Paradoxical Striatal Cellular Signaling Responses to Psychostimulants in Hyperactive Mice*

Jean-Martin Beaulieu1, Tatyana D. Sotnikova, Raul R. Gainetdinov, and Marc G. Caron2

From the Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

Recent investigations have shown that three major striatal-signaling pathways (protein kinase A/DARPP-32, Akt/glycogen synthase kinase 3, and ERK) are involved in the regulation of locomotor activity by the monoaminergic neurotransmitter dopamine. Here we used dopamine transporter knock-out mice to examine which particular changes in the regulation of these cell signaling mechanisms are associated with distinct behavioral responses to psychostimulants. In normal animals, amphetamine and methylphenidate increase extracellular levels of dopamine, leading to an enhancement of locomotor activity. However, in dopamine transporter knock-out mice that display a hyperactivity phenotype resulting from a persistent hyperdopaminergic state, these drugs antagonize hyperactivity. Under basal conditions, dopamine transporter knock-out mice show enhanced striatal DARPP-32 phosphorylation, activation of ERK, and inactivation of Akt as compared with wild-type littermates. However, administration of amphetamine or methylphenidate to these mice reveals that inhibition of ERK signaling is a common determinant for the ability of these drugs to antagonize hyperactivity. In contrast, psychostimulants activate ERK and induce hyperactivity in normal animals. In hyperactive mice psychostimulant-mediated behavioral inhibition and ERK regulation are also mimicked by the serotonergic drugs fluoxetine and 5-carboxamidotryptamine, thereby revealing the involvement of serotonin-dependent inhibition of striatal ERK signaling. Furthermore, direct inhibition of the ERK signaling cascade in vivo using the MEK inhibitor SL327 recapitulates the actions of psychostimulants in hyperactive mice and prevents the locomotor-enhancing effects of amphetamine in normal animals. These data suggest that the inhibitory action of psychostimulants on dopamine-dependent hyperactivity results from altered regulation of striatal ERK signaling. In addition, these results illustrate how altered homeostatic state of neurotransmission can influence in vivo signaling responses and biological actions of pharmacological agents used to manage psychiatric conditions such as Attention Deficit Hyperactivity Disorder (ADHD).

Dopaminergic neurotransmission mediates a series of physiological functions ranging from the control of locomotion and cognition to attention, emotion, and reward (1–3). This neurotransmitter system is also the main target for the actions of psychostimulants. By interfering with dopamine transporter (DAT)3 functions, compounds like methylphenidate and amphetamine normally increase extracellular levels of dopamine, leading to their well known locomotor-promoting/stimulant effect. However, under certain conditions psychostimulants can antagonize hyperactivity through a mechanism of action that is not well understood. Indeed, psychostimulants are used therapeutically to manage the symptoms of hyperactivity, impulsivity, and inattention associated with Attention Deficit Hyperactivity Disorder (ADHD), a common psychiatric condition affecting a significant portion of pediatric and adult populations (4–6). The therapeutic efficacy of psychostimulants that typically enhance monoaminergic neurotransmission has led to the postulate that imbalance in dopamine (DA), noradrenaline, and/or serotonin (5HT) synaptic transmission may contribute to the etiology of ADHD (7–9). In this regard, mice lacking the DAT (DAT-KO) have been previously shown to exhibit behavioral abnormalities that recapitulate some endophenotypes of ADHD (10). When placed in a novel environment, DAT-KO mice develop perseverative hyperlocomotor activity and cognitive abnormalities consistent with enhanced DA neurotransmission (2, 10–12). Moreover, the same psychostimulants that induce locomotor hyperactivity in normal mice exert a paradoxical antihyperkinetic effect in DAT-KO mice (10), thus providing a model system to identify potential mechanisms by which psychostimulants, instead of exerting their normal stimulatory action, can counteract these behavioral manifestations.

Recent investigations on the mechanisms of action of psychostimulants and other psychoactive compounds have shown that many of these drugs can act simultaneously on multiple neurotransmitter systems (13), thus suggesting that distinct cellular signaling mechanisms may determine their pharmacological actions (14–20). Three major signaling pathways have been shown to be associated with striatal DA neurotransmission and concomitant locomotor responses to psychostimulants. First, activation or inhibition of the cAMP pathway through D1 and D2 dopamine receptors leads to regulation of protein kinase A and modulation of the dopamine and cAMP-regulated phosphoprotein, 32 kDa (DARPP-32), an inhibitor of

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1 A NARSAD Southwest Florida Investigator and the recipient of a fellowship from the Canadian Institutes of Health Research (CIHR).
2 To whom correspondence should be addressed: 487 CARL Bldg., Box 3287, Duke University Medical Center, Research Dr., Durham, NC 27710. Tel.: 919-684-5433; Fax: 919-681-8641; E-mail: m.caron@cellbio.duke.edu.

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protein phosphatase-1 (21). Phosphorylation of DARPP-32 on Thr-34 has been associated with locomotor activation and biochemical responses to multiple psychotropic drugs, including psychostimulants (14, 16, 17). Second, a distinct signaling pathway involves the serine/threonine kinase Akt that is negatively regulated by D2-class receptors through a signaling complex involving protein phosphatase-2A and β-arrestin 2 (14). Stimulation of dopamine D2-class receptors leads to a dephosphorylation of Akt on its regulatory Thr-308 residue (14, 15). Inactivation of Akt by DA results in activation of glycogen synthase kinase 3, which in turn contributes to the development of locomotor hyperactivity (14, 15). In addition, DA also positively regulates ERK by acting through multiple signaling mechanisms that involve D1 and/or D2-class dopamine receptors (18–20, 22, 23). Although ERK has been shown to participate in acute responses to cocaine (19), it appears to be mostly involved in the development of long-term changes of gene expression, synaptic plasticity, and locomotor responses following repeated exposure to this drug (19, 24, 25).

Here we have used DAT-KO mice to investigate the involvement of striatal signaling events in the inhibitory action of psychostimulants on hyperactivity. We demonstrate that in the presence of an overactive brain dopaminergic system created by the absence of DAT, inhibition of ERK signaling is a common property that accounts for the action of psychostimulants to

FIGURE 1. Elevated DA-associated cellular signaling in DAT-KO mice. Western blot analysis of dopamine-associated PKA/DARPP32 (A), Akt (B), and ERK2 (C) signaling as measured by phosphoprotein levels in the striatum of DAT-KO mice as compared with WT littermates. n = 5–10 mice/group. Data are average ± S.E. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.005.

FIGURE 2. Psychostimulants antagonize ERK activity and locomotion in hyperactive mice. Locomotor activity and phosphoprotein levels following administration of methylphenidate 30 mg/kg intraperitoneal (A, B) or amphetamine 2 mg/kg intraperitoneal (C, D) to DAT-KO mice. For locomotor activity measurements, mice were placed in an activity monitor, habituated for 30 min, and monitored for 90 min following drug administration. A and C, locomotor activity was continuously recorded as total distance traveled in blocks of 5 min. Arrows indicate drug administration; n indicates the number of animals used per experiment. Data are average ± S.E. B and D, Western blot analyses of pDARPP32, pAkt, and pERK2 were carried out from striata collected at time points for which drugs showed significant behavioral action: methylphenidate 90 min post-injection, amphetamine 60 min post-injection. V, vehicle; M, methylphenidate; A, amphetamine. B and D, n = 5 mice/group. Data are average ± S.E. *, p ≤ 0.05.
diminish the hyperactivity of DAT-KO mice. These findings may have implications for the mechanisms by which psychostimulants achieve their pharmacological effects in ADHD.

MATERIALS AND METHODS

Experimental Animals—C57BL/129SvJ DAT-KO and WT littermates were described previously (11, 26). These mice have been inbred for more than 30 generations to generate mice sharing a similar genetic background between KO and WT control animals. For all experiments mice of 3 to 4 months of age were used. Before experiments, animals were housed four or five to a cage at 23 °C on a 12-h light/12-h dark cycle with ad libitum access to food and water. Animal care was approved by the Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines.

Antibodies—The anti-phospho-Akt (Thr-308), anti-total-Akt, antiphospho-ERK1/2 (Thr-202/Tyr-204), anti-ERK were purchased from Cell Signaling Technology (Beverly, MA). The anti-phospho-DARPP-32 Thr-34 was from Phosphosolutions (Aurora, CO). The anti-DARPP-32 was obtained from BD Transduction Laboratories (Lexington, KY).

Drug Administration—Amphetamine (Sigma), methylphenidate (Sigma), and 5-carboxamidotryptamine (5CT; Sigma) were dissolved in saline and injected intraperitoneally. Fluoxetine (Tocris Cookson Inc., Ellisville, MO) was dissolved in water and injected subcutaneously. SL327 (Tocris Cookson Inc.) was injected intraperitoneally after suspension in a minimal amount of Tween and made up to volume with distilled water. Corresponding vehicle solutions were administered to control animals.

Western Blot Analyses—Western blot analyses were performed as described in Beaulieu et al. (15). For quantitative analysis, total proteins were used as loading controls for phosphoprotein signals.

Measurement of Locomotor Activity—Locomotion was evaluated under illuminated conditions in an automated Omnitech Digiscan apparatus (AccuScan Instruments, Columbus, OH). Locomotor activity was measured in terms of the total distance covered (horizontal activity), and the stereotypy time refers to the total time that stereotypic behaviors (repetitive beam breaks of a given beam or beams with intervals <1 s) were observed (10).

Statistical Analyses—Data were analyzed by two-tailed t test or one-way analysis of variance. Values in graphs were expressed as mean ± S.E. n represents the number of animals used for each experiment.
RESULTS

To examine the activity of DA-associated signaling molecules in hyperactive DAT-KO mice under basal conditions, we performed Western blot analysis on striatal extracts prepared from DAT-KO mice and control wild-type (WT) littermates. This analysis revealed an elevation of Thr-34-DARPP-32 phosphorylation in DAT-KO mice (Fig. 1A). As previously reported (14, 15), DAT-KO mice also exhibited reduced phosphorylation of Akt on its regulatory Thr-308 residue (Fig. 1B). Finally, enhanced phosphorylation/activation of ERK2 was detected using an anti-phospho-ERK1/2 antibody (Fig. 1C), thus indicating that all three signaling pathways were responding to persistently enhanced DA neurotransmission in the striatum of DAT-KO mice.

Administration of the psychostimulants methylphenidate and amphetamine to DAT-KO mice resulted in a marked reduction of locomotor activity (Fig. 2, A and C). To identify common signaling events underlying this behavioral effect, we examined the impact of psychostimulants on phospho-DARPP-32, phospho-Akt, and phospho-ERK2 levels in hyperactive DAT-KO mice. Relative phosphoprotein levels were measured by Western blots in striatal extracts obtained from DAT-KO mice treated either with methylphenidate (30 mg/kg) or amphetamine (2 mg/kg) under conditions where a maximal effect on locomotion was observed. As shown in Fig. 2B, administration of methylphenidate to DAT-KOs resulted in a modest dephosphorylation of Thr-308-Akt and in a more pronounced inhibition of ERK2 while leaving DARPP-32 essentially unaffected. In comparison, treatment of DAT-KO mice with amphetamine led to a slight increase in Akt phosphorylation and a reduction of ERK2 phosphorylation (Fig. 2D), thus indicating that inactivation of ERK is a common effect of psychostimulants in DAT-KO mice.

The paradoxical behavioral action of psychostimulants in hyperactive DAT-KO mice has been related to changes in 5HT neurotransmission (10, 27). We therefore evaluated the effect of serotonergic drugs on behavior and signaling protein phosphorylation. The selective serotonin reuptake inhibitor fluoxetine exerts its pharmacological action by blocking 5HT transporter-mediated reuptake, thus enhancing extracellular 5HT levels (28). As shown previously (10), fluoxetine (20 mg/kg) had a powerful inhibitory effect on hyperactivity in DAT-KO mice (Fig. 3A). Western blots prepared from fluoxetine or vehicle-treated DAT-KO mice showed that this specific serotonin reuptake inhibitor produced a potent inhibition of ERK2 under conditions leading to suppression of locomotor hyperactivity (Fig. 3B). Notably, fluoxetine had no significant effect on Akt or DARPP-32 phosphorylation (Fig. 3B). To further support these observations we then used 5CT a non-selective 5HT receptor agonist (29–31). Administration of 5CT (0.1 mg/kg) produced marked and prolonged suppression of hyperactivity in DAT-KO mice (Fig. 3C). Remarkably, this behavioral effect of 5CT was correlated with reduced ERK phosphorylation without any effect on Akt or DARPP-32 (Fig. 3D).

To examine the dynamic of ERK regulation and its correlation with the behavioral action of psychostimulants, phospho-ERK levels were measured at different time points following administration of amphetamine (2 mg/kg) or methylphenidate (30 mg/kg). Amphetamine caused a sustained dephosphorylation of ERK that reached its maximum at 60 min and lasted over a period of 120 min post-injection (Fig. 4A), thus correlating with the long lasting action of this drug on hyperactivity in DAT-KO mice. In contrast, the action of methylphenidate on ERK phosphorylation was progressive and reached its maximum at 90 min post-injection (Fig. 4B). Taken together, these results indicate that inhibition of ERK represents a common biochemical outcome of psychostimulants and 5HT drugs that suppress locomotor hyperactivity in DAT-KO mice.

Psychostimulants are potent inducers of locomotor hyperactivity in normal rodents. Furthermore, administration of the psychostimulant cocaine is known to activate ERK in the striatum (18, 19), thus suggesting that changes in ERK signaling induced by psychostimulants in hyperactive mice may differ

FIGURE 4. Time course of striatal ERK2 regulation by psychostimulants and fluoxetine. ERK2 phosphorylation was measured by Western blot analysis in the striatum of DAT-KO mice after injection of amphetamine (2 mg/kg, intraperitoneal) (A) or methylphenidate (30 mg/kg, intraperitoneal) (B). n = 4–10 mice/group. Data are average ± S.E. *, p ≤ 0.05.
FIGURE 5. Behavioral and biochemical actions of psychostimulants in normal mice. Locomotor activity and phospho-ERK levels following administration of methylphenidate 30 mg/kg intraperitoneal (A, B), amphetamine 2 mg/kg intraperitoneal (C, D) to WT mice. For locomotor activity measurements, mice were placed in an activity monitor, habituated for 30 min, and monitored for 90 min following drug administration. A and C, locomotor activity was continuously recorded as total distance traveled in blocks of 5 min. Arrows indicate drug administration; n indicates the number of animals used/experiment. Data are average ± S.E. Western blot analyses of pERK2 levels from striatal extracts were collected under conditions used in previous experiments: methylphenidate 90 min post-injection, amphetamine 60 min post-injection. B and D, V, vehicle; M, methylphenidate; A, amphetamine; n = 5 mice/group. Data are average ± S.E. *, p ≤ 0.05.

FIGURE 6. Behavioral and biochemical actions of serotonergic drugs in normal mice. Locomotor activity and phospho-ERK levels following administration of fluoxetine 20 mg/kg subcutaneously (A, B) or 5-carboxamidotryptamine (5CT) 0.1 mg/kg intraperitoneally (C, D) to WT mice. For locomotor activity measurements, mice were placed in an activity monitor, habituated for 30 min, and monitored for 90 min following drug administration. A and C, locomotor activity was continuously recorded as total distance traveled in blocks of 5 min. Arrows indicate drug administration; n indicates the number of animals used/experiment. Data are average ± S.E. Western blot analyses of pERK2 levels from striatal extracts were collected under conditions used in previous experiments: fluoxetine 20 min post-injection, 5CT 30 min post-injection. B and D, V, vehicle; F, fluoxetine; 5CT, 5-carboxamidotryptamine; n = 5 mice/group. Data are average ± S.E. *, p ≤ 0.05.
from their action in normal animals. To test this possibility, we proceeded to evaluate the action of psychostimulants and 5HT drugs on locomotion and ERK signaling in WT animals. As expected, administration of methylphenidate or amphetamine to WT mice resulted in a marked increase of locomotor activity (Fig. 5, A and C). Moreover, instead of inhibiting ERK activity like in DAT-KO mice, these two psychostimulants enhanced ERK2 phosphorylation in the striatum of WT littermates (Fig. 5, B and D). In contrast, administration of the 5HT drugs fluoxetine and 5CT to WT mice resulted in noticeable reduction in basal locomotor activity and in a significant reduction of ERK2 phosphorylation at least in the case of 5CT (Fig. 6, A–D). These results indicate that psychostimulants not only induce opposite behavioral locomotor effects in WT and DAT-KO mice but that these effects are paralleled by paradoxical changes in ERK-mediated signaling. Moreover, both the behavioral and signaling effects of psychostimulants in hyperactive mice are similar to those triggered by 5HT drugs.

To determine the impact of ERK signaling in the development of DA-dependent locomotor hyperactivity, DAT-KO mice were treated with SL327 (100 mg/kg), a blood brain barrier-permeable inhibitor of the ERK kinase mitogen-activated protein kinase/ERK kinase (MEK) that has been used to reduce ERK activity in WT mice (18, 19, 32). As shown by Western blot analysis, SL327 produced a robust reduction of phospho-ERK level in the striatum of DAT-KO mice (Fig. 7, A and B). Measurement of locomotor activity in vehicle or SL327-treated DAT-KO mice also revealed a reduction of hyperactivity and stereotypy in SL327-treated mice (Fig. 7, C and D). To extend our observations to a pharmacological model of DA-induced hyperactivity associated with increased ERK phosphorylation (Fig. 5, C and D), we assessed the role of ERK on the development of behavioral locomotor responses to amphetamine in WT mice. As shown in Fig. 8A, administration of SL327 (100 mg/kg) had little effect on locomotion in habituated WT mice. However, pretreatment of WT mice with SL327 markedly reduced locomotor activation by amphetamine (Fig. 8, A and B). Amphetamine-induced stereotypy was similarly reduced by SL327 (Fig. 8C), indicating that differences in locomotor activity were not due to a competing enhancement of stereotypy. Moreover, inhibition of ERK signaling specifically antagonized DA-associated hyperactivity because SL327 administration had...
no significant effect upon locomotor activation induced by the glutamate NMDA receptor antagonist MK801 in WT mice (Fig. 9).

**DISCUSSION**

The molecular mechanisms by which psychostimulants can induce antihyperkinetic effects in ADHD has remained unexplained. We have used DAT-KO mice that display altered behavioral responses to psychostimulants (10) to investigate the effect of these drugs on the three major striatal signaling pathways (ERK, protein kinase A/DARPP-32, and Akt) known to participate in the development of hyperlocomotor responses to DA (14–16, 19). The results presented here show that in the striatum of hyperactive animals psychostimulants negatively regulate ERK signaling as opposed to their ability to activate this pathway in normal animals. Moreover, pharmacological enhancement of 5HT neurotransmission reduced hyperactivity and also mimicked the effect of psychostimulants on ERK-mediated signaling in DAT-KO mice. Finally, direct inhibition of ERK signaling reduced hyperactivity in DAT-KO mice and abolished locomotor responsiveness to amphetamine in WT animals. These findings indicate that psychostimulant action on hyperactivity can be associated with distinct signaling responses to these drugs in the brain of hyperactive animals.

DA exerts its functions by acting on two classes of G protein-coupled receptors. The D1-class receptors (D1 and D5) are mostly coupled to Gs, whereas D2-class receptors (D2S, D2L, D3, and D4) are coupled to Gi/Go (33, 34). Furthermore, D2-class receptors have recently been shown to regulate the formation of a β-arrestin 2 signaling complex regulating Akt-mediated signaling responses (14). At least three independent or partially independent signaling pathways, the protein kinase A/DARPP32, Akt/glycogen synthase kinase 3, and ERK pathways, have been shown to participate in the regulation of locomotion by DA in rodents (14, 15, 21, 35). These same pathways are also involved in the responsiveness to other neurotransmit-
potentially and psychoactive drugs (36, 37), thus underscoring their function as signal integrators that participate in the control of behavior. The observation that psychostimulants inhibit ERK signaling in hyperactive DAT-KO mice as opposed to their normal activation of this pathway in WT animals indicates that changes in the regulation of DA neurotransmission can lead to dramatic alterations in the response of signaling pathways to specific psychotropic drugs. It is highly possible that such changes affecting DA or other neurotransmitter systems may also occur in the brains of subjects with psychiatric conditions or addicted to drugs, leading to development of distinct responses to different psychoactive compounds. In conclusion, the existence of a paradoxical regulation of ERK-mediated signaling by psychostimulants in DA-dependent hyperactivity underscores the need to explore cellular signaling mechanisms in model systems of altered neurotransmission. A re-examination of psychotrophic drug action with regard to integrative signaling processes in such model systems could help identify cellular mechanisms responsible for their therapeutic effects on specific endophenotypes of mental disorders.  

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Psychostimulant-induced Paradoxical Signaling

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