Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum

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Many drugs of abuse exert their addictive effects by increasing extracellular dopamine in the nucleus accumbens, where they likely alter the plasticity of corticostriatal glutamatergic transmission. This mechanism implies key molecular alterations in neurons in which both dopamine and glutamate inputs are activated. Extracellular signal-regulated kinase (ERK), an enzyme important for long-term synaptic plasticity, is a good candidate for playing such a role. Here, we show in mouse that d-amphetamine activates ERK in a subset of medium-size spiny neurons of the dorsal striatum and nucleus accumbens, through the combined action of glutamate NMDA and D1-dopamine receptors. Activation of ERK by d-amphetamine or by widely abused drugs, including cocaine, nicotine, morphine, and Δ9-tetrahydrocannabinol was absent in mice lacking dopamine- and cAMP-regulated phosphoprotein of Mr 32,000 (DARPP-32). The effects of d-amphetamine or cocaine on ERK activation in the striatum, but not in the prefrontal cortex, were prevented by point mutation of Thr-34, a DARPP-32 residue specifically involved in protein phosphatase-1 inhibition. Regulation by DARPP-32 occurred both upstream of ERK and at the level of striatal-enriched tyrosine phosphatase (STEP). Blockade of the ERK pathway or mutation of DARPP-32 altered locomotor sensitization induced by a single injection of psychostimulants, demonstrating the functional relevance of this regulation. Thus, activation of ERK, by a multilevel protein phosphatase-controlled mechanism, functions as a detector of coincidence of dopamine and glutamate signals converging on medium-size striatal neurons and is critical for long-lasting effects of drugs of abuse.

dopamine D1 receptor | drug addiction | NMDA receptor | nucleus accumbens | protein kinase

Many drugs of abuse share the ability to stimulate dopamine transmission in the nucleus accumbens (1). They are thought to mimic the effects of naturally reinforcing stimuli and divert the normal role of dopamine neurons in coding reward prediction errors (2). Dopamine modulates long-term depression and potentiation at glutamatergic corticostriatal synapses, and current models of striatal circuits suggest that the regulation of the plasticity of corticostriatal transmission is a central mechanism of dopamine-controlled-learning (3, 4). One prediction of these models is that key biochemical events should occur specifically in neurons in which both dopamine and glutamate inputs are activated. Activation of extracellular signal-regulated kinase (ERK) is a candidate for such a role because it depends on both dopamine and glutamate receptors (5). In addition, ERK activity is known to be important for long-term synaptic plasticity (6), and its pharmacological blockade prevents the transcriptional and rewarding effects of cocaine and Δ9-tetrahydrocannabinol (THC) (5, 7). To determine the role of ERK in the long-term action of dopamine in the striatum, it is critical to identify in which neurons it is activated and the mechanism of this activation. In the present study, using a variety of pharmacological tools and mutant mice, we show that activation of ERK occurs in a specific subset of striatal neurons and requires the stimulation of D1 dopamine receptors (D1Rs) and NMDA glutamate receptors (NMDARs). We found that this coincidence detection results from the striatal-specific regulation of both serine/threonine and tyrosine protein phosphatases and is initiated by phosphorylation of the protein phosphatase-1 (PP-1) inhibitor dopamine- and cAMP-regulated phosphoprotein of Mr 32,000 (DARPP-32) on a single threonine residue (Thr-34). Our results provide evidence that this mechanism has important consequences for the long-term effects of psycho-stimulants and may have a general role in the action of many drugs of abuse.

Methods

Experiments were carried out in accordance with the guidelines of the French Agriculture and Forestry Ministry for handling animals (Decree 78849, License 01499) in either male 8-week-old C57BL/6J mice or male and female mutant mice and matched controls from previously described lines (8–10). Mice were habituated to daily saline i.p. injections during the 3 days preceding the experiments. For immunoblotting, microdisks were prepared from sections of rapidly frozen brains and lysed in 1% (vol/vol) SDS solution at 100°C. For immunofluorescence, mice were perfused transcardially with 4% paraformaldehyde in 0.1 M Na3HPO4/NaH2PO4 buffer (pH 7.5). Cells with fluorescence above background were counted by using a computerized image analyzer, in two to four brain sections per animal, bilaterally for each region of interest. Locomotor activity was measured by consecutive interruption of two adjacent beams (¼ tour) in a circular corridor with four infrared beams placed at every 90° (Imetronic, Pessac, France) in a low-luminosity environment. Statistical analyses were done by using one-way ANOVA followed by Bonferroni’s test or Dunnett’s test. Detailed procedures and reagents are described in Supporting.

See Commentary on page 253.

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Experimental Procedures, which is published as supporting information on the PNAS web site.

Results

Psychostimulants Activate ERK in a Subset of Striatal Medium-Size Spiny Neurons (MSNs). To investigate the molecular basis for ERK regulation, we examined its activation by phosphorylation in mouse striatum in response to acute injections of d-amphetamine (d-amph) by using antibodies specific for the active form of the kinase. In agreement with previous results obtained with cocaine (5, 11), we found a robust increase in phospho-ERK (P-ERK) immunoreactivity in the striatum of mice treated with d-amph. P-ERK was detected in neuronal nuclei and perikarya, as well as in the surrounding neuropil, in the dorsal striatum, and in the shell (Fig. 7, which is published as supporting information on the PNAS web site) and core (not shown) of the nucleus accumbens. P-ERK immunoreactivity was found in MSNs, the principal type of striatal neurons, because it colocalized with several markers of these neurons: DARPP-32 (12), striatal-enriched tyrosine phosphatase (STEP) (13) (Fig. 1a and Table 1, which is published as supporting information on the PNAS web site), and Goalpha (14) (data not shown). No P-ERK immunoreactivity was found in cholinergic interneurons identified with antibodies against choline acetyltransferase (Fig. 1a).

MSNs are chemically heterogeneous: some express dynorphin and high levels of D1Rs, whereas others express enkephalin and high levels of D2 receptors (15). After d-amph injection, P-ERK was found in ~60% of dynorphin-positive neurons, but in only ~3% of enkephalin-positive neurons (Fig. 1a and Table 1). Localization of P-ERK in D1R-expressing cells was also supported by their double labeling with D1R antibodies (Fig. 1a).

Because D1R acts mainly by raising cAMP levels and activating cAMP-dependent protein kinase (PKA), we compared the distribution of P-ERK and phospho-Thr-34-DARPP-32, a major PKA substrate in MSNs (12), by using phosphorylation state-specific antibodies (Fig. 8, which is published as supporting information on the PNAS web site). P-ERK was detected in a subpopulation of phospho-Thr-34-DARPP-32-positive neurons (~65%), and virtually never in other striatal neurons (Fig. 1b and c and Table 1). Thus, in response to d-amph, ERK phosphorylation is more restricted than PKA activation, within a subpopulation of dynorphin- and D1R-positive MSNs, indicating that it requires a stimulus in addition to dopamine.

ERK Activation Selectively Requires a Combined Action of Glutamate NMDARs and Dopamine D1Rs. Using immunoblotting of brain samples obtained after rapid freezing, we compared the phosphorylation of ERK to that of the glutamate receptor 1 (GluR1) subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, a well characterized PKA substrate, phosphorylated on Ser-845 in response to D1R stimulation (16). A robust increase in phosphorylation of GluR1 and both ERK isoforms (predominantly ERK2) was observed in response to d-amph (Figs. 2a and 7b). ERK and GluR1 phosphorylation was rapid (maximal at 10 min), transient (return to baseline at 30 min), and dose-dependent (Fig. 7c and d). Phosphorylation of both ERK and GluR1 was blocked by prior administration of the D1R antagonist SCH23390 (Fig. 2a). These results were confirmed by using D1R-knockout mice (Fig. 2b).

The requirement for glutamate stimulation was then examined. Pretreatment of mice with an NMDA receptor antagonist, MK801, prevented ERK phosphorylation, whereas it did not alter GluR1 phosphorylation (Fig. 2c). Similarly, as judged by quantification of the immunofluorescence data, MK801 prevented phosphorylation of ERK, but not of DARPP-32 (Fig. 1c). Taken together, these results indicate that phosphorylation of GluR1 and DARPP-32 through the PKA pathway requires activity of D1R but not of NMDAR. In contrast, ERK phosphorylation requires stimulation of both D1R and NMDAR, showing its dependence on a coincident activity of dopamine and glutamate transmission in a subset of D1R-containing MSNs.

DARPP-32 Is Required for Activation of the ERK Pathway in the Striatum by Drugs of Abuse. MSNs express high levels of DARPP-32, a protein that regulates several phosphorylation cascades (17). In DARPP-32-knockout mice, ERK activation in response to d-amph was dramatically reduced (Fig. 3a). Psychostimulants...
activate ERK in several other brain regions (11) such as the prefrontal cortex, in which DARPP-32 expression is much lower than in the striatum (12). As illustrated in Fig. 3b, activation of ERK by d-amph was similar in the prefrontal cortex of wild-type and DARPP-32-knockout mice, suggesting that DARPP-32 plays a specific role in ERK activation in the striatum. DARPP-32 has a high degree of sequence similarity with inhibitor-1, another PKA-regulated inhibitor of PP-1, also expressed in MSNs (18). However, ERK activation was unchanged in inhibitor-1 knockout mice (data not shown), supporting a selective role of DARPP-32 in this response.

We have recently shown that activation of ERK in the nucleus accumbens shell is a shared property of diverse drugs of abuse (11). We compared the role of DARPP-32 in ERK phosphorylation in response to d-amph, cocaine, morphine, nicotine, and THC in the nucleus accumbens shell (Fig. 3c). ERK activation was blocked for all these drugs in DARPP-32-knockout mice. These results demonstrate that DARPP-32-dependent activation of ERK in the striatum is a general mechanism common to a variety of drugs of abuse.

**DARPP-32 Phosphorylation on Residue Thr-34 Is Specifically Required for Activation of the ERK Pathway by Cocaine in the Striatum.**

DARPP-32 has several regulatory functions that depend on the phosphorylation of distinct residues by different kinases (17), and the absence of DARPP-32 could alter the activation of ERK in the striatum by several mechanisms. Phosphorylation of DARPP-32 on Thr-34 is necessary and sufficient for high-affinity inhibition of PP-1 (19, 20). To investigate the precise role of PP-1 inhibition in ERK activation by psychostimulants, we used mutant mice in which Thr-34 of DARPP-32 was replaced by alanine (10). Cocaine-induced phosphorylation of ERK was prevented in the dorsal striatum and the nucleus accumbens core and shell of Thr-34 → Ala-DARPP-32 mutant mice (Fig. 4a and Fig. 9a, which is published as supporting information on the PNAS web site). In contrast, the mutation had no effect on ERK phosphorylation in the prefrontal cortex. Similar results were observed when the mice were treated with d-amph (data not shown). On the other hand, in mice bearing a point mutation of Thr-75, a residue phosphorylated by CDK5 and responsible for inhibition of PKA (21), ERK activation in the dorsal striatum, the nucleus accumbens core and shell, and the prefrontal cortex was similar to that in wild-type littermates (Figs. 4b and 9b). When we examined the levels of several proteins involved in striatal signal transduction we found that levels of ERK1 and ERK2 were selectively increased in the striatum of Thr-34 → Ala, but not Thr-75 → Ala mutant mice (Fig. 10, which is published as supporting information on the PNAS web site). This change suggested a compensatory reaction to the alteration of the ERK pathway in the striatum.

The above results were obtained by measuring the level of ERK phosphorylation on its activation loop. Although this phosphorylation is highly correlated with ERK activity, it was
important to determine whether increased ERK phosphorylation actually resulted in its functional activation. To test this possibility, we examined the *in vivo* phosphorylation of a major substrate of ERK, the transcription factor Elk-1 (22). As previously reported (7, 23), d-amph and cocaine induced a strong phosphorylation of Elk-1 on Ser-383 in the dorsal striatum, the nucleus accumbens shell and core, and the prefrontal cortex (Fig. 4c). Phosphorylation of Elk-1 was still detected at 30 min (data not shown). We verified that the observed Elk-1 phosphorylation resulted from ERK activation by pretreating mice with SL327, an inhibitor of mitogen-activated protein (MAP)- kinase/ERK kinase (MEK) that crosses the blood–brain barrier (24). SL327 pretreatment completely prevented Elk-1 phosphorylation (data not shown). DARP-32 was necessary for the regulation of Elk-1, because drug-induced phosphorylation of Elk-1 was prevented in the dorsal striatum and nucleus accumbens of DARP-32 Thr-34→Ala mutant mice (Fig. 4c). In contrast, DARP-32 mutation had no effect within the prefrontal cortex (Fig. 4c). These results show that DARP-32 phosphorylation on Thr-34 is a critical element in the regulation of the ERK pathway *in vivo* in response to drugs of abuse.

**Role of DARP-32 and ERK Activation in Behavioral Sensitization.**

Psychostimulants have been reported to alter behavioral responses in rats weeks after a single injection (25). To explore the contribution of DARP-32-mediated ERK activation in lasting behavioral effects of these drugs, we used an experimental protocol allowing analysis of the delayed consequences of a single acute administration of psychostimulant (25). Mice received a first injection of drug and were challenged with a test injection either 2 or 7 days later (Fig. 5 and Fig. 11, which is published as supporting information on the PNAS web site). Wild-type mice displayed increased locomotion in response to the second injection of cocaine, and this sensitization was more pronounced in the group of mice that received the challenge injection at the later time point (Figs. 5 and 11). To evaluate the role of ERK in this response, mice were treated with SL327 before the first injection of cocaine. We selected a dose of SL327 (30 mg/kg) that decreased ERK activation by >80% in the striatum but had no major effect on spontaneous locomotion (data not shown) or acute response to cocaine (Fig. 5a). The increased responsiveness to the challenge injection of cocaine (Fig. 5a) or d-amph (Fig. 11a) was dramatically reduced in SL327-pretreated mice, indicating a role for ERK in the induction of sensitization. In contrast, SL327 had no effect on the expression of sensitization, i.e., it did not decrease the enhanced locomotor response when it was administered just before the challenge injection (data not shown). Locomotor sensitization to cocaine was blocked in DARP-32-knockout (Fig. 11 b and c) and Thr-34→Ala mutant mice (Figs. 5b and 11d). In contrast sensitization was not altered in Thr-75→Ala mutant mice (data not shown). These experiments indicate that the ERK pathway, as well as DARP-32-mediated PP-1 inhibition, is critical for the establishment of long-term alterations of behavioral responses to a second exposure to cocaine.

**DARP-32 Regulates ERK by Preventing Its Dephosphorylation and Increasing Its Phosphorylation.**

We next examined the mechanism by which DARP-32 alters the ERK pathway, taking into account the fact that ERK is likely not a substrate for PP-1 (26). The observed increase in psychostimulant-induced ERK phosphorylation could be the result of either a decrease in phosphatase activity or an increase in kinase activity. Both of these possibilities were examined. ERK activation occurred in MSNs that express high levels of STEP (Fig. 1a), a protein tyrosine phosphatase that inactivates ERK by dephosphorylating the tyrosine residue of its activation loop (27, 28). Phosphatases of the STEP family lose their ability to dephosphorylate ERK upon activation by dopamine (29). Therefore, the decrease in ERK dephosphorylation observed in MSNs after cocaine (30) is unlikely to have been due to a decrease in STEP activity. It is therefore possible that DARP-32 may increase phosphorylation of ERK by forming a complex with its substrate, thus increasing its activity. Increasing phosphorylation of ERK results in increased phosphorylation of Elk-1, which in turn increases the expression of sensitization, i.e., it did not decrease the enhanced locomotor response when it was administered just before the challenge injection (data not shown). Locomotor sensitization to cocaine was blocked in DARP-32-knockout (Fig. 11 b and c) and Thr-34→Ala mutant mice (Figs. 5b and 11d). In contrast, SL327 had no effect on the expression of sensitization, i.e., it did not decrease the enhanced locomotor response when it was administered just before the challenge injection (data not shown). Locomotor sensitization to cocaine was blocked in DARP-32-knockout (Fig. 11 b and c) and Thr-34→Ala mutant mice (Figs. 5b and 11d). In contrast sensitization was not altered in Thr-75→Ala mutant mice (data not shown). These experiments indicate that the ERK pathway, as well as DARP-32-mediated PP-1 inhibition, is critical for the establishment of long-term alterations of behavioral responses to a second exposure to cocaine.
phorylate ERK when they are phosphorylated by PKA on a regulatory serine residue within their ERK-binding domain, termed the kinase interaction motif (28). Dephosphorylation of this site, a reaction catalyzed by several phosphatases, including PP-1 (29), permits STEP to bind to and dephosphorylate ERK. d-amph stimulated the phosphorylation of the 46-kDa isoform of STEP in the striatum, as shown by an increase in its apparent molecular weight (Fig. 6a). This effect was prevented in DARPP-32-knockout mice (Fig. 6a), supporting the regulation of STEP by PP-1 in vivo.

We also investigated the regulation of the signaling cascade that leads to ERK activation. ERK is phosphorylated on both threonine and tyrosine by the dual-specificity protein kinase MEK. MEK is itself activated by phosphorylation. d-amph increased the phosphorylation of MEK in the striatum of wild-type mice, but not of DARPP-32-knockout mice (Fig. 6b). These data indicate that DARPP-32 controls the activation of ERK through at least two mechanisms (Fig. 6c): (i) by preventing its dephosphorylation by STEP, and (ii) by increasing its phosphorylation by MEK.

Discussion

Our results show that drugs of abuse activate ERK in a subpopulation of D1R-containing medium-sized striatal neurons. This activation requires stimulation of both D1R and NMDAR because it is prevented by blocking either type of receptor. In contrast, in vivo activation of PKA in these neurons, as monitored by the phosphorylation of two of its substrates, GluR1 and DARPP-32, necessitates activation of D1R, but not of NMDAR. The regulation by the two types of receptors accounts for the selective phosphorylation of ERK in a subpopulation of the neurons in which DARPP-32 is phosphorylated, as a consequence of the more widespread activation of PKA. Activation of ERK in MSNs thus requires the concomitant activity of two types of receptors and behaves as a functional coincidence detector.

We have identified the molecular mechanisms of this functional interaction between the D1R- and the NMDAR-controlled signaling pathways necessary to activate ERK. DARPP-32, the PP-1 inhibitor that is highly enriched in MSNs, is necessary for this cross-talk, because the activation of ERK was blocked in DARPP-32-knockout mice. The use of a knockin point mutation demonstrated that a single residue of DARPP-32, Thr-34, was responsible for its ability to control ERK phosphorylation in response to psychostimulants, providing evidence for the role of PKA-regulated inhibition of PP-1. Inhibition of PP-1 appeared to be important at several levels for activating the ERK cascade. On the one hand, it prevented ERK dephosphorylation by STEP, by maintaining this tyrosine phosphatase in a phosphorylated, inactive, state. On the other hand, it was also critical upstream of ERK, because MEK phosphorylation in response to psychostimulants was dramatically reduced in DARPP-32-null mice. This latter effect might result from the inhibition by phospho-Thr-34-DARPP-32 of a negative regulation exerted by PP-1 at the level of MEK or upstream.

Electrical stimulation of corticostriatal neurons can activate ERK in the striatum (30), presumably through NMDAR and a Ca\(^{2+}\)/calmodulin-dependent pathway (31, 32). Several synergistic mechanisms coupling NMDAR and the ERK pathway have been reported, including stimulation of Ras-GRF (33), a guanine-nucleotide-exchange factor that activates Ras, and inhibition of SynGAP (34, 35), a GTPase-activating protein that inactivates Ras. Our results suggest that after administration of psychostimulants, stimulation of D1R and the resulting DARPP-32-mediated inhibition of PP-1 potentiate the activation of the ERK pathway depending on NMDAR, insufficient by itself to give rise to a detectable phosphorylation of ERK in these experimental conditions. This mechanism appears selective for striatal neurons because DARPP-32 was not required for ERK activation in the prefrontal cortex. Importantly, the control of the ERK pathway by DARPP-32 in the nucleus accumbens shell was common to all drugs of abuse tested, indicating that it may play a critical role in long-term effects of these drugs.

Abundant evidence indicates that the ERK pathway is required for long-term synaptic plasticity at both transcriptional and posttranscriptional levels in many brain regions (6, 36). Regulation of transcription is also known to play a central role in the long-term effects of drugs of abuse (37). We found that activation of Elk-1, a substrate of ERK, and an essential component of the ternary complex factor involved in the regulation of many immediate early genes (38), was altered after either pharmacological blockade of the ERK pathway or mutation of DARPP-32 Thr-34. This finding supports a role of this pathway for gene regulation in vivo. Moreover, the long-lasting behavioral consequences of a single exposure to drugs, as revealed by locomotor sensitization, were dependent on both DARPP-32 and NMDAR.
neurons to alter the plasticity of corticostriatal synapses. Previous
evidence suggests that such regulatory mechanisms may be
altered after chronic dopamine depletion (39). On the other
hand, this mechanism is likely to be exaggerated after exposure
to drugs of abuse that induce a marked and diffuse increase
in extracellular dopamine. ERK activation may facilitate long-
altering alterations in a subset of striatal neurons activated by
converging corticostriatal inputs triggered by environmental and
internal stimuli concomitant with or preceding drug intake. We
suggest that this mechanism may play a role in the development
of addiction to drugs of abuse, as well as in physiological
reward-controlled learning.

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