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An Akt/β-Arrestin 2/PP2A Signaling Complex Mediates Dopaminergic Neurotransmission and Behavior

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Summary

Dopamine plays an important role in the etiology of schizophrenia, and D2 class dopamine receptors are the best-established target of antipsychotic drugs. Here we show that D2 class-receptor-mediated Akt regulation involves the formation of signaling complexes containing β-arrestin 2, PP2A, and Akt. β-arrestin 2 deficiency in mice results in reduction of dopamine-dependent behaviors, loss of Akt regulation by dopamine in the striatum, and disruption of the dopamine-dependent interaction of Akt with its negative regulator, protein phosphatase 2A. Importantly, canonical cAMP-mediated dopamine-receptor signaling is not inhibited in the absence of β-arrestin 2. These results demonstrate that, apart from its classical function in receptor desensitization, β-arrestin 2 also acts as a signaling intermediate through a kinase/phosphatase scaffold. Furthermore, this function of β-arrestin 2 is important for the expression of dopamine-associated behaviors, thus implicating β-arrestin 2 as a positive mediator of dopaminergic synaptic transmission and a potential pharmacological target for dopamine-related psychiatric disorders.

Introduction

Neurotransmitters exert their functions by binding to two broad classes of receptors that are associated with different dynamic modalities of synaptic transmission. Ionotropic receptors consist of ligand-gated ion channels that mediate fast synaptic transmission (Unwin, 1993). In contrast, metabotropic receptors respond to neurotransmitters by acting through signaling pathways and are associated with slow synaptic transmission (Greengard, 2001; Jessell and Kandel, 1993). A major group of metabotropic receptors consists of seven transmembrane domain proteins (GPCR) that activate different types of heterotrimeric G proteins (Gainetdinov et al., 2004; Vassilatis et al., 2003).

Dopamine (DA) receptors represent prototypic examples of GPCR mediating neurotransmission (Missale et al., 1998). DA is a monoamine neurotransmitter involved in the regulation of locomotion, reward, and affect (Carlsson, 2001; Gainetdinov and Caron, 2003; Zhou and Palmiter, 1995). Dysregulation of dopaminergic neurotransmission is associated with multiple neurological and psychiatric conditions such as Parkinson's disease, Huntington's disease, attention deficit hyperactivity disorder (ADHD), mood disorders, and schizophrenia (Carlsson, 2001; Gainetdinov and Caron, 2003). In the mammalian brain, most dopaminergic neurons project from the substantia nigra to the striatum, a major area of basal ganglia mostly populated by medium spiny neurons that are responsive to DA (Greengard, 2001). Two classes of GPCR have been shown to mediate all DA functions. D1 class receptors (D1R and D5R) are mostly coupled to Gαs and positively regulate the production of the second messenger cAMP and the activity of its main neuronal target, protein kinase A (PKA) (Missale et al., 1998). D2 class receptors (D2R, D3R, and D4R) couple to Gαi/o, thus downregulating cAMP production and PKA activity (Missale et al., 1998). Moreover, this class of DA receptors can also modulate intracellular Ca^{2+} levels (Missale et al., 1998; Nishi et al., 1997), leading to changes in the activity of Ca^{2+}-regulated signaling proteins such as the protein phosphatase calcineurin (PP2B) (Nishi et al., 1997).

We recently demonstrated that prolonged stimulation of D2 class receptors leads to specific dephosphorylation/inactivation of the serine/threonine kinase Akt on its regulatory Thr308 residue (Beaulieu et al., 2004). Interestingly, phosphorylation of Akt on its second regulatory residue (Ser473) was not affected by DA (Beaulieu et al., 2004). Regulation of Akt by DA was observed in the striatum of mice that display persistently elevated levels of extracellular DA due to a lack of the DA transporter (DAT-KO mice). Similar changes in Akt phosphorylation also occurred in normal mice (wt) treated with direct or indirect DA agonists such as apomorphine or amphetamine (Beaulieu et al., 2004). Moreover, depletion of striatal DA in DAT-KO mice or D2 class-receptor blockade resulted in enhanced phosphorylation/activation of Akt, thus indicating that D2 class receptors are responsible for the regulation of Akt by DA (Beaulieu et al., 2004; Emamian et al., 2004).

Dephosphorylation of Akt in response to DA leads to a reduction of kinase activity and a concomitant activation of its substrates glycogen synthase kinase 3 α and β (GSK3α and GSK3β) since both are negatively regulated by Akt (Beaulieu et al., 2004; Cross et al., 1995). Consequently, pharmacological activation of Akt or inhibition of GSK3α/β results in reduction of DA-associated locomotor activity in both DAT-KO mice and wt mice treated with amphetamine (Beaulieu et al., 2004; Gould et al., 2004). Moreover, mice lacking one allele of the GSK3β gene show markedly reduced locomotor responses to amphetamine (Beaulieu et al., 2004), while mice lacking the Akt isoform Akt1 display behavioral manifestations generally associated with enhanced dopaminergic responses (Emamian et al., 2004), thus sup-

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porting a role for the Akt/GSK3 signaling pathway in the expression of DA-associated behaviors.

The mechanism by which D2 class-receptor activation results in a reduction of Akt activity remains to be clarified. Previous investigations have shown that the time course of inactivation of Akt following administration of DA agonists to wt mice is more sustained and less rapid than responses generally observed for biochemical events regulated through canonical G protein/cAMP/PKA pathways (Beaulieu et al., 2004). Moreover, increase in striatal cAMP levels or inhibition of PP2B did not affect the activity of the Akt/GSK3 signaling pathway in the striatum under conditions of enhanced DA receptor stimulation (Beaulieu et al., 2004). Taken together, these observations suggest that a novel GPCR signaling mechanism may be involved in the regulation of Akt by DA.

The scaffolding proteins β-arrestin 1 and β-arrestin 2 have been traditionally associated with the termination of GPCR signaling and with receptor internalization (Attramadal et al., 1992; Ferguson et al., 1996). Following GPCR activation and their phosphorylation by GPCR kinases (GRKs), β-arrestins bind to the receptors to uncouple them from G proteins and participate in the recruitment of the endocytic protein complex, thus leading to an attenuation of GPCR signaling (Gainetdinov et al., 2004). However, evidence obtained from heterologous cellular systems suggested that β-arrestins can also act as G protein-independent positive mediators of GPCR signaling through scaffolding of signaling molecules such as protein kinases (Lefkowitz and Shenoy, 2005; Luttrell et al., 1999, 2001). For example, stimulation of angiotensin receptors transfected in fibroblasts has been shown to result in the activation of extracellular-signal-regulated kinases (ERK1/2) following the formation of protein complexes including β-arrestin 2, ERK1/2, and ERK regulatory kinases (Luttrell et al., 2001). Moreover, β-arrestin-mediated GPCR signaling displays slower and more sustained kinetics than G protein-mediated signaling in transfected fibroblasts (Ahn et al., 2004). Nevertheless, the involvement of β-arrestin-mediated GPCR signaling in physiological paradigms associated with slow synaptic transmission has remained unexplored. Interestingly, our preliminary observation in mice lacking β-arrestin 2 (βArr2-KO mice) suggested that lack of β-arrestin 2 may reduce the expression of some DA-dependent behaviors (Gainetdinov et al., 2004). Here we used a functional in vivo approach in intact mice to examine the contribution of β-arrestin 2-mediated signaling to the regulation of Akt by DA. Our results show that β-arrestin 2 is essential for the inactivation of Akt by DA and the full expression of behavioral responses to the dopaminergic drugs apomorphine and amphetamine. Moreover, the function of β-arrestin 2 in this system is mediated through the formation of a new type of signaling complex involving β-arrestin 2, Akt, and protein phosphatase 2A (PP2A) in response to DA receptor activation. These results provide in vivo evidence for a function of β-arrestin 2 as a kinase/phosphatase scaffold involved in DA receptor signaling and the biology of slow synaptic transmission.

Results

Behavioral Responses to Dopaminergic Drugs Are Attenuated in the Absence of β-Arrestin 2

We first examined the role of β-arrestin 2 in the expression of DA-dependent behaviors. Mice lacking β-arrestin 2 (Bohn et al., 1999) and wt littermates were placed in locomotor-activity monitors and left to habituate for a period of 1 hr prior to drug injection and then monitored for changes in locomotor activity (Figure 1A). Two different dopaminergic drugs, the indirect DA agonist amphetamine, which exerts its action by raising extracellular DA levels, and the direct D1/D2 receptor agonist apomorphine, were used for these tests. These two drugs were chosen on the basis of their demonstrated ability to reliably inactivate Akt in the striatum at doses compatible with behavioral analysis (Beaulieu et al., 2004). While a lower locomotor activity was observed in βArr2-KO versus wt in the initial (first 15 min) phase of habituation (Figure 1A), no difference in either horizontal or vertical activity was observed in vehicle-treated habituated mice (Figures 1B and 1C). However, habituated βArr2-KO mice exhibited markedly less pronounced (~75% less) locomotor activation than wt littermates following administration of two doses of amphetamine (2 or 3 mg/kg, i.p.) (Figures 1A and 1B). Importantly, amphetamine-induced stereotypy was similar in both genotypes, suggesting that differences in locomotor activity were not due to a competing enhancement of stereotypy in βArr2-KO mice (Figure 1C). Assessment of climbing responses in rodents to high doses of the D1/D2 DA receptor agonist apomorphine is classically used to evaluate responsiveness of postsynaptic DA receptors (Wilcox et al., 1980). Injection of apomorphine (3 mg/kg, s.c.) to habituated βArr2-KO mice led to only a marginal increase in vertical activity (climbing), while wt were strongly responsive to this treatment (Figure 1D).

The role of β-arrestin 2 in DA-dependent behaviors was further examined using DAT-KO mice, a genetic model of enhanced dopaminergic neurotransmission. DAT-KO mice develop DA-dependent locomotor hyperactivity when exposed to a novel environment (Gainetdinov et al., 1999; Giros et al., 1996). Mice lacking both DAT and β-arrestin 2 expression (βArr2-KO/DAT-KO) were generated through crossbreeding of DAT-KO and βArr2-KO mice. βArr2-KO/DAT-KO mice are viable and do not present obvious abnormalities beyond those described in parental strains (Bohn et al., 1999; Giros et al., 1996). Like mice lacking the DAT, these double mutants display spontaneous hyperactivity in a novel environment in comparison to both wt and βArr2-KO littermates. However, locomotor activity of βArr2-KO/DAT-KO mice was significantly lower in comparison to DAT-KO littermates (Figures 1E and 1F), while the level of stereotypy was similar (data not shown).

Altogether, these behavioral observations indicate significant disruption in expression of DA-associated behaviors in the absence of β-arrestin 2. This can hardly be explained by the classical role of β-arrestins as mediators of GPCR desensitization. Under this paradigm, a lack of β-arrestin 2 should result in enhancement of DA receptor signaling and behavioral response to DA (Gainetdinov et al., 2003). Rather, these findings

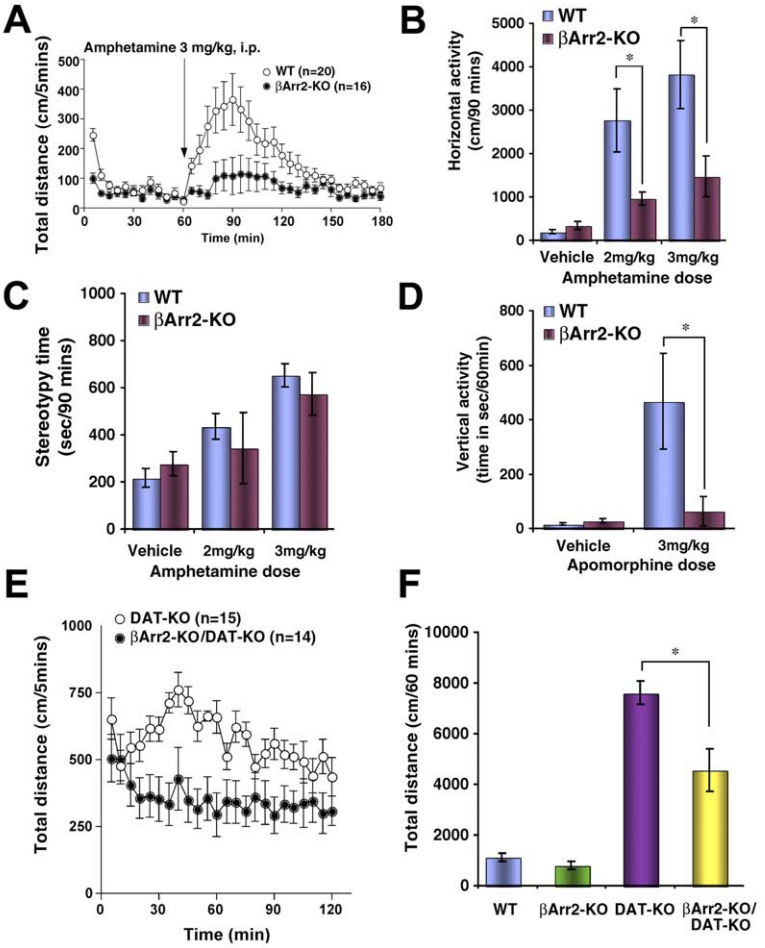

Figure 1. Reduced Responsiveness to Dopaminergic Stimulation in β-Arrestin 2 Knockout Mice

(A) βArr2-KO mice (n = 16) or wt (n = 20) littermates were placed in a locomotor-activity monitor for an initial period of 60 min and were then injected (arrow) with amphetamine (3 mg/kg of body weight, i.p.). Horizontal activity was continuously recorded in blocks of 5 min. Significant differences in horizontal activity were detected over the first 15 min of recording ($p \leq 0.005$). Activity levels of habituated mice were comparable.

(B and C) Horizontal activity (B) and stereotypy time (C) were quantified in habituated βArr2-KO mice or wt littermates for a period of 90 min after injection (i.p.) of amphetamine (vehicle wt, n = 12, βArr2-KO, n = 12; 2 mg/kg wt, n = 12, βArr2-KO, n = 11; 3 mg/kg wt, n = 20, βArr2-KO, n = 16).

(D) Vertical-activity time was measured in habituated βArr2-KO mice or wt littermates for a period of 60 min after injection (s.c.) of apomorphine (vehicle, n = 13; 3 mg/kg, n = 15).

(E) βArr2-KO/DAT-KO mice (n = 14) or DAT-KO (n = 15) littermates were placed in a locomotor-activity monitor for 120 min. Locomotor activity was continuously recorded as total distance traveled in blocks of 5 min.

(F) Total distance travelled in wt (n = 20), βArr2-KO (n = 16), DAT-KO (n = 15), and βArr2-KO/DAT-KO (n = 14) mice for a period of 60 min in the open field revealed significant reduction of hyperactivity in DAT-KO mice lacking β-arrestin 2. Both βArr2-KO/DAT-KO and DAT-KO mice were significantly different from wt and βArr2-KO littermates ($p \leq 0.05$), while no differences were found between wt and βArr2-KO mice. (For all results, data are means ± SEM. *, $p \leq 0.05$.)

suggest that β-arrestin 2 promotes some positive modalities of DA receptor signaling.

β-Arrestin 2 Is Essential for the Regulation of Akt by Dopamine

Since the lack of β-arrestin 2 does not affect striatal DA release (Bohn et al., 2003), we proceeded to evaluate whether β-arrestin 2 could be involved in signaling mechanisms (Figure 2A) known to participate in dopamine action (Beaulieu et al., 2004; Emamian et al., 2004; Greengard, 2001). Apart from the recently described action upon the Akt/GSK3 signaling pathway (Beaulieu et al., 2004), DA has mostly been considered to exert its behavioral actions by regulating the activity of cAMP/PKA signaling pathways (Greengard, 2001; Missale et al., 1998). The protein phosphatase-1 inhibitor dopamine and cAMP-regulated phosphoprotein, 32 kDa (DARPP-32), is a substrate of PKA whose phosphorylation on Thr34 in response to cAMP is regulated by DA receptors (Greengard, 2001; Nishi et al., 1997). Moreover, increased phosphorylation of Thr34-DARPP-32 has been positively associated with the expression of locomotor responses to DA drugs (Fienberg et al., 1998; Nally et al., 2003).

We thus evaluated whether a β-arrestin 2 deficiency could lead to reduced responsiveness/phosphorylation of DARPP-32 as a result of a disruption of PKA-mediated DA receptor signaling. As might be expected, injection of amphetamine (3 mg/kg, i.p.) to wt mice resulted in a robust elevation of phospho-Thr34-DARPP-32 levels in the striatum (Figure 2B). However, a marked increase in striatal DARPP-32 phosphorylation also occurred following administration of amphetamine to βArr2-KO mice (Figure 2B). In fact, the response of DARPP-32 to amphetamine was slightly greater in βArr2-KO as compared to wt mice, possibly as a result of reduced D1 receptor desensitization, a phenomenon that should be associated with enhanced locomotor response to DA drugs. However, despite this slight enhancement in cAMP/PKA/DARPP-32 signaling, βArr2-KO mice displayed profound impairment in DA-associated behaviors (Figure 1), thus suggesting the involvement of other signaling mechanisms.

Regulation of Akt in response to dopaminergic drugs was then investigated in wt and βArr2-KO mice. As shown previously (Beaulieu et al., 2004), significant reduction of phospho-Thr308-Akt occurred in the striatum of wt mice following injection of amphetamine (3

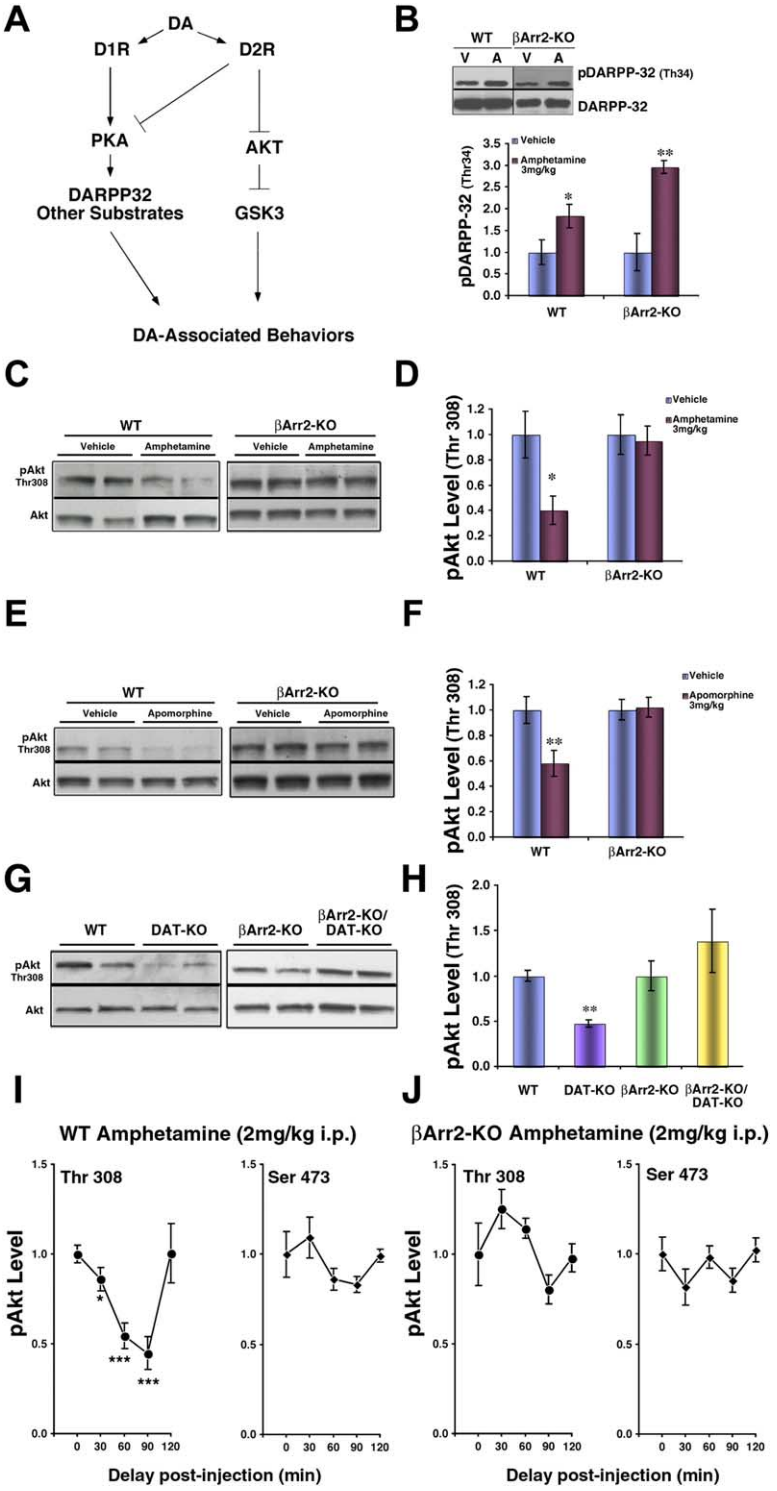

Figure 2. β-Arrestin 2 Deficiency Prevents Inhibition of Akt but Not Activation of DARPP-32 by Dopamine

(A) Schematic representation of signaling pathways associated with DA receptor activation in the striatum. D1R: D1 class DA receptor; D2R: D2 class DA receptor.

(B) Western blots and densitometric analysis of phospho-Thr34 DARPP-32 relative levels in extract prepared from the striatum of wt or βArr2-KO mice 15 min after injection of 3 mg/kg amphetamine. (n = 5 mice.)

(C–F) Western blots (C and E) and densitometric analysis (D and F) of phospho-Thr308 Akt relative levels in extract prepared from the striatum of wt or βArr2-KO mice at 60 min postinjection of 3 mg/kg amphetamine (C and D) or 3 mg/kg apomorphine (E and F). Representative Western blots show results obtained from two separate striatal extracts prepared from different mice. Densitometric analysis was carried out on five mice per group for amphetamine and ten mice per group for apomorphine.

(G and H) Western blots (G) and densitometric analysis (H) of phospho-Thr308 Akt relative levels in extract prepared from the striatum of wt, DAT-KO, βArr2-KO, and βArr2-KO/DAT-KO mice under basal conditions (n = 10 mice).

(I and J) Temporal dynamic of Akt (Thr308 and Ser473) phosphorylation as measured by Western blot analysis at 0, 30, 60, 90, and 120 min after amphetamine injection (2 mg/kg, i.p.) in wt (I) and βArr2-KO (J) mice (n = 5–10 mice).

For all densitometric analyses, results are presented in arbitrary units normalized to phosphoprotein levels observed in vehicle-treated mice. Phosphoindependent antibodies directed against the different proteins were used as loading controls. Data are means ± SEM. *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.001$.

mg/kg, i.p.) (Figures 2C and 2D) or apomorphine (3 mg/kg, s.c.) (Figures 2E and 2F). In contrast, administration of these two drugs had no effect on striatal phospho-Thr308-Akt in βArr2-KO mice.

To substantiate these pharmacological observations, we compared phospho-Thr308-Akt levels in DAT-KO and βArr2-KO/DAT-KO mice. Our previous observations revealed an inhibition of Akt signaling in response to sustained DA receptor stimulation in DAT-KO mice (Beaulieu et al., 2004). In line with this, Akt phosphorylation was reduced in DAT-KO mice as compared to their wt littermates (Figures 2G and 2H). However, no differences in Akt phosphorylation were found between βArr2-KO/DAT-KO mice and their βArr2-KO littermates (Figure 2G and 2H).

To examine whether the dynamic of Akt regulation by

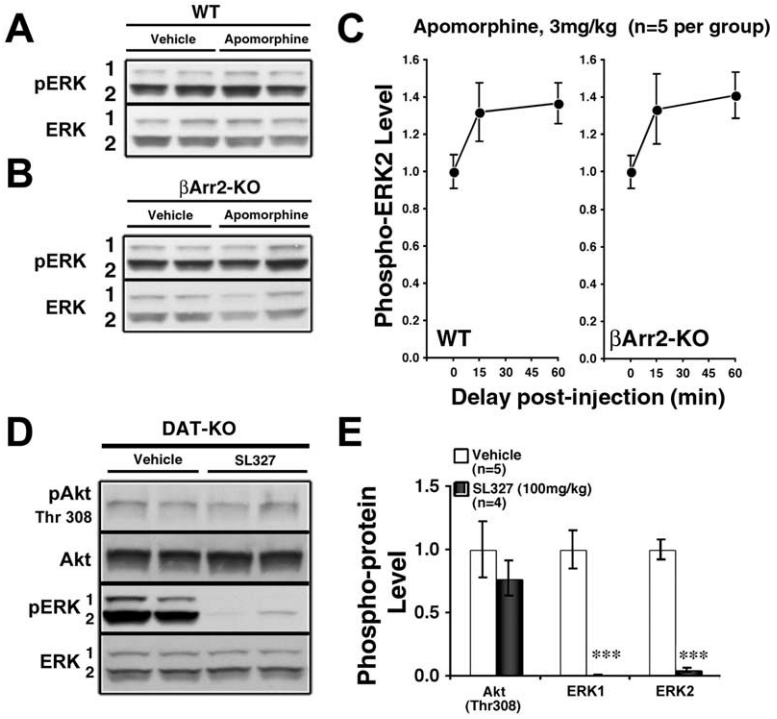

Figure 3. Lack of Regulation of Akt by ERK in Response to Dopamine

(A and B) Western blots of phospho-ERK1/2 (Thr202/Tyr204) in extracts prepared from the striatum of wt (A) or βArr2-KO (B) mice at 60 min postinjection of apomorphine (3 mg/kg). Representative Western blots show results obtained from two separate striatal extracts prepared from different mice.

(C) Densitometric analysis of striatal phospho-ERK2 levels as measured by Western blot analysis at 0, 15, and 60 min following apomorphine injection (3 mg/kg) in wt and βArr2-KO mice. (n = 5 mice).

(D and E) Western blots (D) and densitometric analysis (E) of phospho-Thr308 Akt and phospho-ERK1/2 (Thr202/Tyr204) relative levels in extracts prepared from the striatum of DAT-KO mice 60 min after injection of 100 mg/kg of the MEK inhibitor SL327. (n = 5 mice.)

For all densitometric analyses, results are presented in arbitrary units normalized to phosphoprotein levels observed in vehicle-treated mice. Phosphoindependent antibodies directed against the different proteins were used as loading controls. Data are means ± SEM. ***, $p \leq 0.001$.

DA may be changed in absence of β-arrestin 2, levels of phospho-Thr308 and phospho-Ser473 Akt were measured over time following administration of amphetamine (2 mg/kg) to wt and βArr2-KO mice. In wt mice, amphetamine caused a progressive and sustained dephosphorylation of Akt on Thr308 over a period of 90 min postinjection, while phosphorylation of Akt on Ser473 was not significantly affected (Figure 2I). In contrast, no such dephosphorylation of Akt was observed at any time points in βArr2-KO mice (Figure 2J), thus directly demonstrating that β-arrestin 2 is essential for the regulation of Akt by DA.

Lack of Regulation of Akt by ERK in the Mouse Striatum

Activation of protein kinases ERK1 and ERK2 is a known outcome of β-arrestin 2-mediated signaling following stimulation of angiotensin receptors transfected in fibroblasts (Luttrell et al., 2001). ERK signaling has also been suggested to enhance Akt phosphorylation in embryonic cultured striatal neurons exposed to DA (Brami-Cherrier et al., 2002). To test whether ERK signaling might be involved in the regulation of Akt by DA, Western blot analyses were carried out to assess ERK1/2 phosphorylation in wt and βArr2-KO mice under conditions leading to Akt inactivation. As shown in Figures 3A–3C, similar changes in levels of phospho-ERK were observed in wt (Figures 3A and 3C) and βArr2-KO mice (Figures 3B and 3C) at different time points following administration of a dose of apomorphine (3 mg/kg, s.c.) that produced robust dephosphorylation/inhibition of Akt in wt animals (Figure 2). Thus, DA-dependent ERK signaling does not seem to be affected by a β-arrestin 2 deficiency.

To further substantiate these observations, SL327, a blood-brain-barrier-permeable selective inhibitor of the ERK kinase MEK (Valjent et al., 2000), was used. Under basal conditions, DAT-KO mice exhibit persistently elevated DA tone (Gainetdinov et al., 1999; Jones et al., 1998), leading to sustained DA-mediated inactivation of Akt as compared to normal mice (Beaulieu et al., 2004). Administration of SL327 (100 mg/kg, i.p.) to DAT-KO mice resulted in a marked reduction of striatal phospho-ERK1/2 levels (Figures 3D and 3E). Nevertheless, inhibition of ERK did not result in an enhancement of phospo-Thr308-Akt levels in these animals (Figures 3D and 3E), thus indicating that ERK signaling is not a contributing factor to the deactivation of Akt by DA in the adult mouse striatum.

Inhibition of PP2A Antagonizes the Negative Regulation of Akt by Dopamine

Serine/threonine protein phosphatases have been shown to negatively regulate Akt activity in multiple cellular systems (Andjelkovic et al., 1996; Li et al., 2003; Ugi et al., 2004). We used intracerebroventricular injections (i.c.v.) of protein-phosphatase inhibitors in DAT-KO mice to assess the contribution of phosphatases in the regulation of Akt by DA. Okadaic acid is a potent phosphatase inhibitor acting on both PP1 and PP2A (McCluskey and Sakoff, 2001). Injection of okadaic acid (100 μM, i.c.v.) in the brain of DAT-KO mice resulted in enhanced phosphorylation of Thr308-Akt as revealed by Western blot (Figures 4A and 4B). However, changes in Akt phosphorylation in response to okadaic acid were not limited to the DA-regulated Thr308 residue. Phosphorylation of the second regulatory site of Akt (Ser473) (Alessi et al., 1996) was also enhanced (Figures 4A and 4B), suggesting that multiple mechanisms regulating the phosphorylation of Akt in the striatum

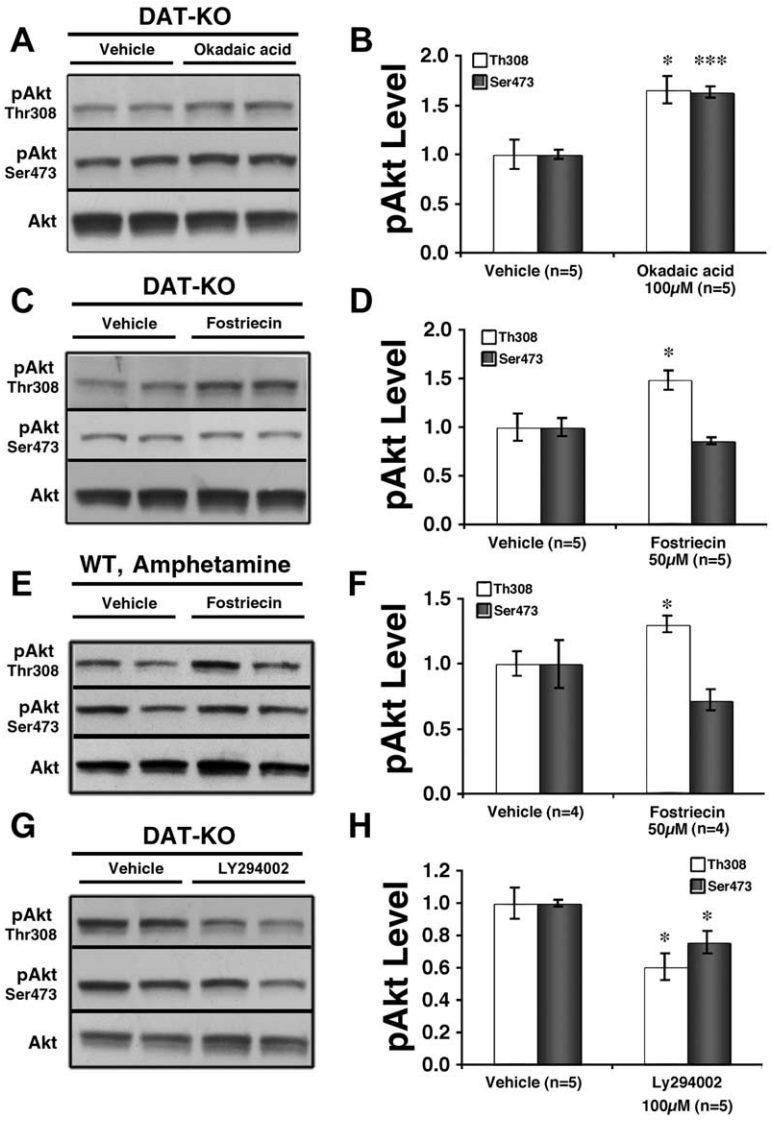

Figure 4. Inhibition of Protein Phosphatase 2A Prevents Inactivation of Akt by Dopamine

(A and B) Western blots (A) and densitometric analysis (B) of phospho-Thr308 and phospho-Ser473 Akt showing increased Akt phosphorylation at both residues 30 min postinjection of the PP1/PP2A inhibitor okadaic acid (100 μM, i.c.v.) to DAT-KO mice.

(C and D) Western blots (C) followed by densitometric analysis (D) revealed a specific increase of phospho-Thr308 but not phospho-Ser473 Akt 30 min after injection of the PP2A-specific inhibitor fostriecin (50 μM, i.c.v.) to DAT-KO mice.

(E and F) Densitometric analysis (F) of Western blots (E) revealed a specific increase of phospho-Thr308 Akt following administration of the PP2A-specific inhibitor fostriecin to amphetamine-treated wt mice. Mice were first systemically treated with amphetamine (3 mg/kg) followed 30 min later by i.c.v. injection of vehicle or fostriecin (50 μM, i.c.v.). Samples were collected 30 min after i.c.v. injection.

(G and H) Western blots (G) and densitometric analysis (H) showing a decrease in phospho-Thr308 and phospho-Ser473 Akt levels 30 min after i.c.v. injection of the PI3K inhibitor LY294002 (100 μM) to DAT-KO mice.

For all analyses, phosphoindependent antibodies directed against the different proteins were used as loading controls for densitometry. Data are means ± SEM. *, $p \leq 0.05$; ***, $p \leq 0.001$. Numbers of animals per group (n) are indicated.

were probably disrupted by this inhibitor. To assess whether more specific inhibition of protein phosphatases could oppose the action of DA on Akt phosphorylation, the specific PP2A inhibitor fostriecin (Walsh et al., 1997) was injected (50 μM, i.c.v.) to DAT-KO mice. As shown in Figures 4C and 4D, inhibition of PP2A resulted in a specific increase in Thr308-Akt phosphorylation in the striatum of DAT-KO mice. To confirm this observation in normal animals, we explored the consequences of a specific inhibition of PP2A in wt mice displaying enhanced DA tones as a result of pharmacological treatment. In this set of experiments, wt mice were first treated with amphetamine (3 mg/kg, i.p.), followed 30 min later by fostriecin (50 μM, i.c.v.). Western blot analysis was conducted on striatal extracts collected 30 min after i.c.v. injections. As shown in Figures 4E and 4F, administration of the PP2A inhibitor fostriecin to amphetamine-treated wt mice resulted in a specific enhancement of Thr308-Akt phosphorylation, thus indicating that β-arrestin 2-mediated deactivation of Akt by DA involves PP2A.

Since Akt might also be positively regulated through phosphoinositide 3 kinase (PI3K) mediated signaling (Alessi et al., 1996), we proceeded to evaluate the effect of PI3K inhibition on Akt phosphorylation in the striatum of DAT-KO mice. Injection of the PI3K inhibitor LY294002 (100 μM, i.c.v.) to DAT-KO mice resulted in a reduction of Akt phosphorylation on both Thr308 and Ser473 residues (Figures 4G and 4H), thus indicating that PI3K is still active in DAT-KO mice and that its inhibition does not recapitulate the selective action of DA upon Akt phosphorylation.

Striatal Akt, GSK3, and PP2A Subunits Form Complexes with β-Arrestin 2 In Vitro

To further examine the relation between Akt, PP2A, and β-arrestin 2, we verified whether recombinant glutathione S-transferase β-arrestin 2 (GST-βArr2) could interact with striatal PP2A and Akt. PP2A is a multimeric protein complex formed by the assembly of a structural A subunit, a catalytic C subunit, and one of a variety

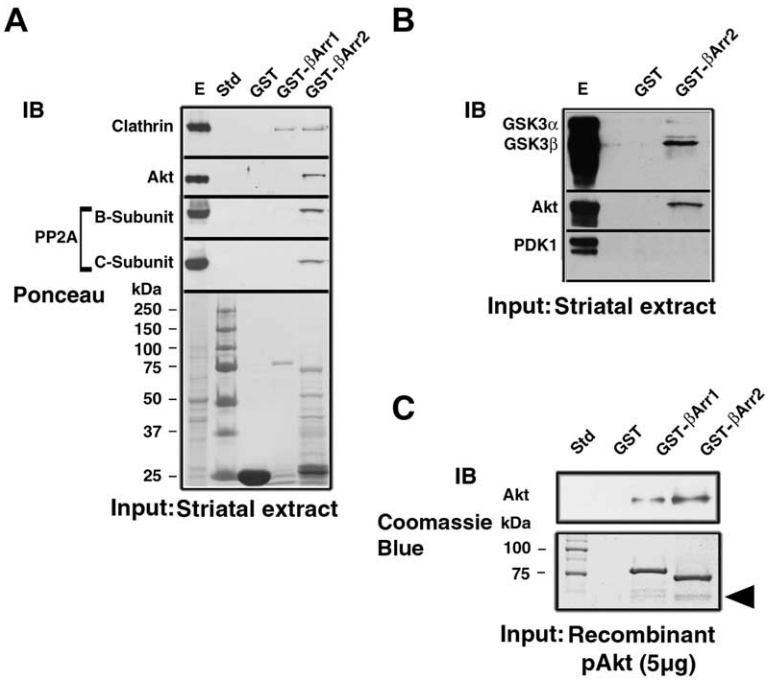

Figure 5. β-Arrestin 2 Specifically Interacts with Akt, GSK3, and PP2A In Vitro

(A) GST pulldown showing interaction of striatal Akt, PP2A, B subunit, and C subunit with β-arrestin 2 but not β-arrestin 1. Clathrin was used as a positive control of interaction with β-arrestins. Experiments were repeated at least two times using separate sets of striatal extracts and recombinant GST fusion proteins.

(B) GST pulldown showing interaction of striatal Akt and GSK3α and β but not PDK1 with β-arrestin 2.

(C) GST pulldown showing interaction of recombinant phospho-Akt (Thr308 and Ser473) with β-arrestin 2 and to a lesser extent β-arrestin 1. Arrowhead indicates a band corresponding to recombinant Akt on SDS-PAGE stained with Coomassie blue. E designates lanes loaded with striatal extract. Std: molecular weight standard. IB: immunoblot.

of targeting subunits belonging to one of four classes termed B, B′, B′′, and B′′′ (Janssens and Goris, 2001; Millward et al., 1999). Affinity purification on columns (GST pulldown) following incubation of GST, GST-βArr1, or GST-βArr2 recombinant proteins with striatal protein extracts revealed a copurification of Akt, a targeting B subunit (55 kDa subunit), and the catalytic C subunit of PP2A (Millward et al., 1999) from extracts incubated with GST-βArr2 (Figure 5A). No such copurification of Akt and PP2A with recombinant proteins was observed when striatal extracts were incubated with either GST or GST-βArr1 recombinant proteins (Figure 5A). In contrast, both GST-βArr1 and GST-βArr2 were copurified to levels comparable with their known direct-interaction partner clathrin (Krupnick et al., 1997), thus suggesting a preferential interaction of striatal Akt and PP2A subunits with β-arrestin 2 (Figure 5A).

Further analysis also revealed the presence of GSK3β and, to a lesser extent, GSK3α but not the phosphoinositide-dependent protein kinase 1 (PDK1) in GST-βArr2 eluates (Figure 5B). GSK3α and GSK3β are both negatively regulated by Akt (Cross et al., 1995) and activated in response to DA receptor stimulation (Beaulieu et al., 2004), while PDK1 is a positive regulator of Akt responsible for the phosphorylation of the Thr308 residue (Alessi et al., 1997). Presence of GSK3α/β but not PDK1 in GST-βArr2 eluates suggests that the GST-βArr2 recombinant protein specifically interacts with a subset of the proteins involved in Akt signaling in these assays.

GST-βArr2 and GST-βArr1 were then incubated with recombinant in vitro-phosphorylated human Akt1 to assess whether Akt can directly interact with β-arrestins in the absence of other proteins. Western blot analysis and Coomassie blue-stained SDS-PAGE from GST-pulldown eluates revealed a copurification of phospho-Akt1 with recombinant β-arrestin 2 and, to a lesser extent, with β-arrestin 1 (Figure 5C). This indicates that, while β-arrestin 2 seems to be the preferred interacting partner of Akt in the striatum, both β-arrestins can interact directly with Akt with different efficiency under in vitro conditions.

Interaction of Akt, PP2A, and β-Arrestin 2 in the Mouse Striatum

We performed immunoprecipitation experiments to confirm the results obtained from GST pulldowns. As shown in Figure 6, immunoprecipitation of Akt from striatum of wt C57BL/6J mice led to a coimmunoprecipitation of endogenous B and C subunits of PP2A (Figure 6A). A separate set of experiments revealed that β-arrestin 2 was preferentially immunoprecipitated over β-arrestin 1 along with Akt (Figure 6B). Moreover, β-arrestin 2 was selectively coimmunoprecipitated with targeting B subunits of PP2A (Figure 6C). Taken together, these results further substantiate that an interaction of Akt and PP2A subunits with β-arrestin 2 occurs in the mouse striatum.

Interaction of Akt with PP2A Is Disrupted in Mice Lacking β-Arrestin 2

To further document a role of β-arrestin 2 as a scaffolding intermediate for the interaction of Akt and PP2A, we performed immunoprecipitation assays in βArr2-KO mice and wt littermates. In line with results from wt C57BL/6J mice (Figure 6A), immunoprecipitation of Akt from the striatum of wt littermates resulted in consistent coimmunoprecipitation of PP2A subunits (Figure 6D). In contrast, immunoprecipitations carried out with mice lacking β-arrestin 2 revealed a dramatic reduction in the amount of PP2A subunits interacting with Akt in the striatum of these mice (Figure 6D). This reduction was not associated with changes in Akt or PP2A subunit levels in βArr2-KO mice, thus indicating that

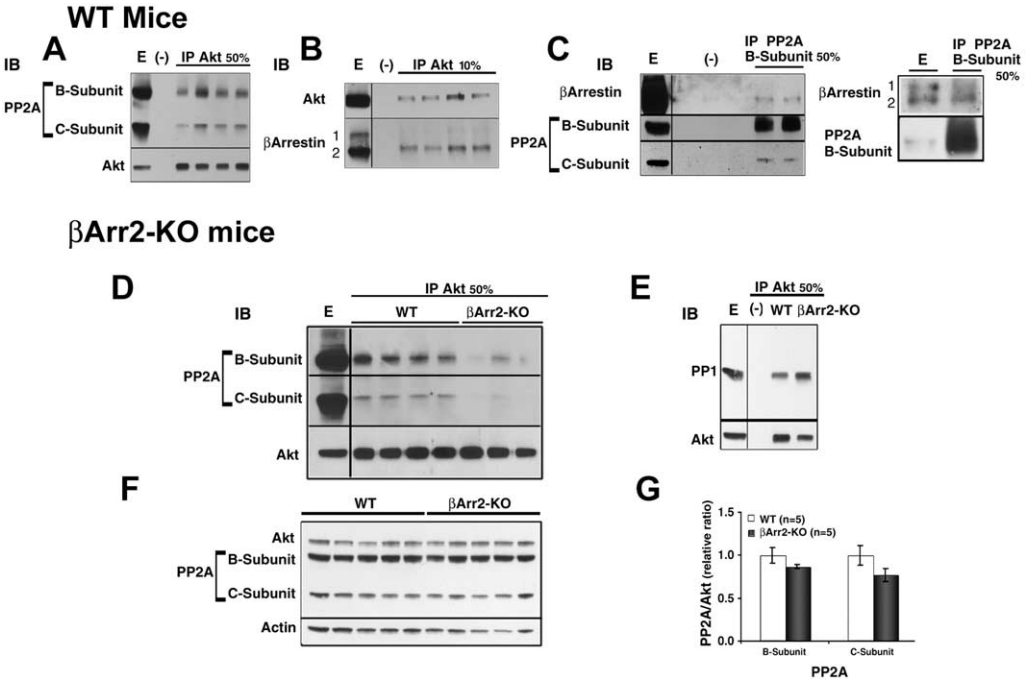

Figure 6. β-Arrestin 2-Dependent Interaction of Akt and PP2A in the Mouse Striatum

(A and B) Akt immunoprecipitation assay showing a coimmunoprecipitation of PP2A B subunit and C subunit (A) as well as β-arrestin 2 (B) along with Akt from protein extracts prepared from the striatum of wt C57BL/6J mice.

(C) Coimmunoprecipitation of β-arrestin 2 and the C subunit of PP2A along with the PP2A B subunit in extracts prepared from the striatum of wt C57BL/6J mice. Right-hand panel shows that the β-arrestin isoform coimmunoprecipitated with PP2A corresponds to β-arrestin 2.

(D) Coimmunoprecipitation of PP2A along with Akt in striatal extracts prepared from littermate wt but not βArr2-KO mice.

(E) Coimmunoprecipitation of the PP1 catalytic subunit with Akt in striatal extracts prepared from βArr2-KO mice and wt littermates.

(F and G) Western blot (F) and densitometric analysis (G) of levels of PP2A B subunit and PP2A C subunit as compared to Akt in the striatum of βArr2-KO mice and wt littermates. Average ratios of PP2A subunit to Akt were normalized to 1.00 in wt animals. Data are means ± SEM. Numbers of animals per group (n) are indicated.

For immunoprecipitations, E designates striatal extract and (–) designates control experiment performed without antibodies. Each track represents independent immunoprecipitation experiments performed from extracts prepared from the striatum of different mice.

β-arrestin 2 is an essential scaffold for the interaction of Akt with PP2A in the mouse striatum (Figures 6F and 6G). Moreover, since PP1 can play some role in Akt regulation (Li et al., 2003), we assessed whether PP1 could also interact with Akt in a β-arrestin 2-dependent fashion. As shown in Figure 6E, immunoprecipitation experiments revealed that the catalytic subunit of PP1 could be coimmunoprecipitated along with Akt from the mouse striatum. However, this interaction was not abolished by the absence of β-arrestin 2 (Figure 6E), thus directly demonstrating that PP2A, but not PP1, is involved in the β-arrestin 2-dependent regulation of Akt by DA.

Akt/β-Arrestin 2/PP2A Interaction Is Regulated by Dopamine

Quantitative immunoprecipitation experiments were carried out to establish whether the formation of an Akt/β-arrestin 2/PP2A signaling complex could be dependent on extracellular DA levels. For these experiments, wt C57BL/6J mice were treated with vehicle or with a dose of amphetamine (3 mg/kg, i.p.) that is known to increase extracellular DA levels and trigger Akt dephosphorylation (Beaulieu et al., 2004). Western blot analysis of immunoprecipitates (Figures 7A and 7D) revealed a marked increase in the amount of PP2A catalytic subunit (Figures 7A and 7B) and β-arrestin 2 (Figures 7C and 7D) precipitated with Akt following amphetamine administration, thus providing compelling evidence for the regulation of the Akt/β-arrestin 2/PP2A interaction by DA.

Discussion

Slow synaptic transmission is for the most part mediated by seven transmembrane domain receptors coupled to G proteins. In the specific case of dopaminergic neurotransmission, most behavioral actions of DA are thought to be associated with the modulation of adenylate cyclase and PKA activity (Greengard, 2001; Missale et al., 1998). Recent investigations have uncovered that stimulation of striatal D2 class receptors also results in a cAMP-independent dephosphorylation/inactivation of Akt (Beaulieu et al., 2004) associated with the expression of DA-dependent behaviors (Beaulieu et al., 2004; Emamian et al., 2004). Here we show, using an in vivo approach, that DA receptors regulate Akt activity and DA-associated behaviors through a β-arrestin 2-mediated

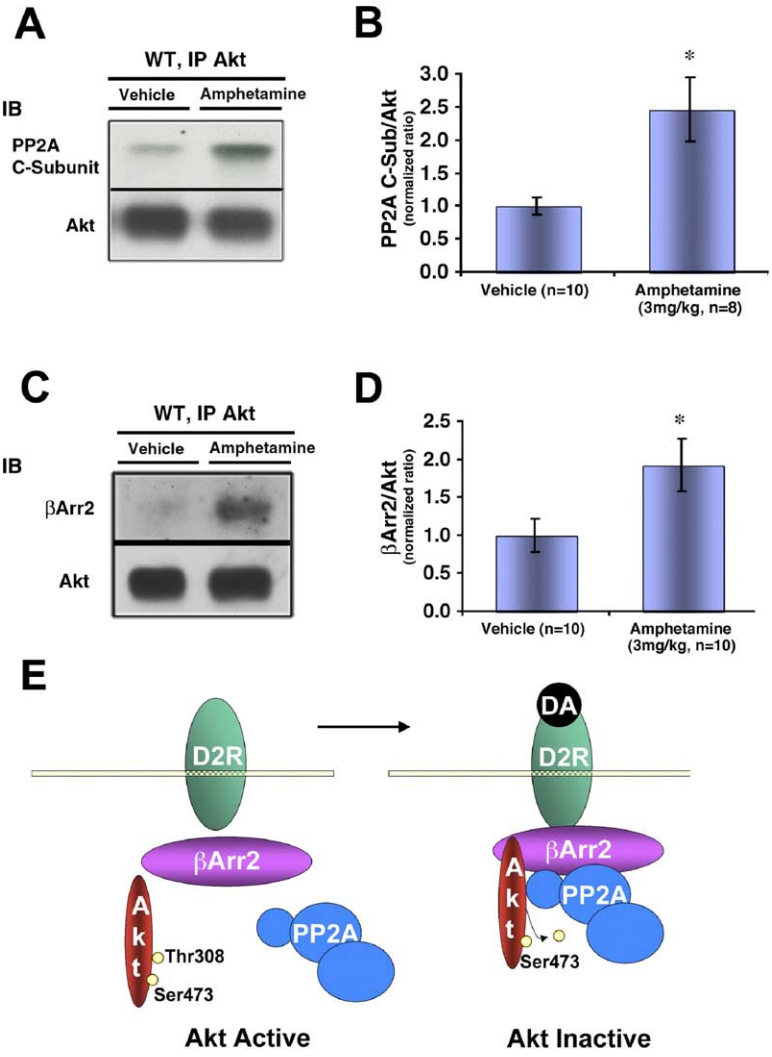

Figure 7. Formation of the Akt/β-Arrestin 2/PP2A Signaling Complex Is Regulated by Dopamine

(A–D) Quantification of the interaction of the catalytic subunit of PP2A (A and B) or of β-arrestin 2 (C and D) with Akt as measured by densitometric analysis (B–D) following coimmunoprecipitation (A and C) from striatal extracts prepared from vehicle and amphetamine (3 mg/kg, 60 min postinjection) treated C57BL/6J wt mice. Average ratios of PP2A to Akt or β-arrestin 2 are normalized to 1.00 in vehicle-treated animals. Data are means ± SEM. Numbers of animals per group (n) are indicated. *, $p \leq 0.05$.

(E) Working model of the Akt/β-arrestin 2/PP2A signaling complex formed in response to activation of DA receptors leading to Akt inactivation in the mouse striatum. D2R indicates D2 class receptors (D2S, D2L, D3, and D4).

signaling mechanism that involves a previously unidentified β-arrestin/kinase/phosphatase signaling complex. These results provide direct physiologically relevant evidence that β-arrestin 2 participates in slow synaptic transmission by acting as a scaffold for signaling molecules in response to GPCR activation.

The signaling mechanism described here involves the formation of a protein complex comprised of at least Akt, β-arrestin 2, and the multimeric protein phosphatase PP2A (Figure 7E). Mice lacking β-arrestin 2 display reduced responsiveness to dopaminergic drugs and exhibit lower locomotor activity during the initial phase of habituation to a novel environment (Figure 1). Moreover, the DA-dependent locomotor activity of DAT-KO mice was reduced in mice lacking both DAT and β-arrestin 2 (Figure 1). These behavioral deficits in βArr2-KO mice are accompanied by loss of Akt regulation in response to D2 receptor stimulation (Figure 2). Inhibition of Akt in response to DA can also be antagonized by specific inhibition of PP2A in mice exhibiting enhanced DA neurotransmission (Figure 4). Furthermore, β-arrestin 2 plays an essential role in mediating the interaction of Akt with a targeting B subunit and the catalytic C subunit of PP2A in response to DA (Figure 5–7).

β-arrestins can act as positive mediators for signaling, leading to activation of protein kinases such as ERK1/2 and Src through protein scaffolding in cell-culture systems (Lefkowitz and Shenoy, 2005). However, the in vivo physiological relevance of β-arrestin-mediated signaling in vivo has remained unclear. Our observations in mice provide direct evidence for the involvement of β-arrestin-mediated signaling in a physiological paradigm, namely, dopaminergic slow synaptic transmission. In doing so, these results also introduce the concept that β-arrestin 2 is not only involved in the positive regulation of kinase activity but can also mediate inactivation of certain kinases, here by scaffolding Akt along with PP2A in response to GPCR stimulation.

Akt has been implicated in the regulation of multiple biological processes ranging from glycogenesis to embryonic development, inflammation, apoptosis, and cell proliferation (Scheid and Woodgett, 2001). Our results show that β-arrestin 2 can play an important function in Akt regulation by acting as a scaffold between this

kinase and its regulatory phosphatase PP2A. Previous observations in cultured fibroblasts have shown that β-arrestin 1 but not β-arrestin 2 participates in signaling pathways resulting in Akt activation in response to insulin growth factor 1 (IGF1) or α-thrombin receptor stimulation (Goel et al., 2002; Povsic et al., 2003). However, no interaction between β-arrestin 1 and Akt has been observed in fibroblasts overexpressing these two proteins (Povsic et al., 2003). Evidence also indicates that β-arrestin 1 would regulate Akt through indirect signaling pathways involving the activation of Src and PI3K rather than through the direct scaffolding of Akt (Goel et al., 2002; Povsic et al., 2003). Nevertheless, our observation in GST-pulldown assays on recombinant phospho-Akt indicate that, under certain conditions, β-arrestin 1 can also interact with Akt, albeit with lower efficacy than with β-arrestin 2. Taken together with previous observations, the results presented here suggest that both β-arrestin 1 and β-arrestin 2 may participate in the regulation of Akt through different mechanisms. This further suggests that, apart from its role in dopaminergic neurotransmission, β-arrestin-mediated signaling may be relevant to many physiological processes associated with Akt functions.

β-arrestins are known to play a critical role in desensitization of GPCRs by binding to receptors following their agonist-dependent GRK-mediated phosphorylation, resulting in termination of receptor-G protein coupling and signaling (Attramadal et al., 1992; Ferguson et al., 1996). Thus, β-arrestin or GRK deficiency would be generally expected to enhance efficacy of GPCR signaling and physiological response (Gainetdinov et al., 2003). In fact, mice lacking β-arrestin 2 display enhanced sensitivity to morphine in tests of pain perception and reward due to impaired desensitization of μ-opiod receptors (Bohn et al., 1999; Bohn et al., 2003). In line with these observations, lack of GRK6, the major GPCR kinase expressed in the striatum, results in markedly enhanced coupling of D2 class receptors to G proteins and behavioral supersensitivity to dopaminergic stimulation (Gainetdinov et al., 2003). However, preliminary investigations also revealed that dopamine-mediated locomotor responses to drugs are reduced in βArr2-KO (Gainetdinov et al., 2004). Here we substantiate these findings by showing profound reduction in responses to amphetamine and apomorphine and genetically elevated DA neurotransmission in the absence of β-arrestin 2. Such remarkable differences in consequences of deficiency in molecules associated with GPCR desensitization highlight the complex role of these molecules in the regulation of GPCR signaling. One possible explanation can be that some receptors are negatively regulated by β-arrestin 2 through receptor desensitization/internalization, while others use this same molecule as a mediator of "positive" signaling. To add to the complexity of this situation, it is likely that the dual roles of β-arrestins in GPCR signaling may be subject to complex regulatory mechanisms in response to different intensity or duration of GPCR stimulation and/or potency of agonists (Nair and Sealfon, 2003).

Most biochemical and behavioral actions of DA have been studied in relation to activation of cAMP-dependent signaling pathways (Greengard, 2001; Missale et al., 1998). Among many PKA substrates, phosphorylation of the PP1 inhibitor DARPP-32 has been positively associated with the expression of DA-associated behaviors (Greengard, 2001). However, mice lacking this molecule display only partial deficits in their behavioral responses to DA drugs (Fienberg et al., 1998; Nally et al., 2003). Here we show that β-arrestin 2 is essential for the expression of a major part of the behavioral response to the dopaminergic drugs amphetamine and apomorphine (Figure 1). Furthermore, double-knockout mice lacking both β-arrestin 2 and DAT have reduced levels of activity in comparison to DAT-KO littermates. As shown in this study, lack of β-arrestin 2 did not result in an inhibition of DARPP-32 phosphorylation in response to DA receptor stimulation. However, cAMP-independent regulation of Akt by DA (Beaulieu et al., 2004) was abolished in βArr2-KO mice (Figure 2), thus revealing a mechanism by which DA modulates Akt activity in striatal cells. It is important to note that, like in mice lacking DARPP-32, DA functions are not fully suppressed in βArr2-KO mice. These findings suggest that an intricate network of signaling pathways in which both cAMP-dependent and -independent events play important and perhaps cooperative functions mediate DA actions. Further studies will undoubtedly be needed to delineate the role of these different pathways in the expression of DA-related functions and psychiatric conditions associated with dysregulation of dopaminergic neurotransmission.

Recent evidence has linked dysregulation of Akt signaling pathways with schizophrenia. Reduced Akt protein levels were shown in the brain of schizophrenic patients, while Akt1 knockout mice developed behavioral manifestations that are generally associated with enhanced dopaminergic functions (Emamian et al., 2004). Furthermore, significant association of Akt1 haplotypes with schizophrenia has been reported following transmission-disequilibrium tests (Emamian et al., 2004; Ikeda et al., 2004). Typical antipsychotics such as haloperidol are thought to exert most of their action by blocking D2 class receptors, thus supporting a role for dopaminergic neurotransmission in the etiology of schizophrenia (Snyder, 1976). Interestingly, administration of haloperidol or the selective D2 class-receptor antagonist raclopride has been shown to prevent the regulation of Akt by DA or enhance Akt phosphorylation in animal models (Beaulieu et al., 2004; Emamian et al., 2004). Moreover, two other drugs used in the management of psychosis, lithium and clozapine, also act as enhancers of Akt signaling in vivo (Beaulieu et al., 2004) or in cell-culture systems (Chalecka-Franaszek and Chuang, 1999; Kang et al., 2004). In light of these observations, involvement of β-arrestin 2-mediated kinase/phosphatase scaffolding in the regulation of Akt by DA indicates that β-arrestin 2-mediated signaling could contribute to the etiology and/or management of schizophrenia.

In conclusion, we have shown that a β-arrestin 2-mediated kinase/phosphatase scaffolding of Akt and PP2A is responsible for the regulation of Akt by DA receptors. These observations provide an alternative pathway by which D2 class-receptor activation leads to the expression of DA-associated behaviors. Moreover, these results provide direct in vivo evidence for the physiologi-

cal relevance of β-arrestin-mediated GPCR signaling while uncovering a previously unappreciated modality, kinase/phosphatase scaffolding, by which β-arrestins can participate in signal transduction. Furthermore, investigations of the function and mechanism of this kinase/phosphatase scaffolding in dopaminergic slow synaptic transmission should provide new interesting research avenues to understand and potentially manage DA-related human disorders.

Experimental Procedures

Experimental Animals

C57BL/129SvJ DAT-KO and βArr2-KO mice were described previously (Bohn et al., 1999; Cyr et al., 2003; Giros et al., 1996). βArr2-KO/DAT-KO mice were derived through a two-step breeding procedure. First, DAT-KO mice were mated to βArr2-KO mice to obtain double-heterozygote mice that were then bred to generate double-KO mice and control littermates having all possible combinations of the DAT and βArr2 mutant alleles. Three-month-old male wt C57BL/6J mice were obtained from Jackson Laboratory. For all experiments involving knockout animals, respective wt littermates were used as controls, and all mice used were of between 3 and 4 months of age. Before experiments, animals were housed four or five to a cage at 23°C on a 12 hr light/12 hr 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 Thr308, anti-phospho-Akt Ser473, anti-total-Akt, anti-PDK1, anti-phospho-DARPP-32 Thr34, and anti-ERK polyclonal antibodies as well as the anti-phospho-ERK1/2 (Thr202/Tyr204) monoclonal antibody were purchased from Cell Signaling Technology (Beverly, Massachusetts). Anti-DARPP-32 and anti-clathrin monoclonal antibodies were obtained from BD Transduction Laboratories (Lexington, Kentucky). The anti-GSK3α/β clone 0011-A and the anti-PP1 C subunit antibodies were from Santa Cruz Biotechnology (Santa Cruz, California). The anti-actin clone c4 was from Chemicon (Temecula, California). The anti-PP2A, B, and C subunit monoclonal antibodies were from Upstate (Charlottesville, Virginia). The polyclonal anti-β-arrestin antibodies have been described previously (Attramadal et al., 1992).

Drug Administration

Amphetamine and apomorphine (Sigma-Aldrich, St. Louis) were dissolved in saline or distilled water containing 0.1% ascorbate, respectively, and injected i.p. (amphetamine) or s.c. (apomorphine). SL327 (Tocris Cookson Inc., Ellisville, Missouri) was injected i.p. after suspension in a minimal amount of Tween and made up to volume with distilled water. Okadaic acid (Calbiochem, Cambridge, Massachusetts) and fostriecin (Sigma-Aldrich) were prepared in 50% DMSO in artificial cerebrospinal fluid and injected i.c.v. in a volume of 4 μl at a rate of 1 μl/min as described (Beaulieu et al., 2004). LY294002 (Sigma-Aldrich) was dissolved in a minimal volume of ethanol prior to being diluted in artificial cerebrospinal fluid and injected i.c.v.. Corresponding vehicle solutions were injected to respective control animals.

Western Blot Analysis

Western blot analyses were performed as described in Beaulieu et al. (2004).

Immunoprecipitations

Immunoprecipitations were carried out as described in Beaulieu et al. (2004) using either 30 μl of immobilized anti-Akt antibody (Cell Signaling Technology) or a monoclonal anti-PP2A B subunit antibody followed by a 2 hr incubation with protein G Sepharose (Amersham Bioscience).

GST Affinity Purification

GST-tagged constructs in pGEX-4T-1 expression vector (Amersham Bioscience) were transformed in *Escherichia coli* competent cells. Overnight cultures were grown from a single bacterial clone in LB medium supplemented with carbenicillin (50 μg/ml), diluted to an A_{600} of 0.2 in the same medium, and then induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 5 hr at 37°C. Cells were then pelleted; washed once with phosphate-buffered saline (PBS); resuspended in PBS containing protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 100 mM $MgCl_2$, 1 mg/ml lysozyme, and 1.7 U/ml DNase; and incubated for 30 min on ice. Cells were lysed by two freeze-thaw cycles followed by incubation for 30 min on ice and centrifuged at 16,000 rpm in a Sorvall SS-34 rotor for 45 min.

For experiments involving striatal protein extracts, ten mouse striata were homogenized in TE buffer (10 mM Tris-HCl [pH 7.4], 2 mM EDTA, 1.0% Triton X-100) containing protease inhibitors, incubated for 30 min on ice, and spun at 16,000 rpm in a Sorvall SS-34 rotor for 45 min. Recombinant phosphorylated Akt1 was purchased from Upstate.

Binding assays were performed using GSTrap FF affinity columns (Amersham Bioscience). Bacterial lysates containing either GST rat GST-β-arrestin 1 or rat GST-β-arrestin 2 constructs as well as striatal extracts or recombinant Akt1 were filtered through a 22 μm filter before they were sequentially applied to the columns using a peristaltic pump. The GST fusion proteins were eluted with 5 column volumes of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione [pH 8]). Samples were collected and protein concentration was determined using a DC protein-assay kit. Equal amounts of proteins were subjected to SDS-PAGE under denaturating conditions, transferred to nitrocellulose, analyzed by Ponceau staining, and subjected to Western blot analysis. Alternatively, SDS-PAGE gels were stained using Biosafe Coomassie (Bio-Rad Laboratories, Inc.).

Measurement of Locomotor Activity

Various parameters of locomotor activity in mice were evaluated as previously described (Beaulieu et al., 2004; Gainetdinov et al., 1999; Wang et al., 1997).

Statistical Analysis

Data were analyzed by two-tailed t test or one-way ANOVA. Values in graphs were expressed as mean ± SEM.

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