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cAMP and Extracellular Signal-Regulated Kinase Signaling in Response to D-Amphetamine and Methylphenidate in the Prefrontal Cortex in Vivo: Role of β1-Adrenoceptors

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ABSTRACT

D-Amphetamine and methylphenidate are widely used in the treatment of attention-deficit/hyperactivity disorder. Both drugs increase extracellular norepinephrine and dopamine in the prefrontal cortex, where they are believed to exert their therapeutic effects. However, the molecular mechanisms underlying their action are poorly understood. To investigate the intracellular signaling pathways activated by D-amphetamine and methylphenidate in the prefrontal cortex in vivo in mice, we measured the cAMP-dependent Ser845 phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor GluR1 subunit and the active form of extracellular signal-regulated kinase (ERK). Administration of D-amphetamine (5–10 mg/kg) or methylphenidate (10–20 mg/kg) increased phosphorylation of GluR1. Basal and D-amphetamine–induced GluR1 phosphorylation was reduced by propranolol, a general β-adrenoceptor antagonist, and betaxolol, a β1-antagonist, but not by (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol (ICI-118,515), a β2-antagonist. The effect of methylphenidate was also blocked by propranolol and betaxolol. The D-amphetamine effect was slightly potentiated by prazosin, an α1-adrenoceptor antagonist, and mimicked by yohimbine, an α2 antagonist. Blockade of dopamine or *N*-methyl-D-aspartate (NMDA) receptors or serotonin depletion had no effect on D-amphetamine–induced GluR1 phosphorylation. D-amphetamine but not methylphenidate increased ERK phosphorylation. This effect required multiple signaling pathways because it was blocked by β1- and α1-adrenoceptor antagonists, by dizocilpine maleate (MK801), an NMDA antagonist, and by serotonin depletion. In contrast, blockade of dopamine receptors had no effect on D-amphetamine–induced ERK phosphorylation. Propranolol and betaxolol increased the hyperlocomotion produced by D-amphetamine and methylphenidate. Thus, both D-amphetamine and methylphenidate potently activate the cAMP pathway in the prefrontal cortex through β1-adrenergic receptors. This activation could have behavioral consequences and contribute to the treatment of attention-deficit/hyperactivity disorder.

D-Amphetamine (D-amph) and methylphenidate (MPH) (here referred to as psychostimulants) are widely used in children and adults for the treatment of attention-deficit/hyperactivity disorder (ADHD) (Wilens et al., 2002). At higher doses, D-amph and, to a lesser degree, MPH have a potential risk for abuse (Swanson and Volkow, 2003). It is therefore important to try and distinguish the molecular basis of therapeutic efficacy and abuse liability of these drugs. D-amph and MPH competitively block monoamine transport at plasma and vesicle membranes with various potencies and promote monoamines release in brain (Sulzer et al., 1995; Gatley et al., 1996). Microdialysis experiments have shown that both D-amph and MPH elevate the extracellular concentrations of dopamine (DA) and norepinephrine

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ABBREVIATIONS: MPH, methylphenidate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AR, adrenergic receptor; ADHD, attention-deficit/hyperactivity disorder; PKA, cAMP-dependent protein kinase; D-amph, D-amphetamine; DA, dopamine; NE, norepinephrine; 5HT, serotonin; ERK, extracellular signal-regulated kinase; NMDA, *N*-methyl-D-aspartate; ANOVA, analysis of variance; ICI-118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; MK-801, dizocilpine maleate; SCH23390, *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SDZ 205,557, 2-methoxy-4-amino-5-chlorobenzoic acid 2-(diethylamino)ethyl ester.

(NE) in various brain regions (Kuczenski and Segal, 1997). D-amph also enhances extracellular serotonin (5HT) with a relatively high efficacy, whereas MPH has a poor affinity for the 5HT transporter and does not alter the extracellular concentration of 5HT, even at high doses (Gatley et al., 1996; Kuczenski and Segal, 1997). Strong evidence implicates DA neurotransmission in the etiology of ADHD and in the therapeutic action of psychostimulants (Wilens et al., 2002). Although less thoroughly investigated, the role of NE is also plausible because selective NE reuptake inhibitors (Biederman and Spencer, 1999; Bymaster et al., 2002) and α2-adrenergic agonists (Arnsten et al., 1996) have therapeutic effects in ADHD. In addition, some evidence suggests the possible involvement of 5HT in the beneficial effects of psychostimulants (Gainetdinov et al., 1999). On the other hand, the addictive properties of these drugs, which are mainly attributed to their action on DA, particularly in the ventral striatum, are also known to be significantly modulated by their effects on NE and 5HT (Di Chiara and Imperato, 1988; Auclair et al., 2004).

The prefrontal cortex is an important site of action of psychostimulants. Neuropsychological tests and brain imaging studies suggest that ADHD is associated with alterations in prefrontal cortex and related subcortical circuits (Wilens et al., 2002). Psychostimulants increase cortical arousal in patients with ADHD, normalizing their brain activity (Berridge and Waterhouse, 2003). Cortical DA and NE have been linked to attention and cognitive functions that are clearly altered in ADHD, supporting their role in the therapeutic effects of psychostimulants (Wilens et al., 2002; Berridge and Waterhouse, 2003). The action of psychostimulants in the prefrontal cortex is also implicated in the development of locomotor sensitization, a behavioral change believed to underlie certain aspects of drug addiction (Vanderschuren and Kalivas, 2000). Thus, psychostimulants could produce their therapeutic effects in ADHD and some of their effects on the development of drug abuse by elevating monoamine extracellular concentrations in the prefrontal cortex. Monoamines activate numerous subtypes of DA, NE, and 5HT receptors that are expressed in abundance in this brain region. Yet little is known about the intracellular signaling events triggered by receptor activation in the prefrontal cortex and about their implications in the functional effects of psychostimulants (Svenningsson et al., 2003).

Here, we studied the effects of D-amph and MPH on two major signaling pathways in the mouse prefrontal cortex in vivo: the cAMP-dependent protein kinase (PKA) and extracellular signal-regulated kinase (ERK) signaling cascades, using a procedure that prevents postmortem dephosphorylation of proteins. We measured the phosphorylation of GluR1 subunit of AMPA receptor on Ser845, a residue specifically phosphorylated by PKA (Roche et al., 1996). Phosphorylation of Ser845 is likely to have important functional consequences because it markedly increases the peak current of AMPA receptors (Roche et al., 1996). Phosphorylation of GluR1 on Ser845 is increased by psychostimulants in the striatum, whereas its regulation in the prefrontal cortex is not known (Snyder et al., 2000). In the same samples, we measured the active form of ERK (i.e., doubly phosphorylated in its activation loop). The ERK pathway is critical for long-lasting alterations of synaptic properties (Derkinderen et al., 1999; Thomas and Huganir, 2004) and is activated in selective brain regions in response to many drugs, including cocaine

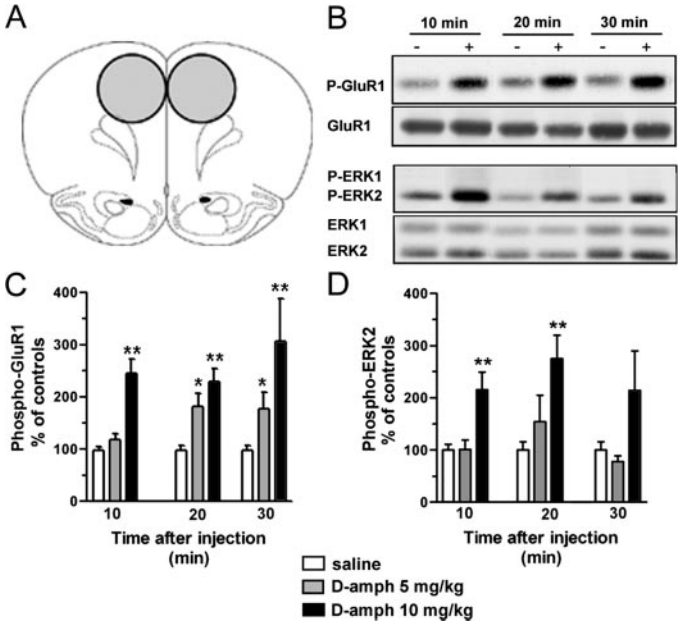

Fig. 1. D-Amphetamine administration stimulates GluR1 and ERK2 phosphorylation in the prefrontal cortex in vivo. A, location of tissue microdisks (1.4 mm diameter) punched out from the median prefrontal cortex. B, Immunoblotting of GluR1 phosphorylated on Ser845 (P-GluR1) and ERK1/2 phosphorylated on Thr183-Tyr185 (P-ERK1/2) in the prefrontal cortex of mice treated with saline (–) or 10 mg/kg D-amph (+). After stripping, the membranes were reprobed with anti-GluR1 (GluR1) or anti-ERK1/2 (ERK1/2) antibodies. C and D, time course of D-amph (5 and 10 mg/kg) effects on the phosphorylation of GluR1 (Phospho-GluR1) (C) and ERK2 (Phospho-ERK2) (D) in the prefrontal cortex. The intensity of phospho-GluR1 and phospho-ERK2-immunoreactive bands was expressed as a percentage of the levels in saline-injected control mice analyzed on the same membranes. Data correspond to means ± S.E.M. (10–12 mice per condition). ANOVA followed by Newman-Keuls test: D-amph-treated versus saline, ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$.

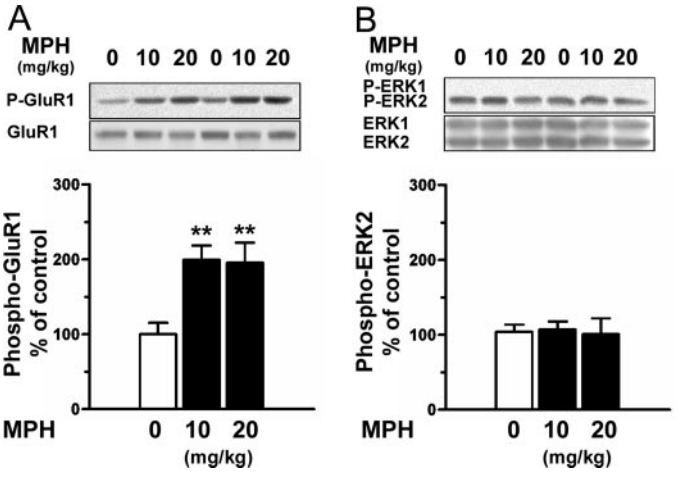

Fig. 2. Methylphenidate administration stimulates phosphorylation of GluR1 but not ERK2 in the prefrontal cortex in vivo. A, immunoblotting (top) and quantification (bottom) of GluR1 phosphorylated on Ser845 (P-GluR1) in the prefrontal cortex of mice 15-min after injection of saline or methylphenidate (MPH, 10 and 20 mg/kg). B, immunoblotting of phospho-ERK1/2 (P-ERK1/2, top) and quantification of phospho-ERK2 (bottom) in the prefrontal cortex of mice after the same treatments administered in A. No significant variation of total amounts of GluR1 or ERK2 was observed between the various groups of mice (A and B, top). Data correspond to means ± S.E.M. (five mice per condition). ANOVA followed by Newman-Keuls test: MPH-treated versus saline, ⋆⋆, $p < 0.01$.

and D-amphetamine (Valjent et al., 2005). We found that D-amph induced a strong phosphorylation of both GluR1 and ERK2, whereas MPH increased only the phosphorylation of GluR1. Using pharmacological tools, we demonstrated an important contribution of NE transmission predominantly via activation of β1-adrenoreceptor (AR). In addition, we found that activation of β1-AR contributed to the behavioral effects of D-amph and MPH, suggesting a possible role of these receptors in the context of ADHD treatment.

Materials and Methods

Animals. Male 8-week-old C57BL/6J mice (Charles River France, L'Arbresle, France) were kept at least 1 week in our animal house in stable conditions of temperature (22°C) and humidity (60%) with a constant 12-h light/dark cycle and free access to food and water. All experiments on mice were performed in accordance with the guidelines of the French Agriculture and Forestry Ministry for handling animals (decree 87849, license A 75-05-22).

Drugs. (+)-α-Methylphenethylamine (D-amphetamine) sulfate salt, methylphenidate hydrochloride, propranolol, betaxolol, ICI-118,515, prazosin, yohimbine, SCH23390, haloperidol, ritanserin, SDZ 205,557, and MK801 were from Sigma-Aldrich (St. Louis, MO). Drugs were dissolved in 0.9% (w/v) NaCl (saline) and i.p. injected.

Treatment. During the 3 days preceding the experiment, mice were habituated to injections by daily i.p. saline administration. Pharmacological treatments were carried out with saline, D-amph (5 or 10 mg/kg), or MPH (10 or 20 mg/kg). The various antagonists were injected 30 min before saline or D-amph (10 mg/kg) injection. Propranolol and betaxolol were administered 15 and 30 min before saline or MPH (10 mg/kg) for biochemical and behavioral analysis, respectively. The 5HT synthesis inhibitor 4-chloro-phenylalanine (300 mg/kg) was i.p. injected daily during the 3 days preceding the D-amph treatment.

Tissue Preparation. At the indicated times after treatment, mice were decapitated, and their heads were immediately frozen in liquid nitrogen (12 s). When the animals were pretreated with receptor antagonists before D-amph or MPH, they were killed 15 min after the D-amph or MPH injection. The frozen heads were cut into 210-μm thick slices with a cryostat, and eight frozen microdisks (1.4 mm diameter) were punched out bilaterally from the median prefrontal cortex (Fig. 1A) and stored at −80°C.

Western Blot Analysis. Micropunches were homogenized by the addition of a hot solution (maintained in a boiling water bath) of 1% SDS (v/v) and 1 mM sodium orthovanadate in water, immediate sonication, and incubation at 100°C for 5 min to inactivate phosphatases and proteases. Equal amounts of protein (100 μg) were separated by SDS-polyacrylamide gel electrophoresis (10%) before electrophoretic transfer onto a nitrocellulose membrane (Hybond Pure; Amersham Biosciences Inc., Piscataway, NJ). Membranes were blocked for 1 h at room temperature in Tris-buffered saline (100 mM NaCl and 10 mM Tris, pH 7.5) with 0.05% Tween 20 for detection of phospho-ERK or 5% nonfat dry milk for phospho-GluR1, respectively. Membranes were then incubated overnight at 4°C with primary antibodies. Bound antibodies were detected with horseradish peroxydase-conjugated anti-rabbit or anti-mouse antibodies (diluted 1:4000; Amersham Biosciences) and visualized by enhanced chemiluminescent detection (ECL; Amersham Biosiences). The same membranes were probed for proteins independently of their phosphorylation state after stripping in a buffer containing 100 mM glycine, pH 2.5, 200 mM NaCl, 0.1% Tween 20 (v/v), and 0.1% (v/v) β-mercaptoethanol for 45 min at room temperature, followed by extensive washing in Tris-buffered saline and incubation in blocking buffer. The relevant immunoreactive bands were quantified with laser-scanning densitometry using Image software (Scion Corporation, Frederick, MD). The results normalized for each membrane were expressed as

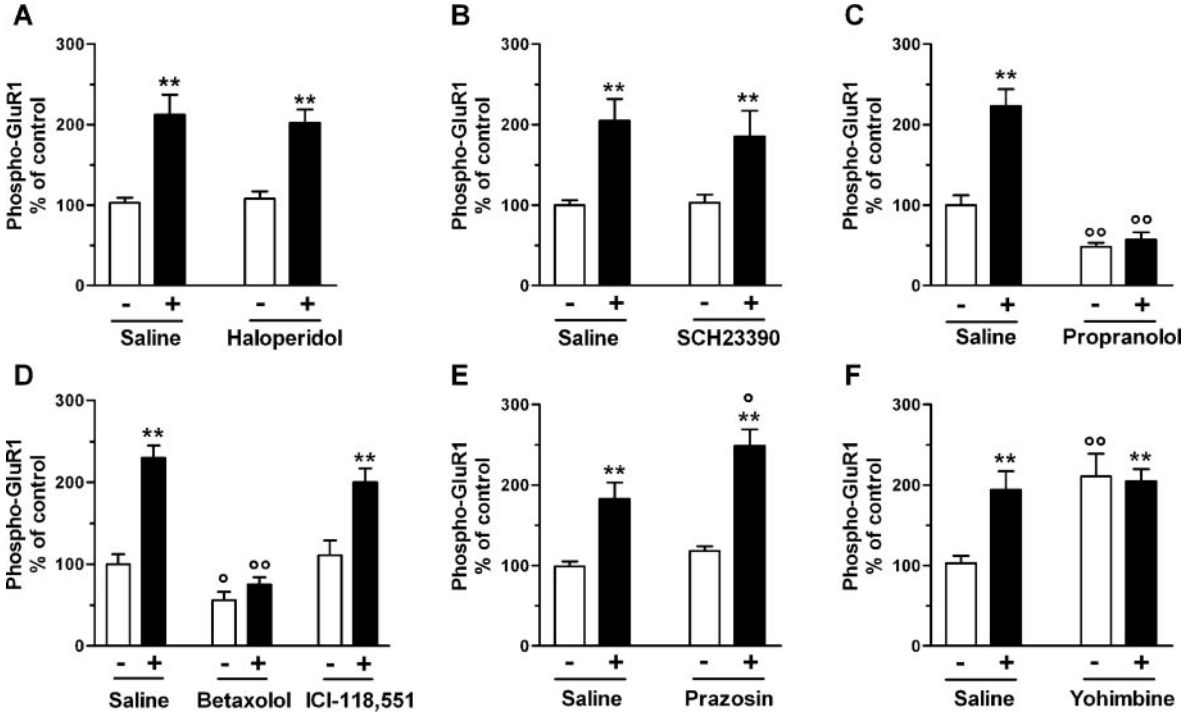

Fig. 3. β1-Adrenoceptors mediate phosphorylation of GluR1 by D-amphetamine in the prefrontal cortex. Various antagonists (or vehicle) were administered 30 min before 10 mg/kg D-amph (+) or saline (–). Mice were killed 15 min after D-amph, and phosphorylation of GluR1 was determined by immunoblotting. A, DA receptor antagonist haloperidol (0.5 mg/kg). B, D1/5 receptor antagonist SCH23390 (0.25 mg/kg). C, β-AR antagonist propranolol (20 mg/kg). D, β1-AR antagonist betaxolol (20 mg/kg) and β2-AR antagonist ICI-118,551 (4 mg/kg). E, α1-AR antagonist prazosin (2 mg/kg). F, α2-AR antagonist yohimbine (3 mg/kg). Data are means ± S.E.M. (at least five mice per group). ANOVA followed by Newman-Keuls test: D-amph-treated versus saline, **, $p < 0.01$; antagonist treatment versus control, °, $p < 0.05$, °°, $p < 0.01$.

percentages of saline-treated controls. The following phospho-specific antibodies were used for Western blotting: rabbit antiphospho-GluR1 (phospho-Ser845, 1:500; Upstate Biotechnology, Lake Placid, NY), and monoclonal antiphospho-ERK1/2 (phospho-Thr183-Tyr185, 1:1000; Sigma-Aldrich). Rabbit polyclonal anti-GluR1 (1:500; Upstate Biotechnology) and anti-ERK1/2 (1:1000; Upstate Biotechnology), were used to measure the total amount of these proteins in tissue samples.

Behavioral Analysis. Mice were injected with saline and placed for 30 min in the locomotor box during 3 consecutive days (days 1–3) for habituation before the actual experiment was performed on day 4. Locomotor responses were evaluated using a circular corridor with four infrared beams placed every 90° (Imetronic, Pessac, France) in a low-luminosity environment. Locomotor activity was counted as travels through one quarter of the circular corridor as detected by consecutive interruption of two adjacent beams. The effects of propranolol (20 mg/kg i.p.) and betaxolol (20 mg/kg) on acute locomotor responses induced by D-amph (2 mg/kg i.p.) and MPH (10 mg/kg i.p.) were evaluated on day 4 as follows: spontaneous activity during 15 min; locomotor activity after administration of saline, propranolol, or betaxolol during 30 min; and locomotor activity after injection of D-amph or MPH during 60 min. Locomotor activity was measured at 5-min intervals, and cumulative counts during the 60 min after D-amph or MPH injection were used for data analysis.

Statistical Analysis. Results are expressed as means ± S.E.M. One-way ANOVA followed by Newman-Keuls and two-way ANOVA with treatment and time were performed with Prism 3.0 software (GraphPad Software Inc., San Diego, CA).

Results

D-amph and Methylphenidate Increase Phosphorylation of GluR1 and ERK in the Prefrontal Cortex. Treatment with 10 mg/kg D-amph increased phosphorylation of GluR1 on Ser845 (P-GluR1) and combined phosphorylation of ERK on Thr183 and Tyr185 (P-ERK) in the prefrontal cortex (Fig. 1B). ERK2 phosphorylation was much more pronounced than phosphorylation of ERK1, which was not always detectable (Fig. 1B). After D-amph injection, GluR1 and ERK2 phosphorylation increased rapidly (10 min) and remained elevated at least until 30 min (Fig. 1, C and D). For each time point, the increased GluR1 phosphorylation was more marked at 10 than at 5 mg/kg D-amph (Fig. 1C), whereas ERK phosphorylation was significant only at 10 mg/kg (Fig. 1D). In all of these experiments, there was no change in the levels of GluR1 and ERK measured with an antibody recognizing both the phosphorylated and unphosphorylated forms of GluR1 or ERK (Fig. 1B).

Fifteen minutes after injection of 10 or 20 mg/kg MPH, GluR1 phosphorylation on Ser845 (P-GluR1) was strongly increased in the prefrontal cortex (Fig. 2, A and B). In contrast, both doses of MPH failed to activate ERK2 phosphorylation, which remained at the baseline level (Fig. 2, C and D). The total levels of GluR1 and ERK were unchanged (Fig. 2, A and C).

***β*-Adrenoceptors Play a Critical Role in the Phosphorylation of GluR1 by D-amph and Methylphenidate.** In the prefrontal cortex, D-amph and MPH share the ability to increase extracellular DA and NE (Berridge and Stalnaker, 2002). The PKA-dependent phosphorylation of GluR1 could result from the stimulation of dopamine D1/D5 receptors or β-AR because both receptor types stimulate adenylyl cyclase activity upon agonist activation and are expressed in significant amounts in the prefrontal cortex (Savasta et al., 1986; Nicholas et al., 1996). To test the contribution of DA receptors, haloperidol (0.5 mg/kg), a nonselective dopamine antagonist, or SCH23390 (0.25 mg/kg), a D1/D5-selective antagonist, was given 30 min before the injection of D-amph (10 mg/kg) or saline. Surprisingly, these treatments had no effect on the basal GluR1 phosphorylation or on its increase by D-amph (Fig. 3, A and B). These results clearly showed that after D-amph administration in vivo, activation of D1/D5 receptors or D2-type receptors (blocked by haloperidol) had no appreciable consequence on the level of GluR1 phosphorylation in the prefrontal cortex.

We then evaluated the contribution of adenylyl cyclase-coupled β-AR to the PKA-dependent phosphorylation of GluR1. For this purpose, 30 min before D-amph or saline injection, mice were pretreated with propranolol at a dose (20 mg/kg i.p.) known to efficiently block all subtypes of brain β-AR (Stone et al., 1996). Propranolol decreased basal GluR1 phosphorylation and completely prevented the effects of D-amph in the prefrontal cortex (Fig. 3C). The role of β1-ARs was examined using a selective antagonist, betaxolol (20 mg/kg i.p.) (Stone et al., 1996). Betaxolol also decreased the basal GluR1 phosphorylation and completely abolished the response to D-amph in the prefrontal cortex (Fig. 3D). In contrast, ICI-118,551 (4 mg/kg i.p.), a selective β2-AR antagonist, failed to alter basal or D-amph–induced GluR1 phosphorylation at a dose known to be efficient in mouse brain (Stone et al., 1996) (Fig. 3D).

The dramatic effects of β1-AR blockers indicated the major role played by NE transmission in the D-amph effect on GluR1 phosphorylation. We also assessed the effects of blockade of α-ARs, which are involved in some behavioral effects of D-amph (Auclair et al., 2004). Pretreatment by the selective α1-AR antagonist prazosin (2 mg/kg i.p.) slightly but significantly enhanced the GluR1 phosphorylation produced by D-amph treatment in the prefrontal cortex without altering the basal GluR1 phosphorylation (Fig. 3E). In α1b-AR-null mice, D-amph effects on GluR1 phosphorylation were also enhanced compared with wild-type littermates (V. Pascoli, E. Valgent, and D. Hervé, unpublished observations), showing that the absence of α1b-AR had effects similar to those observed with prazosin pretreatment. In contrast, after pretreatment with the α2-AR antagonist yohimbine (3 mg/kg i.p.), basal GluR1 phosphorylation was significantly in-

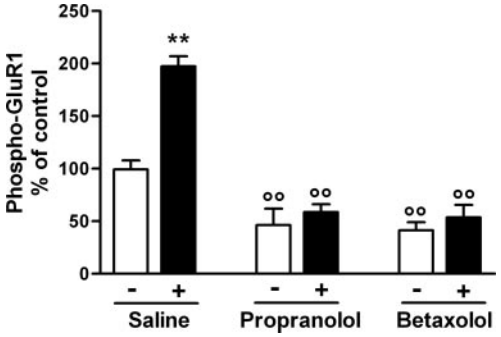

Fig. 4. β1-Adrenoceptors mediate phosphorylation of GluR1 by methylphenidate in the prefrontal cortex. β-AR antagonist propranolol (20 mg/kg), β1-AR antagonist betaxolol (20 mg/kg), or saline was administered 15 min before 10 mg/kg methylphenidate (+) or saline (–). Mice were killed 15 min after the second injection, and phosphorylation of GluR1 was determined by immunoblotting. Data are means ± S.E.M. (at least five mice per group). ANOVA followed by Newman-Keuls test: methylphenidate-treated versus saline, ⋆⋆, $p < 0.01$; antagonist treatment versus control, °°, $p < 0.01$.

creased (Fig. 3F), and no additional effect of D-amph was detected (Fig. 3F).

Given the key role played by β1-AR activation in the D-amph effect, we investigated its implication in MPH-induced phosphorylation of GluR1. We found that the MPH effects were completely prevented by blocking the β1-AR either not selectively with propranolol or selectively with betaxolol (Fig. 4).

Altogether, these results revealed that in the prefrontal cortex, both D-amph and MPH-induced phosphorylation of GluR1 and its basal phosphorylation resulted mainly from the activation of β1-AR but not significantly from that of dopamine receptors. It is interesting that NE neurotransmission seemed also to modulate the levels of GluR1 phosphorylation via α1-AR and α2-AR.

Regulation of ERK2 Phosphorylation in the Prefrontal Cortex by D-amph Involves Multiple Adrenoceptors. We investigated the respective role of dopamine receptors and ARs in the control of D-amph–induced ERK2 phosphorylation in the prefrontal cortex. Pretreatment with haloperidol or SCH23390 slightly decreased the basal phosphorylation of ERK2, because this effect was significant for haloperidol but not for SCH23390 (Fig. 5, A and B). However, these compounds did not prevent the increase in ERK2 phosphorylation produced by D-amph (Fig. 5, A and B). In contrast, pretreatment with the nonselective β-AR antagonist propranolol markedly reduced the effects of D-amph on ERK2 activation (Fig. 5C). This effect resulted from the blockade of β1-AR, because the selective β1-AR antagonist betaxolol (20 mg/kg i.p.) also decreased D-amph response, whereas the selective β2-AR antagonist ICI-118,551 (4 mg/kg i.p.) was ineffective (Fig. 5D). Pretreatment with prazosin (2 mg/kg i.p.) decreased significantly D-amph–stimulated phosphorylation of ERK2 (Fig. 5E) and tended to reduce the basal levels of ERK2 phosphorylation. After blockade of α2-AR by yohimbine (3 mg/kg i.p.), D-amph remained able to increase ERK phosphorylation in the prefrontal cortex, although its effect was slightly reduced (Fig. 5F). Altogether, these results revealed a major role of β1-AR in ERK2 activation in the prefrontal cortex after systemic D-amph administration and a significant contribution of α1-AR and α2-AR. The important role of NE receptors contrasted with the absence of significant implication of dopamine receptors.

Contribution of Serotonin Receptors to the Effects of D-amph. Because D-amph has the capacity to increase the extracellular concentration of 5HT (Kuczenski and Segal, 1997), GluR1 and ERK2 phosphorylation was investigated after inhibition of 5HT synthesis or blockade of 5-HT4 or 5-HT2 receptors (Table 1). These treatments, including blockade of 5-HT4 receptors positively coupled to adenylyl cyclase, did not alter the PKA-dependent phosphorylation of GluR1 in the prefrontal cortex of saline or D-amph–treated animals. In contrast, 5-HT depletion prevented D-amph–induced ERK2 activation without modifying basal ERK2 phosphorylation (Table 1). The blockade of 5-HT4 or 5-HT2 receptors did not alter the basal or D-amph–induced ERK2 phosphorylation (Table 1).

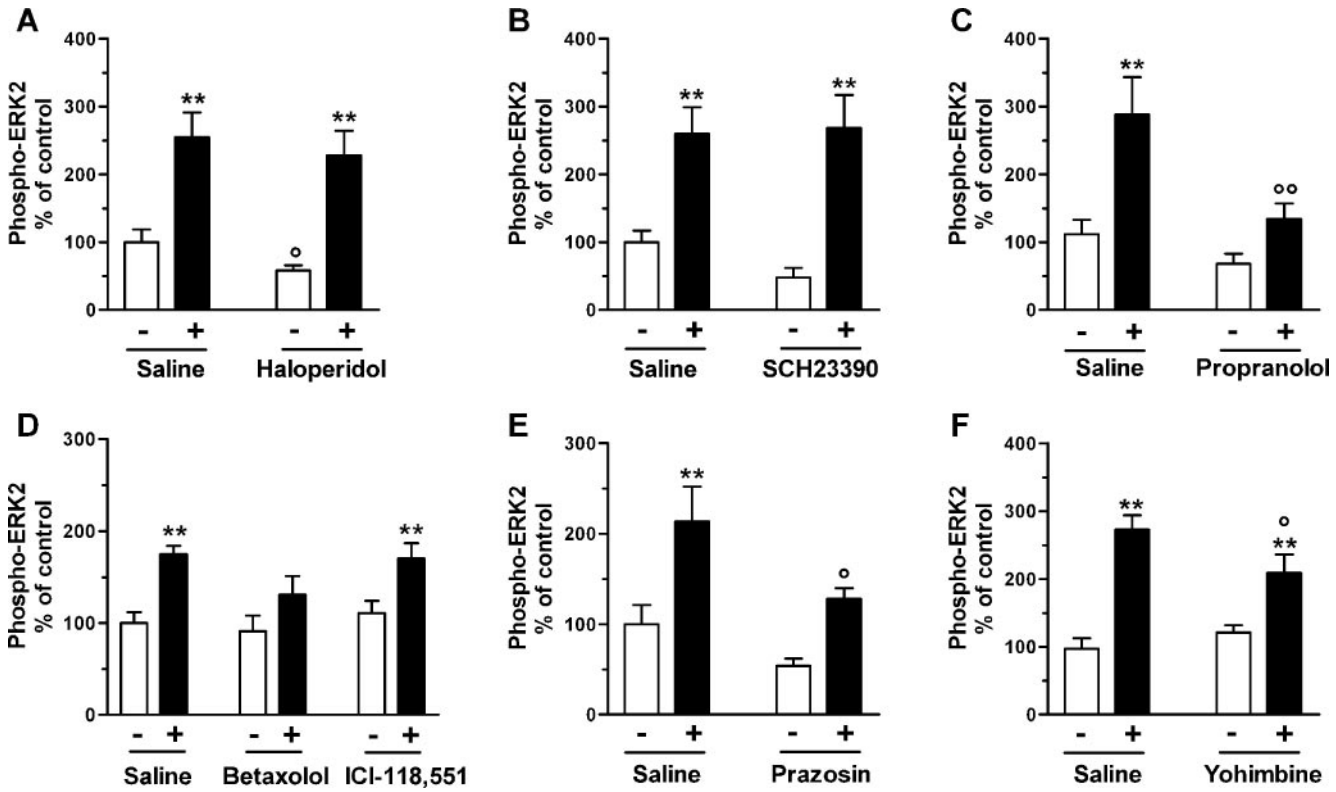

Fig. 5. Role of dopaminergic and adrenergic receptors in D-amphetamine-induced activation of ERK2 in the prefrontal cortex. Various antagonists (or vehicle) were administered 30 min before 10 mg/kg D-amph (+) or saline (–). Mice were killed 15 min after D-amph, and phosphorylation of ERK2 was determined by immunoblotting. A, DA receptor antagonist haloperidol (0.5 mg/kg). B, D1/5-receptor antagonist SCH23390 (0.25 mg/kg). C, β-AR antagonist propranolol (20 mg/kg). D, β1-AR antagonist betaxolol (20 mg/kg) and β2-AR antagonist ICI-118,551 (4 mg/kg). E, α1-AR antagonist prazosin (2 mg/kg). F, α2-AR antagonist yohimbine (3 mg/kg). Data are means ± S.E.M. (at least five mice per group). ANOVA followed by Newman-Keuls test: D-amph–treated versus saline, ⋆⋆, $p < 0.01$; antagonist treatment versus control, °, $p < 0.05$; °°, $p < 0.01$.

Role of Glutamate NMDA Receptor in the Effects of D-amph. We have reported previously that after D-amph administration, ERK activation but not GluR1 phosphorylation was dependent on glutamate NMDA receptor stimulation in the striatum and nucleus accumbens (Valjent et al., 2005). Therefore, we analyzed whether NMDA receptor could also play a role in the effects of D-amph on ERK and GluR1 phosphorylation in the prefrontal cortex. As illustrated in Fig. 6A, pretreatment with MK801 (0.5 mg/kg i.p.), an antagonist of NMDA receptor, decreased the basal phosphorylation of ERK2 and totally prevented the increase of ERK2 phosphorylation produced by D-amph. In contrast, we found that NMDA receptor blockade did not affect significantly basal or D-amph–induced GluR1 phosphorylation in the prefrontal cortex (Fig. 6B).

Role of β1-Adrenoceptors in the Acute Locomotor Response Induced by D-amph and MPH. Because our results revealed the importance of activation of β1-AR in the biochemical effects of D-amph in the prefrontal cortex, we investigated the possible role of these receptors in the behavioral effects of D-amph and MPH. We examined the consequences of β1-AR blockade on locomotor activation induced by these drugs. To avoid the stereotypies seen after the administration of 5 and 10 mg/kg D-amph and to obtain purely locomotor responses, we used 2 mg/kg D-amph for these experiments. Pretreatment with propranolol (20 mg/kg) or betaxolol (20 mg/kg) increased the locomotor activity induced by D-amph (Fig. 7, A and B) or MPH (Fig. 7, C and D). This enhancement seemed especially pronounced on the effects of MPH compared with those of D-amph (Fig. 7). In contrast, pretreatment with the β2-AR–selective antagonist ICI-118,551 (4 mg/kg i.p.) had no effect on acute locomotor responses induced by D-amph (data not shown).

Discussion

In the present work, we sought to gain insight into the mechanisms of action of D-amph and MPH in the prefrontal cortex by studying the effects of these drugs on cAMP- and ERK-mediated signaling pathways. We found that both drugs activated cAMP-regulated phosphorylation of AMPA receptor and that D-amph also activated ERK. Our results revealed a prominent role of NE and β1-AR activation in the actions of D-amph and MPH on AMPA receptor. They also showed that β1-AR activation reduced the hyperlocomotor effects of D-amph and MPH, suggesting its relevance for the therapeutic properties of these drugs in the treatment of ADHD.

Mechanisms of D-amph– and MPH-Induced Phosphorylation in the Prefrontal Cortex: Role of Noradrenergic Transmission. In the prefrontal cortex, D-amph has the ability to elevate the extracellular concentrations of NE, DA, and 5HT, whereas MPH increases only NE and DA without altering 5HT (Kuczenski and Segal, 1997; Berridge and Stalnaker, 2002). Thus, elevation of NE and/or DA could theoretically account for the PKA-mediated phosphorylation of GluR1 in response to D-amph or MPH. In the striatum, GluR1 Ser845 phosphorylation in response to psychostimulants involves the activation of D1/5 DA receptors and inhibition of protein phosphatase-1 by dopamine- and cAMP-regulated phosphoprotein (M_r 32,000) (Snyder et al., 2000). In contrast, our study reveals that D-amph and MPH-induced GluR1 phosphorylation in the prefrontal cortex depends mostly on NE. The effects of D-amph on GluR1 phosphorylation were unaltered by DA receptor antagonists but were mediated by β1-AR, which is the main subtype of β-AR expressed in the cerebral cortex (Nicholas et al., 1996). The role of dopamine- and cAMP-regulated phosphoprotein (M_r 32,000) in these effects in the prefrontal cortex remains to be tested because it has been shown to be phosphorylated at a PKA-specific site after administration of D-amph or various psychotomimetics (Svenningsson et al., 2003). α1-AR stimulation seemed to have a small inhibitory influence on the PKA-dependent phosphorylation of GluR1 because the phosphorylation was enhanced by the blockade of α1-ARs. A possible explanation of this role of α1-AR is that the intracellular Ca^{2+} mobilized by activation of these receptors stimulates a Ca^{2+}-dependent protein phosphatase able to dephosphorylate GluR1 in cortical neurons (Ehlers, 2000). Finally, blockade of α2-AR enhanced basal GluR1 phosphorylation, presumably because NE release is markedly increased when α2-AR presynaptic receptors are blocked (Florin et al., 1994). This explanation is supported by the lack of additivity between the effects of yohimbine and those of D-amph.

D-amph at 10 mg/kg but not MPH activated ERK in the prefrontal cortex. In the present study, the activation of

TABLE 1

Regulation of GluR1 and ERK2 phosphorylation by D-amphetamine in the prefrontal cortex: role of serotonin

Saline, ritanserin (5-HT2 antagonist, 2.5 mg/kg), or SDZ 205,557 (5-HT4 antagonist, 5 mg/kg) was injected intraperitoneally 30 min before D-amph or saline injection. 4-Chlorophenylalanine (p-CPA; 5HT synthesis inhibitor, 300 mg/kg i.p.) was injected daily during the 3 days preceding the D-amph treatment. Mice were killed 15 min after D-amphetamine or saline injection, and phosphorylation of GluR1 at Ser845 (P-GluR1) and of ERK2 at Thr183-Tyr185 (P-ERK2) was analyzed in the prefrontal cortex by immunoblotting with phosphospecific antibodies. Data are presented as means ± S.E.M. (at least five mice per group).

Treatment	P-GluR1		P-ERK2	
	Saline	D-amph	Saline	D-amph
Saline	100 ± 9	212 ± 24**	100 ± 19	255 ± 36**
p-CPA (3 × 300 mg/kg)	91 ± 9	181 ± 19**	102 ± 8	130 ± 9°°
Ritanserin (2.5 mg/kg)	100 ± 5	183 ± 20**	94 ± 18	189 ± 19**
SDZ 205,557 (5 mg/kg)	129 ± 2	223 ± 33**	139 ± 21	328 ± 51**

** $p < 0.01$, D-amph–treated versus saline; ANOVA followed by Newman-Keuls test.

°° $p < 0.01$, antagonist treatment versus control; ANOVA followed by Newman-Keuls test.

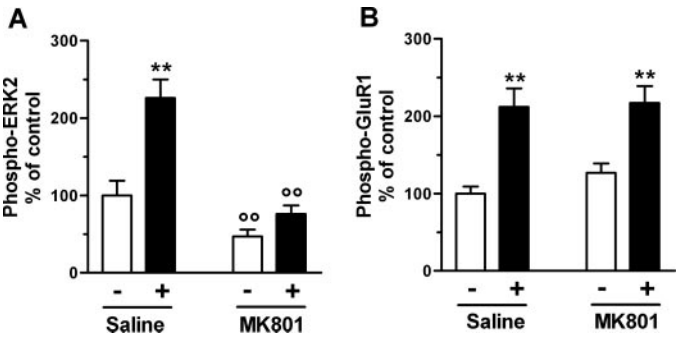

Fig. 6. Role of glutamatergic NMDA receptor in D-amphetamine–induced activation of ERK2 and GluR1 in the prefrontal cortex. The glutamatergic NMDA-receptor antagonist MK801 (0.1 mg/kg) was administrated 30 min before saline (–) or 10 mg/kg D-amph (+). Mice were killed 15 min after D-amph, and phosphorylation of ERK2 (A) and GluR1 (B) was determined by immunoblotting. Data are means ± S.E.M. (at least five mice per group). ANOVA followed by Newman-Keuls test: D-amph–treated versus saline, ★★, $p < 0.01$; antagonist treatment versus control, °°, $p < 0.01$.

β1-AR and α-AR resulting from elevation of extracellular NE seemed to mainly contribute to the ERK activation in the prefrontal cortex in response to D-amph. We cannot exclude a contribution of D1 receptors in the activation of ERK in specific neuronal populations of the prefrontal cortex because we reported previously that ERK activation by cocaine or other drugs of abuse was prevented in the deep layers of the prefrontal cortex by SCH23390 (Valjent et al., 2004). However, the effect was localized and probably quantitatively small because it was not detected in the present study, which used immunoblotting of the whole prefrontal cortex.

In contrast to the phosphorylation of GluR1 Ser845, the regulation of ERK seemed to require active glutamate NMDA receptors in addition to active ARs. Such a requirement has also been reported for the stimulation of ERK phosphorylation by CB1 receptors in the prefrontal cortex and in the hippocampus (Barbara et al., 2003; Derkinderen et al., 2003) and by dopamine D1 receptors in the striatum (Valjent et al., 2000). These results are consistent with the view that ERK activation is a coincidence detector that needs the combination of glutamate with other neurotransmitters (Valjent et al., 2005), the nature of which depends on brain regions: NE or endocannabinoids in prefrontal cortex, DA in the striatum, and endocannabinoids in the hippocampus. Additional mechanisms involving 5HT receptors could also contribute to ERK activation by D-amph in prefrontal cortex because it was prevented by 5HT depletion. The lack of effect of MPH on extracellular 5HT (Kuczenski and Segal, 1997) may account in part for its inability to stimulate ERK.

Possible Functional Consequences of Stimulation of cAMP- and ERK-Controlled Signaling Pathways by β-AR in the Prefrontal Cortex. Several lines of evidence suggest the implication of NE neurotransmission in the ability of psychostimulants to decrease hyperactivity and enhance attention in patients with ADHD. NE acting on β-ARs modulates the firing rate of cortical neurons in a way that increases the signal-to-noise ratio, an effect which may enhance attention and arousal (Berridge and Waterhouse, 2003). Drugs such as atomoxetine, desipramine, or nortriptyline, which block NE transporter more selectively than D-amph or MPH, were reported to be useful for treating hyperactivity in ADHD (Biederman and Spencer, 1999; Bymaster et al., 2002). In rat, MPH was reported to reduce locomotion particularly during the dark (active for rat) phase of the circadian cycle when orally administered at low doses that increase extracellular NE without significantly affecting dopamine (Kuczenski and Segal, 2002). In the present study, we found that blockade of β-ARs enhanced the hyperlocomotor effects of both D-amph and MPH, revealing the clear role of these receptors in the regulation of locomotor behavior in mice. This finding is consistent with previous results showing an increased response to cocaine (Harris et al., 1996) and D-amph (Vanderschuren et al., 2003) after β-AR blockade in the rat. The hyperlocomotor responses to D-amph or MPH are believed to result mostly from the activation of dopamine receptors in the ventral striatum (Di Chiara and Imperato, 1988) and to require also α1b-AR and 5-HT2 receptors (Auclair et al., 2004). The present results suggest that β1-AR activation has an opposite action and tends to reduce the locomotor activity of mice. We propose that this action of β1-AR opposing locomotor activation could be particularly relevant in the context of ADHD and could contribute to the paradoxical calming effects of D-amph and MPH in this condition.

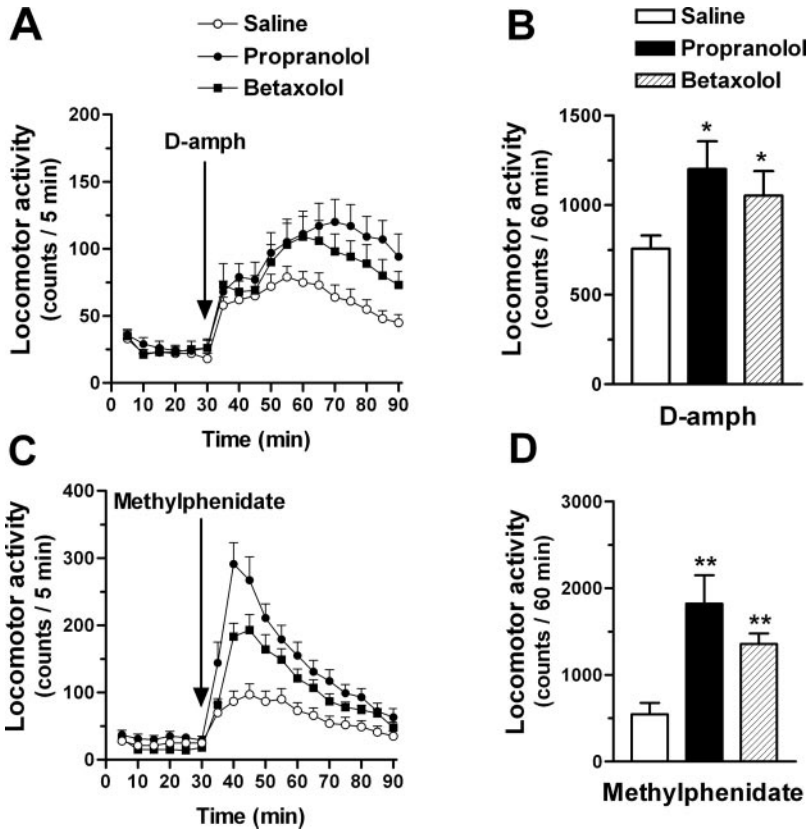

Fig. 7. Blockade of β1-adrenergic receptors increases hyperlocomotor effects of D-amphetamine and methylphenidate. A, time course of locomotor activity induced by D-amph (2 mg/kg i.p.), measured in 5-min intervals. Mice were pretreated with saline, propranolol (20 mg/kg), or betaxolol (20 mg/kg) 30 min before D-amph. Data were analyzed with two-way ANOVA: effects of treatments ($F_{2, 846} = 33.3$, $p < 0.001$) and time ($F_{17, 846} = 28.4$, $p < 0.001$) were significant. B, total locomotor activity during the 60 min after D-amph injection in propranolol- and saline-pretreated mice (ANOVA followed by Newman-Keuls test: $\star$, $p < 0.05$). C, mice were pretreated as in A and received methylphenidate 30 min later (MPH, 10 mg/kg). The effects of treatments ($F_{2, 774} = 87.4$, $p < 0.001$) and time ($F_{17, 774} = 57.70$, $p < 0.001$) were significant (two-way ANOVA). D, total locomotor activity during the 60 min after MPH injection (ANOVA followed by Newman-Keuls test: $\star\star$, $p < 0.01$). Data are means ± S.E.M. (at least 10 mice per group).

β1-AR–dependent phosphorylation of GluR1 could be directly implicated in the therapeutic effects of psychostimulants because it is induced by both D-amph and MPH, two drugs of choice for treating ADHD. GluR1 phosphorylation at Ser845 enhances the AMPA channel currents and thereby could increase neuronal activity in the prefrontal cortex (Banke et al., 2000). In addition, GluR1 phosphorylation is involved in synaptic plasticity, as demonstrated in the hippocampus, in which phosphorylation of GluR1 at Ser845 is modulated during long-term potentiation and long-term depression, and seems to regulate membrane insertion and recycling of GluR1-containing AMPA receptors (Lissin et al., 1999; Lee et al., 2003). Mice bearing point mutations in two GluR1 phosphorylation sites including Ser845 display memory defects in spatial learning tasks (Lee et al., 2003). Thus, by analogy, the increase in GluR1 phosphorylation at Ser845 produced by D-amph and MPH treatment could have positive effects on synaptic plasticity in the prefrontal cortex and on related learning processes.

Several studies have shown that various memory tasks require the activation of ERK within specific brain regions for the consolidation of long-term memory but not for short-term memory or acquisition (Derkinderen et al., 1999; Thomas and Huganir, 2004). Long-term memory storage takes place in the prefrontal cortex and involves ERK activation (Runyan et al., 2004). It is interesting that a recent study in human subjects supports the positive role of D-amph on synaptic plasticity in the cortex (Nitsche et al., 2004). Thus, our results raise the possibility that D-amph–induced ERK activation mimics processes involved in long-term memory formation in the prefrontal cortex. However, this effect was apparent only with the highest dose of D-amph used and was not observed with MPH, a very effective compound for improving cognitive functions in patients with ADHD. In contrast, D-amph seems more addictive than MPH, and it is possible that the effect of D-amph on ERK is more related to its addictive properties than to its therapeutic effects. In support of this hypothesis, a common effect of numerous drugs of abuse is the activation of ERK in neurons of deep layers of the prefrontal cortex, in addition to the nucleus accumbens (Valjent et al., 2004, 2005). This activation is functionally relevant because the blockade of ERK activation in brain abolished the cocaine-conditioned place preference, a test of the rewarding properties of cocaine (Valjent et al., 2000) and D-amph (Gerdjikov et al., 2004). Although it is not possible to determine the relative contributions of prefrontal cortex and ventral striatum in these effects, these results are consistent with a role of ERK in the addictive properties of psychostimulants.

In conclusion, the results reported here provide evidence that peripheral administration of D-amph or MPH potently activates cAMP-dependent intracellular signaling in the prefrontal cortex in vivo. This is caused by the stimulation of noradrenergic transmission, mostly through β1-AR. Our finding that β1-AR blockade potentiates the locomotor activation induced by D-amph or MPH suggests that stimulation of these receptors may contribute to the therapeutic effects of D-amph and MPH in patients with ADHD. In contrast, the activation of ERK by D-amph might be more related to its addictive properties.

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