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Disruption of Akt signaling decreases dopamine sensitivity in modulation of inhibitory synaptic transmission in rat prefrontal cortex

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Abstract

Akt is a serine/threonine kinase, which is dramatically reduced in the prefrontal cortex (PFC) of patients with schizophrenia, and a deficiency in Akt1 results in PFC function abnormalities. Although the importance of Akt in dopamine (DA) transmission is well established, how impaired Akt signaling affects the DA modulation of synaptic transmission in the PFC has not been characterized. Here we show that Akt inhibitors significantly decreased receptor sensitivity to DA by shifting DA modulation of GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs) in prefrontal cortical neurons. Akt inhibition caused a significant decrease in synaptic D2R levels with high-dose DA exposure. In addition, Akt inhibition failed to affect DA modulation of IPSCs after blockade of β -arrestin 2. β -arrestin 2-mediated interaction of clathrin with D2R was enhanced by co-application of a Akt inhibitor and DA. Taken together, the reduced response in DA modulation of inhibitory transmission mainly involved β -arrestin 2-dependent D2 receptor desensitization.

Keywords

Dopamine; Dopamine receptors; Inhibitory synaptic transmission; Akt; Prefrontal cortex; Schizophrenia

INTRODUCTION

Akt, also known as protein kinase B (PKB), is a serine/threonine kinase that plays an important role in the pathogenesis of schizophrenia (SZ) (Bajestan et al., 2006; Emamian et al., 2004; Schwab et al., 2005; Xu et al., 2007). Akt1 protein levels were significantly reduced in brain tissues from patients with SZ, particularly in the prefrontal cortex (PFC)

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SUPPLEMENTAL INFORMATION

The Supplemental Information includes one supplemental figure.

AUTHOR CONTRIBUTIONS

YCL and WJG conceived and designed the experiments. YCL performed electrophysiological experiments, Western blots and Immunoprecipitation; SSY performed part of the electrophysiological experiment; YCL analyzed the data and wrote the manuscript. WJG instructed the experiments and revised the manuscript.

(Emamian, 2012; Emamian et al., 2004; Thiselton et al., 2008; Zhao et al., 2006). The PFC is known to be important in working memory and other cognitive functions, and PFC dysfunction is responsible for many neuropsychiatric disorders, including SZ (Goldman-Rakic and Selemon, 1997; Millan et al., 2012; Seamans and Yang, 2004). In fact, cognitive impairments, particularly working memory deficits, are considered to be a core feature of SZ. Therefore, it is possible that a loss of Akt contributes to PFC dysfunction. Indeed, deletion of Akt1 causes not only a decrease of dendritic architecture in the PFC, but also abnormal working memory performance (Lai et al., 2006). Notably, only under activation of D2 receptors (D2Rs) do Akt knockout mice display working memory deficits, indicating that Akt deficiency makes PFC dysfunction susceptible to tighter regulation by dopamine (DA) transmission (Lai et al., 2006). As a major neurotransmitter in the PFC, DA has long been implicated in SZ. Indeed, all antipsychotic drugs exert their actions by blocking D2Rs (Creese et al., 1976; Seeman and Lee, 1975; Seeman et al., 1976). Recent studies have shown that, apart from classical cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) and phospholipase C (PLC) signaling pathway (Greengard, 2001; Missale et al., 1998; Trantham-Davidson et al., 2004), D2Rs act through a cAMP-independent Akt–glycogen synthase kinase 3 (GSK-3) signaling cascade (Beaulieu et al., 2005; Beaulieu et al., 2004). Activation of D2Rs allows β -arrestin 2 to bind with protein phosphatase 2 (PP2A) and Akt to form a complex in which PP2A dephosphorylates and deactivates Akt, resulting in activation of GSK-3 (Beaulieu et al., 2005; Beaulieu et al., 2004). However, how Akt deficiency affects DA transmission and consequently results in abnormalities in PFC functioning remains unknown.

It is well established that alterations in gamma aminobutyric acid (GABA) receptor signaling is associated with SZ (Benes and Berretta, 2001; Lewis et al., 2005). The modulation of GABA_AR-mediated inhibitory transmission by DA is critical for normal cognitive processing. Furthermore, DA exhibits bidirectional effects on GABA_AR-mediated inhibitory postsynaptic currents (IPSCs); these currents are enhanced by activation of D1Rs and depressed by activation of D2Rs (Li et al., 2011; Li et al., 2012; Seamans et al., 2001; Trantham-Davidson et al., 2004). Our recent findings suggest that activation of GSK-3 β is involved in hyperdopamine/D2R-induced attenuation of GABA_AR-mediated IPSCs (Li et al., 2012). In this study, we further investigate whether and how Akt deficiency affects DA modulation of IPSCs in the PFC. To mimic cortical Akt deficiency, we blocked Akt activity by incubating PFC slices with Akt inhibitors. We found that disruption of Akt decreased DA sensitivity by increasing D2R internalization, which led to a significant change in DA modulation of IPSCs in the PFC.

Materials and Methods

Animals

A total of 112 Sprague Dawley rat pups were used for this study. The pups on postnatal days 10 and their moms were purchased from the Charles River Laboratories (Wilmington, MA) and they were housed in the animal facility with at least two days of accommodation before being used for experiments. Among these animals, 95 were aged between P12-21 (before weaning) with the sex of these animals not identified, and 17 male animals between P22 to

P30 used for electrophysiological recordings were also included. We did not observe significant differences between the young (P12-21) and older male animals (P22-30), so all electrophysiological data were pooled together, as we previously reported (Li et al., 2012). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Drexel University College of Medicine Animal Care and Use Committee.

Preparation of prefrontal cortical slices

The rats were anesthetized with Euthasol (0.2 ml/kg; Virbac AH), and the brains were immediately removed and placed in ice-cold ($\sim 4^{\circ}\text{C}$) sucrose solution (in mM: 2.5 KCl, 1.25 NaH_2PO_4 , 26 NaHCO_3 , 0.5 CaCl_2 , 7.0 MgSO_4 , 213 sucrose, pH 7.4) buffered with 95% O_2 and 5% CO_2 . Neocortex containing medial PFC (PrL) was horizontally sectioned at a thickness of 300 μm using a Leica MT1000 Vibratome (Leica Microsystems). Slices were then transferred to a holding chamber submerged in oxygenated artificial cerebrospinal fluid (ACSF) (in mM: 128 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 2 CaCl_2 , 1 MgSO_4 , 26 NaHCO_3 , and 10 dextrose, pH 7.4.) at 35°C for 1h and then remained at room temperature until used for electrophysiology or Western blotting.

Electrophysiology

Whole-cell patch-clamp recordings were conducted in prefrontal cortical slices through an upright Zeiss Axioskop 2 microscope (Carl Zeiss) that is equipped with optics of infrared-differential interference contrast (IR-DIC) and a digital video camera system. The recordings were conducted at $\sim 35^{\circ}\text{C}$ and the resistance of the recording pipette was 5–7 $\text{M}\Omega$. The inhibitory postsynaptic currents (IPSCs) in layer 5 pyramidal neurons were elicited by stimulating layer 2/3 with either a single pulse or paired pulses at 10 Hz (0.1 ms, 10–100 μA , 10 s inter-stimulus interval) through a bipolar electrode. The miniature IPSCs (mIPSCs) and spontaneous IPSCs (sIPSCs) were recorded at -65 mV in the presence of AP5 (D-(–)-2-Amino-5-phosphonopentanoic acid; 50 μM) and DNQX (6,7-dinitroquinoxaline-2,3-dione; 20 μM) to block glutamate receptor mediated currents with or without TTX (tetrodotoxin; 0.5 μM), respectively. GABA-induced inward currents were recorded at -60 mV in the presence of AP5 (50 μM), DNQX (20 μM) and TTX (0.5 μM) by bath application of GABA (300 μM). A high chloride Cs^+ -based intracellular solution (134 mM CsCl_2 , 2 mM MgCl_2 , 2 mM $\text{Na}_2\text{-ATP}$, 0.5 mM Na_2GTP , 5 mM $\text{Na}_2\text{-phosphocreatine}$, 1 mM EGTA, 10 mM HEPES, pH 7.25) was used for IPSC recordings. All neurons without stable baseline recording of IPSCs for 5 min or with series resistance increases of more than 20%, were discarded from further analysis. All drug effects were then normalized to baseline levels. Statistical analyses were performed using Student t-test between the controls and individual drug treatment groups and one-way or two-way analysis of variance (ANOVA) for multiple-comparisons among the several experimental groups. All data were presented as mean $\pm$ standard error.

Treatment of brain slices and protein extraction

Brain slices were incubated with oxygenated ACSF in the presence of different drugs for 15 minutes. PFC tissue was then dissected from drug-treated brain slices. For total protein extraction, the tissue was homogenized in RIPA buffer (50 mM Tris, 20 mM Tris-HCl, pH

7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 20 mM β -glycerophosphate disodium salt hydrate, 1 mM Na₃VO₄, 1 mg/ml leupeptin, 0.1% SDS, 1% Triton, 1 mM phenylmethanesulfonylfluoride (PMSF), and 1 mM NaF) and then centrifuged at 10,000 g for 10 min at 4°C. The supernatant fraction was aliquoted and stored at -80°C. For synaptosomal membrane protein extraction, tissue was homogenized in sucrose buffer (4 mM HEPES pH7.4, 320 mM sucrose, 2 mM EGTA, 10 mM Na pyrophosphate, 1 mM Na Orthovanadate, 10 mM NaF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin and 0.1 mM PMSF), and then centrifuged at 1000 g for 10 min to remove large cell fragments and nuclear material. The supernatant was centrifuged at 15,000 g for 15 min to yield cytoplasmic proteins in the supernatant. The pellet from this spin was resuspended in homogenization buffer and centrifuged at 15,000 g for an additional 15 min to yield washed synaptosomes. The synaptosomal fraction was then hypoosmotically lysed and centrifuged at 25,000 g for 30 min to yield synaptosomal plasma membranes in the pellet. Pellets were then resuspended in homogenization buffer and aliquoted and stored at -80°C until further use.

Western Blots

Equal amounts of proteins (10–20 μ g) were running on a 7.5% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA,) and blocked with 5% nonfat milk. The membranes were then incubated with the following primary antibodies overnight at 4°C: anti-D2R (1: 250, Santa Cruz Biotechnology, Dallas, TX), anti- β -arrestin2 (1:500, Cell Signaling Technology, Boston, MA), anti-clathrin (1:2000, Abcam, Cambridge, MA) and anti-actin (1: 100,000, Sigma-Aldrich, St. Louis, MO). After three 20 minute washes, the membranes were incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:5000 for 2 h. The immunopositive protein bands were detected with ECL Western Blotting System (GE Healthcare Biosciences, Piscataway, NJ). After the exposure of membranes to HyBlot CL Autoradiography film (Denville Scientific Inc., Holliston, MA), the band densities were measured with NIH Image J software. Final data were normalized to actin. To minimize the interblot variability, each sample was run and analyzed four times. Statistical analyses were similarly performed using Student t-test between the controls and individual drug treatment groups and one-way ANOVA for multiple-comparisons among the several experimental groups. The data were presented as mean $\pm$ standard error.

Immunoprecipitation

PFC tissue was dissected from drug treated brain slices, homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, protease inhibitor mixture) and then centrifuged at 10,000 g for 10 min at 4°C. Supernatant fractions (500 μ g proteins) were incubated overnight with 4–5 μ g of anti-D2R primary antibody (Santa Cruz Biotechnology, Dallas, TX). The immunocomplexes were isolated by the addition of 100 μ l of protein G Magbeads (Genscript, Piscataway, NJ), followed by incubation for 3–4 h at 4°C. The immunoprecipitates were then washed four times with PBS, resuspended in Laemmli sample buffer, and boiled for 5 min at 100°C. Next, samples were centrifuged at 10,000 g for 10 min and the supernatant was collected. The immunoprecipitated proteins were analyzed by

Western blot analysis with antibodies against β -arrestin2 (1:500, Cell Signaling Technology, Boston, MA) and clathrin (1:2000, Abcam, Cambridge, MA).

RESULTS

DA induces dose-dependent and bidirectional effects on GABA_A receptor-mediated inhibitory transmission

We first replicated the effects of different concentrations of DA on GABA_A receptor-mediated IPSCs in mPFC slices. Synaptically evoked IPSCs were recorded from layer 5 pyramidal neurons in the mPFC by stimulating layer 2/3, with a membrane potential held at -65 mV. The GABA_A receptor-mediated IPSCs could be completely abolished by GABA_A receptor antagonist picrotoxin (100 μ M) (data not shown). After recording baseline for at least 5 min to ensure that the recordings were stable, various doses of DA (0.2 μ M, 20 μ M and 200 μ M) were bath-applied to the mPFC slices for 10 minutes. As shown in Figure 1, DA exhibited a bidirectional dose-dependent effect on IPSCs in layer 5 pyramidal neurons. Specifically, at low concentrations of DA (0.2 μ M), IPSC amplitude was significantly increased by $41.38 \pm 9.39\%$ ($n = 10$, $p < 0.05$; Fig. 1A and B). In contrast, at higher doses of both 20 μ M and 200 μ M, DA significantly decreased the amplitude of IPSCs (decreased by $56.49 \pm 8.57\%$; $n = 9$, $p < 0.01$ at 20 μ M; and decreased by $48.16 \pm 8.77\%$; $n = 10$, $p < 0.05$ at 200 μ M; Fig. 1A and B). The suppressing effect on inhibitory currents was long lasting, without recovery ($p < 0.01$ for 20 μ M DA; and $p < 0.05$ for 200 μ M DA) even after 15 minutes washout; whereas the enhancing effect induced by 0.2 μ M DA partially recovered within 15 minutes ($p > 0.05$). Overall, DA exhibited both dose- and time-dependent effects on GABA_A receptor-mediated IPSCs in mPFC slices (two-way ANOVA: main effect of concentration, $F = 8.50$, $p < 0.01$; main effect of time, $F = 17.81$, $p < 0.01$; interaction effect, $F = 16.78$, $p < 0.01$). This dose-dependent effect is consistent with previous reports (Li et al., 2012; Trantham-Davidson et al., 2004), suggesting a bidirectional or inverted-U response (Figure 1B, right panel).

Akt deficiency reduces the sensitivity in DA modulation of inhibitory transmission

Akt is a downstream target of DA receptors, particularly D2Rs, and the Akt signaling pathway plays an important role in dopaminergic transmission to maintain normal PFC function. To examine whether Akt disruption affects DA regulation of inhibitory transmission, Akt inhibitors were bath-applied to mPFC slices for 5 minutes prior to co-application with DA. A selective and membrane permeable Akt inhibitor, 10-DEBC (10 μ M, $IC_{50} \sim 2\text{--}6$ μ M, Tocris), did not show clear effects on the basal amplitudes of IPSCs (increased by $1.42 \pm 0.08\%$; $n = 18$, $p > 0.05$; data not shown). However, co-application of 10-DEBC (10 μ M) with different doses of DA for 10 minutes significantly changed the bidirectional effects of DA on IPSCs. As shown in Figure 1C and D, in the presence of 10-DEBC, 0.2 μ M DA did not increase the IPSCs amplitude, but rather mildly decreased by $25.01 \pm 7.36\%$ without significance after 10 -minute application ($n = 9$, $p > 0.05$); and it significantly decreased the amplitude of IPSCs by $37.05 \pm 8.90\%$ after 15 -minute washout ($n = 9$; $p < 0.05$; One-way repeated ANOVA: main effect of time, $F = 6.49$, $p < 0.01$). In contrast, 20 μ M DA + 10-DEBC dramatically increased IPSC amplitude by $32.11 \pm 6.80\%$ ($n = 10$, $p < 0.05$), whereas 200 μ M DA + 10-DEBC showed similar inhibitory effects on

IPSC amplitude (decreased by $29.83 \pm 9.40\%$; $n = 12$, $p < 0.05$). After 15 minutes of washing, the enhancing effect of $20 \mu\text{M}$ DA could be washed out, but without a further decrease ($n = 10$, $p > 0.05$; One-way repeated ANOVA: main effect of time, $F = 4.01$, $p < 0.05$). The suppressing effect of $200 \mu\text{M}$ DA + 10-DEBC, however, remained to be long lasting ($n = 12$, $p < 0.05$; One-way repeated ANOVA: main effect of time, $F = 6.18$, $p < 0.05$). In addition, we tested lower concentrations of 10-DEBC at $1 \mu\text{M}$ or $3 \mu\text{M}$ and then co-applied them with $20 \mu\text{M}$ DA, respectively. Surprisingly, lower doses of 10-DEBC only prevented the suppressing effect of DA on IPSCs ($n = 6$, $p > 0.05$ for both; Fig. S1), but did not cause an enhancing effect like higher concentration of 10-DEBC ($n = 6$, $p < 0.05$). To verify the effect of Akt on DA regulation of inhibitory transmission, we further examined another structurally different Akt inhibitor. As shown in Figure 1D, Akt inhibitor VI ($5 \mu\text{M}$, $K_d \sim 18 \mu\text{M}$, Calbiochem), an impermeable Akt inhibitor which acts by interfering with Akt-phosphoinositide interaction, caused effects similar to 10-DEBC on inhibitory current responses to different doses of DA. When Akt inhibitor VI was loaded into the recording pipette, $20 \mu\text{M}$ DA produced a strong enhancement of IPSCs amplitude. IPSC amplitude increased by $52.13 \pm 13.74\%$ ($n = 8$; $p < 0.05$), but lost significance after a 15-minute washout (increased by $30.03 \pm 19.41\%$, $p > 0.05$; One-way repeated ANOVA: main effect of time, $F = 4.17$, $p < 0.05$). This enhancing effect was also washed out after 15 minutes, although a mild increase was still shown ($p > 0.05$). Both $0.2 \mu\text{M}$ DA and $200 \mu\text{M}$ DA showed depressant effects on IPSC, but there was no statistical significance after either administrating drugs for 10 minutes or washing for 15 minutes (amplitude decreased by $20.54 \pm 12.21\%$ for $0.2 \mu\text{M}$ DA, $n = 8$, $p > 0.05$; One-way repeated ANOVA: main effect of time, $F = 1.02$, $p = 0.39$ and amplitude decreased by $23.01 \pm 11.28\%$ for $200 \mu\text{M}$ DA, $n = 8$, $p > 0.05$; One-way repeated ANOVA: main effect of time, $F = 0.17$, $p > 0.05$). These results suggest that inhibition of Akt could significantly change the sensitivity in DA modulation of inhibitory transmission, shifting the inverted-U curve of DA response to the left (Figure 1D, right panel).

Akt inhibition-induced decrease in DA sensitivity is associated with a postsynaptic mechanism

The alteration of either pre-synaptic neurotransmitter release or post-synaptic receptor function could change DA regulation of inhibitory transmission. Although the depressant effect of an impermeable Akt inhibitor on the sensitivity in DA modulation of inhibitory transmission directly indicates a postsynaptic mechanism, we further confirmed this assumption by examining paired-pulse ratio, mIPSCs, and bath-GABA-induced currents before and after application of DA ($20 \mu\text{M}$) alone or with Akt inhibitor 10-DEBC ($10 \mu\text{M}$) for 10 minutes (10-DEBC was applied alone for 5 minutes prior to DA). As shown in Figure 2A, IPSCs were elicited by stimulating intracortical fibers in layer 2/3 with two successive stimuli of identical strength at an interval of 100 ms (10 Hz) before and after application of drugs. Although the amplitudes of the first IPSCs were correspondingly decreased or increased by DA or DA co-applied with 10-DEBC, respectively, the paired-pulse ratios were only slightly changed without significant difference (DA alone $n = 11$, $p > 0.05$; 10-DEBC alone or 10-DEBC + DA $n = 12$, $p > 0.05$ for both; One-way repeated ANOVA: main effect of different drugs, $F = 2.59$, $p > 0.05$; Fig. 2A). This finding suggests that there is no significant pre-synaptic change in GABA release. Furthermore, DA caused a significant

reduction in the amplitude of mIPSCs, but had no effect on the frequency ($n = 13$, $p < 0.05$ for amplitude; One-way repeated ANOVA: main effect of time, $F = 5.94$, $p < 0.01$. $p > 0.05$ for frequency; One-way repeated ANOVA: main effect of time. $F = 0.77$, $p = 0.48$; Fig. 2B and C), strongly suggesting a postsynaptic action. However, 10-DEBC could prevent the decrease in mIPSCs amplitude ($n = 11$, $p > 0.05$; One-way repeated ANOVA: main effect of time, $F = 2.25$, $p > 0.05$) and induce an increase of the frequency, potentially obscuring any presynaptic effects ($n = 11$, $p < 0.05$; One-way repeated ANOVA: main effect of time, $F = 1.54$, $p > 0.05$; Fig. 2B and C). Moreover, when bath-application of GABA (300 μM , 1 min) was used to elicit GABA-induced inward current, as shown in Figure 2D, DA (20 μM) significantly decreased the amplitude of GABA-induced current by $45.19 \pm 6.38\%$ ($n = 8$, $p < 0.05$). In contrast, either application of 10-DEBC alone or co-application of 10-DEBC with DA did not influence GABA-induced current. The amplitude of GABA-induced current was increased $0.01 \pm 9.61\%$ by 10-DEBC and was decreased $0.02 \pm 16.65\%$ by co-application of 10-DEBC with DA ($n = 8$, $p > 0.05$ for both; One-way repeated ANOVA: main effect of different drugs, $F = 1.54$, $p > 0.05$; Fig. 2E). All together, these results suggest that either DA or Akt inhibitor's effects on inhibitory transmission is likely attributable to changes in post-synaptic receptors.

Akt inhibition-induced decrease in sensitivity to DA modulation of inhibitory transmission is mediated by DA receptors

To determine whether reduced IPSCs in response to DA, as induced by Akt deficiency, is due to attenuated function of postsynaptic DA receptors, we next examined DA receptor function. Normally, the dose-dependent and bidirectional modulation of IPSCs by DA is dependent on the activation of different DA receptor subtypes (Kroener and Lavin, 2010; Seamans et al., 2001; Seamans and Yang, 2004; Trantham-Davidson et al., 2004). Our results indicate that the enhancement effect of low-dose DA on inhibitory transmission is mediated by D1Rs, whereas the depressant effect of high-dose DA on inhibitory transmission is mediated predominately by D2Rs. As shown in Figure 3A, when we bath applied D1R agonist, SKF 81297 (1 μM), for 10 minutes, IPSCs amplitude increased by $27.70 \pm 4.34\%$ ($n = 10$, $p < 0.05$), similar to the effect of low-dose DA (0.2 μM). However, when SKF 81297 (1 μM) was co-applied with the Akt inhibitor, 10-DEBC (10 μM), for 10 minutes, the D1R agonist's enhancement effect was abolished, replaced with a decreased IPSC amplitude ($21.19 \pm 10.14\%$; $n = 10$, $p > 0.05$). This effect is, again, similar to the effects induced by co-application of low-dose DA and 10-DEBC. This result suggests that Akt deficiency also decreases D1R function under low-DA condition. On the contrary, selective D2R agonist quinpirole (1 μM) mimicked high-dose DA's effect, decreasing IPSC amplitude by $51.40 \pm 6.71\%$ ($n = 10$, $p < 0.01$; Fig. 3A). Co-application of quinpirole with 10-DEBC for 10 minutes eliminated the depressant effect, indicating a reduction of D2R's effect on IPSCs (amplitude decreased by $17.12 \pm 3.47\%$; $n = 12$, $p > 0.05$). Nevertheless, co-application of D2R agonist with Akt inhibitor could not completely mimic Akt inhibition's reversing effects on high-dose DA (20 μM)-mediated depression of IPSCs. One possible reason is that the depressant effect of high-dose DA is mediated by both D1Rs and D2Rs, although D2R plays a dominant effect. Given that an Akt inhibitor specifically decreased D2R function under a high-DA condition, and that this action in turn resulted in elevated D1R activity, the reversing effect of an Akt inhibitor on high-dose DA (20 μM)-induced

enhancement of IPSCs could be mediated by activation of D1R. To test this speculation, we co-applied D1R antagonist, SCH23390 (1 μ M), with 10-DEBC and DA (20 μ M) for 10 minutes. As expected, SCH23390 blocked the reversing effect of Akt inhibition and resulted in a similar effect to that of 20 μ M DA application alone (IPSC amplitude decreased by $49.21 \pm 10.42\%$; $n = 10$, $p < 0.05$; Fig. 3B), suggesting that the enhancement effect of Akt inhibitor with high-dose DA (20 μ M) on IPSC is mediated by D1 receptors. However, co-applied D2 antagonist, L-741626 (10 μ M), with 10-DEBC and DA (20 μ M) for 10 minutes partially blocked the effect of Akt inhibitor with 20 μ M DA on IPSC (IPSC amplitude increased by $2.03 \pm 1.41\%$; $n = 9$, $p > 0.05$), indicating a decrease of D1 function. All together, these results suggest that blocking Akt activity results in a decrease of D2R-mediated high-dose DA stimulation, and might have a mild effect on D1R function as well.

Akt deficiency decreases D2R expression levels on synaptosomal membrane in neurons treated with high-dose DA stimulation

One efficient way to regulate receptor surface expression is to alter the number of receptors expressed on the cell membranes. DA receptors are the prototype example of G protein-coupled receptors (GPCRs). Activation-dependent regulation of the number of receptors presenting on the synaptic membranes is one of the major mechanisms of GPCR desensitization. We therefore examined whether this potential mechanism is associated with Akt deficiency-induced D2R desensitization under high-dose DA stimulation. D2R protein levels expressed on synaptic membranes were measured by Western blots. As shown in Figure 4, neither 10-DEBC (10 μ M) nor DA (20 μ M) had significant effects on synaptosomal membrane D2R protein levels (8 slices from 4 animals for each treatment, $p > 0.05$). However, treatment of mPFC slices with 10-DEBC (10 μ M) for 5 min prior to administration of DA (20 μ M) for 15 minutes dramatically reduced D2R protein levels on synaptosomal membrane (8 slices from 4 animals, $p < 0.01$; One-way ANOVA: main effect of different treatments, $F = 5.96$, $p < 0.01$; Fig. 4). This result suggests that Akt deficiency decreases D2R sensitivity by decreasing its protein levels on synaptosomal membrane and that this action depends on D2R activation.

β -arrestin 2 is required for Akt deficiency-induced desensitization of D2Rs to inhibitory transmission

A recent study demonstrated that β -arrestin 2, a scaffolding protein, is involved in D2R-mediated regulation of Akt activity (Beaulieu et al., 2007; Beaulieu et al., 2005). It has been proposed that activation of D2Rs results in the formation of a binding complex with β -arrestin 2, which in turn deactivates Akt. Therefore, we next tested whether β -arrestin 2 is required for Akt deficiency-induced desensitization of DA in modulation of inhibitory transmission. Because there is no selective β -arrestin 2 protein inhibitor, we loaded anti- β -arrestin 2 antibody (10 μ g/ml; Bioworld Technology) into a recording pipette to determine its effects on evoked IPSCs. To allow diffusion of antibody into the cell, we waited at least 10 minutes before recording evoked IPSCs. β -arrestin 2 antibody did not show any effects on the IPSC after 10 minutes recording ($n = 10$, $p > 0.05$; Fig. 5A). Surprisingly, under this condition, further DA (20 μ M) application did not induce a depressive effect on the IPSCs ($n = 10$, $p > 0.05$; One-way repeated ANOVA: main effect of time, $F = 0.35$, $p > 0.05$; Fig. 5A), suggesting that β -arrestin 2 is required for DA modulation of inhibitory transmission. To

determine whether a blockade of Akt activity still affects DA regulation of inhibitory transmission without activation of β -arrestin 2, we used the same strategy to block β -arrestin 2 with β -arrestin 2 antibody and then applied 10-DEBC for 10 min prior to DA administration. As shown in Figure 5B, 10-DEBC had no significant effect on IPSC amplitude after 10-minute application ($n = 10$, $p > 0.05$). However, further DA ($20 \mu\text{M}$) application failed to enhance inhibitory transmission, and instead, decreased the amplitude of evoked IPSCs by $43.16 \pm 5.49\%$ after the first 5 minutes of DA application and $62.37 \pm 7.62\%$ at 10 min of DA application ($n = 10$ for both, $p < 0.05$; One-way repeated ANOVA: main effect of time, $F = 4.14$, $p < 0.05$; Fig. 5B). These results suggest that Akt deficiency-induced desensitization of DA in modulation of inhibitory transmission is dependent on β -arrestin 2-mediated D2R signaling.

Akt deficiency enhanced β -arrestin 2-dependent interaction of D2R and clathrin

In addition to involvement in Akt signaling, β -arrestin 2 is also known to play an important role in the desensitization of DA receptors. Following stimulation, DA receptors are phosphorylated by G protein kinases. Phosphorylated receptors bind to β -arrestin with a high-affinity, after which an endocytic complex is recruited in order to internalize the receptors, principally in clathrin-coated vesicles. Given that the alteration of β -arrestin 2 is a possible mechanism for Akt deficiency-induced desensitization of D2R following DA stimulation, we first examined the protein levels of β -arrestin 2. As shown in Figure 6, either 10-DEBC or DA ($20 \mu\text{M}$) applied for 10 minutes significantly increased the expression of β -arrestin 2 (8 slices from 4 animals for each treatment, $p < 0.05$ for both). However, co-application of 10-DEBC and DA ($20 \mu\text{M}$) together caused a more dramatic enhancement of β -arrestin 2 protein levels than a single application of either drug alone (8 slices from 4 animals, $p < 0.05$; One-way ANOVA: main effect of different treatments, $F = 8.66$, $p < 0.01$; Fig. 6A and B).

To further determine whether this increase of β -arrestin 2 is the mechanism underlying Akt deficiency-induced desensitization of D2Rs, we detected the interaction between β -arrestin 2 and D2R with co-immunoprecipitation. Surprisingly, 10-DEBC did not change the binding of β -arrestin 2 with D2R following D2R activation by co-applied DA ($20 \mu\text{M}$) compared with application of DA alone (8 slices from 4 animals, $p > 0.05$; Fig. 6C). This result is thus suggesting that the increase of total β -arrestin 2 protein levels may not be directly related to Akt deficiency-induced D2R desensitization. β -arrestin 2-mediated GPCR desensitization is dependent on a direct interaction between β -arrestin 2 and clathrin, which leads to receptor desensitization and endocytosis. Therefore, we also co-immunoprecipitated clathrin with D2R and found that the amount of clathrin interacting with D2Rs was significantly increased by co-application of 10-DEBC and DA compared with DA alone (8 slices from 4 animals, $p < 0.05$; Fig. 6C). In addition, this enhancement is not associated with a change of total protein levels, since the inputs of either clathrin or D2R did not show any changes (8 slices from 4 animals, $p > 0.05$ for both clathrin and D2, $p < 0.05$ for β -arrestin 2; Fig. 6D). Taken together, our finding indicates that Akt deficiency-induced D2R sensitivity change is mediated by the increase of β -arrestin 2-mediated interaction of clathrin and D2Rs, which leads to potentiation of D2R internalization (Fig. 7).

DISCUSSION

Akt1 deficiency has been highly implicated in the pathogenesis of SZ, as well as PFC-dependent cognitive impairments. Akt is also an important downstream substrate of D2R-mediated signaling. However, how Akt affects DA's modulatory action in synaptic transmission and ultimately impairs PFC function has not been investigated. Our results show that pharmacological blockade of Akt activity by Akt inhibitors causes a significant decrease of DA sensitivity in modulation of GABAergic synaptic transmission. This Akt deficiency-induced effect on DA sensitivity is attributable to the increased β -arrestin 2-dependent interaction of D2Rs and clathrin, which in turn results in the internalization and desensitization of D2Rs.

Activation of Akt is mainly controlled by phospholipid binding and phosphorylation. Phosphorylation of the tyrosine and serine residues of Akt is critical for optimal kinase activity. Akt possesses a PH domain, which binds to either PIP2 or PIP3. Once correctly positioned at the membrane via binding to PIP2 or 3, Akt can be phosphorylated by its activating kinases, such as PDK1 and mTOR2. In order to block Akt activity, we have used two different selective inhibitors. One is a membrane permeable inhibitor 10-DEBC, which can inhibit phosphorylation and activation of Akt. The other is a membrane impermeable Akt inhibitor VI. This inhibitor interferes with the PH domain and hence prevents phosphoinositide binding to Akt. Both inhibitors have been reported to significantly reduce phosphorylation at both the tyrosine and serine residues of Akt (Hiromura et al., 2004; Thimmaiah et al., 2005).

Sensitivity of DA receptors is an important indicator of DA function. A number of neuropsychiatric disorders such as SZ, Tourette's syndrome, and drug addiction are associated with imbalanced DA transmission (Jenner and Marsden, 1987; Nestler and Aghajanian, 1997; Sealfon and Olanow, 2000; Seeman et al., 2006; Singer, 2011). We have tested the effects of two selective Akt inhibitors on DA modulation of IPSCs and found that blocking Akt led to a significant left shift (reduction) of DA sensitivity in modulation of IPSCs recorded from layer V pyramidal neurons in the PFC. This result suggests that Akt deficiency decreases the sensitivity in DA modulation of inhibitory synaptic transmission in the PFC. The DA hypothesis of SZ proposes an imbalance of DA with hyperactive subcortical mesolimbic projections and hypoactive mesocortical PFC projections in SZ (Simpson et al., 2010; Tzschentke, 2001). DA with hyperactive subcortical function was based on the established antipsychotic medications that antagonize D2Rs (Creese et al., 1976; Seeman and Lee, 1975) and the psychotogenic effects of DA-like drugs (Lieberman et al., 1987; van Kammen et al., 1984). Consequently, an increase of subcortical D2R sensitivity is implicated in the actions of these drugs (Abi-Dargham et al., 2000; Howes et al., 2009; Kapur et al., 2000; Kessler et al., 2009; Nordstrom et al., 1993; Wong et al., 1986). Although D2R blockers have great efficacy for positive symptoms, they are ineffective for negative and cognitive symptoms that are presumably associated with reduced DA function in the PFC. Numerous brain-imaging studies showed a positive correlation between PFC DA hypoactivity and cognitive impairments (Buchsbaum et al., 1982; Farkas et al., 1984; Ingvar and Franzen, 1974; Liddle et al., 1992; Ragland et al., 2007). Since the majority of DA receptors in the PFC are of the D1 subtype (Bergson et al., 1995; Hall et al., 1994), cortical

D1R hypofunction is believed to be the critical contributor to cognitive impairment in SZ (Abi-Dargham et al., 2002; Karlsson et al., 2002; Okubo et al., 1997). Indeed, blockade of D1Rs in the PFC impairs working memory in nonhuman primates (Vijayraghavan et al., 2007; Williams and Goldman-Rakic, 1995). However, clinical trials with D1 agonists in SZ treatment were not effective. Unlike PFC D1Rs, studies about how PFC D2Rs contribute to cognitive function are rare and inconsistent (Druzin et al., 2000; Wang et al., 2004), indicating that the role of PFC DA in SZ is far more complicated than what was generally proposed.

It has been reported that DA dose-dependently modulates GABA_A receptor-mediated inhibitory transmission by activating different DA receptor subtypes (Kroener and Lavin, 2010; Seamans et al., 2001; Seamans and Yang, 2004; Trantham-Davidson et al., 2004). Specifically, we and others have consistently reported that low DA (< 500 nM) increased the amplitude of IPSCs via activation of D1Rs, whereas high doses of DA (> 10 μ M) decreased the amplitude of IPSCs through activation of D2Rs in juvenile rat PFC (P12-P30) (Li et al., 2012; Seamans et al., 2001; Trantham-Davidson et al., 2004). In this study, we found that blockade of Akt decreased not only the function of D1Rs, but also the sensitivity of D2Rs, depending on the concentration of DA exposed, in the juvenile rat PFC neurons. Previous studies reported that the window of DA exponential growth is P30-P60 revealed by DA immunoreactivity (Dawirs et al., 1993) and DA changes the excitability of GABAergic interneuron in the PFC from adult (PD > 50), but not preadolescent (PD < 36) animals (Tseng and O'Donnell, 2007). However, DA affects inhibitory synaptic transmission much earlier compared with its effect on excitability of GABAergic interneuron (Tseng and O'Donnell, 2007), as discussed above (Li et al., 2012; Seamans et al., 2001; Trantham-Davidson et al., 2004). It is possible that Akt's effect on the D2 modulation of GABAergic transmission exhibited in juvenile period may be age-dependent, and therefore different from that in adolescent or adult neurons. Moreover, we previously reported that in a SZ animal model, overexpression of D2Rs in the striatum resulted in an attenuation of cortical DA's sensitivity in modulation of inhibitory synaptic transmission (Li et al., 2011). However, it is still unknown whether the sensitivity of DA receptors in the PFC will be altered to compensate for a long-term loss of Akt. Nevertheless, the sensitivity change of cortical DA receptors could be one of the important contributors to PFC dysfunction, as the DA imbalance originally hypothesis proposed.

How does Akt affect DA sensitivity? It has been proposed that following activation, DA receptors are usually phosphorylated by G protein-coupled receptor kinases. Phosphorylated receptors present high-affinity binding with multifunctional scaffolding proteins such as β -arrestin. The recruitment of β -arrestin to DA receptors activates two different processes. On one hand, an endocytic complex comprising of β -arrestin, adaptor protein (AP2) and clathrin is recruited for receptor desensitization. The formation of the complex leads to receptor internalization through clathrin-mediated endocytosis. On the other hand, β -arrestin 2 binding with D2R can directly scaffold with Akt and PP2A to form a complex. Once in this complex, PP2A dephosphorylates and deactivates Akt (Beaulieu et al., 2012; Beaulieu et al., 2005). It is thus possible that when Akt is dephosphorylated by an Akt inhibitor prior to DA receptor activation, recruitment of β -arrestin to DA receptors after DA stimulation will increase the probability of creating endocytic complexes rather than Akt signaling

complexes. Our results showed that Akt deficiency indeed enhanced the affinity of D2R and β -arrestin 2 complex with clathrin, which is responsible for the decrease of D2Rs on the synaptosomal membrane by increasing receptor internalization. Since the interaction of clathrin with D2Rs must be through β -arrestin, it appears to be a required mediator for DA sensitization. In fact, our data showed that Akt inhibitor-induced sensitivity decrease in DA modulation of inhibitory transmission could not be detected when we blocked β -arrestin 2 activity. Although a previous study reported that over expression of β -arrestin enhanced receptor internalization (Ferguson et al., 1996) and an Akt inhibitor caused an increase of β -arrestin 2 in our study, the interaction between β -arrestin 2 and D2R was unchanged, indicating that the increase of β -arrestin 2 is not directly associated with D2R internalization. Instead, the interaction between D2R and clathrin appears to be the major player in D2R endocytosis.

It should be noted that DA D2Rs exist in both low- and high-affinity states (D2 Low and D2 High). D2 High is linked to G protein and exhibits high affinity for agonists; D2 Low exhibits low affinity for agonists. D2 Low usually binds to β -arrestin and clathrin, and eventually becomes internalized. Preclinical studies showed that psychosis is associated with an increase of D2 High in the striatum (Seeman, 2006; Seeman et al., 2005), but the proportion of D2 High receptors in the striatum was not affected in GSK3 β knockout mice (Seeman, 2011). Therefore, it is still unclear whether the D2R/Akt/GSK3 pathway is related or linked to factors controlling the D2R state. Here we found that blocking Akt activity increased interaction of D2Rs with clathrin in the PFC, suggesting an increase of D2 Low. Thus, it is likely that the Akt pathway is associated with controlling cortical D2R state without affecting total D2R expression in the PFC. However, it has not been possible to measure cortical D2R state because of very low density of D2R expression in the PFC (Seamans et al., 2008), and thus this assumption remains to be tested.

Based on our findings, here we provide a schematic graph (Figure 7) to show how inhibition of Akt activity affects the DA modulation of inhibitory synaptic transmission via D2R/Akt/ β -arrestin 2/clathrin cascade. Under normal condition, activation of D2Rs negatively regulates GABA_A receptor-mediated transmission via D2R/ β -arrestin 2/Akt complex dependent cascade. However, when Akt is dephosphorylated by an Akt inhibitor prior to DA receptor activation, recruitment of β -arrestin to DA receptors after DA stimulation enhances the interaction between D2R and clathrin. This enhancement causes more D2Rs endocytosis and leads to a diminished D2R-mediated depressive effect on GABA_A receptor-mediated transmission.

In conclusion, we have shown that Akt deficiency attenuated DA sensitivity in regulating GABA_A receptor-mediated inhibitory transmission in the PFC by desensitizing D2Rs. This work suggests that desensitization of D2Rs in the PFC plays a key role in Akt deficit-induced abnormal PFC functioning. Our findings may explain why either D2 antagonist or D1 agonist have not yielded satisfactory results in improving cognitive function, though the hypothesis of hyperactive D2Rs in the striatum and hypoactive D1Rs in the PFC is widely accepted in the field of SZ. Furthermore, a fine tuning between GABAergic and glutamatergic transmission has been proposed to have a critical role in maintaining normal working memory and disruption of such a balance is involved in the pathophysiology of

cognitive deficits in SZ and other related psychiatric disorders (Lewis and Gonzalez-Burgos, 2006). Therefore, future studies should aim to understand whether excitatory transmission is similarly affected by a reduction of DA sensitivity in the Akt deficiency animal model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Dopamine (DA) induces dose-dependent and bidirectional effects on GABA_A receptor-mediated IPSCs in PFC layer V pyramidal neurons; however, blocking Akt activity by Akt inhibitors leads to reduction of DA sensitivity in modulation of GABA_A receptor-mediated IPSCs. **A**, Representative samples show changes of IPSC amplitudes before and after bath application of 0.2, 20, and 200 μ M DA, respectively. **B**, Left panel: Summary graph shows the effects of DA on the amplitudes of IPSCs during application of DA for 10 min and after 15 min washing. Application of 0.2 μ M DA for 10 min significantly increased the amplitude of IPSCs ($n = 10$, $* p < 0.05$). In contrast, 20 or 200 μ M DA dramatically decreased the amplitude of IPSCs without clear recovery even after washing for 15 min (20 μ M DA $n = 9$, $** p < 0.01$; 200 μ M DA $n = 10$, $* p < 0.05$). Overall, DA exhibited both dose- and time-

dependent effects on GABA_A receptor-mediated IPSCs in the mPFC slices (two-way ANOVA: main effect of concentration, $F = 8.50$, $p < 0.01$; main effect of time, $F = 17.81$, $p < 0.01$; interaction effect, $F = 16.78$, $p < 0.01$). Right panel: DA exhibits a dose-dependent and inverted-U effect on IPSCs. **C**, Representative samples show the changes of IPSC amplitudes before and after application of Akt inhibitor 10-DEBC (10 μ M bath) with 0.2, 20, and 200 μ M DA, respectively. **D**, Summary graph shows the effects of DA on the amplitudes of IPSCs after application of drugs for 10 min and washing for 15 min. Co-application of DA with 10-DEBC for 10 min shifted the bidirectional effects on evoked IPSCs with a significant increase and opposite effect at 20 μ M ($n = 10$, $* p < 0.05$), a reduced decrease at 200 μ M ($n = 12$, $* p < 0.05$), and a slight decrease but not significant effect at 0.2 μ M ($n = 9$, $p > 0.05$, left). After 15 min washing following application of drugs for 10 min, co-application of Akt inhibitor with different concentrations of DA showed suppressive effects on IPSCs at 0.2 μ M or 200 μ M (0.2 μ M DA $n = 9$, $* p < 0.05$; 200 μ M DA $n = 12$, $p < 0.05$), and had no significant effect at 20 μ M DA ($n = 8$, $p > 0.05$). Similar to 10-DEBC, 5 μ M Akt inhibitor VI, an impermeable Akt inhibitor, loaded in recording peptide also altered DA-induced bidirectional effects on evoked IPSCs. Far right panel: Application of AKT inhibitor shifted the inverted-U curve of DA's dose-dependent effect on IPSC.

Figure 2.

Akt inhibitor affects DA regulation of mEPSCs and GABA-induced currents, but doesn't show any effects on the paired-pulse ratio. **A**, The paired-pulse ratio of the IPSCs was unaltered before and after administration of different drugs as indicated (DA alone $n = 11$, $p > 0.05$; 10-DEBC alone or 10-DEBC + DA $n = 12$ for both, $p > 0.05$). **B**, Representative traces of mIPSCs before, during and after application of drugs. **C**, Application of 20 μ M DA for 10 min significantly decreased the amplitude of mIPSC ($n = 13$, $* p < 0.05$), but has no effect on the frequency of mIPSCs ($n = 13$, $p > 0.05$). Co-application of 10 μ M 10-DEBC

with 20 μ M DA for 10 min completely blocked DA's effects on the amplitude of mIPSCs ($n = 11$, $p > 0.05$) and even enhanced the frequency of mIPSCs ($n = 11$, $p < 0.05$). **D and E**, left panel: Representative traces of bath-applied GABA (200 μ M)-induced currents; right panel: Bath application of 20 μ M DA for 10 min significantly decreased the GABA-induced currents ($n = 8$, $** p < 0.01$) and this effect was completely blocked by co-application of 10 μ M 10-DEBC, although 10-DEBC alone had no effect on GABA-induced currents ($n = 8$, $p > 0.05$ for both).

Figure 3.

Akt inhibitor dose-dependently desensitizes DA receptors' response to inhibitory transmission. decreases D2 receptor protein levels in synaptic plasma membrane after application of DA **A**, Amplitude of evoked IPSCs was significantly increased by D1 agonist (1 μM SKF81297) or significantly decreased by D2 agonist (quinpirole 1 μM) ($n=10$ * $p < 0.05$ for both). However, co-application of 10-DEBC (10 μM) with either D1 agonist or D2 agonist completely prevented these agonists' effects on IPSCs (SKF + DA $n = 10$ and quinpirole + DA $n = 12$, $p > 0.05$ for both). **B**, 10-DEBC-induced shift of 20 μM DA's effects on IPSCs was completely blocked by D1 antagonist (1 μM SCH23390) ($n = 10$, $p > 0.05$), but only partially blocked by D2 antagonist (10 μM L-741626, $n = 9$, $p > 0.05$).

Figure 4.

Blocking Akt activity decreases D2 receptor protein levels in synaptic plasma membrane after application of DA. **A**, Representative Western blots show the D2 receptor protein levels in synaptic plasma membrane fractions from mPFC slices incubated with Ringer's solution as a control, 10 μM 10-DEBC, or 20 μM DA with and without 10 μM 10-DEBC. **B**, Summary histogram shows that there is no significant difference between incubation with and without application of 10 μM 10-DEBC ($n = 8$ slices from 4 animals, $p > 0.05$). However, D2 receptor protein levels were significantly decreased by co-application of Akt inhibitor with 20 μM DA compared with either control ($n = 8$ slices from 4 rats, $** p < 0.01$) or the 20 μM DA ($n = 8$ slices from 4 rats, $## p < 0.01$).

Figure 5.

Akt inhibitor fails to affect the DA modulation of evoked IPSCs when β -arrestin2 is blocked.

A, blockade of β -arrestin2 by loading β -arrestin2 antibody (10 μ g) into the recording pipette completely prevented 20 μ M DA's inhibitory effects on evoked IPSCs ($n = 10$, One-way repeated ANOVA, main effect of time, $F = 0.35$, $p > 0.05$). **B**, Co-application of 10 μ M 10-DEBC with 20 μ M DA failed to cause enhancement effects on evoked IPSCs in the presence of β -arrestin2 antibody (10 μ g; $n = 10$, One-way repeated ANOVA, main effect of time, $F = 4.14$, $p < 0.05$). The representative evoked IPSC traces are shown at the top of the figure.

Figure 6.

Akt inhibitor enhances β-arrestin 2 protein levels and β-arrestin 2-dependent interaction of D2R and clathrin. **A**, Representative blots show the protein levels of β-arrestin 2 after incubating mPFC slices with Ringer's solution as control or 10 μM 10-DEBC or the 20 μM DA with and without 10-DEBC. **B**, Summary histogram shows that either single application of 10-DEBC or 20 μM DA significantly increased β-arrestin 2 protein levels (n = 8 slices from 4 animals, * p < 0.05 for both). Co-application of 10-DEBC and 20 μM DA caused a greater increase of β-arrestin 2 (n = 8 slices from 4 rats, * p < 0.05) than a single application

of DA (# $p < 0.05$). **C**, D2R immunoprecipitation assay shows that both β -arrestin 2 and clathrin were co-immunoprecipitated with D2R (Left). Co-incubation of 10-DEBC (10 μ M) and DA (20 μ M) with PFC slices significantly increased β -arrestin 2-dependent interaction of D2R and clathrin compared with incubation of DA alone ($n = 8$ slices from 4 rats, * $p < 0.05$). However, the interaction of β -arrestin 2 and D2R exhibited no difference under conditions of with or without administration of 10-DEBC ($n = 8$ slices from 4 rats, $p > 0.05$ right). **D**, Input lysates were detected by western blotting as the representative images showed in the left. Quantitative analysis shows that protein levels of β -arrestin 2 were increased by co-application of 10-DEBC (10 μ M) and DA (20 μ M) compared with administration of DA alone ($n = 8$ slices from 4 rats, * $p < 0.05$). However, neither clathrin, nor D2R was changed ($n = 8$ slices from 4 rats, $p > 0.05$ right).

Figure 7.

Schematic graph shows how Akt mediates the DA modulation of IPSCs in the normal state and Akt deficient state. Under normal conditions, activation of D2Rs negatively regulates GABA_A receptor-mediated transmission via a D2R/ β -arrestin 2/Akt complex- dependent cascade. However, when Akt is dephosphorylated by Akt inhibitor prior to DA receptor activation, recruitment of β -arrestin to DA receptors after DA stimulation enhances the interaction between D2R and clathrin, which causes more D2R endocytosis and leads to a

diminished D2R-mediated depressive effect on GABA_A receptor-mediated transmission, resulting in a net enhancement of IPSCs.

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