Regulation of ERK Kinase by MEK1 Kinase Inhibition in the Brain*

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Background: ERK/MAPK signaling is important in brain function.

Results: MEK1 is inhibited by phosphorylation at Thr-292/286 by Cdk5, ERK, and Cdk1. These mechanisms are regulated by striatal glutamate and dopamine neurotransmission and acute stress in vivo.

Conclusion: Excitatory and metabotropic neurotransmission converge on MEK1 regulation.

Significance: A greater understanding of MEK1/ERK signaling provides insights into brain function, disease, and potential treatment strategies.

Metabotropic (slow) and ionotropic (fast) neurotransmission are integrated by intracellular signal transduction mechanisms involving protein phosphorylation/dephosphorylation to achieve experience-dependent alterations in brain circuitry. ERK is an important effector of both slow and fast forms of neurotransmission and has been implicated in normal brain function and CNS diseases. Here we characterize phosphorylation of the ERK-activating protein kinase MEK1 by Cdk5, ERK, and Cdk1 in vitro in intact mouse brain tissue and in the context of an animal behavioral paradigm of stress. Cdk5 only phosphorylates Thr-292, whereas ERK and Cdk1 phosphorylate both Thr-292 and Thr-286 MEK1. These sites interact in a kinase-specific manner and inhibit the ability of MEK1 to activate ERK. Thr-292 and Thr-286 MEK1 are phosphorylated in most mouse brain regions to stoichiometries of ~5% or less. Phosphorylation of Thr-292 MEK1 is regulated by cAMP-dependent signaling in mouse striatum in a manner consistent with negative feedback inhibition in response to ERK activation. Protein phosphatase 1 and 2A contribute to the maintenance of the basal phosphorylation state of both Thr-292 and Thr-286 MEK1 and that of ERK. Activation of the NMDA class of ionotropic glutamate receptors reduces inhibitory MEK1 phosphorylation, whereas forced swim, a paradigm of acute stress, attenuates Thr-292 MEK1 phosphorylation. Together, the data indicate that these inhibitory MEK1 sites phosphorylated by Cdk5 and ERK serve as mechanistic points of convergence for the regulation of ERK signaling by both slow and fast neurotransmission.

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Numerous signaling pathways initiated at the plasma membrane are mediated by MAPK or ERK family members. These kinases are critical in the transduction of receptor activation signals that affect many cellular functions, including gene transcription and translation, cell differentiation and survival, and modulation of cell structure and metabolism. Within the CNS, ERK signaling is essential to brain development and neurite outgrowth (1), neuronal survival (2, 3), and synaptic plasticity (4, 5). ERK activation is necessary for the consolidation and reconsolidation of hippocampus-dependent memory (6). ERK inhibitors impair contextual fear conditioning memory formation (7–9). Furthermore, dysregulation of ERK signaling has been implicated in both neuropsychiatric and neurological disorders, including schizophrenia (10–12), depression (13–19), addiction (20–23), autism (24, 25), mental retardation (26) and Alzheimer disease (27). Although ERK signaling mediates the actions of many CNS drug therapies, more direct pharmacological interventions that target ERK signaling have been limited chiefly to the field of oncology and may be accompanied by unwanted side effects.

Within the brain, ERK signaling may be initiated by the activation of ionotropic glutamate receptors (4, 5), D1 dopamine receptors (20), or L-type Ca2+ channels (28). Each of these may invoke the cAMP/PKA/Rap1 cascade (29–32). Furthermore, neuronal ERK is activated by the binding of neurotrophins, such as BDNF, to their tyrosine kinase receptors (33). This leads to the activation of the small GTPase Ras. Both Rap1 and Ras activate the protein kinase Raf, which then phosphorylates MEK1/2. MEK1/2 is activated by Raf-dependent phosphorylation at Thr-218/Tyr-222 MEK1 (Fig. 1A). In this phosphorylation state, MEK1/2 then activates ERK1/2 through phosphorylation at Thr-202/Tyr-204. Consequently, active ERK1/2 phosphorylates a myriad of intracellular substrates that contribute to neuronal function.

As the penultimate step in the pathway, MEK1 regulation may be considered the most important element of ERK activation. In its inactive state, MEK1 assumes an autoinhibitory structural conformation (34). Phosphorylation of Ser-218/Ser-
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222 within the activation loop of MEK1 alters this conformation, exposing the enzymatic ATP and substrate binding domains. In addition, MEK1 may be phosphorylated at the proline-directed sites Thr-286 and Thr-292, which occur within its proline-rich domain (Fig. 1A) but not in the homologous region of MEK2. It has been reported that Thr-292 MEK1 may serve as a substrate for ERK (35). In vitro studies have paradoxically suggested that phosphorylation of MEK1 at Thr-286 or Thr-292 either acts as a mechanism of feedback inhibition or has no effect upon MEK1 catalytic activity (36, 37). Also, Cyclin-dependent kinase 1 (Cdk1) has been shown to phosphorylate both Thr-286 and Thr-292 in vitro as an inhibitory mechanism that may be critical for cell cycle progression (38). Finally, it has been suggested that Thr-286 may be phosphorylated by Cdk5 in rat pheochromocytoma cultures (40). This putative Cdk5-MEK1 pathway has also been suggested to contribute to the neurotoxic effects by which aberrant Cdk5 may mediate neurodegeneration.

These studies notwithstanding, the relative contributions of the respective kinases to these phosphorylation events and how they control MEK1 activity remain unclear. Furthermore, the regulation of these sites in brain tissue has not been explored. Here we report the characterization of the site specificity of Cdk5, ERK1, and Cdk1 for the phosphorylation of MEK1, their effects upon MEK1 catalytic activity, their phosphorylation state and distribution within the mouse brain, the relative contributions of these kinases to MEK1 regulation during cAMP/PKA and ionotropic glutamate receptor-mediated signaling, and the effects stress-related behavior has on these MEK1 phosphorylation sites.

Experimental Procedures

Animals and Reagents—Male C57BL/6 mice (The Jackson Laboratory), aged 10–12 weeks, were housed 4 mice/cage in a 12-h light-dark cycle. All experimental procedures were reviewed and approved by the University of Texas southwestern Institutional Animal Care and Use Committee. U0126 was obtained from LC Laboratories (Woburn, MA). CP681301 was provided by Pfizer (New York, NY). Glutathione-agarose resin, glutathione, NMDA, forskolin, adenosine deaminase, protease inhibitor mixture, and ATP were from Sigma. [32P]-ATP was obtained from PerkinElmer Inc. Cdk5/p25 was purchased from EMD Millipore (Darmstadt, Germany) and Sigma. Cdk1 (Cdc2/CycB) was purchased from New England Biolabs, Inc. Active ERK1 was from Millipore. Total ERK1/2, anti-Thr(P)-202/pY204-ERK1/2, and anti-Thr(P)-286-MEK1 antibodies were from Cell Signaling Technologies (Billerica, MA). Anti-Thr(P)-292-MEK1 and anti-Ser(P)-845-GluR1, total NR2B, and Ser(P)-1116-NR2B were from Phosphosolutions (Aurora, CO). Anti-Total MEK1 was from Santa Cruz Biotechnology, Inc. (Dallas, TX). Anti-Cdk1 was from Abcam (Cambridge, UK).

Proteins—The bacterial expression vectors for WT GST-tagged MEK1, constitutively active (CA,2 catalog no. S218E/S222E) GST-tagged MEK1 and His6-tagged ERK2 were a gift from Dr. Melanie Cobb. Using the PfuUltra QuickChange site-directed mutagenesis strategy (Agilent Technologies, Santa Clara, CA), the phosho-mimetic mutations of CA-MEK1 were made using the following primer sequences: 5’ MEK T286D, 5’-GGA GAT GCG GCT GAG GAC CCA CCC AGG AAA 3’; 3’ MEK T286D, 5’-CCT TGG CCT GGG GTG TCT AGC CGC ATC TTC-3’; 5’ MEK T292D, 5’-CCA CCC AGG CCA AGG GAC CCC GGG AGG CCC-3’; and 3’ MEK T292D, 5’-GGG CCT CCC GGG GTG CTC GTG AGC ATC TTC-3’. Site-directed mutagenesis of kinase-dead ERK2-K52R was made using 5’ ERK K52R (5’-GTT CGA GTT CCT GTC CGG AAA ATC AGT CCT TTT GAG CAC C-3’; 3’ ERK K52R, 5’-GGT GCT CAA AAG GAC TGA TTT TCC GGA TAG CAA CTC GAA C-3’). Bacterial expression of GST-MEK1- and His6-ERK-transformed BL21-competent cells was performed at 16°C for 16 h, followed by French press lysis in 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM DTT, and protease inhibitor mixture with 0.2 mM PMSF. For KD-ERK-K52R, the lysis buffer was 20 mM HEPES (pH 7.5), 100 mM KCl, 20% glycerol, and 1 mM 2-mercaptoethanol (for His6-ERK) containing 0.2 mM PMSF and protease inhibitor mixture. Proteins were purified according to GSH-agarose: or Ni2+-agarose product directions and dialyzed in 20 mM HEPES (pH 7.5), 100 mM KCl, 1 mM EDTA, 20% glycerol, 1 mM DTT, and 0.2 mM PMSF.

Mass Spectroscopy—Phosphorylation of recombinant MEK1 and site identification were conducted using similar approaches as those described previously (41, 42). MEK1 was preparatively phosphorylated for site identification. Briefly, MEK1 from in vitro protein phosphorylation reactions with Cdk5 was subjected to SDS-PAGE and in-gel iodoacetamide acylation followed by digestion with trypsin. Nano-LC/MS/MS was conducted on tryptic digests directly. Dried digest mixtures were redissolved in trifluoroacetic acid (0.1% TFA) and loaded onto a ZipTip with C18 resin (Millipore) for purification. After being washed with 0.1% TFA three times, peptides were eluted directly into a nanoelectrospray needle (Proxeon Biosystems). MS analysis was performed on a QSTAR Pulsar I quadrupole time of flight tandem mass spectrometer (Applied Biosystems/ MDS SCIEX) equipped with a nanoelectrospray ion source (MDS Proteomics, Odense, Denmark). For precursor ion scanning, the instrument was set in negative ion mode to detect the PO4 fragment ion at m/z –79. After data acquisition, the instrument was switched to positive ion mode, and the phosphopeptide sequence and site of phosphorylation were determined by nanoelectrospray-QSTAR Pulsar I quadrupole time of flight MS/MS. In MS/MS scan mode, the precursor ion was selected in the quadrupole (Q1) and fragmented in the collision cell (q2) using argon as the collision gas. The protein sequences derived from MS/MS were used to search NCBI non-redundant mouse or mammalian protein databases for confirmation using computer software.

Kinase Assays and Quantitative Immunoblotting—Time course and dose-response in vitro phosphorylation reactions were conducted at 30°C using the protein kinases Cdk5/p25, active ERK1, or Cdk1/cyclin B and the substrates CA-MEK1, CA-T286D-MEK1, CA-T292D-MEK1, CA-T286D/T292D-MEK1, or protein phosphatase inhibitor 1 (positive control). Time course reactions were stopped at 10, 30, 60, 120, or 240 min by the mixture of 10 μl of reaction aliquots with 5 × Laemmlı buffer. Dose-response (Michaelis-Menten kinetic) reac-
tions were conducted under linear conditions. All in vitro phosphorylation reactions were conducted in 20 mM HEPES (pH 7.5), 100 mM NaCl, 200 mM ATP, and 5 mM MgCl₂ containing [³²P-γ]ATP. Reactions were stopped by the addition of 5X Laemmlı buffer and heated at 80 °C for 5 min. Phosphorylation or kinetic parameters were defined by autoradiography or quantitative immunoblot analysis. For autoradiography, samples were subjected to SDS-PAGE. Gels were Coomassie-stained and subjected to PhosphorImager analysis. Standard methods were used for quantitative immunoblotting using nitrocellulose. For quantitation of expression levels and in vivo stoichiometry, total MEK1 levels in various brain regions were first quantified using a total recombinant MEK1 standard. Next, picomoles of phospho-Thr-292 and Thr-286 MEK1 were calculated using phospho-MEK1 standards of defined stoichiometries. In vivo stoichiometries were then obtained by calculating pmols of phospho-MEK1 per picomoles of total MEK1 for each brain region.

Slice Pharmacology—As described previously (43, 44), neonatostria from 10-to 12-week-old wild-type male C57BL/6 mice was acutely microdissected from 350-μm coronal slices and equilibrated for 30 min with adenosine deaminase (10 μg/ml) in Krebs- HCO₃ buffer (124 mM NaCl, 4 mM KCl, 26 mM NaHCO₃, 1.5 mM CaCl₂, 1.25 mM KH₂PO₄, 1.5 mM MgSO₄, and 10 mM D-glucose (pH 7.4)) oxygenated with 95% O₂/5% CO₂

Results

Cdk5 Specifically Phosphorylates Thr-292 MEK1—Previous studies have suggested that Cdk5 phosphorylates Thr-286 of MEK1 (39, 46). However, the efficiency with which this site presumably serves as a substrate for Cdk5 was not demonstrated in vitro, and evidence that Cdk5 phosphorylates this site in cells in vivo or in brain tissue was not provided. Therefore, as an initial evaluation, MEK1 was assessed as a substrate for Cdk5 in vitro (Fig. 1B). Recombinant Cdk5/p25 phosphorylated WT and CA MEK1 under linear conditions in a time-dependent manner to stoichiometries of 0.16 and 0.37 mol/mol, respectively, by 180 min. In other reactions conducted under saturating conditions, phosphorylation stoichiometry routinely reached 0.8 mol/mol, demonstrating MEK1 to be an efficient substrate for Cdk5 in vitro. To positively identify the site of phosphorylation, preparatively phosphorylated MEK1 was digested with trypsin and subjected to nano-LC/MS/MS. This analysis yielded one phosphopeptide encompassing amino acids 292–302, suggesting that Cdk5 phosphorylates Thr-292 (Fig. 1C). Another phosphopeptide containing residues 276–297 and a single phosphorylation site was also detected, leaving the possibility that either Thr-286 or Thr-292 could be phosphorylated on this species. However, another peptide was detected that was not phosphorylated, comprised of amino acids 270–291. In addition, Thr-292 occurs within the context of the sequence “TPGR,” which meets the criteria for ideal Cdk5 consensus sequences (S/TPXH/K/R) (47). Together, these data suggest that Cdk5 phosphorylates a single residue on MEK1, Thr-292.

Proline-directed Kinases Exhibit Different Relative Preferences for Phosphorylation Sites on MEK1—To further investigate the site specificity and preference of Cdk5 as well as that of the other proline-directed kinases, ERK and Cdk1, CA MEK1 was used as a substrate in time course in vitro phosphorylation reactions with each of the kinases. Aliquots of the reaction mixtures were removed at various time points, and reactions were arrested and subjected to quantitative immunoblotting for phospho-Thr-292, phospho-Thr-286, or total MEK1. Blotting was done simultaneously to facilitate comparison across each site (Fig. 2A). Using this approach, Cdk5 was found to phosphorylate MEK1 at Thr-292. However, no phosphorylation was
detected at Thr-286. These data confirm that Cdk5 does not phosphorylate Thr-286 as reported previously but that it is specific for Thr-292. ERK differed from Cdk5 in that it also exhibited a preference for Thr-292, phosphorylating this residue with greater apparent efficiency than Cdk5, but also phosphorylating Thr-286 at comparatively low but detectable levels. Cdk1 exhibited yet a different site preference profile, phosphorylating Thr-292 and Thr-286 MEK1 with nearly equal efficiency over time. Some preference for Thr-286 during early time points when reactions occurred under linear conditions was apparent. Therefore, each protein kinase exhibits a unique profile of specificity and preference for MEK1 phosphorylation.

**Phosphomimetic Substitution at Either Thr-286 or Thr-292 Differentially Affects the Efficiency of Proline-directed Kinases to Phosphorylate Other Sites within MEK1**—Because only five residues separate these two proline-directed sites, it is possible that posttranslational modifications imparting a negative charge at each site affect phosphorylation of the other by Cdk5, ERK or Cdk1. Therefore, the effects of phosphomimetic substitutions on the ability of each kinase to phosphorylate the other site were examined. Recombinant CA-MEK1 and mutants harboring phosphomimetic substitutions (T286D and T292D) were used as substrates for in vitro phosphorylation reactions with Cdk5, ERK, and Cdk1. Phosphorylation of the complementary site was assessed by quantitative immunoblotting (Fig. 2B). Investigation of the effects of mutation of Thr-286 upon phosphorylation of Thr-292 confirmed that Cdk5, again, efficiently phosphorylates Thr-292 (Fig. 2B, top row). Interestingly, phosphorylation increased ~2-fold when Thr-286 was replaced with Asp. Furthermore, both ERK and Cdk1 phosphorylated Thr-292 to appreciable levels, and this was unaffected by the T286D substitution. Next, the effect of phosphomimetic mutation at Thr-292 upon Thr-286 phosphorylation was evaluated (Fig. 2B, bottom row). Again, Cdk5 was unable to phosphorylate Thr-286 MEK1. Also, ERK1 exhibited a moderate capacity to phosphorylate Thr-286. Interestingly, this reaction

**FIGURE 2.** Protein kinases exhibit different site specificities and susceptibilities to intramolecular effects for MEK1 phosphorylation, which inhibits its catalytic activity. A, assessment of protein kinase site specificity. Immunoblots for phospho-Thr-292, phospho-Thr-286, and total CA-MEK1 show the time courses for phosphorylation of each of the two sites by Cdk5, ERK, and Cdk1. B, substrate efficiency effects of phosphomimetic substitution at each site on phosphorylation of the other. Immunoblot quantitation of Thr(P)-292 or Thr(P)-286 from time course reactions comparing phosphorylation of CA-MEK1 (wild-type) to CA-T286D-MEK1 or CA-T292D-MEK1 by Cdk5, ERK, and Cdk1. Quantitative values for each site across different kinases (top and bottom rows) may be compared. C, evaluation of phosphomimetic mutations upon MEK1 catalytic activity. Michaelis-Menten curves and kinetic parameters are shown for phosphorylation of KD-ERK2 by CA-MEK1 or phosphomimetic mutant CA-T286D-MEK1, CA-T292D-MEK1, or CA-T286D/T292D-MEK1. Data represent mean ± S.E. (n = 3), left panel. Data represent mean ± S.E. and nonlinear regression analysis for $K_m$ and $V_{max}$ (right panel).
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Mimic of Phosphorylation at Thr-292 or Thr-286 Inhibits the Catalytic Activity of MEK1—Evaluation of the effects of Thr-292 or Thr-286 phosphorylation has yielded some circumstantial evidence suggesting a possible inhibitory mechanism (35–40, 48). To evaluate this more directly, kinase-dead ERK was used as a substrate for in vitro phosphorylation reactions in which CA-MEK1 or the phospho site mutants T286D, T292D, or T286D/T292D CA-MEK1 served as the protein kinase, and kinetic parameters were derived from quantitative autoradiography (Fig. 2C). Mutation either at each site individually or both together had no effect upon $K_m$ values conducted under linear conditions (Fig. 2C, right panel). Recombinant CA-MEK1 exhibited a $K_m$ value for KD-ERK of 4.1 $\mu$M, comparable with previous reports (49). Furthermore, phosphomimetic mutation of either Thr-292 or Thr-286 or both combined had no significant effect upon $K_m$. In contrast, mutation of either Thr-292 or Thr-286 reduced the $V_{max}$ to ~30% of the value for CA-MEK1. Interestingly, phosphomimetic mutation at both residues only reduced the $V_{max}$ to 60% of the value for CA-MEK1. These results support the notion that phosphorylation of MEK1 at these sites is inhibitory. Moreover, the observed reduction in $V_{max}$ with no effect upon $K_m$ typically characterizes a non-competitive mode of inhibition (50).

Characterization of Phospho-Thr-292 and Thr-286 in the Brain—The above in vitro studies provide support for the regulation of MEK1 through phosphorylation by Cdk5, ERK, and Cdk1. However, evidence of these phosphorylation events occurring under physiological circumstances has been chiefly restricted to cells grown in culture and limited in vitro studies (35–40, 48). Therefore, MEK1 phosphorylation was characterized in the brains of adult mice. For this study, we first assessed the distribution of Cdk5, ERK, and Cdk1 across various brain regions (Fig. 3A). Both Cdk5 and ERK were abundantly expressed in all brain areas examined (olfactory tubercle, caudate putamen, nucleus accumbens, prefrontal cortex, hippocampus, and cerebellum). In contrast, virtually no Cdk1 was detected in any brain region, with the exception of the cerebellum, where some signal could be found.

Next, absolute MEK levels as well as the phosphorylation state and tissue-specific stoichiometry of phospho-Thr-292 and Thr-286 MEK1 were assessed (Fig. 3B). For most brain regions, total MEK1 was expressed at an abundance of ~0.38 – 0.64 nmol/mg of total tissue lysate protein. The nucleus accumbens expressed the highest levels of MEK1, whereas the cerebellum expressed the lowest levels compared with other brain regions. Examination of in vivo stoichiometry indicated that MEK1 was phosphorylated at Thr-292 across the olfactory tubercle, caudate putamen, nucleus accumbens, prefrontal cortex, and hippocampus within a comparable range from 0.04 – 0.057 mol/mol. As an exception, phospho-Thr-292 in the cerebellum achieved a stoichiometry of only 0.006 mol/mol. Examination of phospho-Thr-286 revealed lower stoichiometries ranging between 0.02 – 0.04 mol/mol across all brain regions.

These results demonstrate that, although MEK1 phosphorylated at Thr-292 and Thr-286 occurs throughout brain tissues, it represents a relatively minor portion of most brain MEK1.
kinase, with less than 6% phosphorylated at Thr-292 and less than 5% phosphorylated at Thr-286. Together with the results from Fig. 2, these findings indicate that the majority of MEK1 in most brain regions exists under basal conditions in the non-inhibited form. Whether MEK1 phosphorylated simultaneously at both sites occurs in the brain remains to be determined.

In Vivo MEK1 Phosphorylation Is ERK- and Cdk5-dependent—Given that basal levels of phospho-Thr-292 and Thr-286 were appreciable in mouse brain, the contributions of ERK and Cdk5 to each of these sites were assessed in acutely prepared striatal slices using a pharmaceutical approach. Intact brain slices were either treated with vehicle alone (controls) or incubated for 60 min in the presence of the MEK1 inhibitor U0126, the Cdk5 inhibitor CP681301, or both. Phosphorylation states and total protein levels were then assessed by immunoblotting slice lysates. Either MEK1 or Cdk5 inhibition significantly reduced phospho-Thr-292 levels compared with controls (Fig. 4A). Although combined inhibition of both kinases also induced a reduction in the phosphorylation of Thr-292, the effects were not additive. Furthermore, MEK1 or Cdk5 inhibition significantly reduced phospho-Thr-286 levels, although, again, treatment of slices with both inhibitors combined did not cause a greater reduction than what each drug achieved individually. As expected, MEK1 inhibition, alone or together with Cdk5 inhibition, blocked phosphorylation of ERK1/2 (Fig. 4B). Furthermore, Cdk5 inhibition caused a decrease in the well characterized Cdk5 substrate phospho-Ser-1116 NR2B (45) (Fig. 4C), confirming the efficacy of this pharmacological treatment.

These data support the hypothesis that Cdk5 and ERK1 phosphorylate Thr-292 MEK1 in striatum. Moreover, the data are consistent with ERK1-dependent phosphorylation of Thr-286 MEK1. Interestingly, decreased phospho-Thr-292 and Thr-286 did not result in activation of MEK1 in the absence of pathway stimulation, and, therefore, phospho-ERK1/2 levels did not increase as inhibitory MEK1 phosphorylation was attenuated. Also, because Cdk5 did not phosphorylate Thr-286 MEK1 in vitro (Figs. 1 and 2) and because Thr-286 MEK1 serves as a more efficient substrate for ERK when not phosphorylated at Thr-292 (Fig. 2B), it is surprising that CP681301 treatment and the ensuing reduction in phospho-Thr-292 correlated to decrease in phospho-Thr-286 in intact brain tissue. This raises the possibility that Cdk5 may indirectly regulate or facilitate the phosphorylation of Thr-286 through an undefined pathway or mechanism.

MEK1 Phosphorylation Is Regulated by cAMP-mediated Signaling in the Striatum—The striatum serves as the hub for the mesocorticolimbic circuitry that mediates fine motor control, reward, and emotion through cAMP/PKA signaling (51). Within the striatum, MEK1/ERK signaling is activated through D1 dopamine receptors (52) and by addictive psychomotor stimulants such as cocaine and amphetamine, which raise extracellular dopamine levels (53, 54). Consistent with this body of work, pharmacological activation of adenyl cyclase with forskolin in striatal slices caused a 2.1-fold increase in phospho-ERK1/2 (Fig. 5A). Predictably, inhibition of MEK1 caused a complete loss of the phospho-ERK signal and prevented the detection of a forskolin effect. On the other hand, Cdk5 inhibition resulted in a significantly greater increase in phospho-ERK1/2 levels in response to forskolin compared with forskolin alone. Therefore, Cdk5-dependent phosphorylation of MEK1 appears to govern the efficacy of MEK1/ERK1/2 signaling.

To see whether cAMP signaling might also regulate MEK1 phosphorylation, we examined the effects of forskolin on phospho-Thr-292 and Thr-286 in striatal slices (Fig. 5B). The forskolin-induced ERK1/2 activation corresponded to a 1.6-fold increase in phospho-Thr-292 but had no discernable effect on the phosphorylation state of Thr-286 MEK1. Interestingly, in the presence of forskolin, MEK1 inhibition with U0126 caused a reduction in phospho-Thr-292 to 75% of control levels, whereas combined MEK1 and Cdk5 inhibition resulted in an even greater inhibition (46% of control levels). Furthermore, Cdk5 inhibition in the presence of forskolin stimulation did not result in a significant reduction of phospho-Thr-292 levels compared with forskolin alone. Likewise, phospho-Thr-286 was also reduced in an almost identical manner as phospho-Thr-292 by these treatments. These data demonstrate that MEK1 inhibition, likely as negative feedback from ERK activation, occurs in response to activation of cAMP/PKA signaling chiefly through the phosphorylation of Thr-292 MEK1. Moreover, although the basal phosphorylation states of phospho-Thr-292 and Thr-286 were only moderately affected by MEK1/ERK or Cdk5 inhibition, invocation of cAMP signaling appeared to impart flexibility upon these sights, rendering them
more vulnerable to pharmacological or, possibly, biological modulation. These data also suggest that MEK1 phosphorylation at these inhibitory sites is an additional aspect of cAMP-dependent ERK signaling.

Inhibitory MEK1 Phosphorylation Is Regulated by Protein Phosphatase Activity—Cdk5 and ERK1 are often thought of as constitutively active kinases, and, therefore, the phosphorylation states of many of their sites are balanced through protein phosphatase activity. To gain information regarding the contribution of protein phosphatases to the regulation of MEK1, striatal slices were treated with varying concentrations of the protein phosphatase inhibitor okadaic acid. As shown previously (55), okadaic acid at 200 nM inhibits only 5% of the activity of serine/threonine protein phosphatase 1 (PP1) but 80% of PP2A. However, at 1 μM, PP1 and PP2A are inhibited at 35% and 95% of control phosphatase activity, respectively (55). In striatal slices, ERK1/2 phosphorylation responded to okadaic acid in a dose-dependent manner. Treatment of striatal slices with 200 and 1000 nM okadaic acid caused 2.6- and 8.0-fold increases in phospho-ERK1/2, respectively (Fig. 6A). These dose-dependent effects corresponded to ~2- and 6-fold increases in both phospho-Thr-292 and phospho-Thr-286 levels (Fig. 6B). In contrast, inhibition of the Ca^{2+}-sensitive phosphatase calcineurin (PP2B) with cyclosporin A did not affect the phosphorylation of MEK1 at these sites (data not shown).

The dose-dependent effects of okadaic acid on phospho-Thr-292 and Thr-286 MEK1 suggest that both of these sites may be predominantly dephosphorylated by PP1, although the activation of ERK1/2 by these treatments may have also contributed to the observed increases. Direct dephosphorylation of Thr-202/Tyr-204 of ERK is conducted by its dedicated phosphatases, such as striatal enriched phosphoprotein (56) and MKP-2 (11). However, consistent with the results presented here, both PP1 and PP2A are thought to indirectly regulate ERK (57, 58).

NMDA Regulates MEK1 Phosphorylation—Protein phosphatases are often downstream effectors of excitatory ionotropic glutamate receptors. Furthermore, NMDA treatment of neuronal slices or cultures regulates the MEK/ERK pathway (59–61). Therefore, we evaluated the effects of glutamatergic signaling upon phospho-Thr-292 and phospho-Thr-286 MEK1. In low-Mg^{2+} Krebs buffer, acute striatal slices were incubated in vehicle alone or 50 μM NMDA for 5 min. Activation of NMDA receptors reduced phospho-Thr-292 and Thr-286 MEK1 levels significantly compared with controls (Fig. 6C). Likewise, NMDA treatment reduced phospho-ERK1/2 levels (Fig. 6D). As a control, phospho-Ser-845 GluR1, which is reduced by addition of NMDA to neuronal slices or cultures (62–64), was also reduced by NMDA treatment (Fig. 6C). These results suggest that, in addition to modulation by cAMP, MEK1 inhibition is regulated through excitatory glutamatergic neurotransmission.

Acute Stress Induces Inhibitory MEK1 Phosphorylation—To complement the results derived for inhibitory MEK1 phosphorylation in vitro and in intact brain tissue, we also assessed these mechanisms at the systemic level. Glutamatergic neurotrans-
mission and NMDA receptor activation mediate synaptic plasticity and memory formation (65). Furthermore, systemic NMDA receptor-dependent activation is required for stress-facilitated learning (66, 67). Forced swim is one commonly used paradigm of stress-induced learning that is also used to model depression-related behavior (68). To examine the effect of this form of acute stress on MEK1 regulation, mice were placed in an inescapable container of water and left to swim for 6 min. One hour after rescue, the striatum was dissected, and lysates were analyzed by immunoblotting for phospho-Thr-292 and Thr-286. Forced swim caused reductions in phospho-Thr-292 but not Thr-286 MEK1 (Fig. 7A). Despite the reduction in phospho-Thr-292, forced swim had no effect on phospho-ERK1/2 (Fig. 7B). These data suggest that acute stress lowers the basal state of MEK1 inhibition via phospho-Thr-292 without a corresponding increase in the activation of ERK1/2.

**Discussion**

Here we report the site-specific phosphorylation of MEK1 by Cdk5, ERK1, and Cdk1. We definitively demonstrate that Cdk5 only phosphorylates Thr-292 MEK1 and not Thr-286, as reported previously (39, 40). Possible reasons for this discrepancy may be attributed to the fact that the previous studies were based on cotransfections and site-directed mutagenesis, which may have imparted indirect effects upon Thr-286. We also demonstrated that ERK robustly phosphorylated Thr-286-MEK1, whereas Cdk1 was capable of phosphorylating both sites. In addition to demon-
stratizing kinase-specific interactions between the two phosphorylation sites, we found that phosphomimetic mutation of either or both these MEK1 sites reduced MEK1-dependent phosphorylation of ERK. Previous studies have suggested that phosphorylation of MEK at Thr-292 diminished the duration and intensity of ERK activation (37, 48). It should be noted that MEK1 may also be phosphorylated by other protein kinases. For example, in addition to activation of MEK1 through Raf-dependent phosphorylation, PAK1 phosphorylates MEK1 at Ser-298, thereby facilitating Raf-mediated activation (69–71). MEK1 has also been suggested to be phosphorylated at Thr-386 by ERK (48). Furthermore, MEK1 has been shown to undergo inhibitory phosphorylation in its activation loop at Ser-212 by an unknown protein kinase (72). The functions of these phosphorylations and the possibility of additional posttranslational modifications of MEK1 remain to be explored further. Overall, these studies point to MEK1 as a convergence point for the integration of various positive and negative inputs affecting cellular and neuronal function.

Invocation of cAMP-dependent signaling activated ERK1/2 and also led to increased phosphorylation of MEK1 in a manner consistent with feedback inhibition. D1 dopamine receptor/PAK1/DARPP32 signaling has been shown to cause ERK activation (73). Several mechanistic pathways may contribute to these effects. For example, DARPP-32-dependent inhibition of PP1 may attenuate striatal enriched phosphoprotein phosphatase-mediated inactivation of ERK (53, 74). Furthermore, cross-talk between D1 and NMDA receptors may invoke Ras-MEK/ERK signaling (52). Inhibitory phosphorylation of MEK1 by both Cdk5 and ERK likely integrates into these signaling cascades and may contribute meaningfully to the important role of ERK signaling in brain function and disease.

Experiments with phosphatase inhibitors implicated both PP1 and PP2A as contributors to the basal phosphorylation state of striatal Thr-292 and Thr-286 MEK1. However, we also found that these phosphatases contributed to ERK phosphorylation and activity state. ERK may be indirectly activated by PP1 phosphorylation activity through the regulation of Raf (57). For example, PP1 inhibition with tautomycetin prevents Raf/MEK/ERK activation (57). Conversely, the inhibition of PP2A phosphorylation activity has been demonstrated to enhance the MEK/ERK pathway (58). Therefore, evidence has been reported to suggest that these phosphatases simultaneously activate and inhibit the MEK/ERK pathway. More studies are needed to delineate the intricacies by which phosphatases regulate MEK1 and ERK activity.

In general, environmental experience is thought to trigger fast or excitatory neurotransmission, which is then integrated with slow or metabotropic neurotransmission to mediate synaptic remodeling and memory formation. ERK signaling has been shown to be a critical component of learning and memory. We demonstrated here that NMDA receptor activation and acute stress exposure caused a reduction in inhibitory phosphorylation of MEK1. Treatment of striatal slices with 50 μM NMDA also reduced phospho-ERK1/2. This effect is consistent with a previous report showing that lower concentrations of NMDA (1–30 μM) enhanced pERK, whereas higher concentrations of NMDA (30–250 μM) inhibited pERK in primary neuronal cultures (61). Because NMDA receptor activation is required for stress-facilitated learning (75), it is possible that MEK1 regulation through these mechanisms contributes to aversive memory formation. In addition, stress may contribute to the pathogenesis of depression through the perturbation of MEK/ERK signaling.

In humans with major depression, the MEK/ERK pathway is less active and has reduced expression (13). Here we found that acute stress induced a decrease in inhibitory phospho-Thr-292 MEK1 without inducing an increase in phospho-ERK1/2. In other studies, administration of antidepressants in rodent models enhanced ERK activation (14). Injection of the MEK inhibitors U0126, PD184161, and SL327 in rodents induced depression-like behaviors (15–19). However, acute administration of MEK/ERK inhibitors can, reportedly, also cause anti-depression-like behaviors in the forced swim test (76–78). Inhibition of MEK1 in the striatum has been shown recently to prevent the antidepressant effects of ketamine in the forced swim test (79). Therefore, the precise role of MEK/ERK in depression pathogenesis and effective antidepressant therapies remains unclear. Although it may be that MEK1 inhibitory regulation mediates antidepressant responses or could serve as the target for therapy development, this study does not escape similar apparent dualities. For example, we demonstrate here that phosphorylation of Thr-292 inhibits MEK1 activity, in agreement with earlier reports. However, in intact brain tissue or whole animals, manipulations that reduce these sites did not always result in increased ERK1 phosphorylation, which may be attributable to the paradoxical nature of negative feedback mechanistic loops and the upstream state of the MEK1/ERK activation cascade.

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