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Original Article

# Modulation of $A_{2a}$ receptor antagonist on $D_2$ receptor internalization and ERK phosphorylation

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**Aim:** To explore the effects of heterodimerization of $D_2$ receptor/$A_{2a}$ receptor ($D_2R/A_{2a}R$) on $D_2R$ internalization and $D_2R$ downstream signaling in primary cultured striatal neurons and HEK293 cells co-expressing $A_{2a}R$ and $D_2R$ *in vitro*.
**Methods:** Primary cultured rat striatal neurons and HEK293 cells co-expressing $A_{2a}R$ and $D_2R$ were treated with $A_{2a}R$- or $D_2R$-specific agonists. $D_2R$ internalization was detected using a biotinylation assay and confocal microscopy. ERK, Src kinase and β-arrestin were measured using Western blotting. The interaction between $A_{2a}R$ and $D_2R$ was detected using bioluminescence resonance energy transfer (BRET) and immunoprecipitation.
**Results:** $D_2R$ and $A_{2a}R$ were co-localized and formed complexes in striatal neurons, while both the receptors formed heterodimers in the HEK293 cells. In striatal neurons and the HEK293 cells, the $D_2R$ agonist quinpirole (1 μmol/L) marked increased Src phosphorylation and β-arrestin recruitment, thereby $D_2R$ internalization. Co-treatment with the $A_{2a}R$ antagonist ZM241385 (100 nmol/L) significantly attenuated these $D_2R$-mediated changes. Furthermore, both ZM241385 (100 nmol/L) and the specific Src kinase inhibitor PP2 (5 μmol/L) blocked $D_2R$-mediated ERK phosphorylation. Moreover, expression of the mutant β-arrestin (319–418) significantly attenuated $D_2R$-mediated ERK phosphorylation in HEK293 cells expressing both $D_2R$ and $A_{2a}R$, but not in those expressing $D_2R$ alone.
**Conclusion:** $A_{2a}R$ antagonist ZM241385 significantly attenuates $D_2R$ internalization and $D_2R$-mediated ERK phosphorylation in striatal neurons, involving Src kinase and β-arrestin. Thus, $A_{2a}R/D_2R$ heterodimerization plays important roles in $D_2R$ downstream signaling.

**Keywords:** $D_2$ receptor; $A_{2a}$ receptor; striatum; Parkinson's disease; receptor heterodimerization; receptor internalization; Src kinase; β-arrestin; ERK; ZM241385

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## Introduction

Dopamine receptors are G protein-coupled receptors (GPCRs). These receptors can be divided into $D_1$-like and $D_2$-like families. $D_2$ receptors belong to the $D_2$-like family. The activation of $D_2R$ reduces cAMP expression[1], extracellular signal-regulated kinase1/2 (ERK) phosphorylation[2], phospholipase C activation[3], and receptor internalization[4–6].

Adenosine receptors are also GPCRs[7]. Dopamine $D_2$ and Adenosine $A_{2a}$ receptors form heterodimers[8–10]. In $D_2R$-cotransfected neuroblastoma cells, long-term exposure to $A_{2a}R$ and $D_2R$ agonists resulted in the co-aggregation, co-internalization, and co-desensitization of $A_{2a}R$ and $D_2R$[9]. However, the modulation of $D_2R$ downstream signaling and internalization through $A_{2a}R$ has not been fully demonstrated.

The $A_{2a}R$ antagonist has been demonstrated to improve the effects of L-dopa and to reduce "off" time in clinical trials[11–13]. Because dopamine receptors play a key role in Parkinson's disease[14], characterizing the modulation of $D_2R$ downstream signaling through $A_{2a}R$ would increase our understanding of the role of $D_2R/A_{2a}R$ heterodimers in Parkinson's disease.

In the present study, we explored the ability of the $A_{2a}R$ antagonist ZM241385 in $A_{2a}R/D_2R$ cotransfected cells and striatal neurons to attenuate the receptor internalization and ERK phosphorylation induced by the $D_2R$ agonist quinpirole; we also demonstrated the participation of Src kinase and β-arrestin in $D_2R$ internalization and ERK phosphorylation.

## Materials and methods

### Primary striatal neurons culture

Dissociated primary cultures of striatal neurons from embryonic d 18 (E18) rats were prepared from timed-pregnant Sprague Dawley rats as described previously, with minor modifications[15]. Briefly, the fetuses were removed under sterile conditions and maintained on iced D-Hank's solution

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for microscopic dissection of the striatum. The meninges were removed. The tissue was briefly minced and triturated using a fire-polished Pasteur pipette. The cells were counted and plated on six-well culture plates in DMEM containing 10% fetal bovine serum (FBS). After 5 h, the medium was replaced with serum-free B27/neurobasal medium supplemented with 0.5 mmol/L glutamine and antibiotics. The cultures were maintained at 37°C in a humidified 5% $CO_2$ atmosphere.

### HEK293 cell culture

HEK293 cells were cultured at 37 °C and 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS.

### Co-immunoprecipitation and immunoblotting

Cell lysates were prepared after incubation in modified radioimmunoprecipitation assay (RIPA) lysis buffer (Tris-HCl 50 mmol/L, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.25% Na-deoxycholate, 1% NP-40, 1 mmol/L PMSF, 1 mmol/L $Na_3VO_4$, 1 mmol/L NaF, 10 μg/mL aprotinin, 5 μg/mL leupeptin, and 5 μg/mL pepstatin). The clarified lysates were subjected to SDS-PAGE and electrophoretically transferred to PVDF membranes as described previously[15]. The blots were incubated overnight at 4 °C with primary antibodies. After probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, the immunoreactive proteins were visualized using an enhanced chemiluminescence kit (Pierce, Rockford, Illinois, USA). In some cases, the blots were stripped and reprobed with other antibodies.

Interactions between $D_2R$ and β-arrestin or $A_{2a}R$ were examined by coimmunoprecipitation. Briefly, the cells were prepared after incubation in Tx/G lysis buffer (300 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1.5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mmol/L EDTA and 50 mmol/L Tris, pH 7.4) containing 100 mmol/L iodoacetamide, 10 μg/mL aprotinin and 5 μg/mL leupeptin for 45 min on ice[16]. The clarified lysates were immunoprecipitated through overnight incubation at 4 °C using anti-$D_2R$ (Millipore, Bedford, MA, USA) or anti-$A_{2a}R$ antibodies (Millipore, Bedford, MA, USA), followed by incubation with Protein A/G-Sepharose beads (Santa Cruz, CA, USA). Equivalent amounts of protein were analyzed for each condition. The beads were washed three times with lysis buffer, and the immune complexes were boiled in SDS sample buffer and loaded onto SDS-PAGE gels for immunoblot analysis. The immunoreactive protein bands were detected using the enhanced chemiluminescence kit (Pierce, Rockford, Illinois, USA).

### Biotinylation assays

Living striatal neurons or HEK293 cells were labeled for 20 min at 37 °C with EZ-link NHS-SS-biotin (300 μg/mL, Pierce, Rockford, Illinois, USA) to biotinylate the surface proteins[17]. After washing in PBS+MC, the cells were incubated in medium alone or medium containing different compounds for various times. Trafficking was terminated after rapid cooling to 4 °C. The biotinylated proteins remaining on the cell surface were stripped of biotin using the non-permeant reducing agent glutathione (150 mmol/L glutathione and 150 mmol/L NaCl, pH 8.75). Glutathione was subsequently neutralized with 50 mmol/L iodoacetamide in PBS+MC. The cells were incubated in RIPA buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% SDS) for 30 min at 4 °C. After centrifugation at 16 000×*g*, supernatants containing equal amounts of total protein were incubated with streptavidin beads overnight at 4 °C to capture biotinylated proteins. After washing in extraction buffer, the samples were boiled in sample buffer to elute the biotinylated proteins from the streptavidin beads. The proteins were separated by SDS-PAGE and immunoblotted using antibodies against $D_2R$ (1:600), $A_{2a}R$ (1:4000, Millipore Bedford, MA, USA) or Myc (1:1000 Cell Signaling Technology, Beverly, MA, USA). The immunoblots were processed using a standard chemiluminescence protocol. Equal amount of proteins untreated with streptavidin beads were used for normalization.

### Fluorescence internalization assay

HEK293 cells co-transfected with $D_2GFP$/$A_{2a}RFP$ were first treated for 1 h with the indicated compounds, after which they were fixed with 4% paraformaldehyde (PFA). Fluorescence images were acquired using confocal microscopy (Olympus, Lake Success, NY, USA) and quantified with Image Pro Plus. Total fluorescence intensities (a) and cytoplasmic fluorescence intensities (b) were quantified separately. The percentage of cell surface protein was quantified as (a–b)/a×100%.

### Immunofluorescence

Primary cultured striatal neurons were fixed with acetone, and after washing twice with PBS, the cells were blocked for 1 h in blocking buffer (5% normal goat serum and 1% BSA in PBS) before incubation with anti-$D_2R$ and anti-$A_{2a}R$ antibodies diluted in blocking buffer at 4 °C overnight. The cells were washed three times with PBS and incubated with Alexa Fluor 488 conjugated goat anti-rabbit IgG and Alexa Fluor 555 conjugated goat anti-mouse IgG (1:100) (Molecular probes, Eugene, OR, USA) for 1.5 h at room temperature. The images were captured using a confocal microscope (Olympus, Lake Success, NY, USA).

### BRET assay

The plasmids were transfected into HEK293 cells. At 48 h after transfection, the culture medium was removed, and the cells were washed twice with PBS and subsequently resuspended in DPBS at a density of $3.2\times10^5$/mL. The cells were added into 384-well plate at 25 μL/well, and 25 μL of DeepBlueC solution was added to obtain a final concentration of 5 μmol/L DeepBlueC (Biotium, Hayworth, CA, USA). The plate was immediately read on a POLARstar Omega Microplate Reader (BMG GmbH, Offenburg, Germany) at EM 460 nm and 510/20 nm. The BRET ratio was calculated using the formula below (cells co-transfected with $A_{2a}$Rluc/pGFP were used as a blank):

BRET ratio=[(emission at 510/20 nm)–(emission at EM 460 nm)×Cf]/(emission at EM 460 nm)

(Cf=emission at 510 nm of blank/emission at 460 nm of blank).

### Plasmid construction

Prof Jerrey L BENOVIC (Thomas Jefferson University) kindly provided β-arrestin (319–418). $A_1$R-pcDNA3.1 was generated in our lab[18]. The other constructs were produced by PCR using specific primers. For $D_2$GFP, the $D_2$R fragments were generated by PCR, ligated into the expression vector pCDNA3.1 (Invitrogen, Carlsbad, CA, USA), and sequenced. The primers used were forward 5′-CCAAGCTTGCCACCATGGATCCACTGAATCTGTCCTGGT-3′ and reverse 5′-CCGGAATTCTCAGCAGTGGAGGATCTTCAGGA-3′.

The amplified fragments were fluorescently tagged at the carboxyl terminus using GFP. For $D_2$Myc, the same $D_2$R fragment was generated by PCR using the same primers and inserted into pcDNA3.1/myc-His A (Invitrogen, Carlsbad, CA, USA). For $A_{2a}$RFP, the $A_{2a}$R fragment was generated by PCR, ligated into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), and sequenced. The primers used were forward 5′-CGGAATTCATGCCCATCATGGGCTCCTC-3′ and reverse 5′-GCTCTAGACTACAGCTCGTCCATGCCGA-3′. The amplified fragments were fluorescently tagged at the carboxyl terminus using RFP. Prof Jing-gen LIU (Shanghai Institute of Materia Medica, Chinese Academic of Science) kindly provided pGFP2-N3 and pRluc-N3. For D2pGFP2-N3, the $D_2$R fragment was generated by PCR, ligated into the expression vector pGFP2-N3, and sequenced. The primers used were forward 5′-CCGGAATTCATGGATCCACTGAATCTGTCCTGGT-3′ and reverse 5′-CCAAGCTTGCAGTGGAGGATCTTCAGGAAGGCCTTG-3′. For $A_{2a}$Rluc-N3, the $A_{2a}$R fragment was generated by PCR, ligated into the expression vector pRluc-N3, and sequenced. The forward and reverse primers were 5′-CCCTCGAGATGCCCATCATGGGCTCCTC-3′ and 5′-CCAAGCTTGGACACTCCTGCTCCATCCT-3′, respectively.

### Statistics

The data were presented as the means±SEM and compared with one-way ANOVA followed by the *Bonferroni post hoc* test using the GraphPad InStat statistical program. The level of statistical significance was set at $P<0.01$ and $P<0.05$. The data were obtained from at least three separate experiments.

## Results

### $D_2$R and $A_{2a}$R form heterodimers

$D_2$R and $A_{2a}$R were highly expressed on striatopallidal neurons. To confirm the interaction between $D_2$R and $A_{2a}$R, immunofluorescence experiments were performed using primary cultured striatal neurons. The results showed that $D_2$R and $A_{2a}$R were co-localized on striatal neurons (Figure 1A). The co-immunoprecipitation results indicated that $D_2$R and $A_{2a}$R formed complexes on striatal neurons (Figure 1B). Furthermore, the BRET assay demonstrated that $D_2$R and $A_{2a}$R formed heterodimers on HEK293 cells (Figure 1C). These results suggested that $D_2$R and $A_{2a}$R co-localize and form heterodimers.

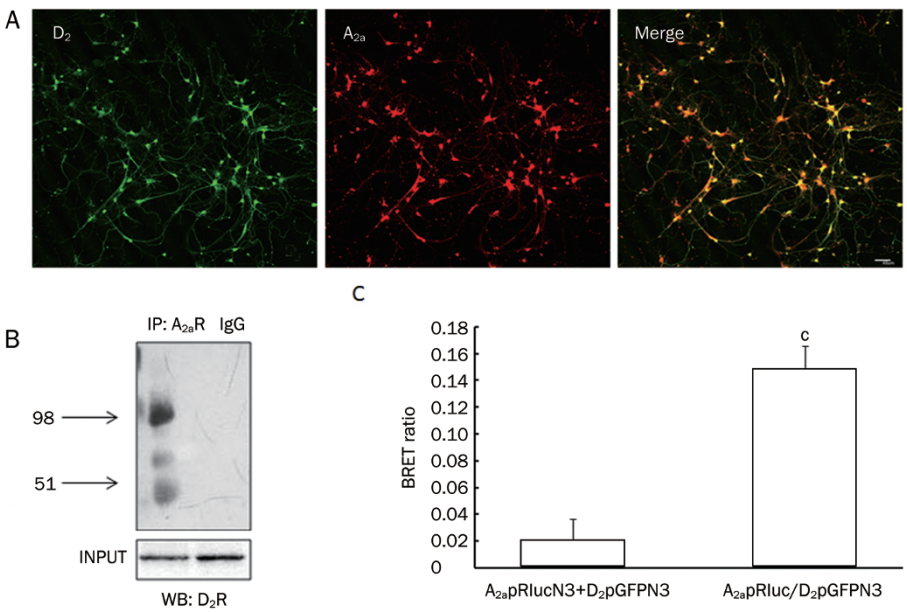



**Figure 1.** $D_2$R and $A_{2a}$R could form heterodimers. (A) Cells were processed for immunostaining using rabbit anti-$D_2$R and mouse anti-$A_{2a}$R antibodies and analyzed by confocal microscopy. The scale bar represents 40 μm. (B) Primary striatal neurons were lysed, and whole cell extracts were immunoprecipitated using an anti-$A_{2a}$R antibody or corresponding normal IgG and subsequently immunoblotted with antibodies to $D_2$R. (C) BRET ratios for HEK293 cells co-expressing $A_{2a}$pRluc and $D_2$pGFP were measured as described in the Methods. A mixture of cells expressing $A_{2a}$pRluc or $D_2$pGFP was used as a negative control. Cells co-expressing $A_{2a}$pRluc and pGFP were used as a blank. Data are presented as the means±SEM of three experiments. $^cP<0.01$ compared with the negative control.

### Quinpirole-induced internalization is attenuated upon combined treatment with ZM241385 and quinpirole

The long-term activation of $D_2R$ could lead to $D_2R$ internalization[6]. Internalization is important for regulating GPCR downstream signaling. A biotinylation assay and confocal microscopy were used to investigate the modulation of the $A_{2a}R$ agonist or antagonist on $D_2R$ internalization. DIV 9 striatal neurons were treated with the $A_{2a}R$ agonist CGS21680 or the $D_2R$ agonist quinpirole alone or in combination for 3 h, and the subsequent induction of $A_{2a}R$ and $D_2R$ co-internalization were observed (Figure 2A, 2B). Because $A_{2a}R$ and $D_2R$ form heterodimers, the activation of either the $D_2$ or $A_{2a}$ receptor could induce the co-internalization of $D_2R/A_{2a}R$ heterodimers, consistent with previously reported data[9]. The results also showed that the $A_{2a}$ receptor antagonist ZM241385 reduced $D_2$ receptor internalization (Figure 2C).

To further confirm this result, the effects of $A_{2a}R$ on $D_2R$ internalization were also examined in HEK293 cells. Plasmids carrying $D_2$GFP and $A_{2a}$RFP were co-transfected into HEK293 cells. After treatment with different compounds, the cells were fixed with 4% paraformaldehyde, and images were captured using confocal microscopy (Olympus). The results showed that treatment with CGS21680 or quinpirole, separately or in combination, induced the co-internalization of the two receptors. In addition, ZM241385 reduced $D_2R$ quinpirole-induced internalization (Figure 3), consistent with the result obtained using striatal neurons.

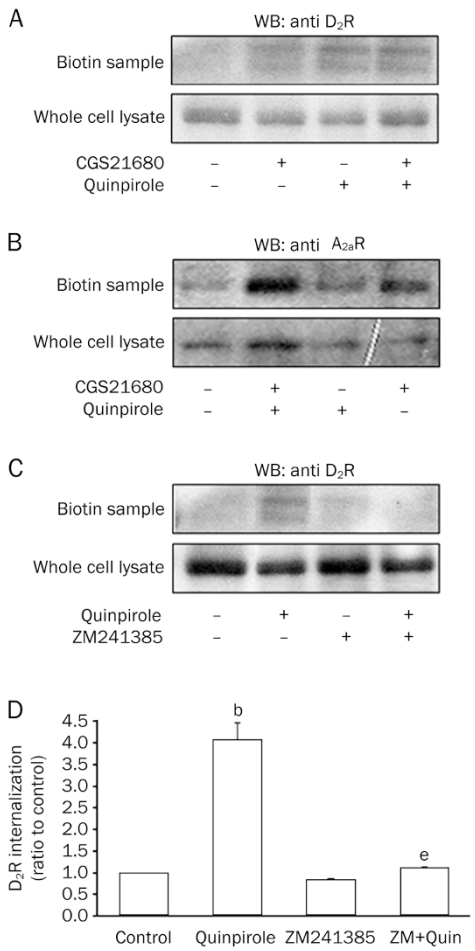



**Figure 2.** Biotinylation assay of $A_{2a}R$ and $D_2R$ endocytosis. (A, B) Primary cultured striatal neurons were labeled with biotin, and the cells were subsequently treated with CGS21680 (1 μmol/L) or quinpirole (1 μmol/L) alone or in combination. After 3 h, the cells were analyzed as previously described. The samples were analyzed by SDS-PAGE and immunoblotting for $D_2R$ (A) or $A_{2a}R$ (B). (C) Neurons were treated with ZM241385 (100 nmol/L) or quinpirole (1 μmol/L) alone or in combination. After 3 h, the cells were analyzed as previously described. The samples were analyzed by SDS-PAGE and immunoblotting for $D_2R$ (C). The data from C were quantitated, and the mean±SEM are shown in the right panel ($n$=3). $^bP<0.05$ compared with the untreated control. $^eP<0.05$ compared with the quinpirole-treated group.

### $D_2R$ internalization occurs in a β-arrestin/clathrin- and Src kinase-dependent manner

Src kinase and β-arrestin are important for GPCR internalization. We used a surface biotinylation assay to characterize the role of Src kinase in $D_2R$ internalization. The results showed that striatal neurons treated with PP2, an Src kinase inhibitor, obviously inhibited quinpirole-induced $D_2R$ internalization (Figure 4A), suggesting that $D_2R$ internalization is Src kinase dependent.

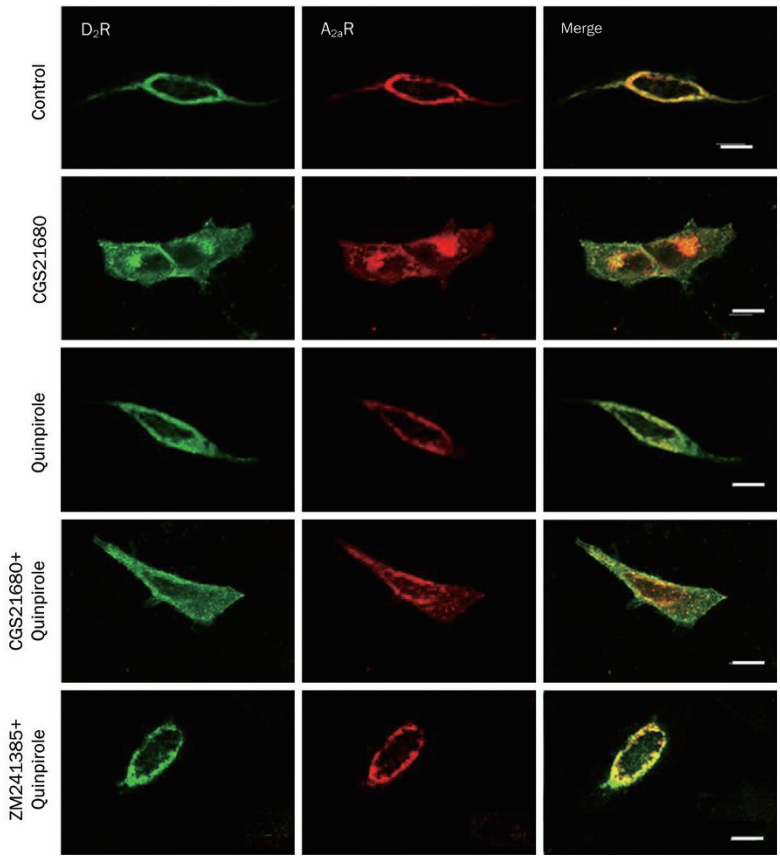
Many GPCRs are internalized in a β-arrestin/clathrin-dependent manner. The dominant negative β-arrestin (319–418) mutant was used to determine the roles of β-arrestins in $D_2R$ internalization. This plasmid inhibited β-arrestin/clathrin-dependent receptor internalization through blocking the formation of the β-arrestin/clathrin complex[19]. We treated β-arrestin (319–418)-transfected HEK293 cells with the $D_2R$ agonist quinpirole to detect $D_2R$ internalization using Western blotting. The results showed that the expression of β-arrestin (319–418) in HEK293 cells inhibited quinpirole-induced $D_2R$ internalization, thereby demonstrating that β-arrestin was involved in $D_2R$ internalization (Figure 4B). These results were consistent with previously reported data[2, 4, 6].

### ZM241385 reduced $D_2R$-induced Src kinase phosphorylation and β-arrestin 2 recruitment

Because Src kinase is involved in $D_2R$ internalization, this protein might also participate in the ZM241385-mediated modulation of $D_2R$ internalization. We used Western blotting to determine whether Src plays a role in the internalization of $A_{2a}R$ and $D_2R$. The results indicated that the activation of $D_2R$ induces Src kinase phosphorylation in striatal neurons. ZM241385 also attenuated $D_2R$-induced Src kinase phosphorylation (Figure 5A).

Moreover, the co-immunoprecipitation data showed that activated $D_2R$ recruited β-arrestin 2 to striatal neurons and that ZM241385 reduced $D_2R$ and β-arrestin 2 interactions (Figure 5B).

These results demonstrated that both Src kinase and β-arrestin are involved in $D_2R$ internalization and that ZM241385 reduces $D_2R$-induced Src kinase phosphorylation



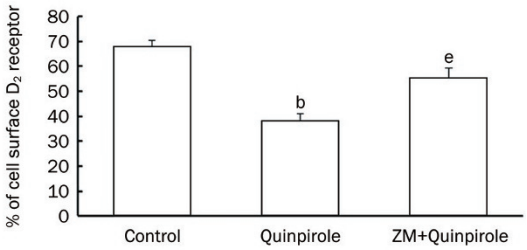




**Figure 3.** Endocytosis of $D_2R$ on HEK293 cells. HEK293 cells were transiently transfected with $D_2$GFP and $A_{2a}$RFP. At 24–36 h after transfection, the cells were treated with CGS21680 (1 μmol/L), quinpirole (1 μmol/L), or ZM241385 (100 nmol/L) alone or in combination for 1 h, and subsequently, the cells were fixed and processed for confocal microscopy. The quantification of cell surface $D_2$ receptors is shown in the panel. $^bP<0.05$ compared with the untreated control. $^eP<0.05$ compared with the quinpirole-treated group. Data are presented as the mean±SEM of at least three independent experiments. The scale bar represents 10 μm.

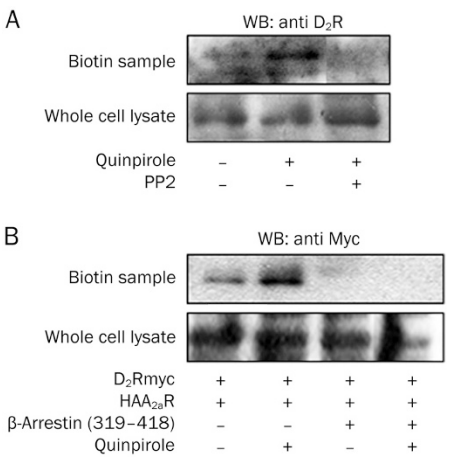



**Figure 4.** Effects of Src kinase and β-arrestins on $D_2R$ endocytosis. (A) Primary cultured striatal neurons were labeled with biotin, and the cells were pretreated with PP2 (5 μmol/L) for 30 min, followed by stimulation with quinpirole (1 μmol/L) for 3 h. Subsequently, the cells were analyzed as previously described, using SDS-PAGE and immunoblotting with a $D_2R$ antibody (Millipore). (B) HEK293 cells were transfected with $D_2$Rmyc, $HAA_{2a}$, or β-arrestin (319–418) alone or in combination. At 48 h after transfection, the cells were treated with quinpirole (1 μmol/L) for 1 h after labeling with biotin and analyzed by SDS-PAGE and immunoblotting using the Myc antibody (Cell Signaling Technology). The data represent three independent experiments.

and β-arrestin recruitment, resulting in the eventual reduction of $D_2R$ internalization.

### ZM241385 reduced $D_2R$-induced ERK phosphorylation

Extracellular regulated protein kinases (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) family, which are involved in many important events, such as neuron survival and cell differentiation[4]. The activation of $D_2R$ induces ERK phosphorylation[20]. Thus, the roles of $A_{2a}R/D_2R$ heterodimerization in $D_2R$-induced ERK activation were also investigated.

Western blot analysis showed that the activation of $A_{2a}R$ did not affect $D_2R$-induced ERK phosphorylation (Figure 6A), whereas ZM241385 reduced $D_2R$-induced ERK phosphorylation in a dose-dependent manner (Figure 6B). These results were confirmed in HEK293 cells (Figure 6D).

### Src kinase and β-arrestin participated in $D_2R$-induced ERK phosphorylation when $D_2R$ was co-expressed with $A_{2a}R$

$D_2R$ induces ERK activation in a G protein-dependent manner, and Src kinase participates in this activation[2, 4]. However, the mechanism of ERK activation might be different under different conditions. The formation of heterodimers might affect receptor-induced ERK activation[21]. In some cases, signal proteins, such as β-arrestin, not only participate in receptor inter-

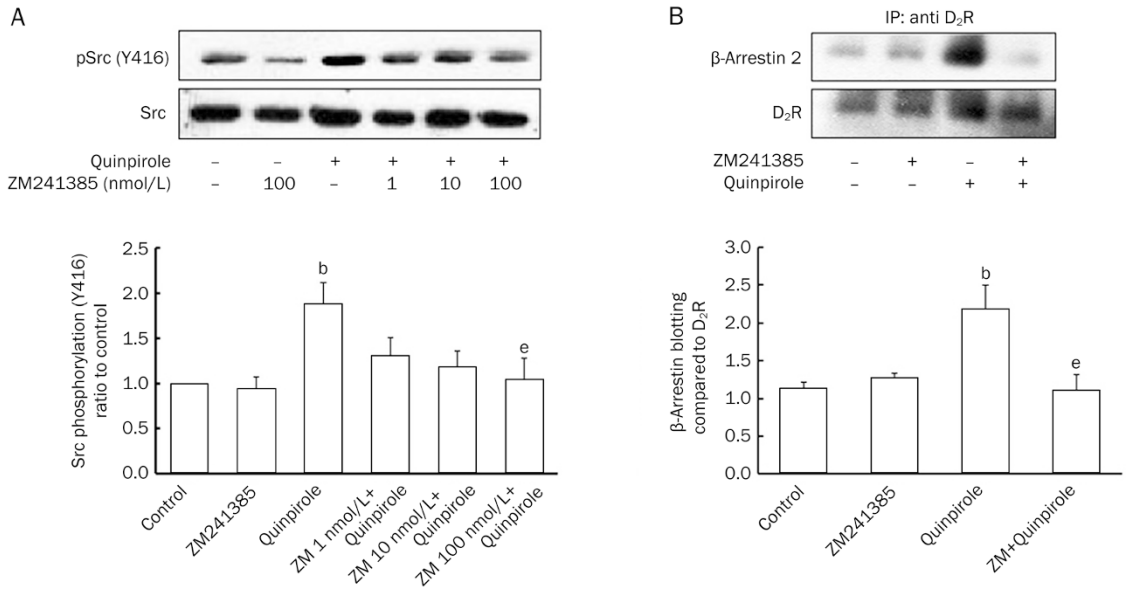



**Figure 5.** Effects of ZM241385 on Src kinase phosphorylation and recruitment of β-arrestin 2 induced by $D_2R$ activation. (A) Neurons were treated with ZM241385 at the indicated concentrations and with the $D_2R$ agonist quinpirole (1 μmol/L) for 10 min. The cell lysates were analyzed by SDS-PAGE and immunoblotting to detect Src phosphorylation using a phospho-specific (Tyr416) antibody. (B) The neurons were treated with ZM241385 (100 nmol/L) or quinpirole (1 μmol/L) alone or in combination for 10 min. The cells were lysed with Tx/G buffer, and the lysates were immunoprecipitated using a $D_2R$ antibody (Millipore). The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-$D_2$ or anti-β-arrestin 2 antibodies. The data were quantitated, and the mean±SEM are shown in the lower panel ($n$=3). $^bP<0.05$ compared with the untreated control. $^eP<0.05$ compared with the quinpirole-treated group.

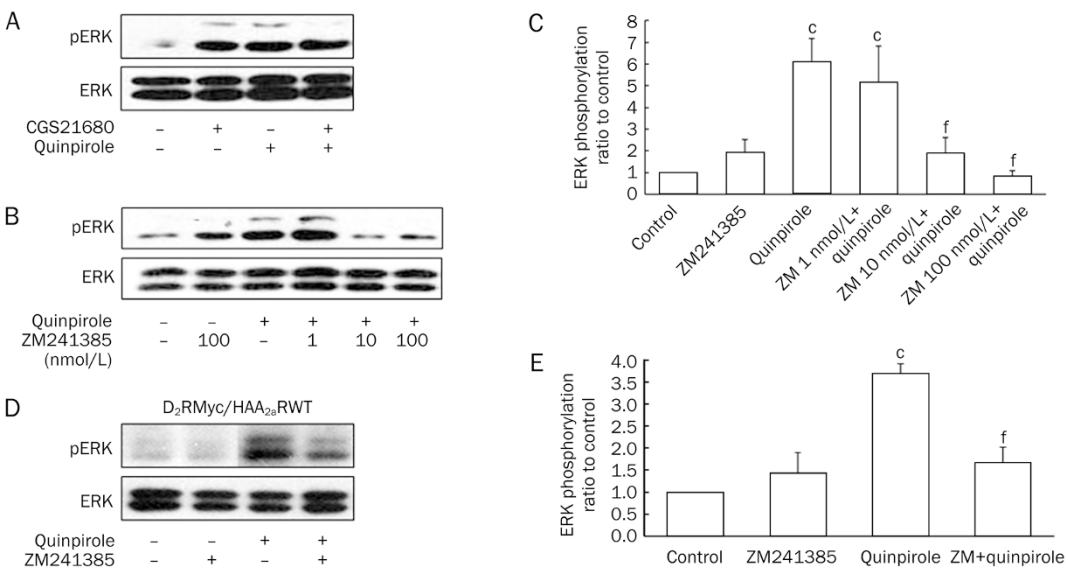



**Figure 6.** Effect of ZM241385 on quinpirole-induced ERK1/2 activation. (A–C) Neurons were treated with the $A_{2a}R$ agonist CGS21680, the $D_2R$ agonist quinpirole (100 nmol/L), and the $A_{2a}R$ antagonist ZM241385 at the indicated concentrations alone or in combination for 10 min. (D–E) HEK293 cells were co-transfected with $D_2R$Myc and $HAA_{2a}R$. At 48 h after transfection, the cells were treated with ZM241385 (100 nmol/L) or the $D_2R$ agonist quinpirole (1 μmol/L) alone or in combination for 5 min. The cells were lysed, and the cell lysates were analyzed by SDS-PAGE and immunoblotting for ERK1/2 phosphorylation using a phospho-specific (Thr202/Tyr204) antibody. The data from B and D were quantitated, and the mean±SEM are shown in C and E, respectively ($n$=3). $^bP<0.05$ compared with the untreated control. $^eP<0.05$ compared with the quinpirole-treated group.

nalization but also are involved in ERK activation[22, 23].

Striatal neurons were treated with the Src kinase-specific inhibitor PP2, followed by treatment with the $D_2R$ agonist quinpirole. PP2 inhibited quinpirole-induced ERK phosphorylation (Figure 7), suggesting that Src kinase is involved in $D_2R$-induced ERK phosphorylation, consistent with previously

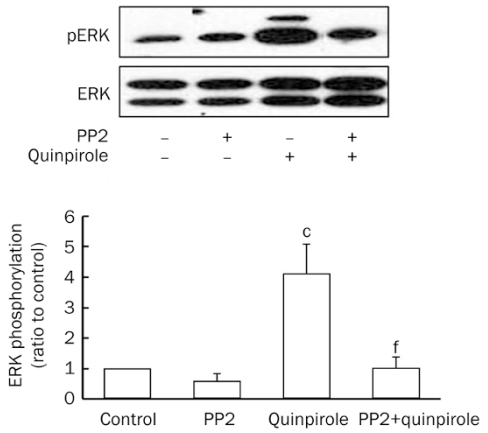



**Figure 7.** Effects of Src kinase on ERK phosphorylation induced by $D_2R$ activation. The neurons were treated with PP2 (5 μmol/L) for 30 min before stimulation with the $D_2R$ agonist quinpirole (1 μmol/L) for 10 min (A). The cell lysates were analyzed by SDS-PAGE and immunoblotting for ERK1/2 phosphorylation using a phospho-specific (Thr202/Tyr204) antibody. The data from B were quantitated, and the mean±SEM are shown in the lower panel ($n$=5). $^cP$<0.01 compared with the untreated control. $^fP$<0.01 compared with the quinpirole-treated group.

reported data[2].

Some studies have implicated β-arrestin in mechanisms of the GPCR downstream signaling pathway, such as ERK phosphorylation[21, 22, 24]. The mutant β-arrestin (319–418) was used to investigate effects of β-arrestins in $D_2$ receptor-induced ERK activation. The data showed that in HEK 293 cells expressing $D_2R$ alone, the expression of β-arrestin (319–418) did not affect quinpirole-induced ERK phosphorylation (Figure 8A), consistent with previous reports[20]. Interestingly, in HEK293 cells co-expressing $D_2R$ and $A_{2a}R$, the expression of β-arrestin (319–418) attenuated quinpirole-induced ERK phosphorylation (Figure 8B), whereas β-arrestin (319–418) expression in HEK293 cells co-expressing $D_2R$ and $A_1R$ did not affect quinpirole-induced ERK phosphorylation (Figure 8C). These results suggested that $D_2R/A_{2a}R$ heterodimerization changed $D_2R$-induced ERK phosphorylation.

Taken together, these results showed that Src kinase (Figure 7) and β-arrestin (Figure 8) are involved in $D_2R$-induced ERK activation and that ZM241385 reduces $D_2R$-induced Src kinase phosphorylation and β-arrestin recruitment (Figure 5), thereby reducing $D_2R$-induced ERK activation through ZM241385 (Figure 6).

## Discussion

$D_2R$ and $A_{2a}R$ form heterodimers, and the activation of either $D_2R$ or $A_{2a}R$ results in the co-internalization of $D_2R/A_{2a}R$ heterodimers[9]. The results of the present study confirmed these observations in striatal neurons and HEK293 cells co-expressing $D_2R/A_{2a}R$. The activation of $A_{2a}R$ induced the co-internalization of $D_2R/A_{2a}R$ heterodimers, whereas ZM241385 reduced $D_2R$ internalization, indicating that $D_2R/A_{2a}R$ receptor heterodimerization affects $D_2R$ internalization.

The present study also demonstrated that after activation, $D_2R$ is internalized through a β-arrestin/clathrin-dependent pathway involving Src kinase. Western blot analysis revealed that ZM241385 reduces $D_2R$-induced Src kinase phosphorylation, and co-immunoprecipitation showed that ZM241385 reduces the $D_2R$-mediated recruitment of β-arrestin2. These results indicated that ZM241385 reduces $D_2R$-induced Src kinase phosphorylation and the recruitment of β-arrestin2, thereby reducing $D_2R$ internalization. Fuxe *et al* demonstrated that $A_{2a}R$ activation enhances $D_2R$ binding with β-arrestin2 and that $D_2R$, $A_{2a}R$, and β-arrestin2 might form a complex[25]. In the present study, we showed that ZM241385 reduces the $D_2R$-mediated recruitment of β-arrestin2. Thus, it is possible that $A_{2a}R$ is involved in the interaction of β-arrestin2 and $D_2R/A_{2a}R$ heterodimers. ZM241385 maintains $A_{2a}R$ in an inactive status[26, 27] and affects the interaction between $D_2R/A_{2a}R$ het-

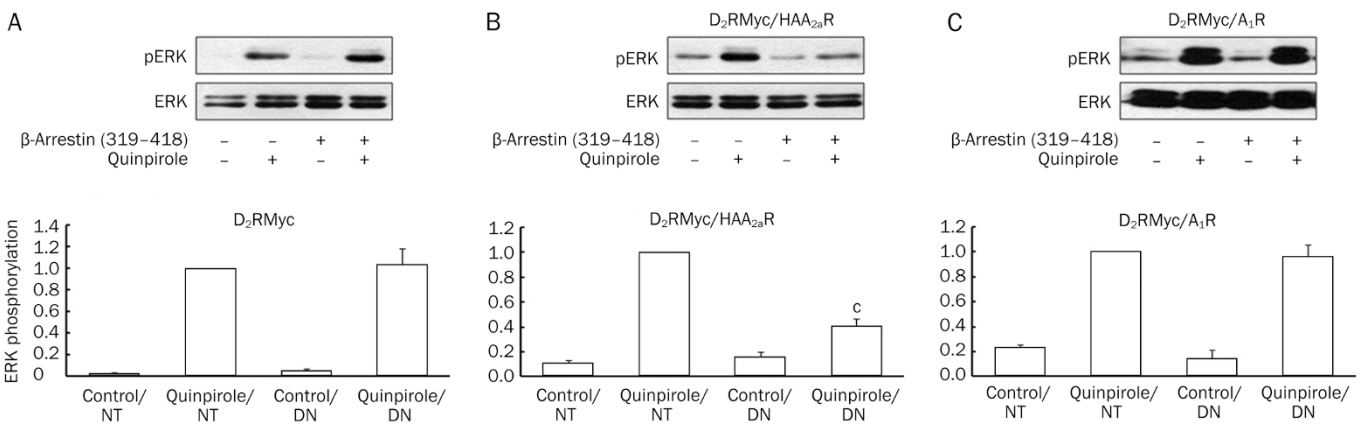



**Figure 8.** Effects of β-arrestins on $D_2R$-induced ERK phosphorylation. HEK293 cells were transfected with $D_2R$Myc (A), $D_2R$Myc and $HAA_{2a}R$ (B), $D_2R$myc or $A_1R$ (C), alone or in combination with β-arrestin (319–418). At 48 h after transfection, the cells were treated with quinpirole (1 μmol/L) for 5 min, followed by lysis and analysis by SDS-PAGE and immunoblotting for ERK1/2 phosphorylation using a phospho-specific (Thr202/Tyr204) antibody. The data were quantitated, and the mean±SEM are shown ($n$=3). NT represents cells not transfected with β-arrestin (319–418). DN represents cells transfected with β-arrestin (319–418). $^cP$<0.01 compared with the quinpirole-treated non-transfected β-arrestin (319–418) cells in each group.

erodimer and β-arrestin2, thereby reducing $D_2R$ internalization. Clinical studies have shown that the $A_{2a}R$ antagonist prolongs the effective time and shortens the "off time" of L-dopa[11, 28], potentially reflecting $D_2R$ internalization.

In this study, we showed that the $A_{2a}R$ antagonist reduces $D_2R$-induced Src kinase phosphorylation and β-arrestin2 recruitment; thus, the $A_{2a}R$ antagonist might not only reduce $D_2R$ internalization but also modulate $D_2R$ downstream signaling. The results in the present study also showed that $D_2R$-induced ERK activation is Src kinase dependent. Moreover, the expression of the mutant β-arrestin (319–418) also reduced $D_2R$-induced ERK activation when $D_2R$ was co-expressed with $A_{2a}R$, suggesting that both Src kinase and β-arrestin participate in $D_2R$-induced ERK activation. The $A_{2a}R$ antagonist reduces $D_2R$-induced Src kinase phosphorylation and β-arrestin2 recruitment; thus, the $A_{2a}R$ antagonist might also reduce $D_2R$-induced ERK activation.

Interestingly, β-arrestin was also involved in $D_2R$-induced ERK phosphorylation when $D_2R$ was co-expressed with $A_{2a}R$, but not when $D_2R$ was expressed alone. ERK phosphorylation might be influenced by many factors, and receptor dimerization might also influence ERK activation[21]. To our knowledge, most studies on $D_2R$-induced ERK activation have utilized cells expressing $D_2R$ alone, showing that the expression of β-arrestin (319–418) had no effects on $D_2R$-induced ERK activation[20]; these results were also confirmed in the present study. However, when $D_2R$ and $A_{2a}R$ were co-expressed, β-arrestin participated in $D_2R$-induced ERK activation. *In vivo*, particularly in the striatum, $D_2R$ exists not only as a monomer or homodimer but also as a $D_2R/A_{2a}R$ heterodimer[9]. Thus, the results of the present study enhance the current understanding of the mechanism underlying $D_2R$-induced ERK activation under physiological conditions. Different mechanisms of ERK activation might exert different downstream effects. Distinguishing $D_2R$ monomer-induced ERK phosphorylation from $D_2R/A_{2a}$ R heterodimer-induced ERK activation is difficult, as the development of a system containing only $D_2R/A_{2a}R$ receptor heterodimers and no $D_2R$ monomers is not easy. However, this study showed the importance of heterodimers in receptor downstream signaling pathway.

Thus, this study showed that $D_2R$ and $A_{2a}R$ co-internalize. The $A_{2a}R$ antagonist reduced $D_2R$-induced Src kinase phosphorylation and β-arrestin2 recruitment, thereby reducing $D_2R$ internalization and ERK phosphorylation. These observations showed that $D_2R/A_{2a}R$ heterodimers play an important role in $D_2R$ signaling pathway and trafficking.

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## Author contribution

Lin-yin FENG and Li HUANG designed the research; Li HUANG, Dong-dong WU, and Lei ZHANG performed the research; Li HUANG analyzed the data; and Lin-yin FENG and Li HUANG wrote the paper.

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