMSK1 antibody (e) or anti-p90RSK antibody (f).

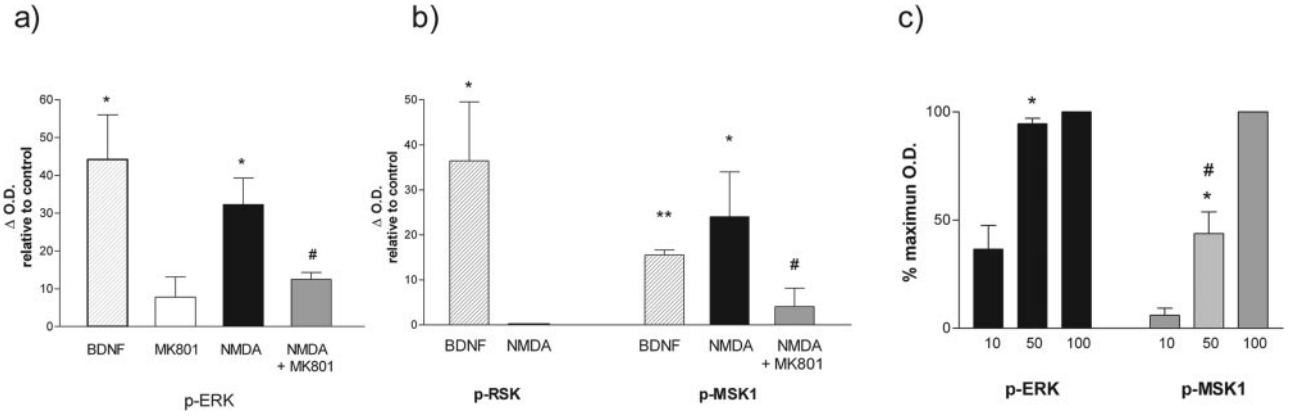

Fig. 5. Semiquantitative analysis of the effect of BDNF and NMDA on phosphorylation of ERK, MSK1, and RSK. Western blot band intensities were measured as described under *Materials and Methods*, and significance was assessed by ANOVA with post hoc Fisher's test. a and b, effect of 100 ng/ml BDNF, 100 μM NMDA, 10 μM MK801, or 100 μM NMDA + 10 μM MK801 on pERK (a), or pRSK and pMSK1 (b). a, $F(3,11) = 5.27$, $p < 0.05$; b, $F(3,8) = 4.91$, $p < 0.05$; ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$ versus vehicle treatment; #, $p < 0.05$ versus NMDA treatment. c, effect of increasing doses of NMDA on pERK or pMSK1 signal. Data are expressed as a percentage of the signal intensity achieved by 100 μM NMDA. Two-way ANOVA (factors, dose and pMSK/pERK): $F(1,6,9) = 25.82$. pMSK-1 signal overall was significantly less than pERK signal ($p < 0.01$), and there was a significant effect of dose ($p < 0.001$), but no significant interaction between dose and pMSK/pERK was observed. ⋆, $p < 0.05$ versus vehicle treatment; #, $p < 0.05$ versus effect of corresponding NMDA dose on pERK signal.

The finding that NMDA receptor activation leads to MSK1 phosphorylation is highly relevant in the context of a recent report demonstrating that increased MSK1 phosphorylation is observed during excitotoxicity in cultured neurons and that the activation of MSK1 may, in fact, contribute to the neurotoxicity observed (Hughes et al., 2003). Neurotoxicity in this model is linked to excessive glutamate receptor activation, including activation of NMDA receptors. Hence, our data indicate that the phosphorylation of MSK1 observed after an excitotoxic stimulus is likely to be a direct result of NMDA receptor stimulation and further implicate MSK1 in the long-term manifestations of NMDA receptor-dependent plasticity.

In this study, we also observed that MSK1 can be phosphorylated by BDNF. Hence, BDNF joins a growing list of growth factors, acting through distinct but related tyrosine kinase signaling pathways, that seem to be able to activate MSKs. Hippocampal neurons exposed to BDNF show activation of ERK and CREB, along with an enhanced synaptic sensitivity that is sustained in the long term by mechanisms dependent on protein synthesis (Messaoudi et al., 2002; Ying et al., 2003). This is very similar to the physiological effects of NMDA receptor stimulation. Hence, commonalities in the signaling pathways activated by NMDA and BDNF may be components of the plasticity response. The fact that MSK1 is phosphorylated in response to both NMDA receptor stimulation and BDNF treatment raises the possibility that MSK1 may be involved in the signaling pathways linked to synaptic plasticity.

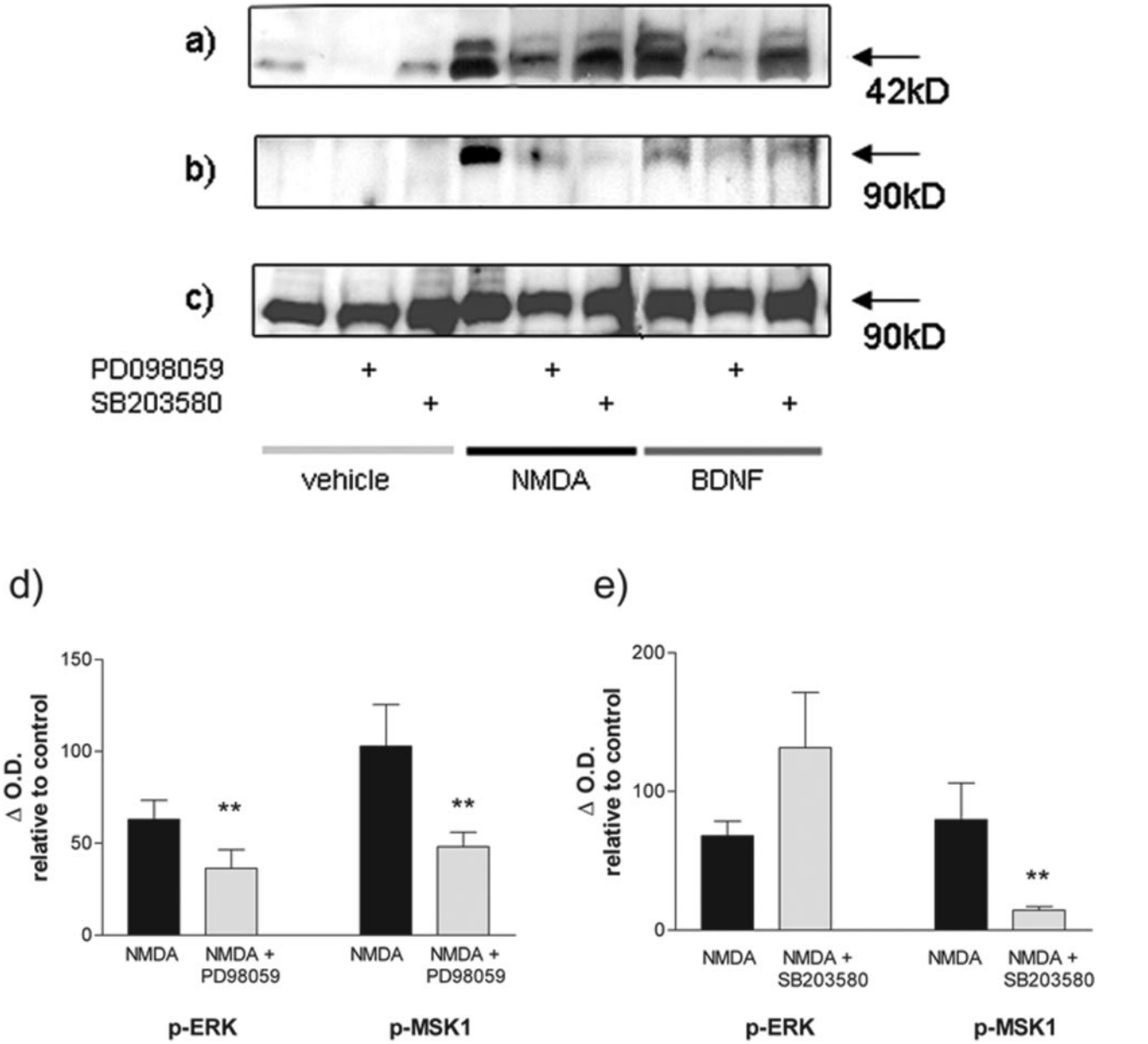

Fig. 6. Effect of PD98059 (10 μM) or SB203580 (5 μM) on phosphorylation of MSK1 and ERK. Cortical neurons were exposed to 100 μM NMDA or 20 ng/ml BDNF in the absence or presence of the inhibitors, and the whole-cell lysates were processed for Western blotting with anti-pERK antibody (a), anti-pMSK1 antibody (b), or total MSK1 antibody (c). Results shown are from a typical experiment that was replicated three times. d and e, semiquantitative analysis of the effect of 10 μM PD98059 (d) or 5 μM SB203580 (e) on phosphorylation of MSK1 and ERK by NMDA. Western blot band intensities were measured as described under *Materials and Methods*, and significance was assessed by paired *t* test. $\star\star$, $p < 0.01$ versus NMDA treatment; $n = 3$ to 5 per group.

The potency of NMDA and BDNF in inducing ERK phosphorylation in cortical neurons in our hands was very similar to the results of other groups (Iida et al., 2001). It is interesting to note that we observed that NMDA seemed to be slightly more potent at inducing ERK phosphorylation relative to MSK phosphorylation. This might implicate MSK activation in the response to higher levels of glutamate receptor stimulation, such as during excitotoxicity.

It is interesting that NMDA-induced ERK phosphorylation within the cytoplasm is active over at least 2 h. The pattern of phosphorylation of MSK1 seems to peak and decline within 30 min, with maximal activation at 10 min. This is in agreement with findings in HeLa cells in which TNF induced peak MSK1 activity at 15 min, and this reached basal levels by 30 min (Deak et al., 1998). This transient phosphorylation of MSK1 compared with the longer time that its upstream activator, ERK, remains phosphorylated suggests that although pERK may switch upon initial phosphorylation of MSK, the duration it remains phosphorylated is not dependent on the continued presence of pERK.

When cortical neurons were probed for pRSKs, NMDA was not seen to generate pRSK. The antibody used here was against p90 RSK of RSKs 1 to 3, phosphorylated at Ser380, and phosphorylation of this amino acid is known to be important for its activation (Dalby et al., 1998). The activation mechanism of the RSKs involves initial phosphorylation and activation of the C-terminal kinase by ERK (Dalby et al., 1998; Frodin and Gammeltoft, 1999). The activated C-terminal kinase autophosphorylates RSK on sites that are important for the activation of the N-terminal kinase, which is then responsible for further autophosphorylation of RSK and substrate phosphorylation. Mutation studies showed that Ser221, Ser363, Ser380, and Thr573 are essential for the activation of RSK1, and there is evidence indicating that ERK catalyzes the phosphorylations at Thr363 and Thr573, whereas Ser380 is phosphorylated by the C-terminal kinase (Dalby et al., 1998; Frodin and Gammeltoft, 1999). Hence it seems likely that we would have detected any activation of RSK with the antibody used here. Indeed, the antibody detected activation of RSK by 8BrcGMP and BDNF in the cortical neurons and by NGF in PC12 cells as predicted (Finkbeiner et al., 1997; Xing et al., 1998; Ho et al., 2003). Thus, our data suggest that NMDA receptor stimulation does not produce any dramatic RSK phosphorylation and activation in cortical neurons.

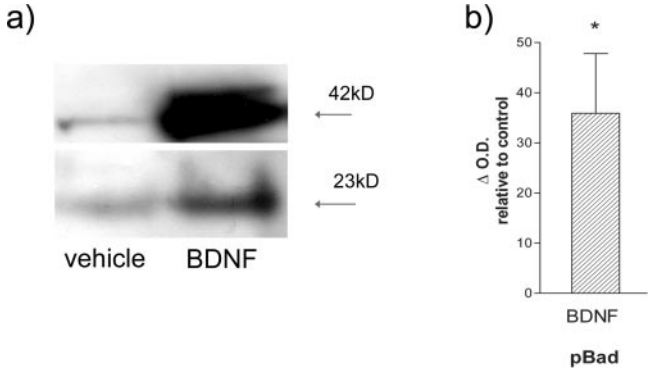

Fig. 7. a, effect of BDNF (100 ng/ml) on pERK and (top) and pBad (bottom) levels in cortical neurons. b, semiquantitative analysis. ⋆, $p < 0.05$ versus vehicle treatment (t test).

The lack of phosphorylation of RSKs after NMDA treatment was surprising, because it has been widely assumed that RSK activation would result from the profound ERK activation induced by NMDA receptor stimulation in neurons (West et al., 2002). To our knowledge, however, RSK phosphorylation in response to NMDA in neurons has not been reported.

There have been several studies emphasizing the emerging physiological importance of MSK1. For instance, a recent report shows that EGF-induced c-jun expression required MSK1 phosphorylation (Gupta and Prywes, 2002), whereas nuclear factor-κB can be transcriptionally activated by MSK1 (Vermeulen et al., 2003). MSK1 was also shown to phosphorylate the N terminus of histone H3, suggesting roles in modulation of gene expression, chromatin remodeling, and chromosome condensation (Thomson et al., 1999; Zhong et al., 2001; Soloaga et al., 2003). There is substantial overlap between the protein targets phosphorylated by RSKs and MSK1, and it is not entirely clear at present how the consequences of cellular MSK1 activation differ from those of RSK activation. It is well-known that exposure to BDNF, or stimulation of NMDA receptors, in cortical, hippocampal, or striatal neurons leads to phosphorylation of the transcription factors CREB and Elk-1 (Finkbeiner et al., 1997; Hardingham et al., 1999; Iida et al., 2001; Tong et al., 2001; Minichiello et al., 2002; Arthur et al., 2004). There is evidence that, at least in some cell types, CREB-dependent transcription may be activated by MSKs rather than by RSKs (Wiggin et al., 2002), whereas Elk-1/serum-response factor-dependent transcription may be activated by RSKs rather than by MSKs (Bruning et al., 2000). It seems likely that as our understanding of these kinases increases, it will become evident that they perform distinct roles, either in cell-specific activation pathways and/or in the activation of distinct target proteins.

Because this study used cultured neurons, the results may provide an indication of MSK responsiveness to NMDA receptor stimulation and BDNF in immature neurons, such as during developmental processes. However, the results are also likely to be applicable to the mature central nervous system. Indeed, we have obtained recently evidence for MSK1 phosphorylation after high-frequency stimulation in mature hippocampal slices (C. J. Clark, C. Guilding, C. T. O'Shaughnessy, and B. J. Morris, manuscript in preparation).

Our data therefore support a scheme whereby both NMDA- and BDNF-activated pERK, with some p38 involvement, are able to phosphorylate MSK1. The lack of RSK phosphorylation observed suggests that MSKs rather than RSKs may be the more important intermediates downstream of ERKs activated by NMDA receptor stimulation, and MSKs may link NMDA- and BDNF-induced ERK phosphorylation to transcriptional activation in neurons.

References

Arthur JSC and Cohen P (2000) MSK1 is required for CREB phosphorylation in response to mitogens in mouse embryonic stem cells. *FEBS Lett* **482:**44–48.

Arthur JSC, Fong AL, Dwyer JM, Davare M, Reese E, Obrietan K, and Impey S (2004) Mitogen- and stress-activated protein kinase 1 mediates cAMP response element-binding protein phosphorylation and activation by neurotrophins. *J Neurosci* **24:**4324–4332.

Bruning JC, Gillette JA, Zhao Y, Bjorbaeck C, Kotzka J, Knebel B, Avci H, Hanstein B, Lingohr P, Moller DE, et al. (2000) Ribosomal subunit kinase-2 is required for growth factor-stimulated transcription of the c-Fos gene. *Proc Natl Acad Sci USA* **97:**2462–2467.

Clerk A and Sugden PH (1998) The p38-MAPK inhibitor, SB203580, inhibits cardiac stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs). *FEBS Lett* **426:**93–96.
Dalby KN, Morrice N, Caudwell FB, Avruch J, and Cohen P (1998) Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. *J Biol Chem* **273:**1496–1505.
Davies SP, Reddy H, Caivano M, and Cohen P (2001) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351:**95–105.
Deak M, Clifton AD, Lucocq JM, and Alessi DR (1998) Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38 and may mediate activation of CREB. *EMBO (Eur Mol Biol Organ) J* **17:**4426–4441.
English JD and Sweatt JD (1996) Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. *J Biol Chem* **271:**24329–24332.
English JD and Sweatt JD (1997) A requirement for the MAP kinase cascade in hippocampal LTP. *J Biol Chem* **272:**19103–19106.
Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, and Greenberg ME (1997) CREB: a major mediator of neuronal neurotrophin responses. *Neuron* **19:**1031–1047.
Frodin M and Gammeltoft S (1999) Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol* **151:**65–77.
Fuller G, Veitch K, Lai Kwan H, Cruise L, and Morris BJ (2001) Activation of p44/p42 MAP kinase in striatal neurons via kainate receptors and PI3 kinase. *Mol Brain Res* **89:**126–132.
Gupta P and Prywes R (2002) ATF1 phosphorylation by the ERK MAPK pathway is required for epidermal growth factor-induced c-jun expression. *J Biol Chem* **277:**50550–50556.
Hardingham GE, Chawla S, Cruzalegui FH, and Bading H (1999) Control of recruitment and transcription-activating function of CBP determines gene regulation by NMDA receptors and L-type calcium channels. *Neuron* **22:**789–798.
Ho AK, Mackova M, Cho C, and Chik CL (2003) Regulation of 90-kilodalton ribosomal S6 kinase phosphorylation in the rat pineal gland. *Endocrinology* **144:**3344–3350.
Hughes JP, Staton PC, Wilkinson MG, Strijbos PJLM, Skaper SD, Arthur JSC, and Reith AD (2003) Mitogen and stress response kinase-1 (MSK1) mediates excitotoxic induced death of hippocampal neurones. *J Neurochem* **86:**25–32.
Iida N, Namikawa K, Kiyama H, Ueno H, Nakamura S, and Hattori S (2001) Requirement of Ras for the activation of mitogen-activated protein kinase by calcium influx, cAMP and neurotrophin in hippocampal neurons. *J Neurosci* **21:**6459–6466.
Impey S, Mark M, Villacres EC, Poser S, Chavkin C, and Storm DR (1996) Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. *Neuron* **16:**973–982.
Impey S, Obrietan K, Wong ST, Poser S, Yano S, Wayman G, Deloulme JC, Chan G, and Storm DR (1998) Cross talk between ERK and PKA is required for Ca^{2+} stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron* **21:**869–883.
Lali FV, Hunt AE, Turner SJ, and Foxwell BMJ (2000) The pyridinyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. *J Biol Chem* **275:**7395–7402.
Lee JC, Kassis S, Kumar S, Badger A, and Adams JL (1999) p38 Mitogen-activated protein kinase inhibitors—mechanisms and therapeutic potentials. *Pharmacol Ther* **82:**389–397.
Markou T and Lazou A (2002) Phosphorylation and activation of mitogen- and stress-activated protein kinase-1 in adult rat cardiac myocytes by G-protein-coupled receptor agonists requires both extracellular-signal-regulated kinase and p38 mitogen-activated protein kinase. *Biochem J* **365:**757–763.
Messaoudi E, Ying S-W, Kanhema T, Croll SD, and Bramham CR (2002) Brain-derived neurotrophic factor triggers transcription-dependent, late phase long-term potentiation in vivo. *J Neurosci* **22:**7453–7461.
Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R, and Korte M (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. *Neuron* **36:**121–137.
Morris BJ (1995) Stimulation of immediate-early gene expression in striatal neurons by nitric oxide. *J Biol Chem* **270:**24740–24744.
Morris BJ (2004) Neuronal plasticity, in *Molecular Biology of the Neuron* (Davies RW and Morris BJ eds) pp 357–386, Oxford University Press, Oxford.
New L, Zhao M, Li Y, Basset WW, Feng Y, Ludwig S, Padova FD, Gram H, and Han J (1999) Cloning and characterization of RLPK, a novel RSK-related protein kinase. *J Biol Chem* **274:**1026–1032.
Pierrat B, Correia JS, Mary JL, Tomas-Zuber M, and Lesslauer W (1998) RSK-B, a novel ribosomal S6 kinase family member, is a CREB kinase under dominant control of p38 mitogen-activated protein kinase (p38MAPK). *J Biol Chem* **273:**29661–29671.
She Q-B, Ma W-Y, Zhong S, and Dong Z (2002) Activation of JNK1, RSK2 and MSK1 is involved in serine 112 phosphorylation of bad by ultraviolet B radiation. *J Biol Chem* **277:**24039–24048.
Simpson CS and Morris BJ (2000) Regulation of neuronal cell adhesion molecule (NCAM) expression by NF-kB. *J Biol Chem* **275:**16879–16884.
Soloaga A, Thomson S, Wiggin GR, Rampersaud N, Dyson MH, Hazzalin CA, Mahadevan LC, and Arthur JSC (2003) MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. *EMBO (Eur Mol Biol Organ) J* **22:**2788–2797.
Tan Y, Ruan H, Demeter MR, and Comb MJ (1999) p90RSK blocks bad-mediated cell death via a protein kinase C-dependent pathway. *J Biol Chem* **274:**34859–34867.
Thomson S, Clayton AL, Hazzalin CA, Rose S, Barratt MJ, and Mahadevan LC (1999) The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. *EMBO (Eur Mol Biol Organ) J* **18:**4779–4793.
Tomas-Zuber M, Mary JL, Lamour F, Bur D, and Lesslauer W (2001) C-terminal elements control location, activation threshold and p38-docking of ribosomal S6 kinase B. *J Biol Chem* **276:**5892–5899.
Tomas-Zuber M, Mary JL, and Lesslauer W (2000) Control sites of ribosomal S6 kinase B and persistent activation through tumor necrosis factor. *J Biol Chem* **275:**23549–23558.
Tong L, Thornton PL, Balazs R, and Cotman CW (2001) β-Amyloid-(1–42) impairs activity-dependent cAMP-response element-binding protein signaling in neurons at concentrations in which cell survival is not compromised. *J Biol Chem* **276:**17301–17306.
Vermeulen L, De Wilde G, Van Damme P, Vanden Berghe W, and Haegeman G (2003) Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *EMBO (Eur Mol Biol Organ) J* **22:**1313–1324.
West AE, Griffith EC, and Greenberg ME (2002) Regulation of transcription factor by neuronal activity. *Nat Rev Neurosci* **3:**921–931.
Wiggin GR, Soloaga A, Foster JM, Murray-Tait V, Cohen P, and Arthur JSC (2002) MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. *Mol Cell Biol* **22:**2871–2881.
Xing J, Kornhauser JM, Xia Z, Thiele EA, and Greenberg ME (1998) NGF activates RK and p38 MAK kinase pathways to stimulate CREB serine133 phosphorylation. *Mol Cell Biol* **18:**1946–1955.
Ying SW, Futter M, Rosenblum K, Webber MJ, Hunt SP, Bliss TVP, and Bramham CR (2003) Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. *J Neurosci* **22:**1532–1540.
Zhong S, Jansen C, She Q-B, Goto H, Inagaki M, Bode AM, Ma W-Y, and Dong Z (2001) Ultraviolet B-induced phosphorylation of histone H3 at serine 28 is mediated by MSK1. *J Biol Chem* **276:**33213–33219.

Address correspondence to: Dr. B. J. Morris, Institute of Biomedical and Life Sciences, West Medical Building, University of Glasgow, Glasgow G12 8QQ, UK. E-mail: b.morris@bio.gla.ac.uk