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Novel Regulatory Mechanism of Canonical Wnt Signaling by Dopamine D_2 Receptor through Direct Interaction with β-Catenin[S]

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ABSTRACT

Classical G protein-coupled receptors (GPCRs) and canonical Wnt pathways were believed to use distinct signaling pathways. However, recent studies have shown that these two pathways interact each other by sharing several intermediate signaling components. Recent in vivo studies showed that antipsychotic drugs, which block dopamine D2-like receptors, increase the cellular levels of downstream signaling components of canonical Wnt pathways, such as dishevelled (Dvl), glycogen synthase kinase 3β (GSK3β), and β-catenin. These results suggest that some functional interactions might exist between Wnt pathway and D2-like receptors. In this study, we show that among five different dopamine receptor subtypes, D_2 receptor (D_2R) selectively inhibited the Wnt signaling, which was measured by lymphoid enhancing factor-1 (LEF-1)-dependent transcriptional activities. D_2R-mediated inhibition of Wnt signaling was agonist- and G protein-independent and did not require receptor phosphorylation or endocytosis. D_2R inhibited the LEF-1-dependent transcriptional activities, and this inhibitory activity was not affected by the inhibition of GSK-3β, suggesting that D_2R inhibited the Wnt signaling by acting on the downstream of GSK3β. D_2R directly interacted with β-catenin through the second and third loops, leading to a reduction of β-catenin distribution in the nucleus, resulting in an inhibition of LEF-1-dependent transcription. This is a novel mechanism for the regulation of canonical Wnt signaling by GPCRs, in which receptor proteins recruit β-catenin from cytosol to the plasma membrane, resulting in the decrement of the β-catenin/LEF-1-dependent transcription in the nucleus.

Introduction

Seven transmembrane receptors are divided into classic and atypical families. The former represents GPCRs, and the latter includes Frizzled (FZD). FZD is the receptor for Wnt, a family of highly conserved secreted glycoproteins (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). Until recently, it has been believed that these two receptor systems use distinct downstream signaling pathways; however, some recent studies have shown that two pathways might interact each other by sharing intermediate signaling components such as G proteins, GPCR kinase (GRKs), β-arrestins, Axins (axis inhibitor I), or scaffolding proteins such as Dvl family proteins (Force et al., 2007).

The canonical Wnt pathway is activated when Wnt proteins bind to FZD and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) located on the plasma membrane, causing the receptors to activate Dvl family proteins, which are cytoplasmic signaling proteins (Moon, 2005). These series of events ultimately alter the level of β-catenin in the cytosol, which enters the nucleus and acts as a transcriptional coactivator.

The cellular level of β-catenin is tightly controlled by a multiprotein complex, called a destruction complex, which is composed of glycogen synthase kinase 3β (GSK3β), casein kinase Iα (CKIα), Axin, and adenomatous polyposis coli (Liu

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ABBREVIATIONS: FZD, Frizzled; Wnt, Wingless/int; GRK, G protein-coupled receptor kinase; LRP, lipoprotein receptor-related protein; GSK, glycogen synthase kinase; CKI, casein kinase I; LRP, low-density lipoprotein receptor-related protein; LEF, lymphoid-enhancing factor; GFP, green fluorescent protein; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; RNAi, RNA interference; CMV, vytomegalovirus; PAGE, polyacrylamide gel electrophoresis; GST, glutathione transferase; CM, conditioned medium; GPCR, G protein-coupled receptor; Dvl, disheveled; $I3D_2$, third intracellular loop of D_2.

et al., 2002; Kimelman and Xu, 2006). In the absence of Wnt, β-catenin is phosphorylated by CKIα and GSK-3β in the destruction complex and undergoes ubiquitin-mediated proteasomal degradation (Cadigan and Liu, 2006). In the presence of Wnt, Dvl, which is activated through FZD and LRP5/6, causes the dissociation of destruction complex and inhibition of GSK3β, leading to an accumulation of β-catenin in the cytosol. Accumulated β-catenin moves into the nucleus, where it binds to the transcription factor known as lymphoid-enhancing factor (LEF)/T-cell factor, and activates the transcription (Brunner et al., 1997; Cadigan and Liu, 2006). *Cyclin D1* and c-*myc* are typical target genes of Wnt (Cadigan and Nusse, 1997; Moon, 2005).

Dopamine is involved in the control and coordination of the movement, cognition/emotion, and the hormone release from the pituitary via three major dopaminergic pathways, nigrostriatal, mesocorticolimbic, and tuberoinfundibular, respectively. The importance of proper dopaminergic function is evident when any of these systems becomes compromised as in Parkinson's disease (Lee et al., 1978), schizophrenia (Seeman, 1987), or hyperprolactinemia (Cunnah and Besser, 1991). The dopamine receptors differ in their pharmacological profiles and tissue distributions. On the basis of their pharmacological and functional characteristics, the dopamine receptors are classified into two subfamilies, D1- and D2-like receptors (Brown and Makman, 1972; De Camilli et al., 1979; Kebabian and Calne, 1979). The D1-like receptors have been subdivided into the D_1 and D_5 receptors (D_1R, D_5R) (Dearry et al., 1990; Sunahara et al., 1990, 1991), and the D2-like receptors have been subdivided into the D_2, D_3, and D_4 receptors (D_2R, D_3R, D_4R) (Bunzow et al., 1988; Sokoloff et al., 1990; Van Tol et al., 1991). D_1R and D_5R are positively coupled to adenylyl cyclase by the $G\alpha_s$, whereas the D_2R, D_3R, and D_4R inhibit this enzyme through coupling to $G\alpha_{i/o}$ (Missale et al., 1998).

Functional interactions between dopaminergic nervous system and Wnt have not been reported at cellular or molecular level. It is noteworthy that recent studies in vivo showed an increase in cellular levels of Dvl, GSK3β, and β-catenin after long-term treatment with antipsychotic drugs, which are the blockers of D2-like receptors (Alimohamad et al., 2005a,b). In addition, roles of the Wnt signaling pathways in schizophrenia and antipsychotic drug action are recently being emphasized (Freyberg et al., 2010). These results suggest that there could be functional interaction between Wnt pathway and D_2R/D_3R, which are the major targets of currently used neuroleptics.

In this study, roles and molecular mechanisms of dopamine receptors on the Wnt signaling were determined. Our results show that among five subtypes of dopamine receptors (D_1R–D_5R), only D_2R inhibits canonical Wnt signaling. D_2R interacted with β-catenin through the second and third intracellular loops and inhibited the entry of β-catenin into the nucleus, leading to an inhibition of the LEF-1-dependent transcription. These findings demonstrate that the functional regulation of Wnt signaling by GPCRs could occur through direct interaction with β-catenin independently of the upstream signaling components. It is expected that these results could be extended to other GPCRs to establish common paradigms for their functional interactions. In addition, these results could provide a new understanding for the etiology of dopamine- and Wnt-related disorders and novel strategies for their treatments.

Materials and Methods

Materials. (−)Quinpirole, forskolin, anti-FLAG antibody-conjugated agarose beads, pertussin toxin, haloperidol, clozapine, antibodies to green fluorescent protein (GFP) and FLAG, and LiCl were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). Alexa Fluor 594-labeled anti-mouse antibodies were purchased from Invitrogen (Carlsbad, CA). Antibodies to β-catenin, lamin B1, actin, and horseradish peroxidase-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to caveolin-1 were from BD Biosciences (San Jose, CA). Antibodies to β-arrestin and GRK2 were kindly provided by Dr. Robert Lefkowitz and R. Premont, respectively (Duke University, NC).

Plasmid Constructs. Human dopamine D_1–D_5 receptors (D_1R–D_5R) in pCMV5 were described previously (Cho et al., 2006). Receptor expression levels of D1- and D2-like receptors were determined as described previously (Kim et al., 2001, 2004). D_2R and D_3R tagged with M2-FLAG epitope at the N-terminal tail or with GFP at the C-terminal tail were described previously (Kim et al., 2001, 2005). Chimeric receptors between D_2R and D_3R in which the second and third intracellular loops are exchanged were described previously (Robinson and Caron, 1996; Kim et al., 2001). A phosphorylation-deficient D_2R mutant, D_2R–IC2/3, was described previously (Cho et al., 2010). In this mutant, all the serine and threonine residues located within the second and third intracellular loops were altered to alanine and valine residues, respectively. GFP-tagged and various deletion mutants of β-catenin constructs were described previously (Kim et al., 2000). N-terminal–deleted β-catenin (β-catenin ΔN) lacks the first 86 amino acids, and Arm β-catenin lacks both the N-terminal 86 amino acids and the C-terminal 123 amino acids. Kinase dead form of GSK3β and 14-3-3 were provided by K.Y. Lee (Chonnam National University, Korea). FLAG-tagged β-catenin was provided by K.Y. Choi (Yonsei University, Korea). Small hairpin RNA for GFP, β-arrestin2, and GRK2 were described previously (Cho et al., 2010). Small hairpin RNA for β-catenin was obtained from Addgene (Cambridge, MA).

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells, SH-SY5Y cells, and Wnt3a-producing L929 cells, were obtained from the American Type Culture Collection (Manassas, VA). Cell culture reagents were obtained from either Mediatech (Herndon, VA) or Invitrogen. HEK-293 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml gentamicin in a humidified atmosphere containing 5% CO_2. Human neuroblastoma SH-SY5Y cells and L929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum.

Primary Brain Cell Culture and Immunocytochemistry. Primary cortical neuronal cultures have been described previously (Kwon et al., 2011), and the cells were transfected using Lipofectamine 2000 (Invitrogen). For immunocytochemistry, cells were fixed with ice-cold 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 for 1 h. Cells were incubated with phosphate-buffered saline containing 3% fetal bovine serum and 1% bovine serum albumin for 1 h, then incubated with FLAG antibody overnight at 4°C. After three washes, cells were incubated with Alexa Fluor 594 conjugated secondary antibody for 2 h at room temperature. After three washes with washing buffer, the cells were mounted in Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and viewed with a fluorescence microscope (Olympus, Japan).

Preparation of Stable Cell Lines. To establish stable RNAi cell lines, HEK-293 cells were transfected with GFP (control), β-arrestin2, GRK2, or β-catenin RNAi plasmid. After 2 days, cells were selected with 500 μg/ml G418 (GFP, β-arrestin2, and GRK2 RNAi) or 1 μg/ml puromycin (β-catenin RNAi). For D_2R-expressing cell lines,

HEK-293 cells were transfected with pRC/CMV vector or D_2R-pRC/CMV and then selected with 500 μg/ml G418. Knockdown of target protein was confirmed by immunoblotting and the receptor expression was determined by [^{3}H]sulpiride binding.

Determination of Wnt Signaling. Cells were transfected with TOP-FLASH reporter gene or its control FOP-FLASH reporter gene along with combinations of pRL/TK control vector (Kim et al., 2000). Mouse Wnt1 and Dvl-1 constructs were cotransfected or cells were treated with the conditioned medium containing Wnt3a for 16 h for the activation of TOP-FLASH. Reporter gene activity was determined using dual luciferase assay kit (Promega, Madison, WI). The total amount of DNA transfected in each experimental group was adjusted to be equal.

Immunoprecipitation. Cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), centrifuged at 12,000*g* for 30 min at 4°C. Supernatants were incubated with 35 μl of FLAG-agarose beads for 2 h and 30 min on the rotation wheel, and beads were washed with washing buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, and 1% NP-40) three times for 5 min each. Immunoprecipitates were analyzed on the SDS-PAGE gel and immunoblotted.

In Vitro Binding Studies. The third cytoplasmic loop of D_2R was bacterially expressed as a fusion protein with glutathione transferase (GST). Because the whole third intracellular loop of D_2R ($I3D_2$) was not expressed as soluble protein (data not shown), the third loop of D_2R was divided into two regions: $I3D_2$-N covers Arg227 to Ile304 and $I3D_2$-C covers Glu2250 to Lys342. A GST fusion protein with the third intracellular loop of rat D_3R ($I3D_3$) was described previously (Cho et al., 2003). BL21 bacterial cells were treated with 0.5 mM isopropyl β-D-thiogalactoside for 2 h, lysed, and centrifuged, and the resulting supernatant was separated into aliquots and stored at −70°C until use.

Lysates of HEK-293 cells expressing wild-type or deletion mutants of GFP-β-catenin were incubated with the glutathione agarose beads that had been bound to GST fusion proteins. Agarose beads were washed and retained proteins were eluted with SDS sample buffer, and the eluents were analyzed with SDS-PAGE gel and immunoblotted with antibodies to GFP.

Immunocytochemistry and Confocal Microscopy in HEK-293 Cells. One day after transfection with FLAG-β-catenin and GFP-D_2R, the cells were seeded onto 35-mm dishes containing a centered, 1-cm well that was formed from a glass coverslip-sealed hole in plastic confocal dishes and allowed to recover for 1 day. The next day, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100. Cells were labeled with M2 FLAG antibodies (Sigma) at the 1:500 dilutions and Alexa 594-conjugated anti-mouse antibodies at 1:250 dilutions. Cells were examined by Zeiss laser scanning confocal microscopy.

Preparation of Wnt3a-Conditioned Medium. The Wnt3a-producing L929 cells were cultured with DMEM containing 10% fetal bovine serum for 24 h. The medium was changed to serum-free DMEM, and the cells were cultured for 36 h. The culture medium [Wnt3a-conditioned medium (Wnt3a-CM)] was then harvested, centrifuged at 1000*g* for 10 min, and filtered through a nitrocellulose membrane with 0.22-μm pore size. The activity of Wnt3a-CM was assayed on the normal L929 cells by examining the β-catenin level.

Subcellular Fractionation. Cell lysates were fractionated into cytosolic, membrane, and nuclear fraction according to a previous report (Pan et al., 2005). In brief, cells were incubated with buffer 1 (10 mM HEPES/KOH, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4) for 20 min and centrifuged at 2000*g*. The supernatants were centrifuged for another 10 min at 15,000*g*, and the supernatant was saved as cytosolic fraction. The pellets were lysed with radioimmunoprecipitation assay buffer to constitute the membrane fraction. The pellet from the first centrifugation step was washed with buffer 1 for 15 min and centrifuged at 15,000*g* for 10 min, and the resulting pellet was incubated with buffer 2 (20 mM HEPES/KOH, pH 7.8, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4) for 20 min. After centrifugation at 24,000*g* for 10 min, the supernatant was collected as nuclear extract.

Statistics. All of the results are expressed as mean ± S.E.M. Comparisons between experimental groups were performed by Student's *t* test.

Results

D_2R constitutively Inhibits Canonical Wnt Signaling Pathways. The Wnt signaling was measured by TOP-FLASH assay, which measures LEF-1-dependent gene transcription. FOP-FLASH was used as negative control. When Wnt1 and Dvl-1 were coexpressed in HEK-293 cells, the LEF-1-dependent gene transcription increased around 15 times above that of the mock-transfected group. Among five different dopamine receptor subtypes tested, Wnt signaling was selectively inhibited in cells expressing the D_2R (Fig. 1A). There are two different isoforms of alternatively spliced D_2R: a short form and a long form in which 29 extra amino acids are inserted in middle of the third cytoplasmic loop (Bunzow et al., 1988; Giros et al., 1989). Wnt signaling was inhibited to a similar extent by short and long alternatively spliced forms of D_2R (Supplemental Fig. 1), suggesting that the 29-amino acid insert in the third cytoplasmic loop did not contribute to the inhibition of Wnt signaling. It is noticeable that D_3R, which is closely related to D_2R, had no effect on the Wnt signaling. Virtually the same results were obtained when the LEF-1-dependent transcriptional activity was induced by treating the cells with Wnt3a-CM (Fig. 1B). The selectivity of D_2R on the Wnt signaling was also confirmed in dopaminergic human neuroblastoma SH-SY5Y cells (Fig. 1C). The effect of D_2R in Wnt signaling was agonist-independent. Treatment with increasing concentrations of quinpirole (0.01–100 nM), a D_2R agonist, did not alter the magnitude of the inhibitory effect of D_2R on Wnt signaling compared with vehicle group (Fig. 1D). Although previous in vivo results have shown that antipsychotic drugs elevated cellular levels of Wnt signaling components, our results show that haloperidol or clozapine have no effect on the inhibition of Wnt signaling by D_2R (Fig. 1E).

Characterization of D_2R-Mediated Inhibition of Wnt Signaling. Next we examined whether D_2R-mediated regulation of Wnt signaling occurs through G protein coupling or receptor endocytosis. When cells were treated with 100 ng/ml pertussin toxin, at which concentration the coupling between D_2R and $G\alpha_{i/o}$ proteins is completely abolished (Kim et al., 1995), the inhibitory activity of D_2R on the Wnt signaling was not affected (Fig. 2A). These results were corroborated by using a point mutant of D_2R in which the arginine or aspartic acid residue of DRY (Asp-Arg-Tyr) motif was altered to histidine (R132H-D_2R) or asparagine (D131N-D_2R). R132H-D_2R lacks both G protein coupling and agonist-induced endocytosis but shows intact ligand binding properties (Kim and Caron, 2008), whereas D131N-D_2R shows intact G protein coupling and ligand binding properties but reveals a 4-fold increase of agonist-induced endocytosis (Supplemental Fig. 2A) (Kim et al., 2008). As shown in Fig. 2, B and C, R132H-D_2R and D131N-D_2R showed a similar extent of inhibitory activities on Wnt signaling, suggesting that D_2R

exerts constitutive inhibitory activity toward Wnt signaling without the involvement of G protein coupling or receptor endocytosis.

Next, we tested the involvement of receptor phosphorylation on the D_2R-mediated inhibition of Wnt signaling. For this, D_2R–IC2/3, a mutant of D_2R in which all the possible phosphorylation sites located within the second and third intracellular loops were mutated, was used. As shown in Fig. 2D, wild-type D_2R and D_2R–IC2/3 showed a similar extent of inhibition of Wnt signaling, suggesting that receptor phosphorylation is not a critical cellular event for the inhibition of Wnt signaling by D_2R.

It was has been reported that GRK2 inhibits the canonical Wnt signaling (Wang et al., 2009). The involvement of GRK2 in the D_2R-mediated inhibition of Wnt signaling was tested using GRK2 knockdown cells. Wnt signaling was moderately ($p < 0.05$) increased in GRK2 knockdown cells; however, the inhibitory activities of D_2R on Wnt signaling were similar in control and GRK2 knockdown cells (Supplemental Fig. 2B).

β-Arrestin and Akt are reported to be involved in the regulation of canonical Wnt pathway (Chen et al., 2001; Fukumoto et al., 2001; Bryja et al., 2007). A recent study has shown that D_2R forms a signaling complex, β-arrestin2/Akt/protein phosphatase 2A, and regulates Akt/GSK3β signaling in a G protein-independent and β-arrestin2-dependent manner (Beaulieu et al., 2005). These results suggest that β-arrestin might play some roles in the D_2R-mediated inhibition of Wnt signaling. Wnt1/Dvl1-induced (Fig. 2E) but not

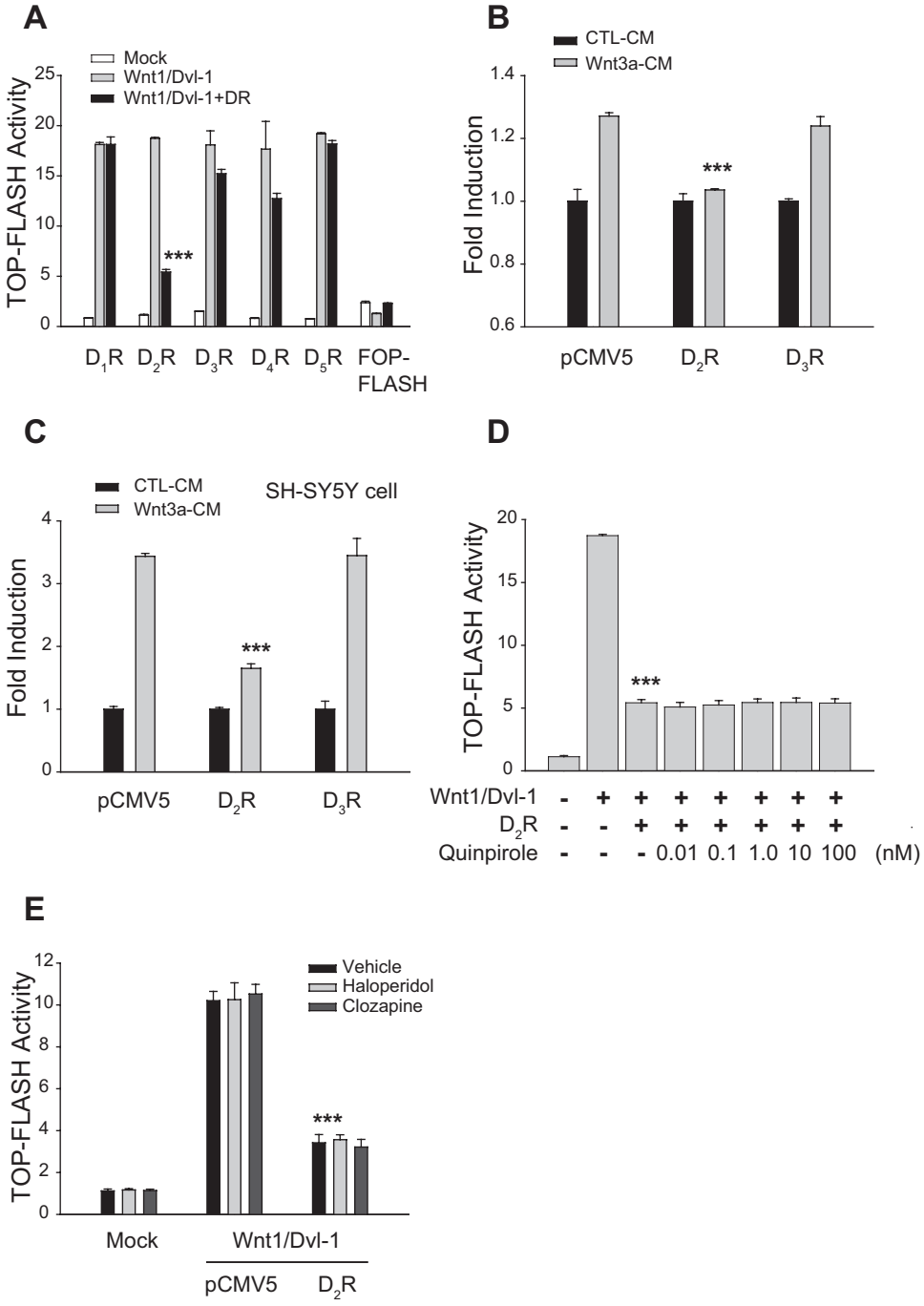

Fig. 1. Effects of dopamine receptor subtypes on the Wnt signaling. A, effects of dopamine receptor subtypes on the Wnt signaling were determined by TOP-FLASH as described under *Materials and Methods*. Cells were transfected with Wnt1 in pCDNA3.0 (1 μg) and Dvl-1 in pRC/CMV (1 μg) together with 2 μg of each dopamine receptor subtypes in pCMV5 for 100-mm culture dishes. Mock groups were transfected with only reporter gene/pRL-TK control vector. D_2R represents the short form of alternatively spliced D_2R unless specified otherwise. Receptor expression levels were maintained between 1.7 and 1.9 pmol/mg membrane proteins. ***, $p < 0.001$ compared with Wnt1/Dvl-1 group. B, effect of D_2R and D_3R on the Wnt3a-induced TOP-FLASH activity. HEK-293 cells expressing D_2R or D_3R were treated with control-CM or Wnt3a-containing CM for 16h. ***, $p < 0.001$ compared with pCMV5 group. C, effect of D_2R and D_3R on the Wnt signaling was confirmed in SH-SY5Y human neuroblastoma cell lines. ***, $p < 0.001$ compared with pCMV5 group. D, effects of overnight treatment with quinpirole, a selective agonist of D_2R, were tested on the D_2R-mediated inhibition of Wnt signaling. ***, $p < 0.001$ compared with Wnt1/Dvl-1 group. E, effects of the antagonists of D2-like receptors on the D_2R-mediated inhibition of Wnt signaling. Cells were treated with 10 μM haloperidol or clozapine for 16 h before reporter gene assay. ***, $p < 0.001$ compared with Wnt1/Dvl-1/pCMV5 group.

β-catenin-induced (Fig. 2F) increase in TOP-FLASH activity was reduced when endogenous β-arrestin2 was lowered (Supplemental Fig. 2C). In both cases, however, the inhibitory activities of D_2R on TOP-FLASH activity were similar. These results suggest that D_2R inhibits Wnt signaling independently of β-arrestin and confirm a previous study showing that β-arrestins synergistically activate Wnt signaling through interaction with Dvl (Chen et al., 2001).

The Second and Third Intracellular Loops of D_2R Are Involved in The Inhibition of Wnt Signaling. The D_2R and D_3R possess high homology, the two sharing 46% overall amino acid homology and 78% identity in the transmembrane domains (Giros et al., 1990). Likewise, D_2R and D_3R share many signaling and regulatory properties when they are expressed in mammalian cells (Kim et al., 2001). In this sense, it was unexpected that only D_2R but not D_3R selectively inhibited the Wnt signaling. To determine the receptor regions of D_2R involved in the regulation of Wnt signaling, chimeric receptors between D_2R and D_3R in which the second and third cytoplasmic loops were exchanged (Robinson and Caron, 1996; Kim et al., 2001) were used. Schematic diagrams of the chimeric receptors between D_2R and D_3R are shown in Fig. 3A. As shown in Fig. 3B, the inhibitory activities of D_2R decreased as the second and third intracellular loops were replaced with those of D_3R. In addition, sequential replacement of the second and third loop of D_3R

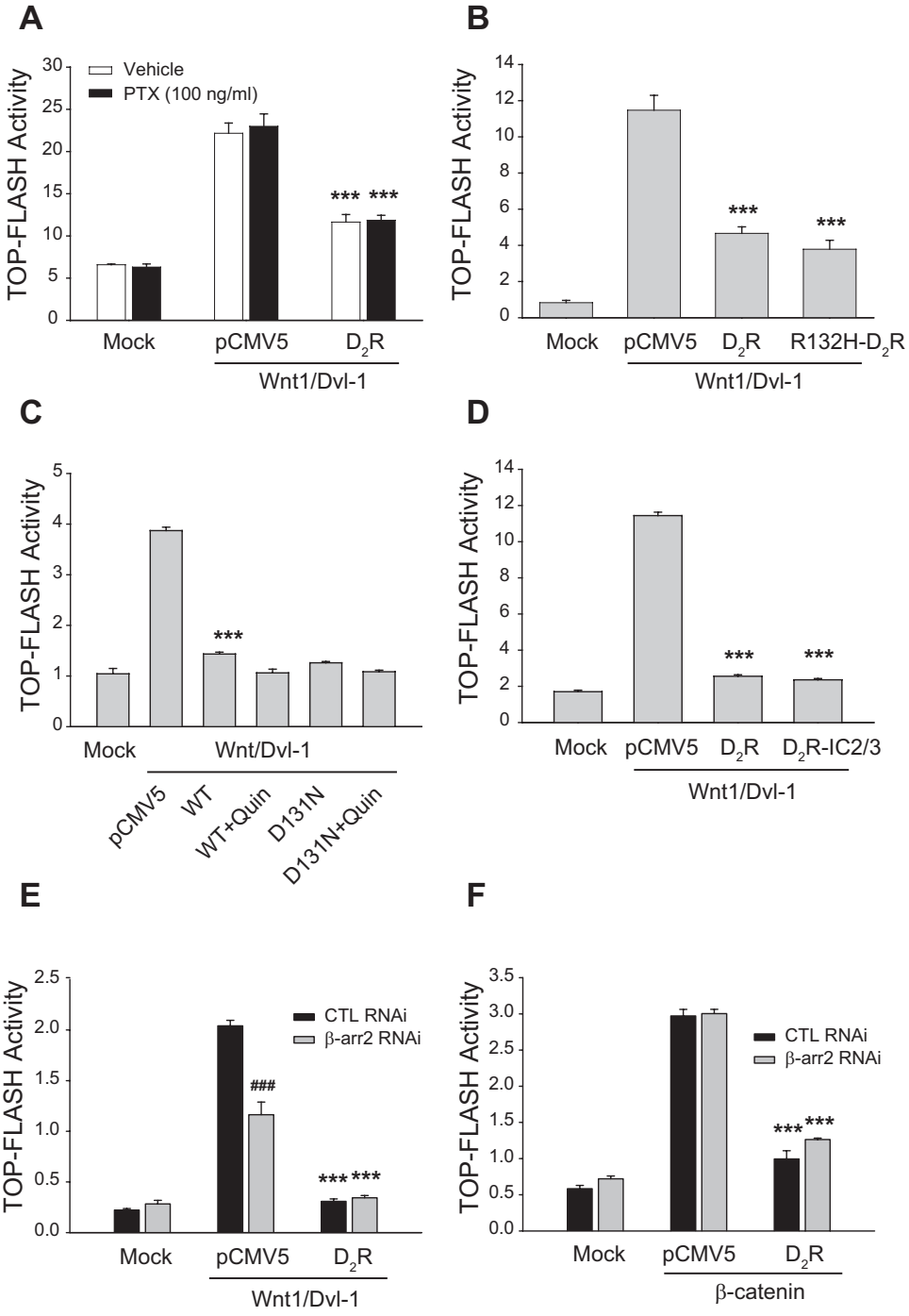

Fig. 2. Characterization of D_2R-mediated inhibition of Wnt signaling. A, involvement of D_2R signaling through G protein in the regulation of Wnt signaling was determined by inhibiting the coupling between D_2R and $G\alpha_{i/o}$. Cells were treated overnight with 100 ng/ml pertussis toxin and the TOP-FLASH activity was determined. ***, $p < 0.001$ compared with pCMV5 group. B, involvement of receptor signaling and endocytosis in the inhibition of Wnt signaling were tested using a point mutant of D_2R in which 132th Arg residue located within the Asp-Arg-Tyr motif was changed to His. ***, $p < 0.001$ compared with pCMV5 group. C, the relationship between the endocytosis of D_2R and the inhibitory activity on the Wnt signaling was tested using D131N-D_2R. Cells were treated with 100 nM quinpirole for 6 h to induce receptor endocytosis before reporter gene assay. ***, $p < 0.001$ compared with pCMV5 group. D, relationship between receptor phosphorylation and inhibition of Wnt signaling were tested using a mutant of D_2R, D_2R–IC2/3 in which all the serine and threonine residues located within the second and third intracellular loops were mutated to alanine and threonine residues, respectively. ***, $p < 0.001$ compared with pCMV5 group. E and F, roles of β-arrestin on the D_2R-mediated inhibition of Wnt signaling were studied using HEK-293 cells which stably express either control RNAi plasmid or β-arrestin2 RNAi plasmid. Wnt signaling was stimulated by coexpressing either Wnt1/Dvl-1 (D) or β-catenin (E). ***, $p < 0.001$ compared with pCMV5 group; ###, $p < 0.001$ compared with control RNAi group.

with those of D_2R changed the phenotype of D_2R to that of D_3R (Fig. 3C), suggesting that both the second and third loops are involved in the inhibition of Wnt signaling.

D_2R Regulates Canonical Wnt Signaling at the Level of β-Catenin. The point of the involvement of D_2R for the regulation of Wnt signaling was determined by testing the inhibitory activity of D_2R for canonical Wnt signaling, which was initiated at different levels of Wnt cascade. D_2R inhibited Wnt- or Dvl-1-induced LEF-1-dependent transcriptional activities (Fig. 4A), suggesting that D_2R acts at the downstream of FZD and LRP5/6. D_2R also inhibited the TOP-FLASH activity that was induced by β-catenin (Fig. 4B), regardless of the blockade of GSK3β (Fig. 4C), suggesting that D_2R act at the level of β-catenin or downstream. In accordance with this, D_2R also inhibited the TOP-FLASH activity that was raised by treatment with LiCl, an inhibitor of GSK3β (Fig. 4D). D_3R, which was employed as negative control, had no effect. Cellular levels of β-catenin were elevated by coexpression of Wnt1/Dvl-1 (Fig. 4E) or by treatment with lithium (Fig. 4F); however, D_2R did not affect the total cellular amount of β-catenin, suggesting that D_2R does not affect the transcription/translation or the stability of endogenous β-catenin.

Interaction between D_2R and β-Catenin Mediates the Inhibition of Wnt Signaling. To understand the molecular mechanisms involved in the D_2R-mediated inhibition of Wnt signaling, cellular components that are involved in the Wnt signaling, such as GSK3β, β-catenin, and 14-3-3, were tested for the interactions with D_2R. Along with Chibby, an antagonist of β-catenin, 14-3-3 is known to form a trimolecular complex with β-catenin (Li et al., 2008) to control the subcellular localization and stabilization of β-catenin. Among the proteins tested, only β-catenin interacted with D_2R (Supplemental Fig. 3A). In accordance with the TOP-FLASH assay, interaction between D_2R and β-catenin did not change after agonist (quinpirole) or antagonist (haloperidol) treatment (Supplemental Fig. 3B).

Because the second and third intracellular loops of D_2R were responsible for the inhibition of Wnt signaling (Fig. 3) and D_2R interacted with β-catenin (Supplemental Fig. 3, A and B), we tested whether β-catenin interacted with this receptor region. Both wild-type D_2R and D_3R-(D_2-IC23), a chimeric D_3R that contains the second and third intracellular loops of D_2R, interacted with endogenous β-catenin (Fig. 5A), suggesting that the inhibition of Wnt signaling by D_2R is mediated through the interaction between β-catenin and the second and third cytoplasmic loops. In contrast, D_3R, which was employed as a negative control, did not interact with β-catenin. Involvement of the intracellular loops of D_2R for the interaction with β-catenin was further confirmed by testing the interaction between β-catenin and the third intracellular loop of D_2R fused to GFP (GFP-I3D_2). As shown in Fig. 5, B and C, GFP-I3D_2 interacted with β-catenin, and coexpression of GFP-I3D_2 significantly inhibited the β-catenin-induced increase in TOP-FLASH activity. Inhibitory activity of D_2R on the β-catenin-induced increase in TOP-FLASH activity, was confirmed by confocal microscopic studies in HEK-293 cells, which stably express D_2R. Coexpression of D_2R prevented the entry of β-catenin into the nucleus (Fig. 5D). As expected, coexpression of β-catenin inhibited the D_2R-mediated decrement of Wnt signaling in a dose-dependent manner (Fig. 5E).

Because D_2R interacts with β-catenin and inhibits Wnt signaling, we were curious whether stimulation of Wnt pathway reciprocally regulates D_2R signaling. Stimulation of Wnt

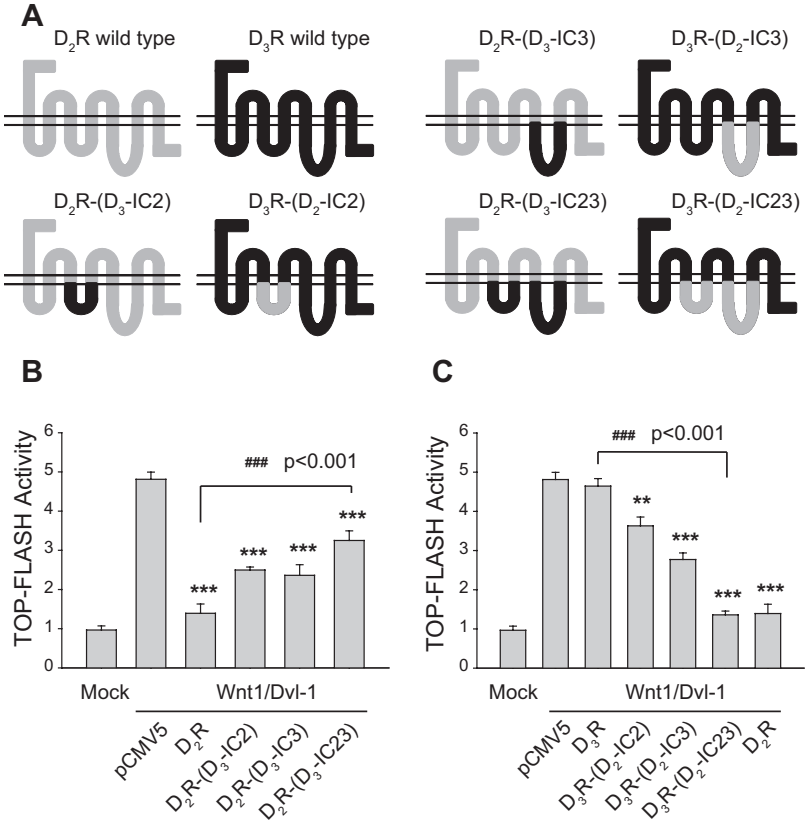

Fig. 3. Receptor regions responsible for the D_2R-mediated inhibition of Wnt signaling were determined using chimeric receptors between D_2R and D_3R. A, scheme for chimeric receptors between D_2R and D_3R. Either the second intracellular loop, the third intracellular loop, or both loops were switched between D_2R and D_3R. B, TOP-FLASH activity was determined in cells which express wild-type or chimeric D_2Rs in which the second and third intracellular loops were switched with those of D_3R. C, TOP-FLASH activity was determined in cells which express wild-type or chimeric D_3Rs in which the second and third intracellular loops were switched with those of D_2R (C). Receptor expression levels were maintained between 1.7 and 1.9 pmol/mg proteins. **, $p < 0.01$, ***, $p < 0.001$ compared with pCMV5 group. ###, $p < 0.001$ when D_2R-(D_3-IC23) group was compared with D_2R group, or when D_3R-(D_2-IC23) group was compared with D_3R group.

pathway by coexpression of Wnt1/Dvl-1 (Supplemental Fig. 4A) or β-catenin (Supplemental Fig. 4B) had no effect on the signaling of D_2R. Similar results were obtained by extracellular signal-regulated kinase activation assay (Supplemental Fig. 4C). In addition, knockdown of endogenous β-catenin did not alter the signaling (Supplemental Fig. 4D) and internalization (Supplemental Fig. 4E) of D_2R. These results together suggest that D_2R-mediated regulation of Wnt signaling is unidirectional and the interaction with β-catenin did not affect G protein coupling efficacy of D_2R.

The Armadillo Repeat Domain of β-Catenin Interacts with the N-Terminal Part of the Third Intracellular Loop of D_2R. Interaction between D_2R and β-catenin was further characterized by GST pull-down assay. For this, the third intracellular loops of D_2R and D_3R were expressed as fusion proteins with GST. As shown in Fig. 6A, only the N-terminal part of the intracellular third loop of D_2R (GST-I3D_2-N) interacted with β-catenin. On the other hand, GST, GST-I3D_2-C, or GST-I3D_3 (the whole third intracellular loop of D_3R) failed to bind β-catenin. To determine the domain of β-catenin that binds to the third intracellular loop of D_2R, GST pull-down assayd were performed for three deletion constructs of β-catenin (Fig. 6B). The central part of β-catenin, the armadillo repeat domain, seems to be the region responsible for the interaction with the third intracellular loop of D_2R. As shown in Fig. 6C, the N-terminal deletion mutant of β-catenin (β-catenin-ΔN) or the armadillo repeat domain itself was enough to bind to the third intracellular loop of D_2R.

D_2R Alters the Subcellular Distribution of β-Catenin to Prevent the Nuclear Translocation. Because D_2R binds to β-catenin (Fig. 6) and inhibits LEF-1-dependent transcriptional activity by acting at downstream of GSK3β (Fig. 4), we determined whether D_2R inhibits the TOP-FLASH activity by altering the subcellular localization of β-catenin. The cellular levels of β-catenin were raised by transfecting with Wnt1/Dvl-1, because the cellular content of β-catenin was too low to manipulate. Cell lysates from different experimental groups were fractionated into cytosolic, plasma membrane, and nuclear fraction by employing actin, caveolin-1, and lamin B1, respectively, as internal control. As

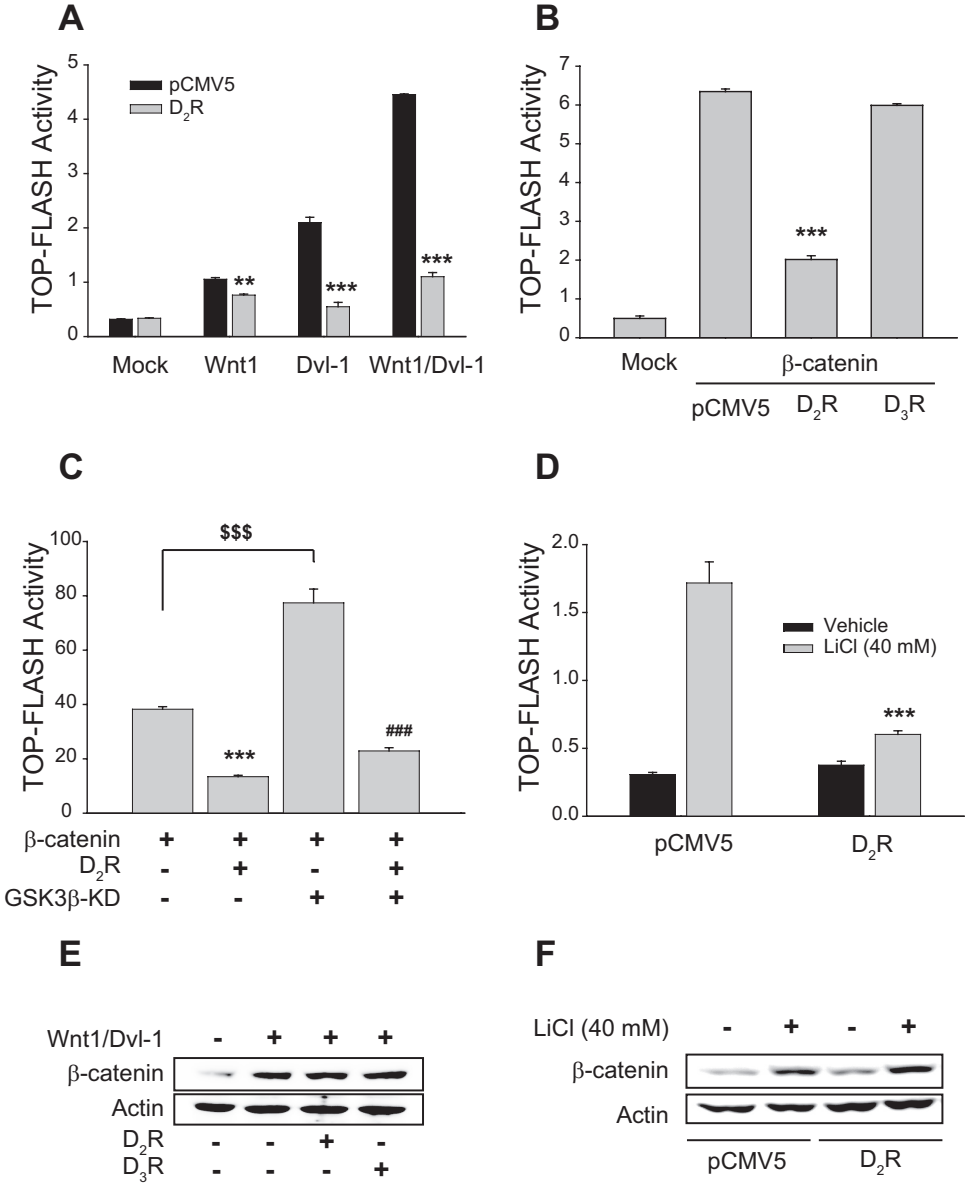

Fig. 4. Determination of the molecular target of D_2R for the inhibition of Wnt signaling. Reported gene assay was conducted as in Fig. 1A. A, cells were transfected with Wnt1 or/and Dvl-1 along with pCMV5 or D_2R. **, $p < 0.01$, ***, $p < 0.001$ compared with pCMV5 group. B, cells were transfected with GFP-β-catenin along with pCMV5, D_2R, or D_3R. ***, $p < 0.001$ compared with β-catenin/pCMV5 group. C, cells were transfected with kinase-dead form of GSK3β (GSK3β-KD) along with Mock plasmid or D_2R. ***, $p < 0.001$ compared with β-catenin only group. ###, $p < 0.001$ compared with β-catenin/GSK3β-KD group. $$$, $p < 0.001$ when β-catenin/GSK3β-KD group compared with β-catenin group. D, cells transfected with pCMV5 or D_2R were treated with vehicle or 40 mM LiCl overnight. ***, $p < 0.001$ compared with pCMV5 group. E, cells were transfected with Wnt1/Dvl-1 along with Mock plasmid, D_2R, or D_3R. F, cells transfected with pCMV5 or D_2R, were treated with 40 mM LiCl overnight. Cell lysates were analyzed with SDS-PAGE gel and immunoblotted with antibodies for β-catenin and actin. Experiments in E and F were conducted three independent times, and representative results are shown.

shown in Fig. 7A, D_2R alone increased β-catenin level in the membrane and cytosolic faction by 2.1- and 1.6-fold compared with the mock group, respectively (compare lanes 1 and 3). Coexpression of Wnt1/Dvl-1 resulted in increases in β-catenin levels in all the fractions tested (compare lanes 1 and 2). Compared with mock group, 8.6-, 1.7-, 1.7-, and 1.5-fold increases in β-catenin levels were observed for total lysate, membrane fraction, cytosolic fraction, and nuclear fraction, respectively. When D_2R was additionally expressed with Wnt1/Dvl-1, the Wnt1/Dvl-1-induced elevation of the cellular β-catenin levels in the nuclear (Fig. 7A4) and cytosolic fraction (Fig. 7A3) reverted to the basal levels (compare lanes 2 and 4). On the other hand, β-catenin levels in the membrane fraction (Fig. 7A2) and total cell lysate (Fig. 7A1) rather increased or remained the same, respectively. These results suggest that D_2R on the plasma membrane binds to β-catenin, shifting its distribution to the plasma membrane, and prevents the β-catenin/LEF-1 complex from moving into the nucleus as shown in Fig. 5D. These results were corroborated by immunocytochemical studies. β-Catenin was largely distributed in the cytosolic compartment and to a lesser extent in the nuclear fraction (Fig. 5D). When D_2R was coexpressed, β-catenin and D_2R colocalized on the plasma membrane and in the cytosol of HEK-293 cells (Fig. 7B, top). Similar results were obtained from primary cultured brain cortical neurons (Fig. 7B, bottom). These results suggest that the interaction between two proteins could be the major cellular mechanism for the regulation of Wnt signaling.

Discussion

This study is the first to answer whether and how the functional interactions between dopaminergic nervous system and Wnt signaling occur. Among five dopamine receptor subtypes characterized, only D_2R inhibited the canonical Wnt signaling (Fig. 1A). The mode of regulation was one-directional; D_2R inhibited Wnt signaling but not vice versa. Our study also shows how D_2R controlled canonical Wnt signaling. D_2R did not

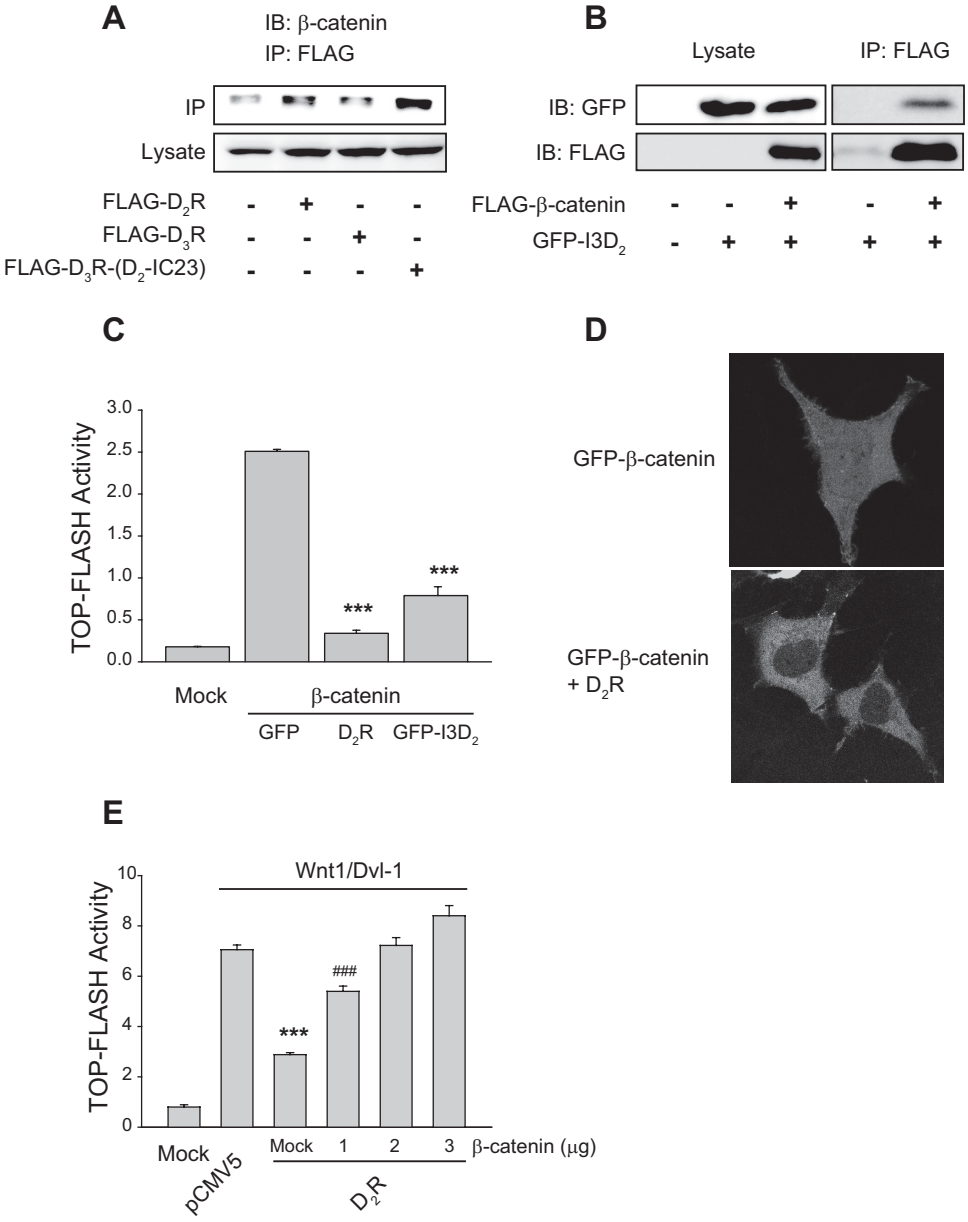

Fig. 5. Specific and functional interaction between D_2R and β-catenin. A, identification of the second and/or third intracellular loops of D_2R as the interacting regions with β-catenin. Cells transfected with FLAG-D_2R, -D_3R, or –D_3R-(D_2-IC23), were treated with 40 mM LiCl overnight to elevate cellular levels of β-catenin. Cell lysates were immunoprecipitated with anti-FLAG beads, analyzed on the SDS-PAGE gel, and immunoblotted with antibodies to β-catenin. Experiments were conducted three independent times, and representative results are shown. B, interaction between β-catenin and isolated fragment of the third intracellular loop of D_2R ($I3D_2$). Cells were transfected with FLAG-β-catenin and GFP-$I3D_2$. Cell lysates were immunoprecipitated with anti-FLAG beads, analyzed on the SDS-PAGE gel, and immunoblotted with antibodies to GFP or FLAG. Experiments were conducted three independent times, and representative results are shown. C, effect of isolated fragment of the third intracellular loop of D_2R on the TOP-FLASH activity. Reporter gene assay was conducted in cells transfected with D_2R or GFP-$I3D_2$. ***, $p < 0.001$ compared with β-catenin/GFP group. D, effects of D_2R on the subcellular distribution of β-catenin. HEK-293 cells which were stably transfected either with pRC/CMV or D_2R-pRC/CMV were transiently transfected with GFP-β-catenin. E, effects of over-expression of β-catenin on the inhibitory activities of D_2R on Wnt signaling. Increasing amounts of β-catenin was co-expressed along with D_2R. **, ###, $p < 0.001$ compared with pCMV5 group and Mock/D_2R group, respectively.

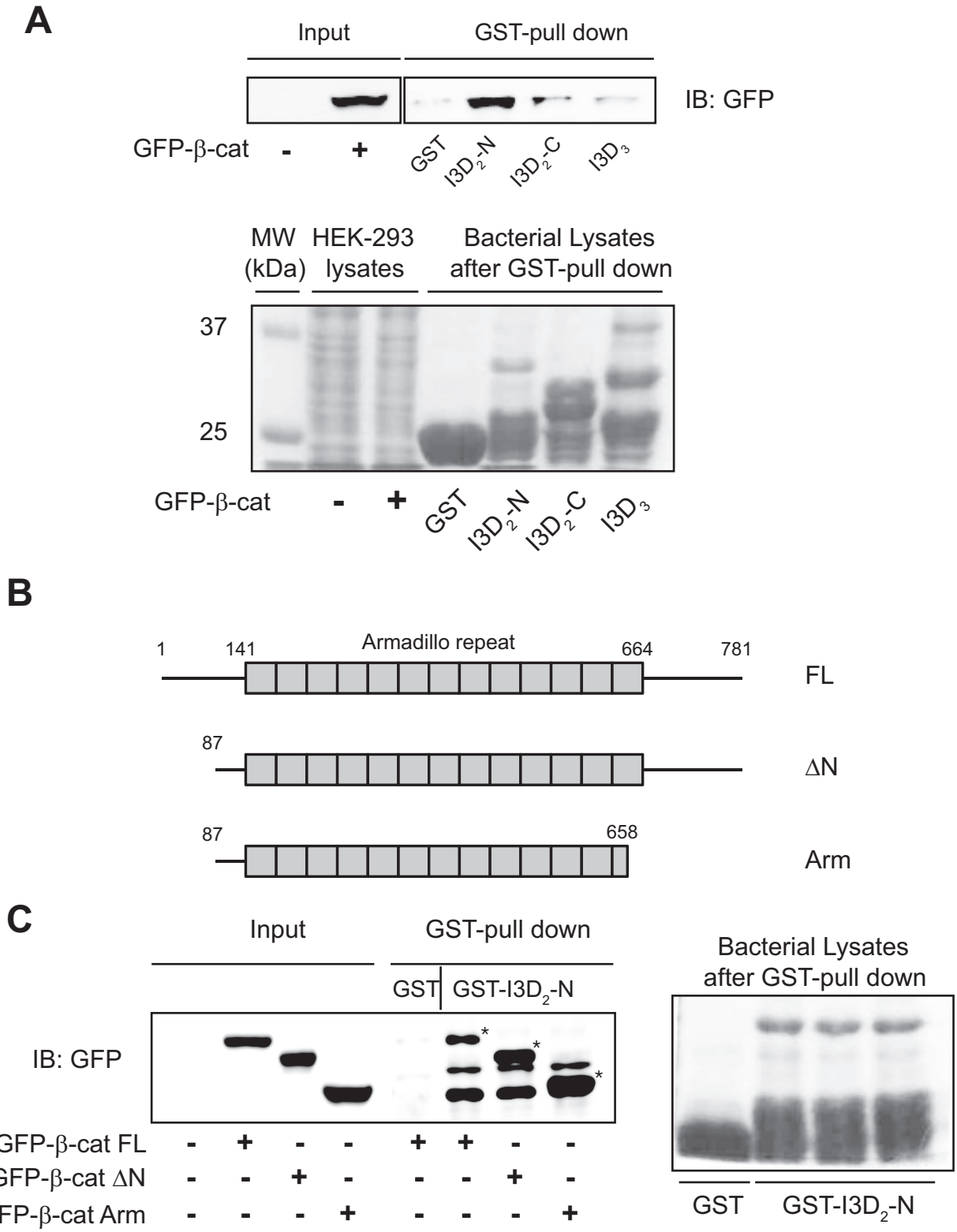

Fig. 6. Determination of interaction between D_2R and β-catenin by GST pull-down assay. A, bacterial lysates containing the GST fusion proteins of the third intracellular loops of D_2R (I_2D_2-N, the N-terminal part; I_2D_2-C, the C-terminal part) or D_3R (I_3D_3) were mixed with the cell lysates of HEK-293 cells transfected with Mock plasmid or GFP-β-catenin. After three washes, GST beads were incubated with SDS sample buffer. The eluents were analyzed with SDS-PAGE gel, and blotted with antibodies to β-catenin (upper figure, GST pull-down part). Blot from HEK-293 cell lysates is shown in the input part. The figure in the bottom shows the SDS-PAGE gel which contains lysates of HEK-293 cells (HEK-293 lysates) and "after-wash" of bacterial cell lysates. B, schematic representation of the β-catenin constructs. FL indicates the construct containing full-length β-catenin. ΔN indicates the construct containing arm repeats and C-terminal domain (amino acids 87–781). Arm indicates the construct containing only 12 arm repeats domain (amino acids 87–658). C, bacterial lysates containing the GST-I_3D_2-N was mixed with lysates of HEK-293 cells containing the full-length, N-terminus-deleted (ΔN), the armadillo repeat domain of β-catenin (Arm). GST pull-down was conducted as in A except that it was blotted with antibodies to GFP. The figure in the bottom of each blot shows the SDS-PAGE gel which contains 'after-wash' of bacterial cell lysates. All experiments were conducted three independent times, and representative results are shown. Asterisk represents the main band of β-catenin.

cross-talk with FZD or LRP5/6 on the plasma membrane. A direct and selective regulation of canonical Wnt signaling through interaction with β-catenin is the focus of this study.

In the absence of Wnt signaling, β-catenin is included as a member of the destruction complex, which is composed of adenomatous polyposis coli, Axin, CKIα, and GSK3β. Under these conditions, β-catenin is phosphorylated by CKIα and GSK-3β and then undergoes ubiquitination-dependent proteasomal degradation. When Wnt signal is initiated, this destruction complex is disassembled, and the degradation of β-catenin is inhibited, facilitating its entry to the nucleus. Most of the previous studies have shown that the cross-talk of the signal transduction pathways between Wnt and classic GPCR occurs at GSK3 level (Shevtsov et al., 2006; Force et al., 2007). Results from this study show that the interaction between certain GPCRs and β-catenin could also control the canonical Wnt signaling pathway independently of the destruction complex. Considering that β-catenin is a well known multifunctional protein, the interaction between D_2R and β-catenin might exert additional functional roles other than D_2R-mediated inhibition of Wnt signaling. For example, β-catenin is usually bound to Cadherin, a cell adhesion molecule, and is involved in the formation of adherin junctions or assembly of synaptic vesicle (Bamji et al., 2003; Xu and Kimelman, 2007). Therefore, it is possible that β-catenin, through interaction with D_2R, might play certain roles in the control of dopaminergic synaptic transmission in which D_2R acts as an autoreceptor.

Wnt signaling is reported to play important roles in dopamine-related adult brain functions, such as Parkinson's disease (Inestrosa and Arenas, 2010), a neurodegenerative disease in the dopaminergic nervous system, and schizophrenia (Inestrosa and Arenas, 2010), an affective disorder that is caused by an overactivation of dopaminergic transmission. However, the functional relationship between Wnt and the dopaminergic nervous system has not been conducted except for a couple of indirect in vivo studies. For example, it was reported that long-term treatment with antipsychotic drugs results in the elevation of intermediate components involved in the Wnt signaling such as Dvl-3, GSK3β, and β-catenin (Alimohamad et al., 2005a,b; Sutton et al., 2007). Results from our study could provide mechanistic explanation for these in vivo results as follows. Long-term treatment with antipsychotics blocks dopaminergic neurotransmission by blocking D2-like receptors. A decrease in dopaminergic transmission results in an elevation of cellular levels of D2-like receptors through negative feedback mechanism. Axin 2, a target gene of β-catenin, acts as a central player in the negative feedback loop of Wnt cascade by decreasing the stability of β-catenin (Jho et al., 2002). Because the β-catenin activity will be constitutively inhibited when the cellular levels of D2-like receptors are elevated, long-term treatment with antipsychotics will increase Wnt signaling by removing the negative feedback imposed on it by Axin2, resulting in an accumulation of intermediate signaling components such as Dvl-3, GSK3β, and β-catenin. In accordance with this, it was

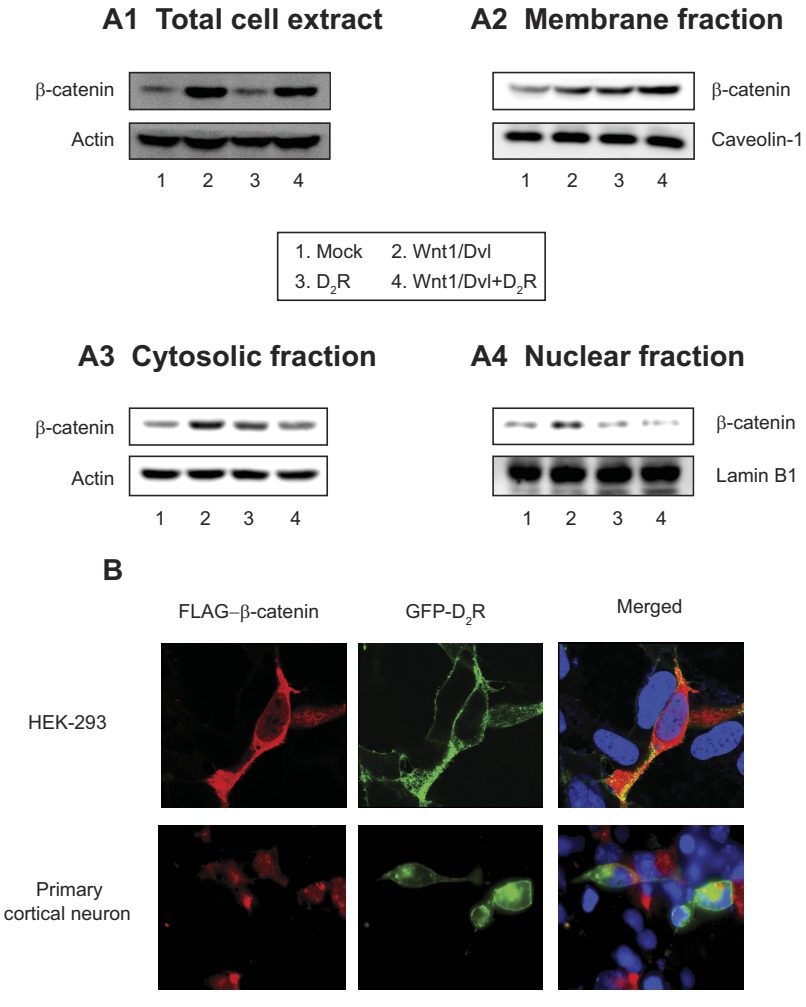

Fig. 7. Effects of D_2R on the subcellular distribution of β-catenin. A, cells were divided into four different groups and were transfected with corresponding constructs. Cell lysates were prepared from each experimental group, and fractionated into membrane, cytosolic, and nuclear fraction, as described under *Materials and Methods*. After analysis on the SDS-PAGE gel, they were blotted with antibodies to β-catenin. Actin was used as reference proteins for total cellular extract and cytosol fraction; caveolin-1 and lamin B1 were employed as reference proteins for plasma membrane and nuclear fraction, respectively. All experiments were conducted three independent times, and representative results are shown. B, colocalization between β-catenin and D_2R was determined by confocal microscopy. Top, HEK-293 cells were transfected with FLAG-β-catenin along with GFP-D_2R. Cells were labeled with FLAG-β-catenin and with Alexa Fluor 594-conjugated anti-mouse secondary antibodies. 4′,6-Diamidino-2-phenylindole was included in the mounting solution to stain nuclear regions (shown blue). Bottom, cortical neurons were plated at 5×10^5 cells per well. After 2 days, cells were transiently transfected with FLAG-β- catenin and D_2R-GFP using Lipofectamine 2000 (Invitrogen). Immunocytochemistry was conducted as described under *Materials and Methods*.

reported that Wnt pathway is activated in schizophrenic patients (Miyaoka et al., 1999). Therefore, it is expected that elucidation of the functional interactions between Wnt and GPCRs such as dopamine receptors will provide the fundamentals for the pathophysiological basis of the related diseases.

This study is the first to show that classic GPCRs regulate Wnt signaling through direct interaction with β-catenin without involvement of upstream components of Wnt signaling pathway. More detailed studies (e.g., for example, identification of characteristic protein motif involved for their interactions) will make it possible to apply these findings to other GPCRs.

Authorship Contributions

Participated in research design: K.-M. Kim, K.-S. Kim, and Shin.
Conducted experiments: Min, Cho, and Kwon.
Performed data analysis: Min and K.-M. Kim.
Wrote or contributed to the writing of the manuscript: Min and K.-M. Kim.

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