Brief Communications

Regulation of Akt Signaling by D_2 and D_3 Dopamine Receptors *In Vivo*

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The serine/threonine kinase Akt is a downstream target of dopamine receptor signaling that is inhibited/dephosphorylated in response to direct and indirect dopamine receptor agonists. Although pharmacological studies uncovered the involvement of D_2-class dopamine receptors in Akt regulation, they did not identify the role of individual receptor subtypes in this process. Here we used knock-out mice lacking the D_1, D_2, D_2 long, or D_3 dopamine receptors as well as a D_4 receptor-selective antagonist to address the function of each of these receptors in the regulation of Akt *in vivo*. Under basal conditions, D_2, D_2 long, and D_3 knock-out mice display enhanced striatal Akt activation, whereas D_1 knock-out mice and mice treated with the D_4 receptor antagonist L745870 (3-[[4-(4-chlorophenyl)piperazin-1-yl]methyl]-1*H*-pyrrolo[2,3-b]pyridine trihydrochloride) have phospho-Akt levels comparable with those of normal control animals. Furthermore, both amphetamine and apomorphine lose their ability to inhibit Akt in D_2 knock-out mice but retain their normal effect on this signaling molecule in D_1 knock-out animals. Finally, D_3 knock-out mice show a reduced sensitivity of Akt-mediated signaling to dopaminergic drugs but retain the action of these drugs on Akt at high dose regimens. These results indicate that D_2 receptors are essential for the inhibition of Akt by dopamine and that D_3 receptors also participate in this signaling potentially by enhancing D_2 receptor response. Identification of the functions of individual dopamine receptor subtypes in Akt regulation may help the development of new pharmaceutical approaches for mental disorders related to abnormal dopamine transmission such as bipolar disorder and schizophrenia.

Key words: **dopamine; Akt; D_2 receptors; D_3 receptors; amphetamine; apomorphine; knock-out; signaling**

Introduction

The monoaminergic neurotransmitter dopamine (DA) has been implicated in multiple brain disorders, including schizophrenia, affective disorders, addiction, and Parkinson's disease (Snyder, 1976; Carlsson, 1987; Gainetdinov and Caron, 2003). In the brain, the main dopaminergic neuron population arises from the substantia nigra pars compacta and projects to striatal neurons. Two subclasses of G-protein-coupled receptors (GPCRs) mediate the various physiological functions of DA (Kebabian and Calne, 1979). D_1-class receptors (D_1 and D_5 subtypes) are mostly coupled to $G\alpha_s$ and enhance the production of cAMP, whereas D_2-class receptors (D_2, D_3, and D_4 subtypes) are coupled to $G\alpha_{i/o}$ and inhibit this same process (Kebabian and Greengard, 1971; Enjalbert and Bockaert, 1983; Missale et al., 1998). Moreover, an alternate splicing of the D_2 receptor mRNA leads to the expression of two D_2 receptor isoforms, the D_2 short (D_2S) and D_2 long (D_2L), which have been associated with presynaptic and postsynaptic D_2 receptor functions, respectively (Giros et al., 1989; Monsma et al., 1989; Usiello et al., 2000; Lindgren et al., 2003).

Recent *in vivo* studies revealed that striatal D_2-class receptors also exert their action in a cAMP-independent manner by promoting the formation of a signaling complex composed of Akt, protein phosphatase-2A (PP2A), and β-arrestin 2 (Beaulieu et al., 2004, 2005). Formation of this complex leads to the inactivation of Akt after the dephosphorylation of its regulatory threonine 308 (Thr-308) residue by PP2A (Beaulieu et al., 2005). Inactivation of Akt in response to DA results in the activation of glycogen synthase kinase 3 (GSK3), which in turn contributes to the expression of DA-associated behaviors (Beaulieu et al., 2004). Interestingly, reduced Akt functions have been reported in schizophrenic patients, whereas administration of the antipsychotic haloperidol, a D_2-class receptor antagonist, activates Akt and inhibits GSK3 in the mouse brain (Emamian et al., 2004). However, the characterization of Akt and GSK3 regulation by DA receptors has remained limited to the use of pharmacological agents that do not allow the delineation of individual roles played by specific subtypes of DA receptors in this process (Beaulieu et al., 2004; Emamian et al., 2004). Here we used mice lacking D_1, D_2, D_2L, or D_3 dopamine receptors to elucidate the functions of each of these GPCRs in the regulation of Akt *in vivo*. Our results

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indicate that the negative regulation of Akt by DA and dopaminergic drugs is dependent on D_2 receptors and, to a lesser extent, on D_3 receptor activation.

Materials and Methods

Experimental animals. D_1 receptor knock-out (D_1-KO) (Drago et al., 1994), D_2 receptor knock-out (D_2-KO) (Baik et al., 1995), D_2L receptor knock-out (D_2L-KO) (Usiello et al., 2000), D_3 receptor knock-out (D_3-KO) (Joseph et al., 2002), DA transporter knock-out (DAT-KO) (Giros et al., 1996; Cyr et al., 2003), and their respective wild-type (WT) littermates have been described previously. All mice used were from 3 to 4 months of age. For all experiments, test and control groups were composed of age- and sex-matched animals with ~50% mice from each sex. Animals were housed four or five to a cage at 23°C on a 12 h light/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.

Drug administration. Amphetamine (Sigma, St. Louis, MO) and L745870 (Tocris Cookson, Ellisville, MO) were dissolved in saline and injected intraperitoneally. Apomorphine (Sigma) was dissolved in distilled water containing 0.1% ascorbate and injected subcutaneously. Corresponding vehicle solutions were administered to control animals.

Western blot analysis. Western blot were performed as described previously (Beaulieu et al., 2004). Briefly, mice were killed by decapitation, after which the heads of the animals were immediately cooled by immersion in liquid nitrogen for 6 s. The right hemistriatum was rapidly dissected out (within 30 s) on an ice-cold surface and frozen in liquid nitrogen before protein extraction. Tissue samples were homogenized in boiling 1% SDS solution supplemented with 2 μM okadaic acid and boiled for 10 min. Protein concentration was measured by using a DC-protein assay (Bio-Rad, Hercules, CA). Protein extracts (25 or 50 μg) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Blots were immunostained overnight at 4°C with the following primary antibodies: anti-phospho-GSK3/Ser-21/9 (1:200 dilution); anti-phospho-Akt Thr-308 (1:100); anti-phospho-Akt Ser-473 (1:500); anti-GSK3/clone 0011-A (1:5000); and anti-Akt (1:1000). Immune complexes were detected using appropriate peroxidase-conjugated secondary antibodies along with a chemiluminescent reagent (SuperSignal West-Pico; Pierce, Rockford, IL). Densitometric analysis was performed within linear range by using IMAGEQUANT version 1.1 (GE Healthcare, Piscataway, NJ). Total protein signal was used as loading controls for phospho-proteins. Results are normalize to respective control conditions and presented as means ± SEM. Data were analyzed by two-tailed *t* test. Anti-GSK3/clone 0011-A was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other primary antibodies were from Cell Signaling Technology (Beverly, MA). Secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

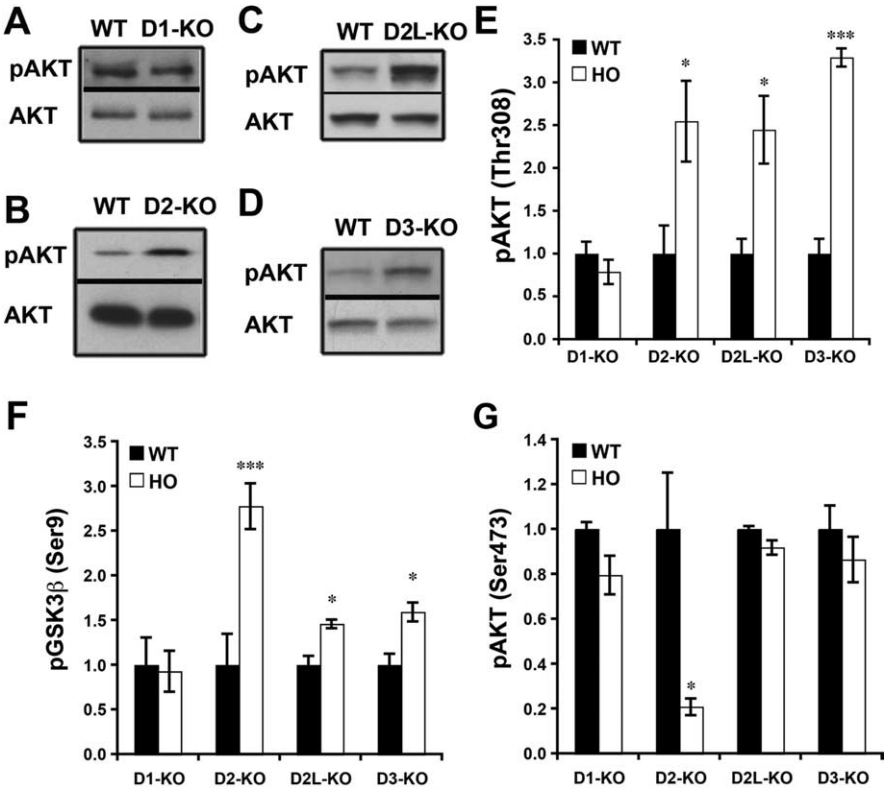

Figure 1. D_2 and D_3 dopamine receptors regulate Akt phosphorylation under basal conditions. ***A–E***, Western blots (***A–D***) and densitometric (***E–G***) analysis of phospho-Thr-308 Akt levels in extracts prepared from the striatum of different drug-naive DA receptor knock-out mice (HO) (***A***, D_1; ***B***, D_2; ***C***, D_2L; ***D***, D_3) and WT littermates. ***E–G***, Phospho-Thr 308 Akt (***E***), Phospho-Ser-9 GSK3β (***F***) or phospho-Ser-473 Akt (***G***) levels in extracts prepared from the striatum of drug-naive DA receptor knock-out mice and WT littermates. Results are presented in arbitrary units normalized to phospho-protein levels observed in WT littermates. Phospho-independent antibodies directed against respective kinases were used as loading controls. $n = 5$–10 mice per group; data are average ± SEM. $^*p \leq 0.05$, $^{***}p \leq 0.005$.

Results

Basal regulation of Akt and GSK3β in dopamine receptor knock-out mice

Western blot analysis of the relative levels of striatal phospho-Thr-308 Akt showed no variation in Akt phosphorylation between D_1-KO mice and WT littermates (Fig. 1*A*,*E*), thus confirming previous pharmacological evidence (Beaulieu et al., 2004) that D_1 receptors play little role in inhibiting Akt activity under basal conditions. In contrast, elimination of either D_2, D_2L, or D_3 receptors in genetically engineered animals led to increased striatal Akt phosphorylation (Fig. 1*B–E*). Furthermore, phosphorylation of the Akt substrate GSK3β was also enhanced in these mice (Fig. 1*F*), indicating the both D_3 and the long postsynaptic D_2 isoform D_2L can both contribute to the regulation of Akt/GSK3 signaling by DA.

Overall Akt activity is the result of an equilibrium between its phosphorylation/activation on Thr-308 and Ser-473 in response to phosphatidylinositol kinase (PI3K)-mediated signaling and its dephosphorylation by protein phosphatases. DA receptor signaling through the Akt:β-arrestin 2:PP2A complex results in a dephosphorylation of Thr-308 Akt by PP2A without affecting its phosphorylation on Ser-473 (Beaulieu et al., 2004, 2005). In contrast, changes in PI3K signaling affect the phosphorylation of both Thr-308 and Ser-473 in a similar manner (Beaulieu et al., 2005). To further establish that changes of Akt phosphorylation observed in DA receptor KO mice result from impaired Akt deactivation by the Akt:β-arrestin 2:PP2A complex and not from enhanced PI3K-mediated signaling, we evaluated relative levels of striatal phospho-Ser-473 Akt in the different DA receptor KO mice and respective littermates. As shown (Fig. 1*G*), phospho-Ser-473 levels were not increased in the different DA receptor KO mice, consistent with the enhanced Thr-308 phosphorylation in D_2, D_2L, and D_3-KO mice resulting from a reduction in β-arrestin 2-mediated DA receptor signaling. Interestingly, Ser-

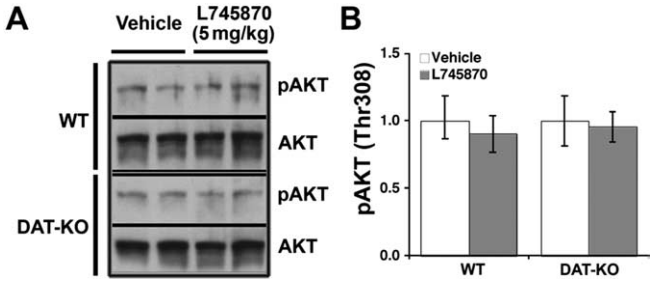

Figure 2. D_4 receptor blockade does not affect striatal Akt phosphorylation. Western blots (***A***) and densitometric analysis (***B***) of phospho-Akt (Thr-308) levels in striatal extracts from WT or DAT-KO mice 30 min after injection of 5 mg/kg of the D_4 receptor blocker L745870. Results are presented in arbitrary units normalized to phospho-Akt levels observed in vehicle-treated mice of the same genotype. Phospho-independent antibodies directed against Akt were used as loading controls. $n = 5$ mice per group; data are average ± SEM.

473 Akt levels were reduced in D_2-KO mice, suggesting unexplored additional roles of D_2 in Akt regulation.

D_4 receptor blockade does not affect striatal Akt regulation

To examine the possible contribution of D_4 receptors, we administered an effective dose of the selective D_4 receptor antagonist L745870 (Ukai and Mitsunaga, 2005) to WT animals. A similar experiment was also performed using DAT-KO mice that display a basal reduction of striatal phospho-Thr-308 Akt as a results of exacerbated dopaminergic neurotransmission (Beaulieu et al., 2004, 2005). DAT-KO mice have persistently increased extracellular dopamine and display high responsiveness to D_2-class receptor antagonists (Gainetdinov and Caron, 2003) and may thus represent a more sensitive experimental system to evaluate the impact of such drugs *in vivo*. As shown in Figure 2, D_4 receptor blockade did not affect striatal Akt phosphorylation in either WT or DAT-KO mice.

Regulation of Akt by dopaminergic drugs in DA receptor knock-out mice

For the D_1/D_2-class DA receptor, the direct agonist apomorphine and the psychostimulant amphetamine, which acts by promoting DA efflux from dopaminergic terminals, both trigger Akt dephosphorylation in the WT mouse striatum (Beaulieu et al., 2004, 2005). Administration of apomorphine (3 mg/kg, s.c.) or amphetamine (3 mg/kg, i.p.) to WT or D_1-KO mice resulted in a similar reduction of phospho-Thr-308 Akt levels (Fig. 3*A–D*), thus further confirming that D_1 receptors are dispensable for Akt inhibition in response to dopaminergic drugs.

In contrast, administration of apomorphine (3 mg/kg, s.c.) or amphetamine (3 mg/kg, i.p.) to mice lacking D_2 receptors failed to reduce striatal Akt phosphorylation (Fig. 3*A–D*). Instead, apomorphine at a dose of 3 mg/kg significantly enhanced the phosphorylation of Akt in the absence of D_2 receptors (Fig. 3*B*), whereas amphetamine had no significant effect on Akt in D_2-KO mice (Fig. 3*D*). Activation of Akt by apomorphine in the absence of D_2 probably resulted from the unmasking of a secondary action of this drug on another receptor(s) that may positively regulate Akt in the striatum (Roth et al., 2004). These observations reveal a central role for D_2 receptors in the inhibition of Akt because the remaining striatal DA receptors are not able to mediate the inhibitory action of dopaminergic drugs on this signaling molecule in D_2-KO mice.

Injection of 3 mg/kg amphetamine to D_3-KO mice resulted in reductions of striatal Akt phosphorylation similar to those observed in WT animals (Fig. 4*A*,*B*). However, elimination of D_3 receptors prevented Akt dephosphorylation in response to a dose of 3 mg/kg apomorphine (Fig. 4*E*,*F*). This discrepancy between the effects of the two drugs led us to explore the action of apomorphine and amphetamine on Akt in D_3-KO mice over a broader range of doses. As shown in Figure 4, *C* and *D*, injection of amphetamine at a dose of 1 mg/kg reduced Akt phosphorylation in the striatum of WT mice but had no effect on Akt activity in D_3-KO animals. Furthermore, administration of apomorphine to D_3-KO mice at a dose of 6 mg/kg resulted in a reduction of striatal Akt phosphorylation comparable with that observed in WT animals in response to a lower dose (3 mg/kg) of this drug (Fig. 4*E*,*F*). Together, these observations indicate that D_3 receptors may play a role in regulating the sensitivity of Akt-mediated signaling to dopaminergic drugs but are dispensable for the action of these drugs on Akt at higher dose regimens.

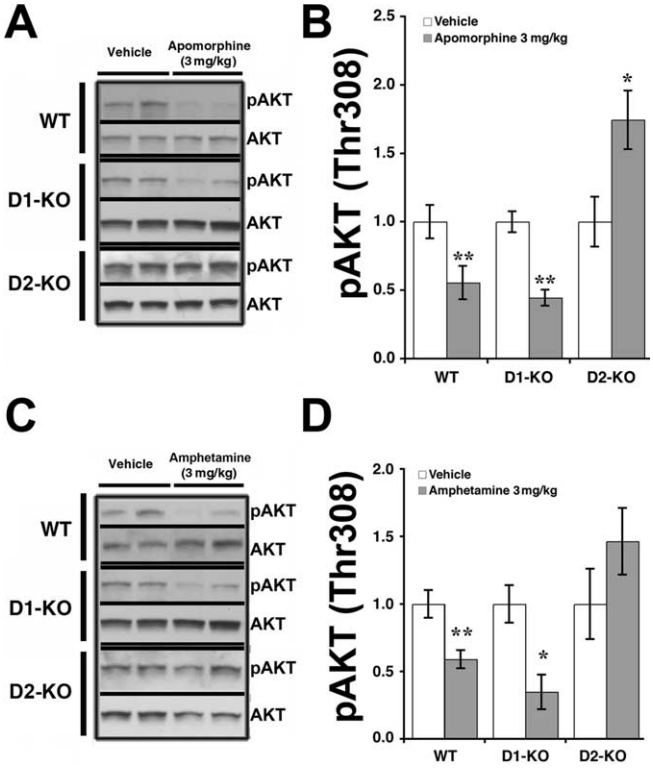

Figure 3. Regulation of Akt by DA drugs in D_1 and D_2 receptor knock-out mice. Phospho-Thr-308 Akt levels in extracts prepared from the striatum of WT, D_1, and D_2 DA receptor knock-out mice injected with apomorphine (3 mg/kg) or amphetamine (3 mg/kg). Representative Western blots (***A***, ***C***) show results obtained from two separate striatal extracts prepared from different mice. Analyses were conducted at 60 min after injection. Results of densitometric analysis (***B***, ***C***) are presented in arbitrary units normalized to vehicle-treated mice of the same genotype. $n = 5$–10 mice per group; data are average ± SEM. $^*p \leq 0.05$, $^{**}p \leq 0.01$.

Discussion

Multiple recent lines of evidence identified an involvement of the Akt/GSK3 pathway in DA receptor signaling and functions (Beaulieu et al., 2004, 2005; Emamian et al., 2004; Gould and Manji, 2005; Beaulieu, 2007). Genetic inactivation of Akt1 or GSK3β, administration of GSK3 inhibitors, or uncoupling of Akt from dopamine receptors in β-arrestin 2 knock-out mice have been shown to affect DA-related changes in locomotor activity or sensory motor gating (Beaulieu et al., 2004, 2005; Emamian et al., 2004). Characterization of Akt-mediated signaling in WT and DAT-KO mice treated with the D_2-class receptor antagonists hal-

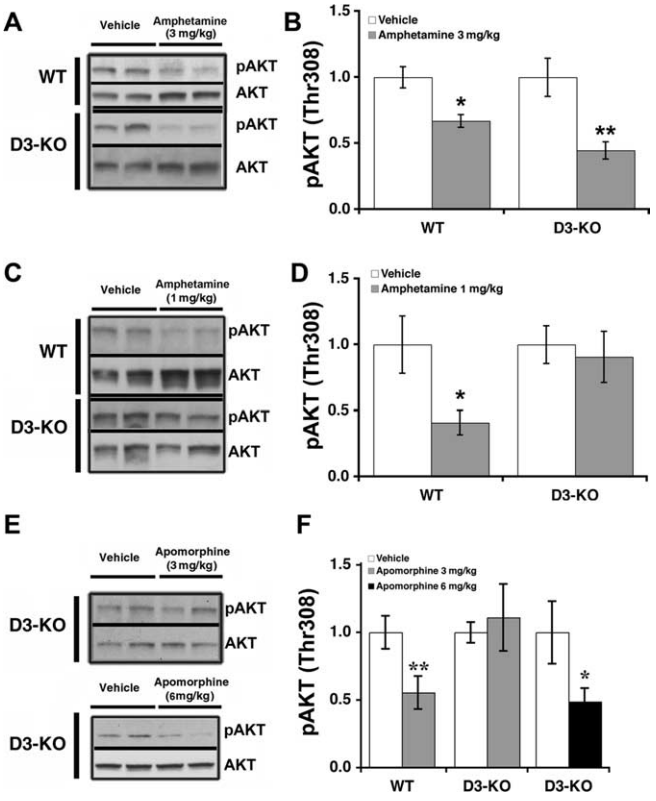

Figure 4. Regulation of Akt by DA drugs in D_3 receptor knock-out mice. ***A***, ***B***, Relative phospho-Akt (Thr-308) levels in extracts prepared from the striatum of WT or D_3 DA receptor knock-out mice 60 min after injection of amphetamine, 3 mg/kg (***A***, ***B***) or 1 mg/kg (***C***, ***D***), or of apomorphine (3 or 6 mg/kg) (***E***, ***F***). Representative Western blots (***A***, ***C***, ***E***) show results obtained from two separate striatal extracts prepared from different mice. Results of densitometric analysis (***B***, ***D***, ***F***) are presented in arbitrary units normalized to vehicle-treated mice of the same genotype. $n = 5$–10 mice per group; data are average ± SEM. $^*p \leq 0.05$, $^{**}p \leq 0.01$.

operidol and raclopride have pointed toward a role of this DA receptor class in the regulation of Akt-mediated signaling (Beaulieu et al., 2004; Emamian et al., 2004). However, the role of individual subtypes of DA receptors in triggering Akt dephosphorylation in dopaminoceptive neurons had not been fully examined. The results presented here reveal that two subtypes of D_2-class receptors, D_2 and D_3, are involved in the inhibition of Akt by DA and dopaminergic drugs in the mouse striatum, whereas D_4 dopamine receptors do not appear to play a role in this phenomena. Furthermore, these data clearly establish that D_1 dopamine receptors are not engaged in this type of signaling.

Genetic inactivation of total D_2 or of the postsynaptic D_2L receptors led to enhanced Akt phosphorylation in the striatum of knock-out mice. Furthermore, absence of D_2 receptors also resulted in a loss of normal striatal Akt regulation by dopaminergic drugs. D_2 receptors exert both presynaptic and postsynaptic functions. In their presynaptic autoreceptor function, mediated by the D_2S isoform, these receptors are responsible for the regulation of DA synthesis and, consequently, impulse-dependent dopamine release (Baik et al., 1995; Missale et al., 1998; Usiello et al., 2000; Benoit-Marand et al., 2001). Postsynaptic D_2 receptors have been associated with, among other effects, stimulation of locomotion and the development of haloperidol-induced catalepsy (Baik et al., 1995; Usiello et al., 2000). A loss of D_2 autoreceptor function thus should result in an increase of DA synthesis and, consequently, release (Benoit-Marand et al., 2001), which would in turn lead to a reduction in striatal Akt phosphorylation as demonstrated in DAT-KO and amphetamine-treated WT mice (Beaulieu et al., 2004, 2005). However, the increased basal Akt phosphorylation in D_2-KO as well as in D_2L-KO mice, which do not display major changes in D_2 autoreceptor function (Usiello et al., 2000), indicate that the negative regulation of Akt by DA is mostly a postsynaptic phenomena regulated by D_2 receptors.

Like D_2, D_3 receptors are believed to exert their actions both presynaptically and postsynaptically (Missale et al., 1998; Schwartz et al., 2000; Joseph et al., 2002). However, despite multiple associations between D_3 receptors and different neuropsychiatric disorders (Schwartz et al., 2000), knock-out studies have yet to produce a clear picture of the behavioral and neurochemical functions mediated by these receptors (Ralph et al., 1999; Joseph et al., 2002; Waddington et al., 2005). Our results suggest that D_3 receptors act as modulators that affect the threshold at which Akt is regulated by D_2 receptors. At relatively high drug doses (e.g., 3 mg/kg amphetamine or 6 mg/kg apomorphine), D_3 receptors are dispensable and D_2 receptors can regulate Akt dephosphorylation in their absence. However, at lower drug doses or under basal conditions, D_3 receptors may also contribute to the negative regulation of Akt activity by D_2 receptors.

Studies conducted in heterologous systems have shown that D_3 receptors possess an affinity for DA that is 100-fold higher than that of D_2 receptors (Sokoloff et al., 1992). This difference in affinity may explain why D_3 receptors can respond to lower doses of dopaminergic drugs and regulate Akt activity. However, this explanation does not account for the inability of D_3 receptors to modulate Akt activity in the absence of D_2. Several possible scenarios can explain the observation that DA inhibition of Akt is dependent on D_2 receptors and only modulated by D_3 receptors. It is known that GPCRs can function as dimers (Angers et al., 2002), and one potential mechanism to explain this conundrum is the possibility of dimerization of D_2 and D_3 dopamine receptors expressed in the same medium spiny neurons. Our results are consistent with the idea that Akt may be regulated by D_2/D_2 homodimers as well as by D_2/D_3 heterodimers in which D_3 would provide a higher affinity for DA receptor agonists, allowing D_2 to inhibit Akt in response to lower drug doses. However, this explanation remains hypothetical, and other possibilities such as an integration of D_2 and D_3 receptors signal at the level of Akt or other signaling intermediates cannot be excluded. Another alternative is that, because our results are derived from a biochemical approach on the whole striatum, the readout coming from a higher number of cells expressing D_2 receptors may also mask a possible D_2-independent action of D_3 receptors in some individuals cells. Characterization of the detailed mechanism by which D_2 and D_3 DA receptors collaborate to inhibit Akt will certainly require protracted studies *in vivo*, in isolated neurons, as well as in heterologous systems and should provide an exciting avenue for future research.

The Akt/GSK3 signaling pathway has recently emerged as a potential culprit and therapeutic target for psychiatric disorders (Beaulieu et al., 2004, 2005; Emamian et al., 2004; Gould and Manji, 2005; Beaulieu, 2007; Li et al., 2007). Brain Akt/GSK3 signaling has been shown to be responsive to DA (Beaulieu et al., 2004, 2005), typical/atypical antipsychotics (Emamian et al., 2004; Beaulieu, 2007; Li et al., 2007), antidepressants (Li et al., 2007), and mood stabilizers (Beaulieu et al., 2004; Gould and Manji, 2005; Beaulieu, 2006). Moreover, deregulation of Akt functions have been reported in schizophrenia (Emamian et al., 2004). Interestingly, D_2 and D_3 receptors have been linked previously to schizophrenia (Snyder, 1976; Schwartz et al., 2000),

whereas D_3 has also been associated with some cases of bipolar disorder (Schwartz et al., 2000). Our present observations that both D_2 and D_3 receptors can contribute to the regulation of Akt *in vivo* may thus be important for the development of more selective pharmaceutical interventions for the management of mental disorders associated with dopaminergic deregulation.

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